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Original article

Distribution and mitochondrial CO1-based genetic diversity of *Aedes aegypti* L (Culicidae: Diptera) in Saudi Arabia

Al Thabiani Aziz

Department of Biology, Faculty of Science, University of Tabuk, Tabuk 71491, Saudi Arabia

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ABSTRACT

Mosquitoes (Diptera: Culicidae) act as vectors for various pathogens and parasites that affect millions of people worldwide. *Aedes aegypti* (Linnaeus, 1762) is one of the devastating pests of humans, acting as a key vector of dengue viruses. Therefore, correct identification of this serious pest to determine its distribution is paramount in its management. Morphological identification is usually based on the maturity and quality of the specimens. This can still yield ambiguous results in distinguishing *Ae. aegypti* species due to limited taxonomic expertise and the presence of cryptic species. In this research, mitochondrial CO1 gene-based identification was adopted to analyze 7 samples, each containing 7 specimens of *Ae. aegypti* from various localities of Saudi Arabia: Jeddah (A1), Makkah (A2), Al Madinah Al Munawwarah (A4), Jazan (A5), Qunfudah (A6), Yanbu (A8), and Najran (A10). DNA barcoding and maximum likelihood (ML) tree analysis revealed that all 49 species belong to *Ae. aegypti* and showed high similarity with specimens of this species worldwide.

1. Introduction

The genus *Aedes* contains more than 190 species of mosquitoes worldwide and is an important subject for studying disease management strategies of pests (Gupta et al., 2012; Martínez et al., 2020). Needed information about its biology, dispersal rate, the ability of disease transmission and major seasons of high prevalence are required (Mourya et al., 2001; Angel and Joshi, 2008). The mosquito fauna of Saudi Arabia contains 49 confirmed mosquito species, including 18 anophelines and 31 culicines species (Alahmed et al., 2019). About 150 insect species act as public health vectors. Among them, the genera *Anopheles*, *Aedes* and *Culex* share a central role in transmitting various diseases in humans causing significant health problems and deaths (Mehlhorn et al., 2012; Severson and Behura, 2012; Taraphdar et al., 2012; Benelli, 2015). *A. aegypti* mosquitoes are recognized as a significant threat to public health when they act as vectors of serious pathogens resulting in dengue and Zika virus (Bhatt et al., 2013; Higgs and Vanlandingham, 2015). Considerable effort has been made to control this devastating pest. However, these arboviruses seem to become cosmopolitan affecting various parts of Africa, and other continents (Weaver and Barrett, 2004; Diallo et al., 2018). Dengue virus can be recognized as the most prevalent arthropod-borne virus as it infects about 390 million persons each year (Bhatt et al., 2013). Due to the lack of a vaccine or antiviral treatment, the control of these mosquitoes depends on knowledge of their distribution based on accurate identification to determine distribution. Most mosquito species can be distinguished by morphological features to identify each species (Bram, 1967; Harrison, 1975). Correct information about the taxonomy and genetic identity of *A. aegypti* is vital for its management. Usually, mosquito species are identified morphologically, but until now, only 10 % of species have been identified worldwide due to a lack of taxonomic expertise (Besansky et al., 2003; Pennisi, 2003). Along with taxonomic characters, mtCOI sequences have been used for species identification of *A. aegypti* in so far as this is faster and reliable (Mousson et al., 2005). This DNA barcoding is useful because it doesn't need a large fragment to act as a specific barcode for individual species. For insects, this DNA fragment corresponds to a sequence of ~ 650 base pairs (bp) located at the 5th end of the cytochrome *c* oxidase subunit I gene (COI) (Joyce et al., 2018). Nuclear genes are considered inferior molecular markers because of introns, more exposure to recombination, and their diploid mode of inheritance (Saccone et al., 1999). Similarly, the mitochon-

E-mail address: aalthbyani@ut.edu.sa
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drial marker CO1 gene is considered the most valuable among the three subunits with a protein sequence containing highly conserved functional domains and having variable regions making it useful for evolutionary studies. Mitochondrial DNA has a high evolution rate, making it most suitable for species identification and analyzing evolutionary processes (Brown et al., 1979). DNA-based identification does not need any gender or particular life stage of the mosquito and gives suitable results whether using the entire body or some part of it (Garros et al., 2004; Ruiz et al., 2005; Kumar et al., 2007). More recently, researchers are depending more on molecular approaches for species identification (Manonmani et al., 2001; Singh et al., 2004; Kang and Sim, 2013), genetic diversity (Pfeiler et al., 2013; Low et al., 2014) and molecular phylogeny (Shepard et al., 2006) due to a higher precision rate. Detailed studies have been done for the *A. aegypti* populations of Southeast Asia, Africa, America, and some Latin American countries, showing variable disease transmission rates due to local genetic variation and gene flow among the same species (Urdaneta-Marquez and Failloux, 2011). Gupta et al. (2016) revealed that *A. aegypti* has extremely high genetic similarity compared to the same species from India and Thailand but showed a high variability to the Madagascar strain. Information about the geographic distribution of mosquito vectors is invaluable for developing management tactics (Engdahl et al., 2014). Meanwhile, the identification of mosquito species and knowledge about their distribution plays a key role in diversity studies and preparing management strategies and can help in determining areas with a high risk of pathogen transmission of public health importance (Laurito and Almirón, 2015). A solid knowledge base is necessary to identify the mosquito species limited to particular habitats along with their pathogen diversities as it provides a primary source for the planning of managing targeted vector control strategies to eradicate disease transmission (Walker et al., 2007). The aim of this research is to explore the genetic diversity of *A. aegypti* population in Saudi Arabia by using the cytochrome oxidase marker. For this purpose, in this study, mtCOI gene sequences from 49 specimens of *Ae. aegypti* distributed in KSA were examined. The identified *Ae. aegypti* mosquito species exhibited significant resemblance in their DNA sequences when compared to the same species from other geographical regions

2. Material and methods

2.1. Collection and identification of specimen

The mosquito specimens were collected from seven different locationsof Saudi Arabia namely: Jeddah (A1), Makkah (A2), Al Madinah Al Munawwarah (A4), Jazan (A5), Qunfudah (A6), Yanbu (A8), and Najran (A10) (Fig. 1). Adults of *Ae. aegypti* were collected from various wetland habitats and places associated with water. Collected specimens were preserved in 99 % Ethanol and identified based on the morphological characters before DNA extraction. Adult mosquitos were examined using an Olympus SZX10 stereomicroscope and identified morphologically by use of valid pictorial keys (Rueda, 2004; Becker et al., 2010; Soltani et al., 2017). For further identification, the tentative specimens of *Ae. aegypti* were selected for molecular studies.

2.2. DNA extraction and gene amplification

According to the protocol described by (Wilson et al., 2012), the genomic DNA was eluted from whole single mosquito species and then stored in a freezer at − 20 °C until use. Further, all extracted samples quantity and quality were examined through Nanodrop 7000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The whole body of each specimen preserved in absolute alcohol was used to extract genomic DNA. For DNA extraction, the Insect Lysis buffer was prepared first according to the instructions of (Wilson et al., 2012). A mixture of the following chemical components was prepared: 16.5 g of GuSCN (Sigma), 12 ml of 0.5 M EDTA pH 8.0, 6 ml of 1 M Tris-HCL pH 8.0, 1 ml Triton X-100 (Sigma), and 10 ml Tween-20 (Fluka). This mixture achieved a final volume of 200 ml with the addition of H_2O (Wilson et al., 2012). Samples were placed in new Eppendorf tubes of 1.5 ml each. First, 20 µl of proteinase K (proteinase K 20 ml/g) was added to 150 µl of Insect Lysis and transferred to the tubes containing crushed mosquitoes and the ingredients were vortexed for one min at room temperature. Then, those tubes were incubated for 2 h at 56 °C. After incubation, the samples were centrifuged at 10,000 rpm for 2 mins and then the supernatant was removed and transferred to a new 1.5 ml Eppendorf tube for further processing. A 100 µl of absolute ethanol was added to the Eppendorf tube and mixed gently together. These contents were then transferred to the spin column containing silica gel as an auxiliary medium attached to the DNA which was centrifuged for 2 mins at 12,000 RPM to get rid of the remaining fluid (Fig. 2). Afterwards, the liquid content was removed from the collecting tube manually before re-installation. A 500 µl of 70 % ethanol was added to the spin column tubes to dispose of completely and then centrifuged at a speed of 12,000 RPM for 2 min. The liquid content from the collecting tube was removed manually and the same step was repeated. The precipitate was then removed from the ethanol. The column tube was then placed in the centrifuge for 3 min at a speed of 13,000 RPM until the ethanol was eliminated, to ensurethe sample drying. The upper part of the spin column tubes was pulled and placed in a new 1.5 ml Eppendorf tube. A 20 µl of Nuclease free water was added to the centre of the silica gel and kept at room temperature for 10 mins to check the DNA loss caused by the spin column. These tubes were placed in a centrifuge at 12,000 RPM for 1 min and 50 µl of Nuclease-free water was added again to the centre of the silica gel and left at room temperature for 10 mins. These tubes were centrifuged at 12,000 RPM for 1 min. After centrifugation, all DNA content was separated from the silica gel into the new Eppendorf tube, and the DNA extraction was ready for testing by gel electrophoresis (Wilson et al., 2012) and the extracted DNA was deposited at − 20 °C for further studies.

2.3. Agarose gel preparation

0.4 g of agarose powder were placed in a glass vial containing 50 ml 1X Tris-Acetate-EDTA buffer (TAE) (illustrated in Fig 4-38A). The sample was heated in a microwave oven for 30 s. After cooling, 3 µl of ethidium bromide was added and stirred before pouring into the gel cube and leave until frozen. The gel cube was held on the electrophoresis device with an appropriate amount of TAE solution with its base dumped well to ensure electric separation. The comb was removed from the gel cube. For size estimation, 2 µl of molecular weight marker was well-mixed with 2 µl of loading dyes and 2 µl of DNA and dropped into one of the injections for each sample separately. Subsequently, the generator was turned on at a voltage of V140 (Fig. 3) and left for 30 mins. In the end, an Ultraviolet (UV) device was used for imaging to ensure the presence of DNA (Appendix 1).

2.4. Gene amplification

In this research, the polymorphism among the various insect populations was examined through mtCOI and then the genes were amplified by polymerase chain reaction (PCR) using the universal barcoding primer pair, LCO1490 (5′-GGTCAACAAATCATAAA GATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGAC CAAAAAATC-3′) (Vrijenhoek, 1994). The band length was between

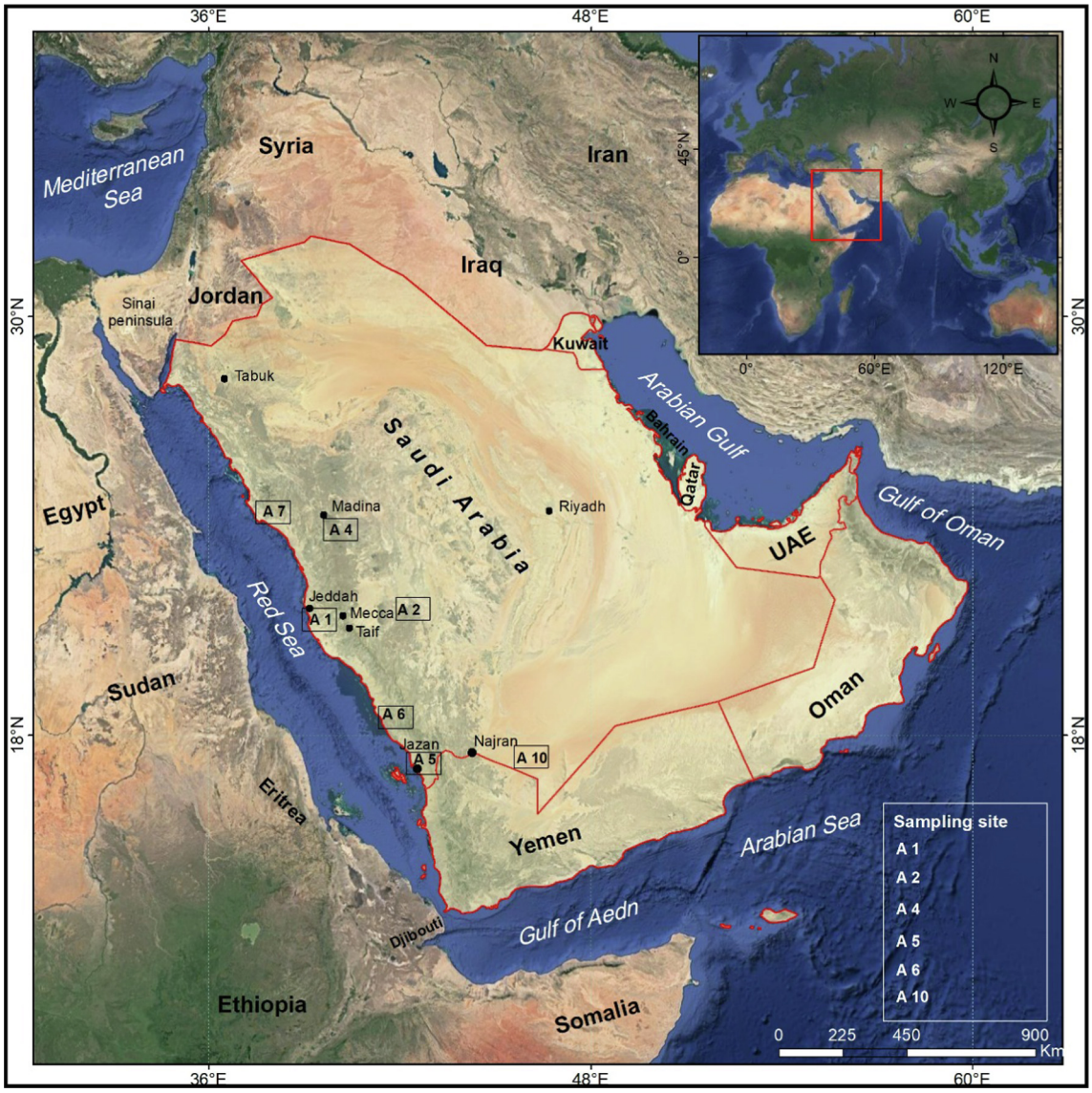

Fig. 1. Sampling location map of *Aedes aegypti* in Saudi Arabia.

600 and 700 bp. The 25 µl of reaction volume containing 15.3 µl 1 × bovine serum albumin (BSA), 2.5 µl 10 × Reaction Buffer, 2 µl dNTPs (2.5 µM),1.25 µl of each primer (10 µM/L), 0.2 µl Taq DNA Polymerase (1.0 U), and 2.5 µl (greater than50 ng) template DNA were used. The PCR conditions were denaturation for 2 mins at 94 °C, followed by 40 cycles for 30 secs at 94 °C, followed by annealing for 45 s at 49 °C and extension for 45 s at 72 °C, then finally for 1 min at 72 °C. In order to validate the results, the PCR products were run at 2 % agarose gel, followed by analyzing the gel under UV light (Appendix 2). From the agarose gel, the amplified bands were purified and sequenced at Macrogen Company, Korea by Applied biosystems sequencer 3730Xl.

2.5. Sequence analysis

Basic Local Alignment Search Tool (BLAST) (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare deduced nucleotide data with previously reported sequences in the Gene Bank database. The obtained nucleotide sequences were aligned using ClustalW tool in MEGA. Finally, by using the same software, the retrieved sequence translation to amino acids was carried out to check mutations. In order to find out the relationship between the individual retrieved sequences, a maximum likelihood tree was generated using MEGAX (version 10.1.8) with boostrap, the NCBI database by using the Basic Local Alignment Search Tool (BLAST).

3. Results

3.1. Molecular characterization

DNA was extracted from seven groups, with each group having seven samples, making it a total of 49 samples. Insect Lysis buffer was used for DNA extraction according to the instructions in [41] and examined on 1 % agarose gel (Fig. 2). For COX1 gene identification, genomic DNA was extracted from 49 randomly selected mosquito samples of all groups. PCR amplification was then carried out using universal primers for the mTCOX-1 gene, as shown in the materials and methods. This resulted in 600–700 bp being examined on 1.5 % agarose gel electrophoresis (Appendix 2).

3.2. Gene sequencing and phylogenetic tree

The amplified mTCOX-1 gene was sequenced at Macrogen Inc., Korea. Further, the sequences were analyzed at NCBI BLAST too.

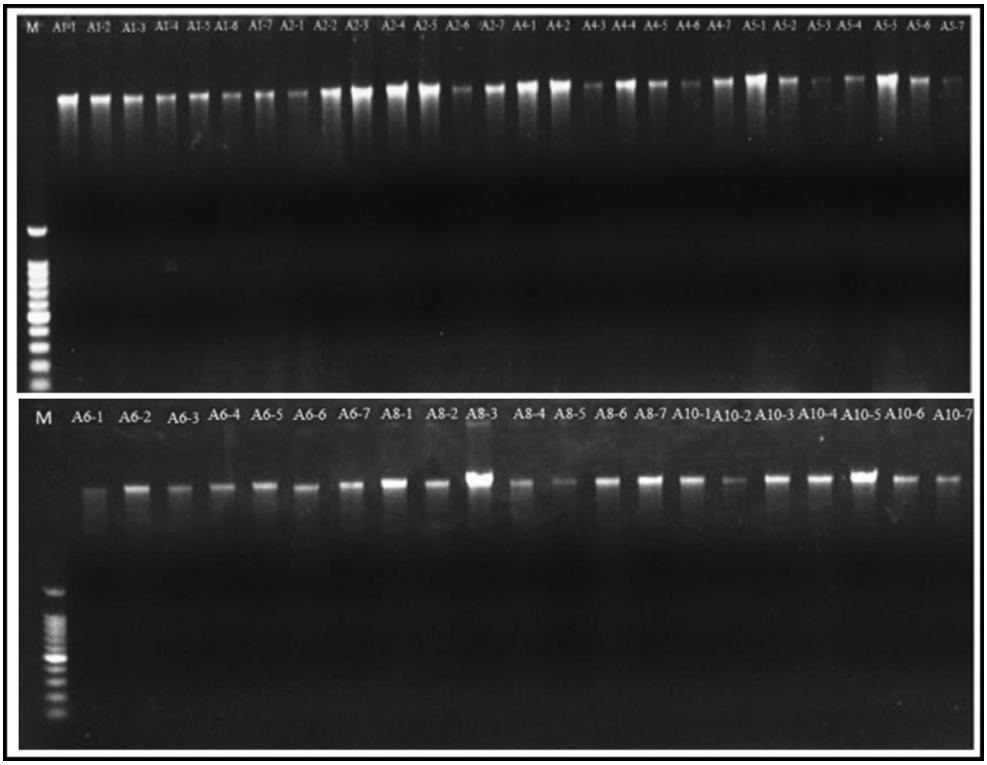

Fig. 2. DNA extraction image on an agarose gel.

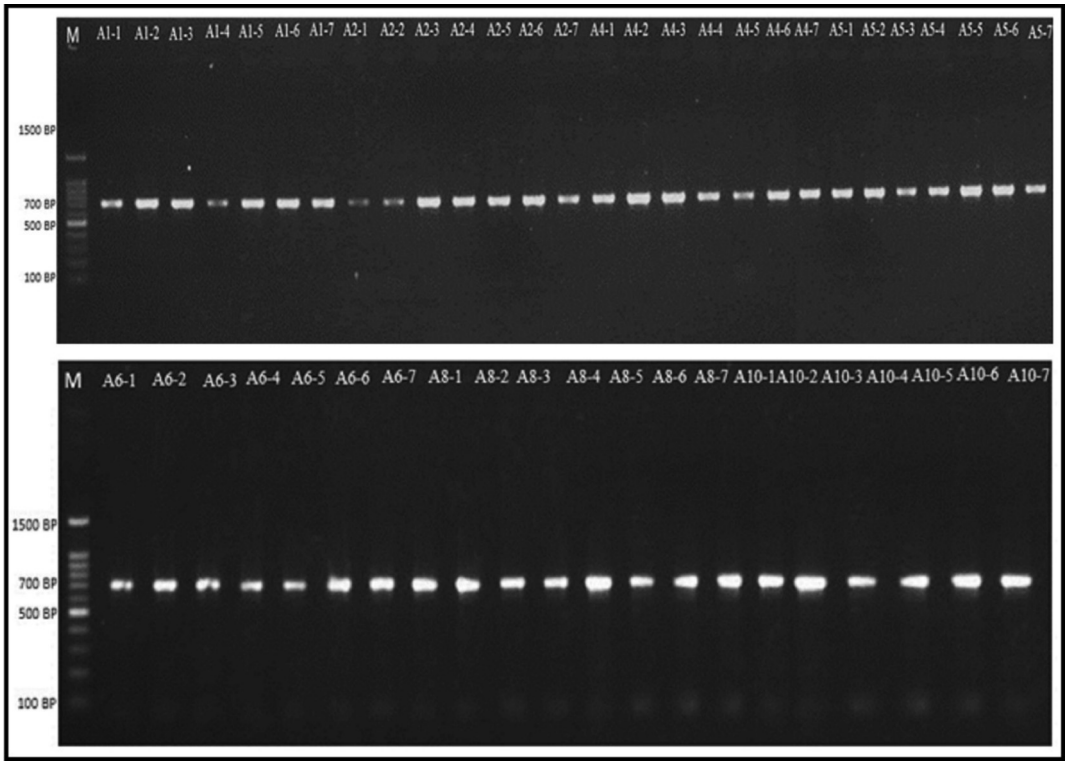

Fig. 3. Agarose gel image of the amplified cox-1 gene of the selected samples (100 bp ladder DNA).

BLAST results showed that all these seven randomly selected mosquitoes were *Ae. aegypti*. To further confirm the above results, the maximum likelihood (ML) method was generated to align the mTCOX-1 genetic sequence and build a phylogenetic tree illustrating genetic evolution by estimating the affinity distance between the investigated samples and available sequences from Gene Bank (NCBI) (with Gene Bank samples (MN229016.1, MN299008.1, MF443397.1, MN299014.1 and MN229016.1) using

Table 1
A comparison of the study samples with the data in the Gene Bank (NCBI).

No. Samples	Genebank Code	Gene Published date	Organism and Gene Definition	Gene Length (bp)	% Identical	Country	References
A1-2, A1-3, A1-4, A1-5, A1-6, A1-7	MF443397.1	04-JUN-2018	*Aedes aegypti isolate C cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial..*	677 bp	100	Canada	Giordano, B.V., Barelli, L., Gasparotto, A., Liang, P., Nelder, M., Russell, C. and Hunter, F.F. (2018): Molecular characterization of Aedes (Stegomyia) albopictus populations collected in Ontario, Canada with notes on the first Canadian record of *Aedes aegypti*. Centre for Biotechnology, Brock University, Canada.
A2-1, A2-2, A2-3, A2-4, A2-5, A2-6, A2-7	MN299016.1	09-JUN-2020	*Aedes aegypti isolate PeCO2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial*	701 bp	100 %	Argentina	Lapadula, W. J., Marcet, P. L., Taracena, M. L., Lenhart, A., & Ayub, M. J. (2020). Characterization of horizontally acquired ribotoxin encoding genes and their transcripts in Aedes aegypti. Gene, 754, 144857.
A4-1, A4-2, A4-3, A4-4, A4-5, A4-6, A4-7	MN299016.1	09-JUN-2020	*Aedes aegypti isolate PeCO2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial*	701 bp	100 %	Argentina	Lapadula, W. J., Marcet, P. L., Taracena, M. L., Lenhart, A., & Ayub, M. J. (2020). Characterization of horizontally acquired ribotoxin encoding genes and their transcripts in Aedes aegypti. Gene, 754, 144857.
A5-1, A5-2, A5-3	MN299016.1	09-JUN-2020	*Aedes aegypti isolate PeCO2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial*	701 bp	100 %	Argentina	Lapadula, W. J., Marcet, P. L., Taracena, M. L., Lenhart, A., & Ayub, M. J. (2020). Characterization of horizontally acquired ribotoxin encoding genes and their transcripts in Aedes aegypti. Gene, 754, 144857.
A5-4, A5-5, A5-6, A5-7	MK890453	06-MAY-2019	*Aedes aegypti voucher ECU22361 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial*	672 bp	100 %	Ecuador	Cevallos, V. and Ponce, P.(2019).Direct Submission. GIDI, National Institute of Public Health Research, Iquique, Ecuador.
A6-1, A6-3, A6-6	MT328866.1	13-APR-2020	*Aedes aegypti isolate Ae. aeg.2020 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial*	647 bp	100 %	Egypt	Elansary, R.E., Eldabaa, S.H. and Bream, A.S. (2020). Identification of Mosquitoes by CO-Biology.
A6-2, A6-4, A6-5, A6-7	MN299016.1	09-JUN-2020	*Aedes aegypti isolate PeCO2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial*	701 bp	100 %	Argentina	Lapadula, W. J., Marcet, P. L., Taracena, M. L., Lenhart, A., & Ayub, M. J. (2020). Characterization of horizontally acquired ribotoxin encoding genes and their transcripts in Aedes aegypti. Gene, 754, 144857.
A8-1, A8-3, A8-6	MN299016.1	09-JUN-2020	*Aedes aegypti isolate PeCO2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial*	701 bp	100 %	Argentina	Lapadula, W. J., Marcet, P. L., Taracena, M. L., Lenhart, A., & Ayub, M. J. (2020). Characterization of horizontally acquired ribotoxin encoding genes and their transcripts in Aedes aegypti. Gene, 754, 144857.
A8-2	GQ143718.1	07-MAY-2009	*Aedes aegypti cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial*	780 bp	99.19 %	Australia	Ballard, J.W.O., Puslednik, L. and Wolff, J.N. (2009). Variation under nature: A sesquicentennial DNA barcoding perspective School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, Australia.
A8-4, A8-5	MT328866.1	13-APR-2020	*Aedes aegypti isolate Ae. aeg.2020 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial*	647 bp	99.35 %	Egypt	Elansary, R.E., Eldabaa, S.H. and Bream, A.S. (2020). Identification of Mosquitoes by CO-Biology.
A8-7	MK300226	14-DEC-2018	*Aedes aegypti isolate NAH11 cytochrome c oxidase subunit 1 (COI) gene, partial cds; mitochondrial*	709 bp	99.35 %	Kenya	Makanda, M., Wahome, L., Onyambu, G., Mutunga, J. and Bargul, J. (2018). Direct Submission. Institute of Basic Sciences Technology and Innovation, The Pan African University, Nairobi, Kenya
A10-1, A10-2, A10-3, A10-4, A10-5, A10-6, A10-7	MN299016.1	09-JUN-2020	*Aedes aegypti isolate PeCO2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial*	701 bp	100 %	Argentina	Lapadula, W. J., Marcet, P. L., Taracena, M. L., Lenhart, A., & Ayub, M. J. (2020). Characterization of horizontally acquired ribotoxin encoding genes and their transcripts in Aedes aegypti. Gene, 754, 144857.

MEGA X software. The phylogenetic tree, constructed by using MEG X software for all samples, illustrates the degree of convergence and divergence between the samples (Appendix 1). The resulting differences between the study samples are due to the presence of mutations between the samples (Appendix 2). The comparison results of the sample groups (A2, A4, and A10) with Gene Bank samples MN229016.1, MN299014.1 and MN299008.1 indicated in this study that they are entirely similar (100 %) to those found in Argentina (Table 1, Appendix 3-8). Also, the samples of group A1 were examined with Gene Bank samples MF443397.1, MN299014.1 and MN299008.1; results show that the samples are 100 % similar to those found in Canada (Table 1, Appendix 9 & 10). Regarding group five, which was identical to two regions, the samples A5-1, A5-2, and A5-3 are similar to MN229016.1, MN299014.1 and MN299008.1 found in Argentina, while the samples A5-4, A5-5, A5-6, and A5-7 are identical to Mk890453.1 found in Ecuador (Table 1, Appendix 11 & 12). Similarly, the sixth group is also similar to two areas, with samples A6-1, A6-3, A6-6 being 100 %

identical to MT328866.1 found in Egypt, and the samples A6-2, A6-3, A6-6, and A6-7 are 100 % similar to the results found in Argentina (Table 1, Appendix 13 & 14). The results for the eighth group revealed high similarity (99.19 % – 100 %) to four countries: Argentina, Australia, Egypt and Kenya (Table 1 and Appendix 15 & 16).

4. Discussion

The identification of *Ae. aegypti* mainly emphasizes adult morphological characteristics. However, morphology alone is insufficient and cumbersome to define species because of their small size at the adult stage and the morphological variations among adults. Thus, the combination of morphological and molecular identification can be fast and efficient in gaining more insights into variations between the populations. More recently, the data sets of mitochondrial genes provide a better opportunity to not only establish the identity of insects, but also to find out their molecular evolution (Cook et al., 2005). Because mitochondrial genes are present in multiple copies, as compared to single-copy nuclear genes, they are easy to amplify (Cook et al., 2005). In this research, both morphological identification and molecular markers were employed to examine the genetic diversity among *Ae. aegypti* populations collected from Saudi Arabia: (Jeddah (A1), Makkah (A2), Al Madinah Al Munawwarah (A4), Jazan (A5), Qunfudah (A6), Yanbu (A8), Najran (A10) (Fig. 1). The sequence of modern molecular markers COI of *Ae. aegypti* from these regions are phylogenetically analyzed with their relatives in SA and worldwide. The sequencing results and their BLAST analysis shows that the samples used in this study were *A. aegypti*. Moreover, with little or no variation, all samples used in this study were significantly identical to those found in Argentina, Canada, Ecuador and Egypt. Moreover, an eighth group was 99.19 % – 100 % identical to *A. aegypti* found in Argentina, Australia, Egypt and Kenya. The variation in this group was due to mutation within sequences, which might be due to differences in sample locations. This also suggests that due to the genetic variations within the samples of this eighth group, they may cause a variable rate of infection, which needs to be further elucidated. Previously, it was reported that ITS-2 regions had homology with isolates in Saudi Arabia (Alam et al., 2007). This close homology could be the result of similar environmental conditions which led to similar evolution within the collected mosquito strains (Alam et al., 2007). A similar study was also carried out in order to identify the same mosquito species in the south of Iran. Similar to the present study, they suggest that females of *Ae. aegypti* mosquitoes exhibit a medium size, with a scale pattern on the head, legs, abdomen and chest. The molecular and evolutionary study suggests that *Ae. aegypti* from the south of Iran are also 100 % identical and are undergoing similar evolution (Dorzaban et al., 2020). However, another study from the Pilani region of Rajasthan, India showed that *A. aegypti* has high similarity with an Indian isolate from Thirumala, Andhra Pradesh. On the other hand, it exhibited high similarity with Thailand's strain but high variability with the Madagascar strain (Gupta et al., 2016). Overall, this study expands the basic knowledge about the molecular evolution of *Ae. aegypti*, which might be useful to further improve the biotechnological applications in order to control the virulence of this vector. Moreover, in this study, it was generated the CO1 barcodes of *A. aegypti* collected from different regions of SA to show the importance and ease of this technique in order to discriminate among the species. Therefore, more detailed investigation and analysis involving in DNA barcoding of other mosquito species which will aid scientists in not only gaining more insight into the identification and molecular evolution of mosquito species but also providing the opportunity to better monitor mosquito-borne diseases in Saudi Arabia particular dengue, West Nile virus and filarial parasite.

5. Conclusions

Ae. aegypti is recognized as a serious pest by acting vector of pathogen transmission and causing diseases. The correct identification and distribution of this mosquito vector play a vital role in the control of resulting diseases. This study provided a comprehensive knowledge of *Ae. aegypti* in Saudi Arabia based on its molecular identification. The occurrence of *Aedes* vector in the studied area also suggests that high risk of local transmission of Dengue. Therefore, conventional and some advanced control measures should be adopted with a precise surveillance system to check the arboviral diseases in Saudi Arabia. The DNA barcoding and maximum likelihood (ML) tree analysis results show that all studied species belong to *Ae. aegypti* with high similarity to specimens of the same species worldwide. It also suggests that there is less chance of variability in infection rates of *Ae. aegypti* in Saudi Arabia due to high genetic similarity among specimens.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2023.103566.

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