Research Article

Phylogenetic Analysis of *Aedes aegypti* Based on Mitochondrial *ND4* Gene Sequences in Almadinah, Saudi Arabia

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Background: *Aedes aegypti* is the main vector of the yellow fever and dengue virus. This mosquito has become the major indirect cause of morbidity and mortality of the human worldwide. Dengue virus activity has been reported recently in the western areas of Saudi Arabia. There is no vaccine for dengue virus until now, and the control of the disease depends on the control of the vector.
Objectives: The present study has aimed to perform phylogenetic analysis of *Aedes aegypti* based on mitochondrial NADH dehydrogenase subunit 4 (*ND4*) gene at Almadinah, Saudi Arabia in order to get further insight into the epidemiology and transmission of this vector.
Materials and Methods: Mitochondrial *ND4* gene was sequenced in the eight isolated *Aedes aegypti* mosquitoes from Almadinah, Saudi Arabia, sequences were aligned, and phylogenetic analysis were performed and compared with 54 sequences of *Aedes* reported in the previous studies from Mexico, Thailand, Brazil, and Africa.
Results: Our results suggest that increased gene flow among *Aedes aegypti* populations occurs between Africa and Saudi Arabia.
Conclusions: Phylogenetic relationship analysis showed two genetically distinct *Aedes aegypti* in Saudi Arabia derived from dual African ancestor.

Keywords: *Aedes*; Mosquito; *ND4* gene; Phylogenetic; Saudi Arabia

1. Background

Aedes aegypti is the main vector responsible for transmission of the yellow fever, dengue virus, as well as, other arboviruses which affect human and numerous other animal species (1). It has become the major indirect cause of morbidity and mortality of human worldwide (2). According to the World Health Organization (WHO, 2007), it has been estimated that 50-100 million cases of the dengue fever occur every year in the world. Another recent study has estimated that 3900 million people, in 128 countries, are at risk of infection (3).

Dengue virus activity has been reported in the western areas of the Saudi Arabia; Jeddah, Makkah, and Almadinah (4, 5).

Until now, there is no vaccine for dengue virus, and the control of the disease mainly remains dependent on the control of the vector. This can explain why the rate of infection has recently increased dramatically. (6). Due to this reason, knowledge of vector dispersion has taken a prime importance and a critical role in controlling vector born disease.

In recent decades, entomologists have developed a number of morphological key characters for taxonomic goals in order to better understand the transmission and epidemiology of these vectors -borne diseases (7,

8). However, with advances in molecular biology, numerous studies have demonstrated that morphological keys are not sufficient. In addition, they are accompanied with several limitations (9) which might be due to several reasons, such as, minor genetic variation due to ecological impact, as well as, the constant use of insecticides, in addition to others factors (10-12). Therefore, numerous molecular researches have been undertaken to find new molecular marker as an alternative tool to identify mosquito species.

1.1. Phylogenetic Marker in Mosquitoes

The idea of genetic marker is based on the principle that every species has a genetic identity which can be used as molecular marker for species identification. Several genetic markers have been studied in the previous studies, such as; ITS2 (Internal Transcribed Spacer), mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene, and mitochocondrial NADH dehydrogenase subunit 4 (*ND4*) gene.

ITS2 has been proven to be a good molecular marker because of its highly conserved region and species specific sequence; as a result, it is largely being used as a phylogenetic marker for mosquito's taxonomy (13, 14).

COI-based DNA barcoding has been also used by Abigail Chan and co-workers to indentify mosquitoes in Singapore (15). This study has demonstrated that *COI*-based DNA barcoding can be also a good molecular marker for mosquitos' taxonomy.

However, mitochondrial NADH dehydrogenase subunit 4 (*ND4*) gene has gained increasing popularity in phylogenetic analysis and population genetic studies. (16) In addition, (*ND4*) gene has shown to be an excellent genetic marker (6, 17-20).

2. Objectives

Phylogenetic analysis regarding dengue vector *Aedes aegypti* is not assessed until now in Saudi Arabia based on the mitochondrial NADH dehydrogenase subunit 4 (*ND4*) gene. In this context, our study has aimed to construct phylogenetic tree of the *Aedes aegypti* mosquito based on *ND4* gene to get more insight into the epidemiology and transmission of these vector born disease.

3. Materials and Methods

3.1. Collection of Mosquito

Eight Mosquitoes were collected from Almdinah in western part of the Saudi Arabia in 2013. Adult mosquitoes specimens were collected using BG-sentinel traps (BioGents AG, Germany), CO_2 light traps, human baited net traps, and human landing catch method. Subsequently mosquitoes were killed through exposure to -20ºC for a few minutes, and were identified morphologically using the key described by Schaffner and co-workers (21). Following to the morphological identification, adults were stored frozen at -20ºC.

3.2. *DNA Extraction and Nad4 Sequencing*

Genomic DNA was extracted from the 8 adult mosquitoes using a QIA amp DNA Mini Kit and re-suspended the DNA in 80 µL of buffer EB (from Qiagen) (22). Primers used to amplify the NADH dehydrogenase subunit 4 (*ND4*) gene were composed of ND4 forward-(5′-TGATTGCCTAAGGCTCATGT-3′) and ND4 reverse-(5′-TTCGGCTTCCTAGTCGTTCAT-3′) (17). The polymerase chain reaction (PCR) amplification of the 344 pb fragment was preceded by a five minute denaturation at 96ºC and subsequent 35 cycles of the amplification consisted of 40 s at 94ºC, 40 s at 56ºC and 40 s at 72ºC, followed by a final extension step of five minutes at 72ºC (6). PCR products were visualized on 1.5% agarose gels stained with GelRed (Biotium Ink., USA). Amplicons were gel-electrophoresed, excised from the gels and recovered with a QIAamp Gel Extraction Kit (Qiagen). Then, PCR products were sequenced in both directions using an automated MegaBACE 1000 Analysis System sequencer (GE Healthcare, UK).

3.3. Phylogenetic Tree and Sequences Analysis

Sequences obtained for mosquitoes were analyzed using BLAST program (http://blast.ncbi.nlm.nih.gov) to confirm the morphological identification. Mitochondrial *ND4* gene sequences from eight *Aedes aegypti* were aligned using ClustalW software program (http://www.ebi.ac.uk/clustalw2/). phylogenetic trees were constructed by using MEGA software version 6 (23), to determine Phylogenetic relationships and Genetic variability.

Two phylogenetic trees were constructed for *Aedes aegypti* collected from Almadinah, Saudi Arabia. The first tree was based on the UPGMA algorithm within the Tamura-Nei genetic distance model. Bootstrap support was calculated by means of 1000 replicates. The Second tree was based on neighbor-joining (NJ) algorithm under the Tamura-Nei genetic distance model. Bootstrap support was calculated by means of 1000 replicates.

All sequences were compared with 54 sequences available in the previous studies, from Brazil-AY906835-AY906853 (20), JQ926718-JQ926719 (24), Mexico- JX297249- JX297259 (deposit in Gen Bank by Pfeiler *et al*. 2012), Thailand- JQ926720-JQ926721 (24), and from Africa-JX427511-JX427525 (25). Phylogenetic tree was constructed based on neighbor-joining (NJ) algorithm under the Tamura-Nei genetic distance model. Bootstrap support was calculated by means of 1000 replicates.

In this analysis *Aedes koreicus* Gen Bank accession number: KJ623732-KJ6237375 (deposit in GenBank by Raharimalala *et al*. 2014 (unpublished) were employed as out-group species. Information regarding sample size and localities are listed in the (Table 1).

3.4. Statistical Analysis

Statistical analyses were performed. The nucleotides diversity, Tajima's *D* (26) and neutrality tests were calculated using MEGA software version 6.

4. Results

4.1. DNA Extraction and Nad4 Sequencing

In this study we report for the first time eight sequences of the *Aedes aegypt* collected from Almadinah, Saudi arabia (KSA). The eight sequences were deposited in the National Center for Biotechnology Information (NCBI) in 2010 GenBank accession number are listed in (Table 2).

4.2. Phylogenetic Tree and Sequences Analysis

The obtained sequences were analyzed using BLAST program to confirm morphological identification, all sequences show high similarities with NADH dehydrogenase subunit 4 (*ND4*) gene with an identity of 100% and E-value: 8e-179.

Two phylogenetic trees were constructed for *Aedes aegypti* collected from Almadinah, Saudi Arabia by using two different algorithm UPGMA and neighbor-joining (NJ) algorithm (Figure 1).

The results obtained from the two models indicate that *Aedes aegypti* S2, S3, S4, S5, S6, and S7 share high similarity and form one group, whereas, this

Table 1. Localization and sample size of *Aedes aegypti*

Ogranism	State	Sample size
Aedes aegypti	Brazil	21
Aedes koreicus	Belgium	6
Aedes aegypti	Mexico	11
Aedes aegypti	Thailand	2
Aedes aegypti	Senegal	14
Aedes aegypti	Saudi arabia	8

Table 2. Fragments' length and GenBank accession Number of *Aedes aegypt* collected from Almadinah, Saudi Arabia

Ogranism	Gen Bank accession Number	Length (pb)	City	state
Aedes aegypti	AB594491.1	344 bp	Al -Madinah	Saudi arabia
Aedes aegypti	AB594490.1	344 bp	Al -Madinah	Saudi arabia
Aedes aegypti	AB594489.1	344 bp	Al -Madinah	Saudi arabia
Aedes aegypti	AB594488.1	344 bp	Al -Madinah	Saudi arabia
Aedes aegypti	AB594487.1	344 bp	Al -Madinah	Saudi arabia
Aedes aegypti	AB594486.1	344 bp	Al -Madinah	Saudi arabia
Aedes aegypti	AB594485.1	344 bp	Al -Madinah	Saudi arabia
Aedes aegypti	AB594484.1	344 bp	Al -Madinah	Saudi arabia

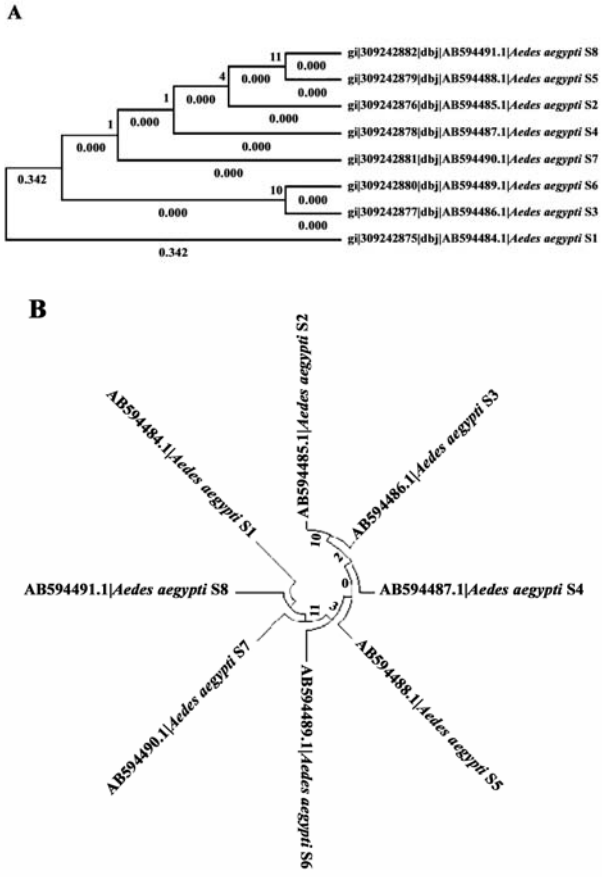

Figure 1. Phylogenetic relationships among *Aedes aegypt collected* from Almadinah, based on the UPGMA method under the Tamura-Nei genetic distance model. A: Bootstrap values are marked on the branches. B: Phylogenetic relationships among *Aedes aegypt* collected from Almadinah, based on the neighbor-joining (NJ) algorithm under the Tamura-Nei genetic distance model. Bootstrap values are marked on the branches

group shares low similarity with S1. All sequences have been compared with 54 sequences available from previous studies. Phylogenetic tree was constructed based on neighbor-joining (NJ) algorithm under the Tamura-Nei genetic distance model. Bootstrap support was calculated by means of 1000 replicates.

The results indicate that among 54 *Aedes* located in different states, the group consisting of *Aedes aegypti* S2, S3, S4, S5, S6, and S7 show close relationship to the *Aedes aegypti* located in Africa, as well as, *Aedes agypti* located in Mexico, On the contrary, the *aedes aegypti* S1 is disclosed to the group (S2, S3, S4, S5, S6, and S7) and is very close to a different group of *Aedes aegypti* located in Africa (Moore *et al*. 2013). (Figure 2).

Statistical analyses were performed and genetic diversity was calculated. The highest value of the nucleotide diversity was found in this study compared to the previous studies (Table 3).

5. Discussion

Our results demonstrate that among the eight *Aedes*

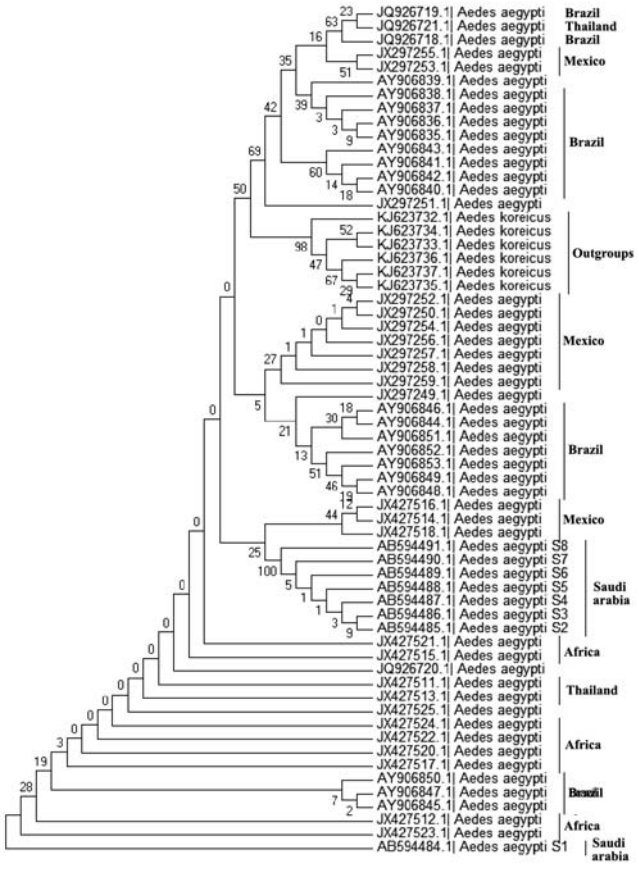

Figure 2. Phylogenetic relationships among *Aedes aegypt*, based on the neighbor-joining (NJ) algorithm under Tamura-Nei genetic distance model. Bootstrap values are marked on the branches

Table 3. Genetic variability and neutrality tests of the ten *Aedes aegypti* samples from Amadinah, Saudi arabia

m	*S*	P_s	θ	π	*D*
8	149	0.438235	0.169016	0.109559	-1.921827

m=number of sequences, *s*= number of segregating sites, P_s= SIN, θ= Ps/a1, π= nucleotide diversity, and *D* is the Tajima test statistic

aegypti collected from Almadina in Saudi Arabia, only seven *Aedes* have shared high genetic similarity and form one unique group consisting of S2, S3, S4, S5, S6, S7, and S8.

In fact, this group discloses and shares low similarity with S1, which is an *Aedes aegypti* collected from the same city (Almadinah) at the same time in 2010.

In 2013, Moor and co-workers have studied *Aedes aegypti* populations from Senegal, West Africa, Kenya, and East Africa based on mitochondrial DNA analysis. Phylogenetic analyses have shown that population of *Aedes aegypti* out of Africa are consisted of mosquitoes that have arisen from one of the two ancestral clades. One clade is associated with the West Africa, while the second has its origin from the first and contains primarily mosquitoes from East Africa.

Unexpectedly, our study shows that *Aedes aegypti* S1 discloses a close relationship with the first ancestral clade, which is associated with the West Africa. Whereas, the second African ancestor, which is associated with the East Africa and arises from the first one, shows a close relationship with *Aedes* group consisting of S2, S3, S4, S5, S6, S7, and S8. However, compiling our results with those described previously by Moor *et al*. (2013), we can state that *Aedes* group consisting of S2, S3, S4, S5, S6, S7, and S8 are derived from *Aedes* S1.

These results suggest that the main vector of yellow fever and dengue virus might have been introduced into Saudi Arabia by African pilgrims and continued to circulate in western Saudi Arabia. Our finding support the results of (Esam *et al*. 2015), as well as, the hypothesis of the dual African Origins for *Aedes aegypti* (Moore *et al*. 2013).

5. Conclusions

As a conclusion, our results suggest that the increased gene flow among *Aedes aegypti* populations occurs between Africa and Saudi Arabia. Phylogenetic relationship analysis shows that the two genetically distinct *Aedes aegypti* in Saudi Arabia have deriven their origin from dual African ancestors. Further supports to our finding come from the fact that commer-

cial exchanges and transports from dengue endemic regions, in addition to foreign pilgrims could play a crucial role in the disease transmission.

Finally, we can express that, it is difficult to determine whether the observed genetic distance in Saudi Arabia *Aedes* population is related to geographic distance, or other factors respectively.

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