Journal of Current Health Sciences. 2025, 5(1): 1-14
DOI:10.47679/jchs.202598
ISSN 2809-3275 (print), ISSN 2809-2236 (online)

RESEARCH ARTICLE

Mitochondrial DNA-based genetic variation of *Anopheles aquasalis*, a malaria vector in Venezuela

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Published online: 13 February 2025

Abstract

The Plasmodium parasites are responsible for the severe public health malaria disease happening in Venezuela. In the year 2022, the estimated malaria cases in Venezuela were 178.000 with 190 deaths. *An. aquasalis* is the main malaria vector in the northern coastal part of Venezuela, distributed along the entire coast, from the Guajira Peninsula in the Zulia State to the Delta Amacuro State. This study aimed to investigate the genetic diversity and population structure of the vector *An. aquasalis* from coastal regions of Venezuela through single strand conformation polymorphism markers of the mitochondrial NADH dehydrogenase (ND5) gene. Adult mosquitoes were collected between November 2017 and December 2018. A 450-bp region of the ND5 gene was amplified by the polymerase chain reaction and tested for variation using SSCP among 358 *An. aquasalis* from seven localities near the coast of the States of Zulia, Falcón, Carabobo, Aragua, Miranda, Sucre, and Delta Amacuro of Venezuela separated by up to 1960 km. This study revealed that the populations of *An. aquasalis* in Venezuela are highly polymorphic, with high genetic diversity among 48 haplotypes detected throughout Venezuela and sorted into four clades. In addition, a significant *An. aquasalis* genetic population structure was found, apparently in response to geographical barriers and ecological differences.

Keywords: malaria, *An. aquasalis*, genetic structure, single strand conformation polymorphism analysis, ND5 haplotype, Venezuela

INTRODUCTION

The Plasmodium parasites are responsible for the severe public health malaria disease happening in different tropical and subtropical regions from Africa, Asia, and America (WHO, 2023). Seventeen countries and one territory in the Region of the Americas are currently at risk of malaria. Four countries accounted for almost 80% of all estimated cases being Venezuela one of them with 28% of the cases (WHO 2023). In the year 2022, the estimated malaria cases in Venezuela were 178.000 with 190 deaths.

The main malaria vectors in the Americas have adapted to the ecological conditions of different regions, making some species prevail over others in a given area (Sinka et al. 2010). *Anopheles aquasalis* is found preferably in high salt concentration waters. The distribution of *An. Aquasalis* in the Americas includes the northern coastal region of the Atlantic and Pacific Oceans (Sinka et al. 2010). The complete genome of *An. Aquasalis* was elucidated recently (Alencar et al., 2023), and this data will serve to look for different options for malaria vector control (Prado et al. 2023). *An. Aquasalis* is the main vector in the northern coastal part of Venezuela, distributed along the entire coast, from the Guajira Peninsula in the Zulia State to the Delta Amacuro State (Osborn et al. 2004).

Anopheles species may differ in several characteristics of epidemiological importance such as biting behavior and vectorial capacity which may be detected through molecular markers (Loaiza et al. 2012). In this sense, the 2La inversion, one of the alternative arrangements of genes on the left arm of chromosome 2 of *An. Gambie*, is a molecular marker associated with adaptation to different microclimates, desiccation resistance, malaria

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susceptibility and resting behavior of mosquitoes (Riehle et al. 2017). A correlation has been observed between markers of insecticide resistance and the 2La inversion, suggesting a potential association between mosquito resting behavior and insecticide resistance (Mwagira-Maina et al 2021).

In general, DNA polymorphism acts as a genetic marker and is followed using different techniques. One simple, sensitive, and cheap method to detect DNA polymorphism is the single strand conformation polymorphism (SSCP) analysis (Orita et al. 1989). This method recognizes the alteration of the three-dimensional structure of a single strand DNA molecule because of changes in its nucleotide sequences (Sharma and Rishi 2021). At present, the SSCP method has a variety of applications (Marwal and Gaur 2020). For instance, it can be used in genotyping to detect homozygous and heterozygous individuals. In the field of virology, It can be used to detect variations in different viral strains. Additionally, it can be used in the identification of variations in the intron region of the β3-adrenergic receptor gene intron. This intron is a candidate gene associated with growth traits.

These markers could be used to determine the relatedness of geographic populations and associate this information with vector movements (Bunmee et al. 2021). Therefore, knowing the genetic diversity, population structure, and migration dynamics of vectors helps to analyze the risk of disease transmission from the introduction of vectors present in areas of high malaria transmission to areas of low transmission (Loaiza et al. 2012). As a result, considerable studies on diversity and genetic structure have been carried out with different vectors of the parasite (Weeraratne et al. 2018; Campos et al. 2019; Bunmee et al. 2021; Carter et al. 2021; Debrah et al. 2023; Odero et al. 2023; Orazbayeva et al. 2024).

This study aimed to investigate the genetic diversity and population structure of the vector *An. Aquasalis* from coastal regions of Venezuela through SSCP markers of the mitochondrial NADH dehydrogenase (ND5) gene.

MATERIALS AND METHODS

Mosquito collection and DNA extraction.

The selection of sampling sites was based on the following information: *An. aquasalis*, a species of mosquito, has been documented in a wide variety of freshwater and brackish water breeding sites situated at altitudes below 550 meters, with seasonal rainfall exceeding 1,000 millimeters and mean annual temperatures ranging from 25 to 27 degrees Celsius (Berti et al. 2010). The immature stages of *An. aquasalis* are predominantly found in breeding sites that are fully or partially exposed to the sun and where the mangrove *Avicenia germinans* predominates. In addition, the sites were previously identified as *An. aquasalis* vector distribution areas and have sufficient water throughout the year to allow sampling in both the dry and wet seasons (Osborn et al. 2004).

Adult mosquitoes were collected using light traps between November 2017 and December 2018 near the coast of the States of Zulia, Falcón, Carabobo, Aragua, Miranda, Sucre, and Delta Amacuro of Venezuela, separated by up to 1960 km. The sample consisted of 358 *An. aquasalis* mosquitoes with taxonomic identification and then stored at -70ºC awaiting DNA extraction. The DNA was extracted from individual specimens using a phenol/chloroform method, resuspended in 60 µL of sterilized water, and stored at - 80ºC (Rivero et al. 2004). The localities of the states, with their coordinates and sample sizes, are indicated in Table 1, and Figure. 1 presents the map of Venezuela showing the sampled localities.

Mitochondrial gene amplification.

A 450-bp region of the ND5 gene was amplified using the oligonucleotides and reaction conditions of De Merida et al. (1999). All PCRs were performed in 25-µL reaction volumes containing 20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.03 units of *Taq* polymerase, 25 pmol each of forward and reverse primer (5′-CTT CCA CCA ATT ACT GCT ATA ACA-3′ and 5′-AGG ATG AGA TGG CTT AGG TT-3′) and 50-100 ng of DNA in a PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA). An initial denaturation of 5 minutes at 95°C was followed by 10 cycles of 92°C for 60 seconds, 48°C for 60 seconds, and 72°C for 90 seconds. Then a second round of 36 cycles of 92°C for 60 seconds, 54°C for 55 seconds, and 72°C for 90 seconds and final extension for 7 min. Negative controls (all reagents except template) were run to detect possible contamination. The amplified products were visualized by electrophoresis in 2% agarose gels stained with SYBR Green.

SSCP analysis

The PCR product (10 µL: 40-50 ng) was mixed with 8 µL of loading buffer (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol), centrifuged and heated to 95ºC for 10 min on a thermal cycler, then transferred immediately into ice (Carrozza et al. 2016). Samples were loaded onto 27 × 20 cm, 1 mm thick, 7% polyacrylamide gels. Gels were run at 4ºC for 20 h at a constant 8 milliamps and silver stained to visualize DNA fragments (Black and DuTeau, 1997). PCR products from 3-6 individuals with the same haplotype were purified and unidirectionally sequenced for both strands. The sequencing reactions were performed with ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit on an Applied Biosystem Model 310 Genetic Analyzer at the Instituto de Estudios Avanzados (Caracas, Venezuela).

Statistical analysis of mitochondrial haplotype frequencies

Analysis of molecular variance (AMOVA) was conducted on the resulting haplotypes within and among regions using Arlequin version 3.5 (Excoffier et al. 2007). The significance of the variance components was computed using a non-parametric permutation test (Excoffier et al. 1992). According to McDermott and McDonald (1993), the genetic flow Nm was calculated from the F_{ST} to estimate the number of individuals that migrate between populations per generation. The DNA sequences were aligned using the Clustal W software package (Thompson et al. 1994). The nucleotide sequence and the frequency of each haplotype for each collection were analyzed using DnaSP version 5.10 (Librado and Rozas 2009). The number of polymorphic sites, the average number of nucleotide differences (k) (Tajima 1983), the nucleotide diversity ($\pi 1$) and the nucleotide diversity with the Jukes and Cantor correction ($\pi 2$) (Nei 1987) were estimated. Effective migration rates (Nm) were calculated

from F_{ST}. Transformed F_{ST} /(1- F_{ST}) were regressed on the natural logarithm of pairwise geographic distances among populations to test for isolation by distance (Slatkin 1993). The Mantel test was performed using FORTRAN program MANTEL (William C. Black IV, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO). Genetic distance matrices were used to construct a cladogram among all collections by means of unweighted pair-group method with arithmetic averaging analysis (UPGMA) in the NEIGHBOR procedure of PHYLIP3.5C (Felsenstein 2004).

Phylogenetic relationships among haplotypes

MEGA version 4.0 was used to perform phylogenetic analyses using maximum parsimony (Tamura et al. 2007). A bootstrap analysis with 1000 replications was done to assess the consistency with which the dataset supported the resolved phylogenies. Sequences of *Drosophila* (GeneBank gi|215259920), *Aedes aegypti* (GeneBank gi|164523399), and *An. gambiae* (GeneBank gi|90657004) were used as outgroups.

RESULTS

Mosquito collection

A total of seven samples, representing 358 wild specimens of *An. aquasalis* were collected in seven states of Venezuela defined as Zulia (SZ), Falcón (BTF), Carabobo (PC), Aragua (MEA), Miranda (HM), Sucre (LPSD) and Delta Amacuro (MDA) (Table 1).

Haplotype frequencies

Forty-eight haplotypes were detected by SSCP analysis and sequencing**.** Table 2 shows the frequencies of these haplotypes in each population studied. Some haplotypes were found in two or more states and unique haplotypes with frequencies> 30% were present in some populations. Table 2 also presents the total frequencies, considering all populations (358 total mosquitoes). It shows a predominance of some haplotypes in the total population that had frequencies ranging between (4.8-13.1) %. The remaining haplotypes had a frequency ≤ 3%. The haplotypes with the higher frequencies, both by state and in the whole country, are shown in Figure 1.

The subpopulations shared only nine haplotypes: H12-H16, H21-H23, and H44. H14 (12.01%), one of the most frequent haplotypes in the total population, distributed along a gradient of decreasing frequency (31.9%-6%) from the Falcón western state passing through the central states Carabobo, Aragua, and to a lesser extent, Miranda state (Figure 1). This last state had two main haplotypes, H29 (38%) and H30 (34%). Comparing the sequences of these haplotypes with the corresponding ones of H15 and H14 showed that H29 had four nucleotides different from H15 and H14 (Supplementary file 1). H30 had four nucleotides different from H15 and only two with H14. All these haplotypes collapsed in the same clade. However, haplotype 12, found in the Zulia (19.1%) and Falcón states (5.8%), was distributed in a different clade. The H44 was the most frequent haplotype in the Sucre state (26.9%), and in the Delta state was also found (15%). The H46 was only found in the Delta at a high frequency (69.8%). Both haplotypes found in lineage one presented a significant difference of 8 nucleotides and are very different from H14 and H15.

Population genetic structure

Geographical analysis of variation in ND5 haplotype frequencies was conducted by AMOVA (Table 3). Most variation (77.0%) arose among mosquitoes in collections, while 22.9% were among collections. The average F_{ST} was 0.2297 (*p=0.0000),* indicating substantial genetic structure among collections.

An analysis of the gene flow level among the populations was also conducted. The parameter Nm was evaluated using the formula $Nm = (1 - F_{ST})/4F_{ST}$. The gene flow value (Nm) obtained showed that approximately 0.83 individuals migrate per generation among the seven populations studied. In addition, the effect of the distance on gene flow was estimated by regressing linearized F_{ST} values on geographic distance. There was no correlation among collections from any particular region (data not shown).

Genetic Diversity

For tests involving polymorphic sites, haplotypes with frequencies <1% were not considered since a haplotype is polymorphic when it appears in the total collection at a rate of at least 1%. There were 24 polymorphic haplotypes, and 9 of them were common between subpopulations (Table 2). A detailed analysis of the nucleotide sequences of the 24 polymorphic haplotypes, was carried out. The number of polymorphic sites (S) and nucleotide diversity indices were determined (Table 4). The haplotypes from SZ, BTF, and LPS presented a high number of polymorphic sites (S) with high polymorphic variability (high k and π) and under positive selection according to Tajima's D test; however, only the results of SZ and BTF were statistically significant. On the other hand, the MEA locality also presented haplotypes with high S but with lower polymorphic variability and under statistically significant negative selection. The analysis of this test for the total population also gave a negative result, however, for all analyses it was statistically non-significant.

Table 1. States, locations per state, geographic coordinates, and sample size

State	Locality	Latitude	Longitude	No. individuals
Zulia	Sinamaica (SZ)	11° 06' 17N	071° 55' 15W	51
Falcón	Boca del Tocuyo (BTF)	11° 03' 47N	068° 20' 54W	47
Carabobo	Patanemo (PC)	10° 26' 18N	067° 55' 05W	51
Aragua	Magdaleno (MEA)	10° 06' 22N	067° 35' 48W	54
Miranda	Higuerote (HM)	10° 23' 44N	066° 05' 20W	50
Sucre	Los Palmares (LPS)	10° 34' 45N	062° 51' 13W	52
Delta Amacuro	La Horqueta (MDA)	09° 13' 09N	062° 12' 07W	53
Total	7			358

Table 2. Haplotypes: Locality and Frequency per population and the total population

Haplotype	Locality	Frequency per population (%)[a]	Frequency total population (%)[b]
1	SZ	33	4.75
2	SZ	1.9	0.28
3	SZ	7.8	1.12
4	SZ	7.8	1.12
5	SZ	1.9	0.28
6	SZ	13.7	1.96
7	SZ	1.9	0.28
8	SZ	1.9	0.28
9	SZ	1.9	0.28
10	SZ	7.8	1.12
11	SZ	5.8	0.84
12	SZ, BTF	5.8 (SZ), 19.1 (BTF)	3.35
13	BTF, MEA	6.3 (BTF), 3.7 (MEA)	1.40
14	BTF, MEA, PC, HM	31.9(BTF), 20.3(MEA), 27.4(PC), 6(HM)	12.01
15	BTF, MEA, PC	25.5(BTF), 17.6(PC), 48.1(MEA)	13.13
16	BTF, MEA	42(BTF), 3.7(MEA)	1.12
17	BTF	2.1	0.28
18	BTF	2.1	0.28
19	BTF	6.3	0.84
20	BTF	2.1	0.28
21	SZ, PC, MEA	7.8(SZ), 7.8(PC), 7.4(MEA)	3.35
22	PC, MEA	1.9(PC), 7.4(MEA)	1.40
23	PC, HM	35.2(PC), 4(HM)	5.59
24	PC	3.9	0.56
25	PC	1.9	0.28
26	PC	3.9	0.56
27	MEA	1.8	0.28
28	MEA	7.4	1.12
29	HM	38	5.31
30	HM	34	4.75
31	HM	4	0.56
32	HM	8	1.12
33	HM	6	0.84
34	LPS	11.5	1.68
35	LPS	5.7	0.84
36	LPS	5.7	0.84
37	LPS	3.8	0.56
38	LPS	7.6	1.12
39	LPS	11.5	1.68
40	LPS	11.5	1.68
41	LPS	3.8	0.56
42	LPS	5.7	0.84
43	LPS	1.9	0.28
44	LPS, MDA	26.9(LPS), 15(MDA)	6.15
45	LPS	3.8	0.56
46	MDA	69.8	10.34
47	MDA	13.2	1.96
48	MDA	1.8	0.28

[a]Nº mosquitoes per population: SZ (51), BTF (47), PC (51), MEA (54), HM (50), LPS (52), MDA (53)
[b]Nº total mosquitoes: 358

Table 3. Analysis of molecular variance in the frequency of ND5 haplotypes among *An. Aquasalis* collections in Venezuela

Source of variation	Degrees of freedom	Variance components	Variation (%)	Fixation index	P
Among populations	6	0.11159	22.97		
Within populations	352	0.37423	77.03	F_{ST}: 0.22970	0.0000
Total	358	0.48582			

FST: correlation among haplotypes within collections relative to the correlation of random pairs drawn from the whole sample

Phylogenetic analysis

Phylogenetic analysis provided a well-supported phylogeny with four maternal lineages, with lineage 1 containing 36 haplotypes (Figure 2). The rest of the 12 haplotypes distributed in the other lineages had low frequency with three exceptions: H6 from the Zulia state (13.7%), H12 from the Falcón (19.1%) and Zulia (5.8%) states, and H39 (11,5%) from the Sucre state.

In addition, a well-supported phylogenetic tree from the ND5 sequences of the 24 haplotypes was generated (Figure. 3). In the two clades obtained, the first was divided into two, and one was subdivided into three more. All these subclades had haplotypes mostly from the Zulia

state. The second clade was divided into two subclades. The first subclade located a single haplotype of the LPS population, and the second one, in turn, was divided into three sub-subclades, with the first one containing only the principal haplotype of MDA. The second subclade had haplotypes from the Sucre state, and the third subclade distributed the majority of the haplotypes from the west and central states, representing 65% of the total haplotypes in the clade. In addition, two haplotypes from the Delta state collapsed in this subclade but separated from the rest in a new subclade.

Table 4. Variability estimates in the mitochondrial genome among *An. aquasalis* collections in Venezuela

Locality	N	S	k	π1	π2	Tajima′s D	P
SZ	51	52	23.1208	0.0521	0.0558	2.8603	< 0.01
BTF	47	49	20.6772	0.0466	0.0500	2.8770	< 0.01
PC	51	13	1.7443	0.0039	0.0040	-1.1842	> 0.10
MEA	54	34	3.1030	0.0070	0.0071	-2.0288	< 0.05
HM	50	9	3.2057	0.0072	0.0073	1.6667	> 0.10
LPS	52	44	15.8786	0.0358	0.0372	1.6950	> 0.10
MDA	53	11	3.7997	0.0086	0.0087	1.6358	> 0.10
Total samples	358	76	14.2500	0.0321	0.0338	-0,2372	> 0.10

N: number of samples; S: polymorphic sites; k: average number of nucleotide differences; π1: nucleotide diversity; π2: nucleotide diversity with Jukes and Cantor correction

DISCUSSION

Molecular markers of mitochondrial genomes have been used for population genetic and phylogenetic studies of mosquito vectors transmitting a wide variety of diseases (Herrera et al. 2006; Bunmee et al. 2021, Lv et al. 2020; Feng et al. 2017, Debrah et al 2023, Weeraratne et al 2018). The present study described an analysis of 444 bp sequence of ND5 mitochondrial DNA in *An. aquasalis* populations obtained from 7 collection sites in seven states of Venezuela. We found 48 haplotypes in total population. The phylogenetic tree determined that 75% of the haplotypes were in lineage one and, because of that, could be the oldest lineage of *An. aquasalis* in Venezuela. The low-frequency haplotypes may be of recent origin. Phylogenetic analysis of the 24 haplotype sequences demonstrated that the haplotypes with higher frequencies belonging to the western-central region of the country were still grouped in the same clade. On the contrary, the H46 haplotype separated into another clade, which suggests that H46 originated or was introduced into the Delta region.

The 24 haplotypes with the higher frequency in the total population were only present in some subpopulations where their frequencies were high enough to remain >1% when joined to the others in the total population. This behavior in different Anopheles vector species was also present in numerous investigations (Molina-Cruz et al. 2004; Angêlla et al. 2007; Makhawi et al. 2013).

The H14 has one nucleotide different from H1 and H23. The relationship among these haplotypes suggests that H1 and H23 (with a difference of two nucleotides between them) originated from H14 (Figure. 1). H1 spread to Zulia state (33%) and H23 to the central state of Carabobo (35.2%) and a minor level to Miranda (4%). H30 had two nucleotides different from H14 which pointed again to H14 as the haplotype from which other haplotypes originated. H15 could also have originated from H14 because there were only two nucleotides different from H14, and both were found in almost the same states. H44 may originate in Sucre and spread to the Delta, while H46 may arise in Delta. It looks like there were haplotypes characteristic for the western-central region and others for the eastern one. That could be due to a lower genetic flow from the west to the east due to geographical barriers, especially in the eastern state of Sucre, which has a mountainous relief (Chiu et al. 2023).

The *An. aquasalis* population of the Delta Amacuro presented only four haplotypes, with H46 being dominant. That may happen because *An. aquasalis* is not the most abundant mosquito in the Delta. In this region, approximately 30 species of mosquitoes compete for the same habitats or food (Berti et al., 2019). This competition can lead to less growth of some mosquito populations regarding others, which could influence the genetic diversity of the populations (Juliano and Lounibos 2005; Zhao et al. 2023). On the other hand, the Delta locality has numerous water currents. The deviation from Delta population expansion may also occur due to the low salt concentration of the water. Bertie et al (2010) demonstrated that the abundance of *An. aquasalis* larvae depended on water salinity. Thus, the Delta population may have evolved a mitochondrial phenotype, which permits an adaptation to water with low salinity. At this juncture, it is salient to acknowledge that *An. aquasalis* is a singular species of mosquito capable of thriving in environments characterized by elevated or diminished salt levels in water. Climate change, characterized by global increases in sea levels, has emerged as a salient concern, particularly with respect to its potential implications for the dissemination of malaria in coastal regions (Ramasamy and Surendran 2011).

The results indicate that the majority of the *An. aquasalis* populations in Venezuela were highly polymorphic in the ND5 gene. These results agree with previous findings on different Anopheles species (Sarma et al. 2012; Makhawi et al. 2013; *Anopheles gambiae* 1000 Genomes Consortium 2017; Weeraratne et al. 2018). Neutrality tests detected significant deviation from neutrality theory in the ND5 gene within three populations of *An. aquasalis*. Two positive selections (SZ and BTF) were found suggesting population expansion. In addition, one negative selection (MEA) was found, suggesting population reduction. *An. aquasalis* was a minority mosquito in the MEA area and had to compete with other more abundant species, such as *Aedes aegypti* and *Aedes albopictus*. In addition, mosquito larvae were placed in freshwater from local lagoons of MEA. Similar to the Delta population, both factors may lower population growth. Both selections could impact the pattern of variance transversely to the entire mtDNA genome. In addition, there were large F_{ST}

(0.2297; p = 0.0000) values among collections, indicating the existence of a genetic structure between populations. The estimated number of individuals that migrate per generation (Nm=0.83) is indicative of low genetic flow among populations, which consequently may lead to high genetic variability among them. This genetic differentiation did not follow the model of isolation by distance.

The geographic distribution of *An. aquasalis* along the Venezuelan coastline constitutes a critical factor in evaluating the potential risk of malaria transmission. However, the true risk is determined by the simultaneous presence of infected individuals, and all sampled areas exhibit a close proximity between the vector breeding habitats and human settlements. Notably, malaria cases have been documented in all these regions (WHO 2023). However, the states of Sucre and the Delta have demonstrated a particularly high incidence of malaria, thus being designated as malaria regions. The Zulia state has experienced outbreak occurrences in specific localities, while the other states have only reported a limited number of cases. The observed variation in the number of malaria cases across the total states could be attributed to a variety of environmental and biological factors. Consistent with other reports (Hume et al. 2007; Edgerton et al. 2020), these variations may be influenced by differences in the capacity of mosquitoes to support parasite development. Indeed, Sucre and Delta states have been observed to exhibit markedly divergent haplotype profiles compared to other states, which may be indicative of a heightened susceptibility to Plasmodium infection in these particular mosquito populations.

Another possible explanation for the different incidence of malaria cases among the states could be that the mosquito populations of Sucre and Delta states have developed insecticide resistance, which is a growing problem for malaria control (Suh et al. 2023). If so, the haplotype profiles of these states could serve as insecticide resistance markers.

An. aquasalis has been identified as a significant contributor to the transmission of malaria in neighboring countries (Sinka ME, et al. 2012; Martins-Campos, et al. 2018). It is plausible to hypothesize that Venezuelan *An. aquasalis* could spread to neighboring countries, facilitating genetic exchange among the vectors and thereby influencing the genetic variability of *An. aquasalis* in nearby countries. For instance, the dissemination of insecticide-resistant haplotypes of *An. aquasalis* from Venezuela to other coastal regions of nearby countries (such as Colombia, Trinidad and Tobago, Guyana, and Brazil) and a rapid fixation of these haplotypes is a plausible scenario. To address this issue, it is imperative to first confirm the presence of insecticide-resistant haplotypes in Venezuelan mosquito populations. In the event of a positive identification, a parallel investigation must be conducted in *An. aquasalis* populations from neighboring countries. Determining the most prevalent haplotypes in these regions is necessary. However, there is a paucity of studies addressing the genetic structure of *An. aquasalis* populations in these countries.

These findings highlight the importance of considering both ecological and genetic factors that influence the vectorial capacity of *An. aquasalis* in different regions of Venezuela. Further research is essential to elucidate the specific mechanisms of these variations in order to develop effective malaria control strategies.

CONCLUSION

This study revealed that the populations of *An. aquasalis* in Venezuela are highly polymorphic within the ND5 gene of mitochondrial DNA with high genetic diversity. In addition, a significant *An. aquasalis* genetic population structure was found, apparently in response to geographical barriers and ecological differences. Moreover, the vectorial capacities or the insecticide resistance of these populations with different genetic compositions may also differ. Further investigations related to the characteristics of the populations would establish efficient vector control actions to diminish the potential impact of the dissemination of crucial genes like insecticide-resistant genes over the *An. aquasalis* populations.

Acknowledgement

To the entomology technicians of the Instituto de Altos Estudios Arnoldo Gabaldon for the collection of *An. aquasalis* mosquitoes and to MSc. Marifel Carrozza for advice us on statistical analysis on preliminary work

DECLARATIONS

Funding. This study was funded by Fondo Nacional de Ciencia, Tecnología e Innovación, Venezuela (grant 2008000911-1).

Conflicts of interest/Competing interests. The authors declare that they have no conflicts of interest.

Ethics approval. It was not necessary

Consent to participate and publication. The named authors read the manuscript and approved it for participation and publication.

Availability of data and material. All data generated during this study are included in this published article

Code availability 'Not applicable'

Authors' contributions. Johanny Ruiz carried out the experimental assay and applied the statistics to analyze study data; César Pacheco contributed to paper review; Flor Herrera conducted the investigation and wrote the initial draft.

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APPENDIX 1

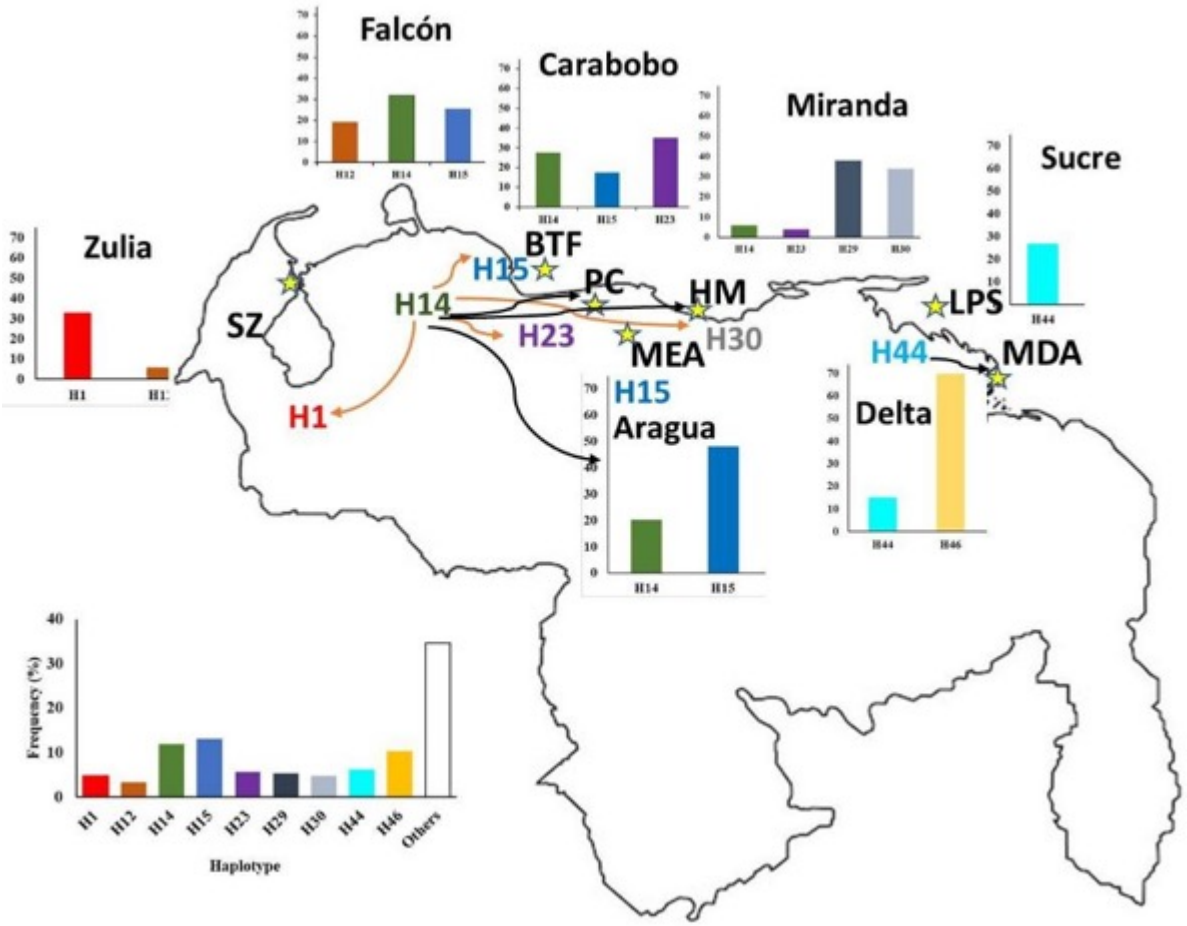


Figure. 1

Map of Venezuela showing the seven states of *Anopheles aquasalis* populations. The bars indicate % of haplotype frequencies in each population and the total % of haplotype frequencies in the whole population. Black arrows indicate the direction of the spatial gradient of the distribution of haplotype H14 and the distribution of H44. Orange arrows show the spreading of the H1, H15, H23, and H30, originating possibly from H14.Yellow stars indicate the collection sites

APPENDIX 2

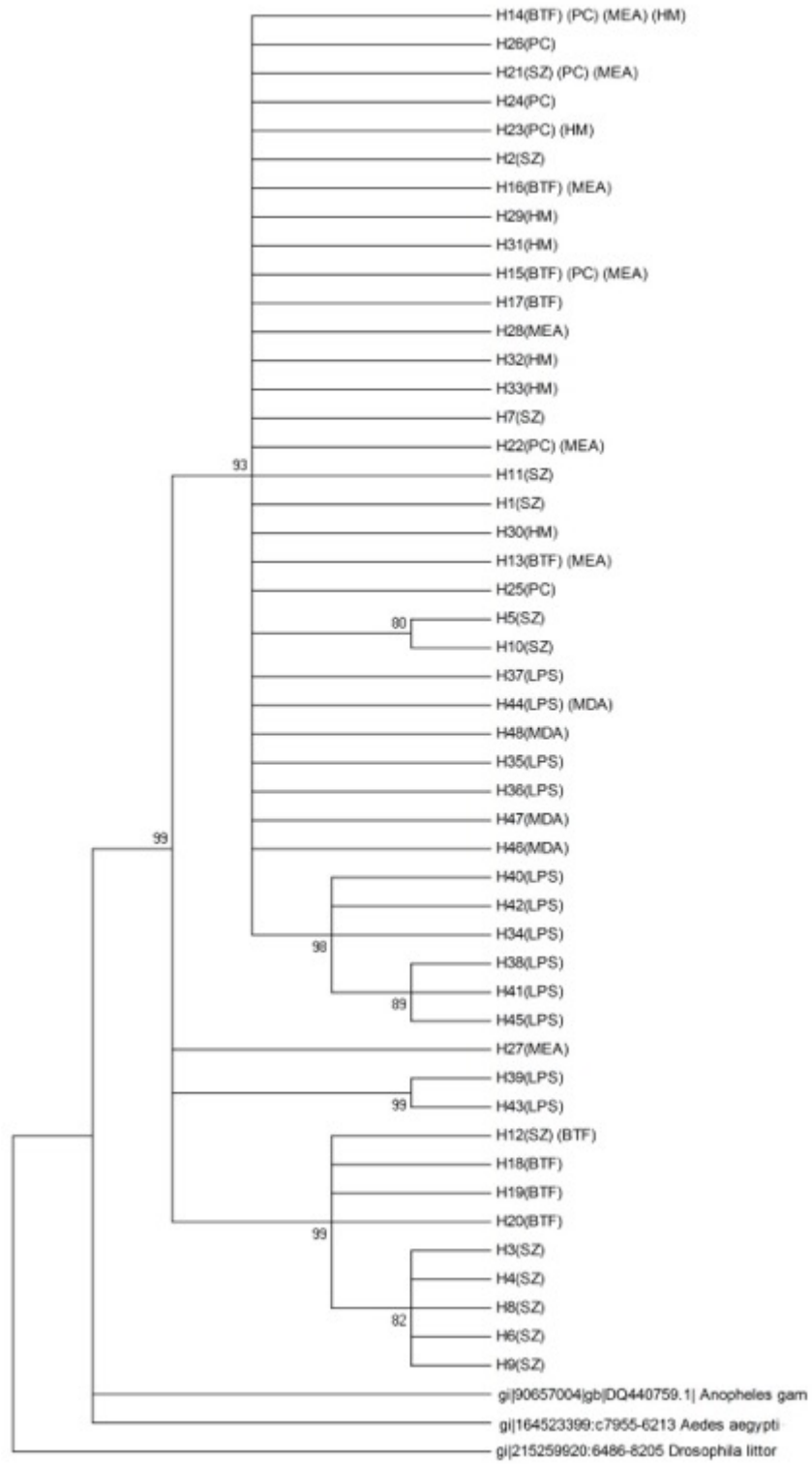


Figure. 2

Maximum parsimony tree among individual haplotypes of *An. aquasalis.* Bootstrap support using maximum parsimony analysis appears above each branch, with the nonrelated genuses (*Drosophila and Aedes)* and other species of the genus Anopheles (*An. gambie*) used as outgroups

APPENDIX 3

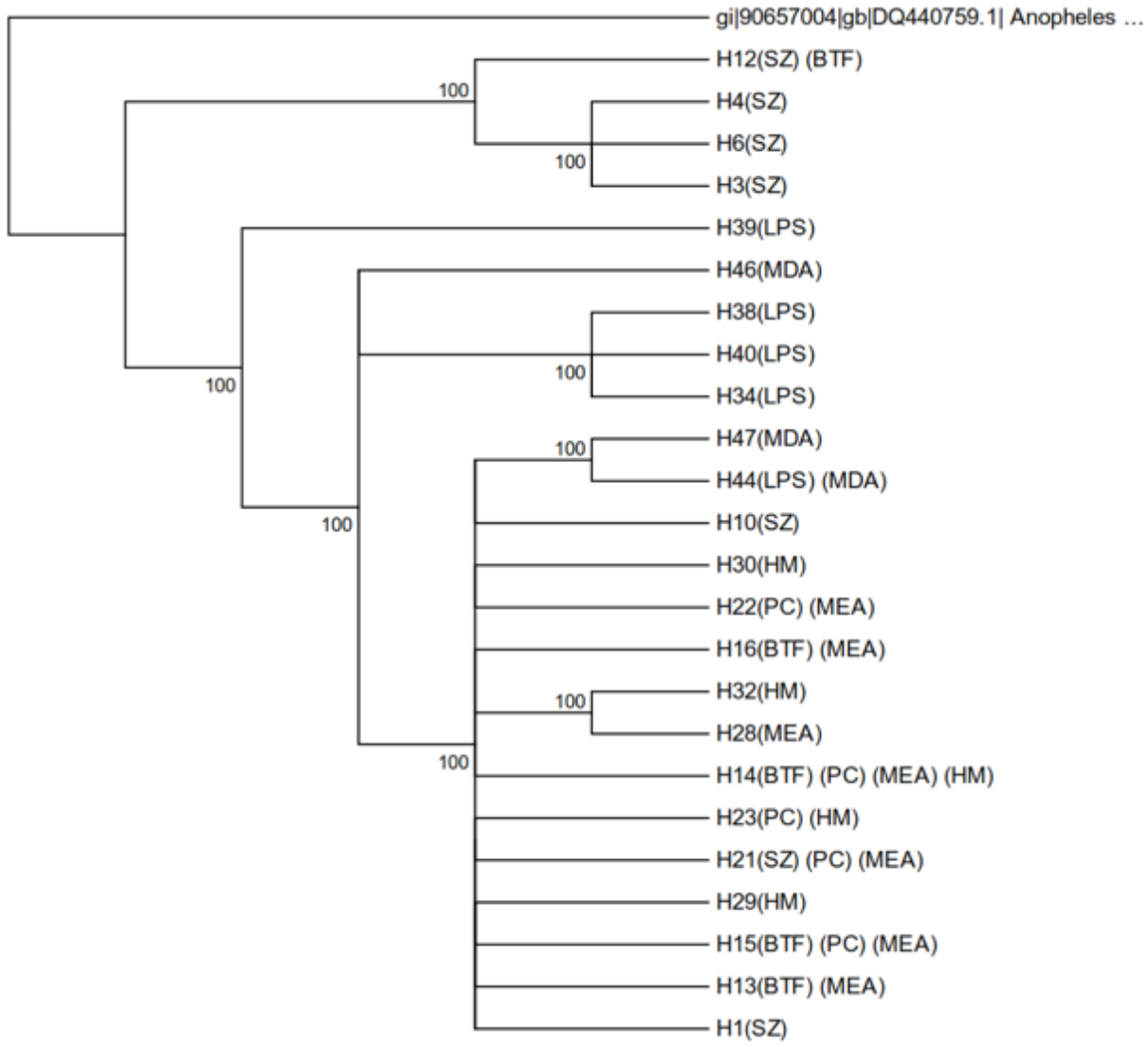
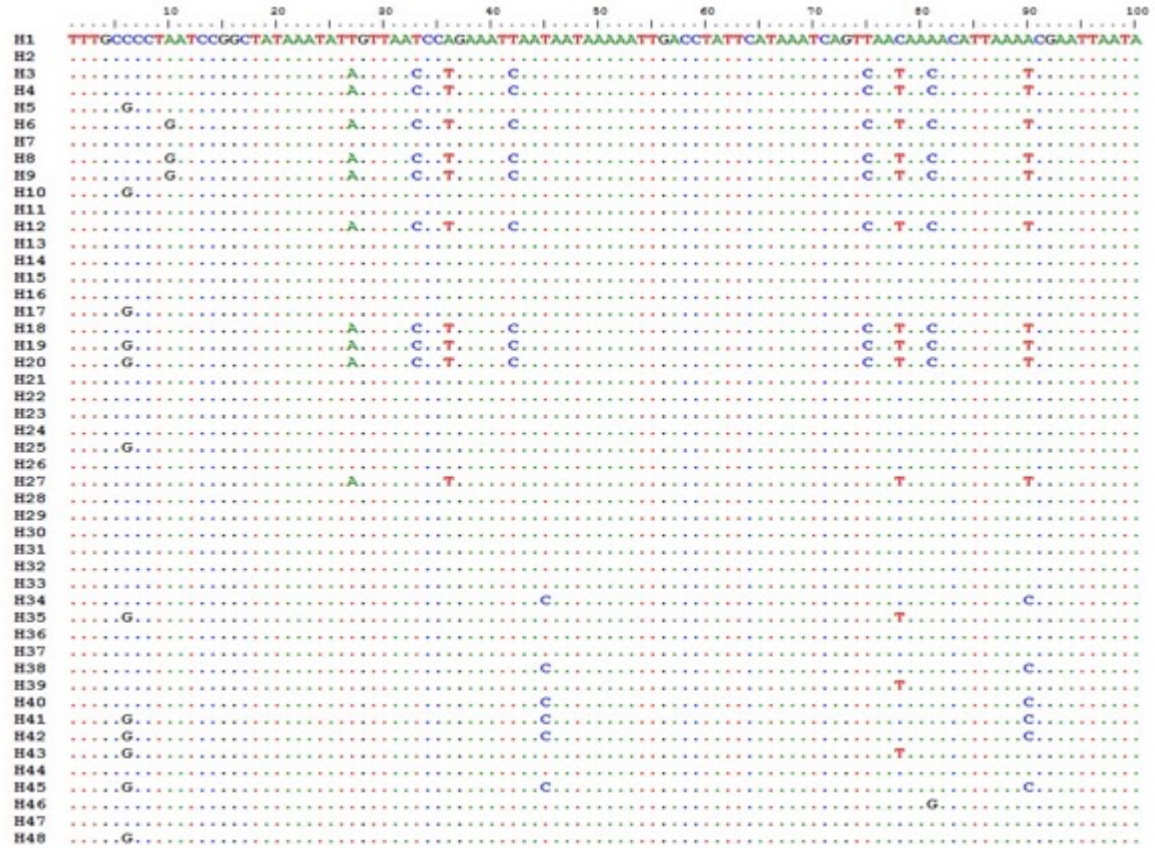


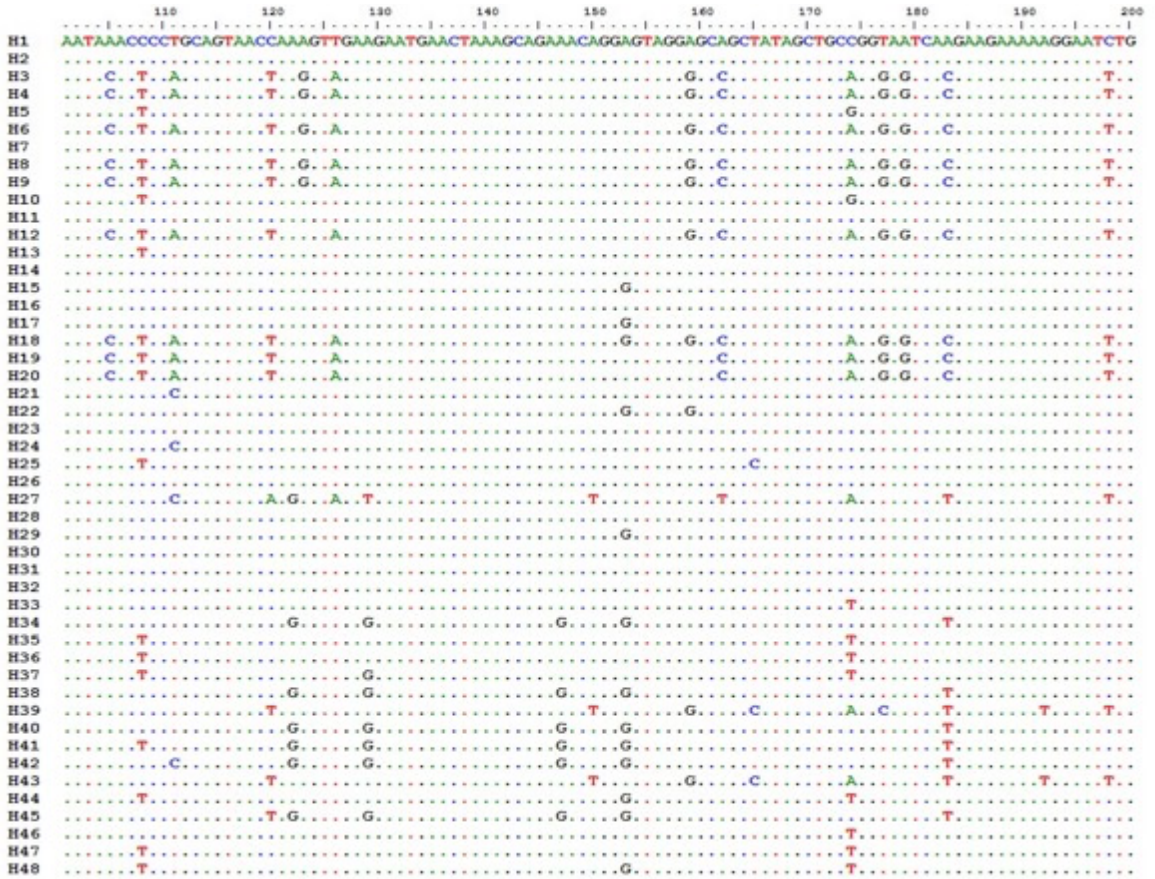
Figure. 3

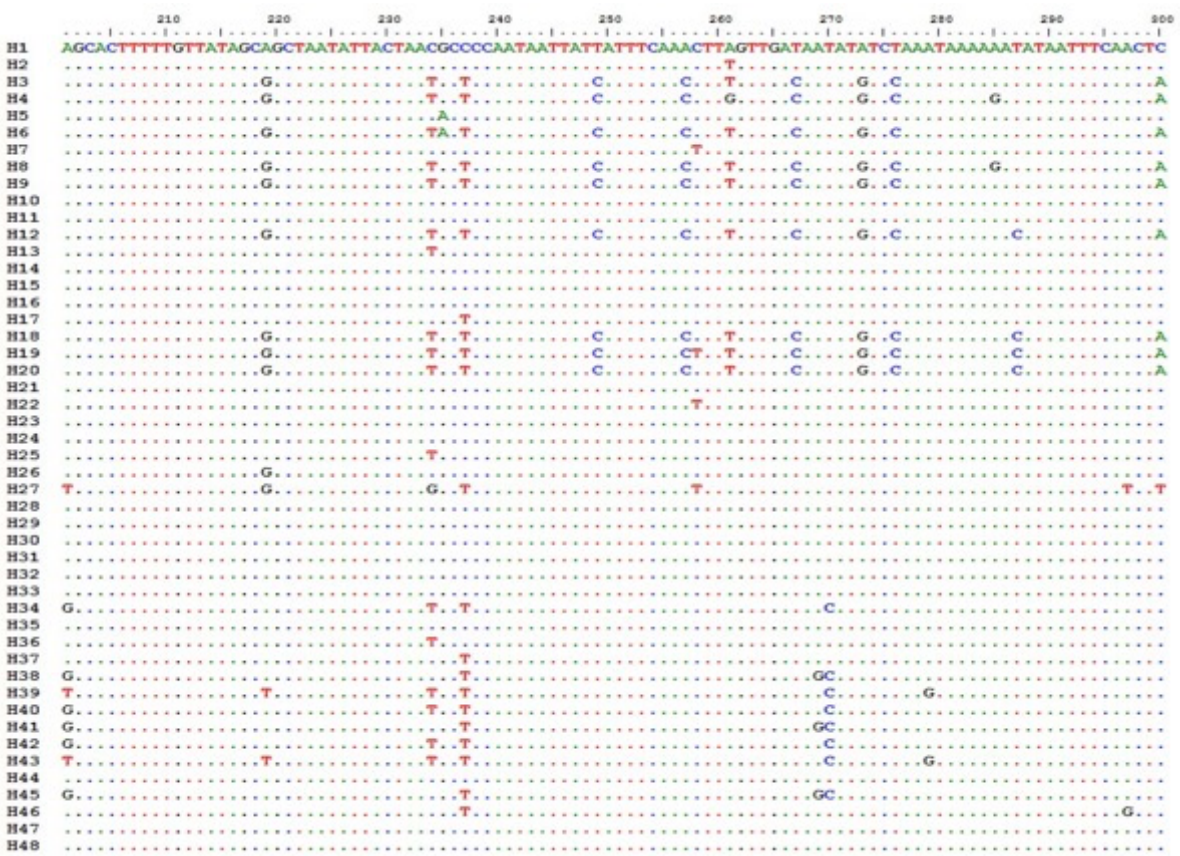
Maximum parsimony tree among the higher frequency haplotypes of *An. aquasalis.* Bootstrap support using maximum parsimony analysis appears above each branch, with the *An. gambie* sequence used as an outgroup

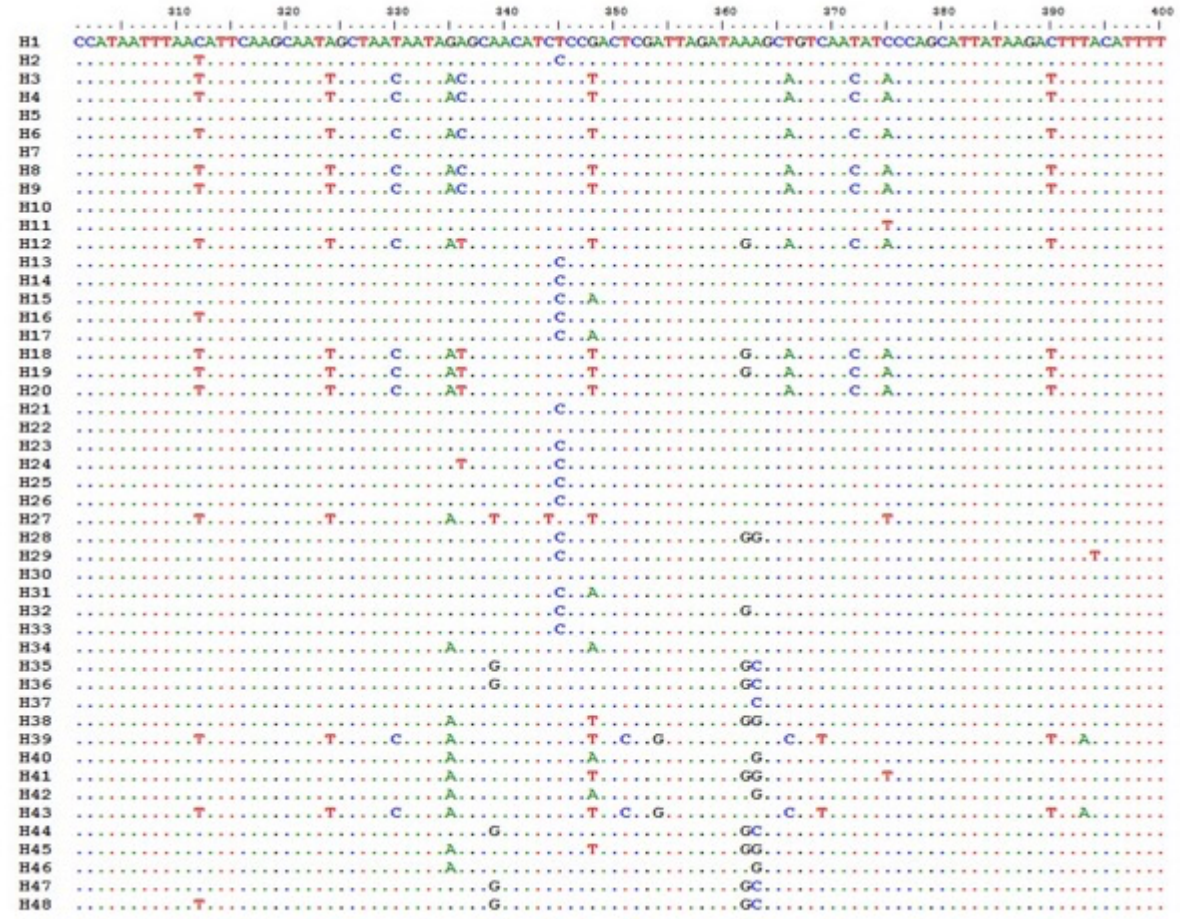
APPENDIX 4

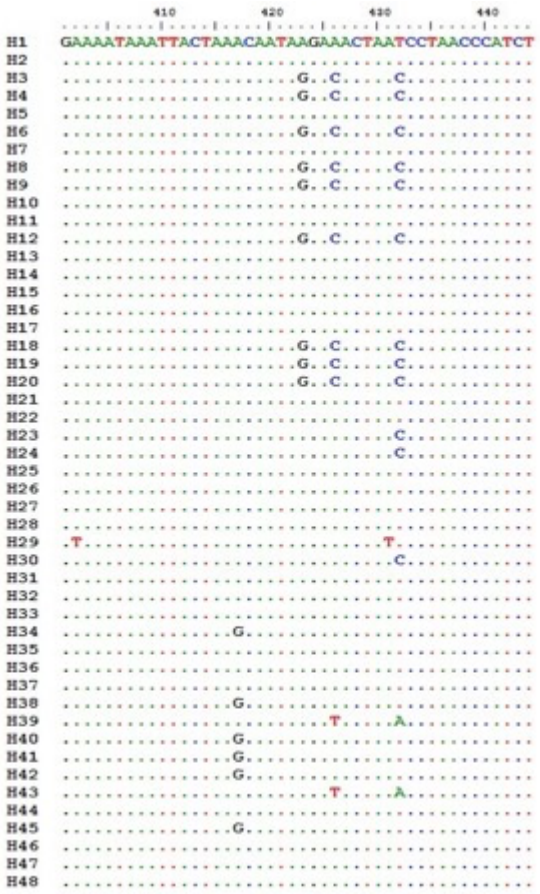
Supplementary File






210 220 230 240 250 260 270 280 290 300
H1 AGCACTTTTTGTTATAGCAGCTAATATTACTAACGCCCCAATAATTATTATTTCAAACTTAGTTGATAATATATCTAAATAAAAAATATAATTTCAACTC
H2 ....T..........
H3 ..........G..........T..T..........C.....C...T.....C.....G..C..........A
H4 ..........G..........T..T..........C.....C...G.....C.....G..C.....G....A
H5 ....A..........
H6 ..........G..........TA.T..........C.....C...T.....C.....G..C..........A
H7 ....T..........
H8 ..........G..........T..T..........C.....C...T.....C.....G..C.....G....A
H9 ..........G..........T..T..........C.....C...T.....C.....G..C..........A
H10 ....
H11 ....
H12 ..........G..........T..T..........C.....C...T.....C.....G..C.....C....A
H13 ....T..........
H14 ....
H15 ....
H16 ....
H17 ....T..........
H18 ..........G..........T..T..........C.....C...T.....C.....G..C.....C....A
H19 ..........G..........T..T..........C.....CT..T.....C.....G..C.....C....A
H20 ..........G..........T..T..........C.....C...T.....C.....G..C.....C....A
H21 ....
H22 ....T..........
H23 ....
H24 ....
H25 ....T..........
H26 ....G..........
H27 T.........G..........G..T..........T..........T..T
H28 ....
H29 ....
H30 ....
H31 ....
H32 ....
H33 ....
H34 G.........T..T..........C.....
H35 ....
H36 ....T..........
H37 ....T..........
H38 G.........T..........GC.....
H39 T.....T...T..T..........C.....G.....
H40 G.........T..T..........C.....
H41 G.........T..........GC.....
H42 G.........T..T..........C.....
H43 T.....T...T..T..........C.....G.....
H44 ....
H45 G.........T..........GC.....
H46 ..........T..........G..
H47 ....
H48 ....


310 320 330 340 350 360 370 380 390 400
H1 CCATAATTTAACATTCAAGCAATAGCTAATAATAGAGCAACATCTCCGACTCGATTAGATAAAGCTGTCAATATCCCAGCATTATAAGACTTTACATTTT
H2 ..........T..........C..........
H3 ..........T.....T.....C....AC.....T.....A.....C..A.....T.....
H4 ..........T.....T.....C....AC.....T.....A.....C..A.....T.....
H5 ....
H6 ..........T.....T.....C....AC.....T.....A.....C..A.....T.....
H7 ....
H8 ..........T.....T.....C....AC.....T.....A.....C..A.....T.....
H9 ..........T.....T.....C....AC.....T.....A.....C..A.....T.....
H10 ....
H11 ....T..........
H12 ..........T.....T.....C....AT.....T.....G...A.....C..A.....T.....
H13 ....C..........
H14 ....C..........
H15 ..........C..A.....
H16 ..........T.....C..........
H17 ..........C..A.....
H18 ..........T.....T.....C....AT.....T.....G...A.....C..A.....T.....
H19 ..........T.....T.....C....AT.....T.....G...A.....C..A.....T.....
H20 ..........T.....T.....C....AT.....T.....A.....C..A.....T.....
H21 ....C..........
H22 ....
H23 ....C..........
H24 ..........T.....C..........
H25 ....C..........
H26 ....C..........
H27 ..........T.....T.....A...T....T...T.....T.....
H28 ..........C.....GG.....
H29 ..........C.....T.....
H30 ....
H31 ..........C..A.....
H32 ..........C.....G.....
H33 ....C..........
H34 ..........A.....A.....
H35 ..........G.....GC.....
H36 ..........G.....GC.....
H37 ....C.....
H38 ..........A.....T.....GG.....
H39 ..........T.....T.....C....A.....T..C..G.....C..T.....T..A.....
H40 ..........A.....A.....G.....
H41 ..........A.....T.....GG.....T.....
H42 ..........A.....A.....G.....
H43 ..........T.....T.....C....A.....T..C..G.....C..T.....T..A.....
H44 ..........G.....GC.....
H45 ..........A.....T.....GG.....
H46 ..........A.....G.....
H47 ..........G.....GC.....
H48 ..........T.....G.....GC.....



Supplementary File

Comparison of the haplotype sequences. The mitochondrial NADH dehydrogenase (ND5) gene region sequence is comprised of five distinct blocks, designated as 1-100, 101-200, 201-300, 301-400, and 401-444, respectively. The H1 ND5 gene sequence is observed in a continuous fashion across the initial line of each block. In the subsequent lines, the sequences of the other haplotypes are arranged in an increasing order up to H48. In instances where the base at a specific position within a given haplotype corresponds to that of H1, a line is inserted. Conversely, if they are distinct, the characteristic base of the particular haplotype is designated.