Morphological and molecular-based identification of *Aedes aegypti* (Diptera: Culicidae), a main vector of Dengue Fever, the first record in Iran after decades.

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Research

Keywords: Aedes aegypti, Dengue Fever, vector surveillance, CO1 Gene, Iran

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Abstract

Background : Aedes aegypti is an important vector for transmission of some dangerous arboviral diseases including Dengue Fever. This study was conducted for the first time in Iran in order to survey the existence of this invasive species in oriental parts of the country located near the Persian Gulf during August 2017 to January 2020.

Methods : Different sampling methods were used to collect all stages of mosquitoes in five selected cities of Hormozgan province, south of Iran. After morphological identification, a molecular study based on Cytochrome Oxidase (CO1) gene-specific primers was performed to identify them more precisely. Then, the CO1 gene were sequenced via the Sanger method.

Results : Totally, 4560 adults and 3520 larvae were collected from all sampling areas. Thirty-one Aedes aegypti specimens were identified from Bandar Khamir and Bandar Lengeh seaports.

Conclusion : Based on the biology and ecology of Aedes aegypti , the possibility of establishment of this species in this region is very high due to the tropical climate of this region. Considering the detection of this invasive vector, high incidence of some arboviral diseases in the neighboring countries, and continuous movements of the settlers of these areas, some potential outbreaks of arboviral diseases can be predicted. Thus, planning and implementing an immediate preventative and surveillance program is vital in order to prevent the establishment of this invasive vector in this area.

Keywords : Aedes aegypti , Dengue Fever, vector surveillance, CO1 Gene, Iran

Background

Mosquito-borne diseases are among the most serious public health problems in the world. The correct diagnosis of vectors is one of the most important factors in identifying the transmission of vector-borne diseases [1, 2]. Before molecular methods were introduced for the classification and identification of mosquitoes, they were identified and categorized based on their morphological characteristics. Owing to the recent advancements in technology, performing cytogenetic analyses and detecting Cytochrome Oxidase (CO1) enzyme indices have become quite feasible [3]. The molecular method can be very useful for identifying mosquitoes, because the samples that are not detectable and well maintained through morphological assay can be easily identified by this method [4]. Molecular method can be employed for the identification of sibling species as well as those that are morphologically similar to a certain population [5]. Up to now, several specific molecular markers with a high level of accuracy, such as Internal Transcribed Spacers (ITSs) of ribosomal DNA genes, 28S rDNA gene, mitochondrial Cytochrome Oxidase C subunit I and II (COI & COII), and Cytochrome Oxidase B, have been employed for the detection of species [4, 6–7].

Mosquito-borne diseases represent one of the most important public health issues all over the world. One of the most dangerous species that can be the vector of some serious and deadly arboviral diseases is

Aedes aegypti [8]. Aedes aegypti lives in tropical and subtropical areas next to human habitats. Aedes aegypti eggs are very resistant to drought, and the mosquitoes usually lay them on the wet inner walls of containers with water or above the waterline. The eggs hatch into larvae within two days at a temperature of 27–30 °C. The larvae turn into pupae after eight days. The larvae of this species are found in human-made containers, such as pottery jugs, water storage tanks, drink cans, empty pots, broken bottles, and old tires, which are all used for keeping water inside and outside farm buildings located 500 meters away from residential areas. The larvae can also be easily found in tree holes, branches, bamboo trees, and even coconut trees. Larvae usually spend long periods of time underwater in deep-water areas to find food.

Female mosquitoes usually suck blood in the shade during daytime and sometimes enter low-light places at nights. Aedes aegypti are highly anthropophilic, such a way that each blood intake occurs with 2-4-day intervals. After the blood-sucking process, the resting phase usually takes place in indoor areas, such as cabinets or behind doors. Adult mosquitoes do not normally fly over long distances and usually spread up to a few hundred meters from their larval breeding places. The main origin of this species was in Africa, which was then gradually spread to other tropical areas.

Ae. aegypti is one of the species that can easily establish in a new region and adapt itself to the new conditions. So far, numerous studies have been conducted on the biological and medical importance of this invasive species in the world [9–12]. Many molecular studies have also been carried out to identify mosquito vectors in Iran. The majority of these studies have been focused on malaria vectors, while limited studies are available regarding Aedes mosquito. In fact, no comprehensive research has been carried out on Aedes mosquito with a focus on Ae. aegypti in Iran. There are some nonofficial reports of Ae. aegypti in southern parts of Iran a few decades ago as the same species or other species such as Ae. argenteus or Stegmyia fassiata [13] but there is no samples r reliable documentary of intent. Considering the reporting of some important arboviral diseases such as yellow fever, chikungunya, and dengue fever and their vectors in neighboring countries, the present study was conducted for the first time in Iran in order to survey this species in Hormozgan province (an oriental region located in the southern part of Iran near the Persian Gulf) during August 2017 to January 2020.

Methods

Study area

Hormozgan province with an area of 68,379 square kilometers (27.1387° N, 55.1376° E, altitude 9.14 m) is located in southern Iran. Hormozgan province borders the Oman Sea and the Persian Gulf, stretching about 10,000 km along the coastline. This province has 12 small and large islands as well as 13 cities, namely Bandar Abbas, Bandar Lengeh, Minab, Rudan, Bandar Khamir, Jask, Parsian, Bastak, Hajiabad, Qeshm, Abu Musa, Bashagard, and Cirik (Fig. 1). The weather is hot and humid. The province receives very little rainfall in most areas, with an average annual precipitation of 150–200 mm. Kishi is one of the villages of the seaport city of Bandar Khamir. This village is located in a valley and has many palm trees,

seasonal streams running through the village, and several seasonal rivers. These conditions are favorable for the growth and development of mosquitoes. Bandar Lengeh is also a seaport of great economic and commercial importance in Hormozgan province with geographical coordinates of 26°54'60.49"N, 54°87'96.63"E (Fig. 1).

Sampling methods

From the beginning of 2017, all stages of Aedes mosquitoes were collected from five selected regions across Hormozgan province (Bandar Abbas, Bandar Lengeh, Bandar Khamir, Jask, and Bashagard). The mosquitoes were caught by different techniques, including total catches (pyrethrum spray catch), hand catch, pit shelters, and light traps, for collecting adults. Larval stages and eggs were collected by WHO dipping method as well as by ovitraps installation. The potential breeding sites that were inspected for Aedes mosquitos were water reservoirs, pots, bottles, drinking water springs, small and large pools, jars, discarded tires, cisterns, and installed ovitraps (Figs. 2 and 3).

Morphological identification

All collected adults and larvae were identified morphologically using valid pictorial keys [9, 14–15]. The specimens that were suspected of Aedes aegypti were selected for further molecular studies to confirm the sample identification.

Molecular identification

DNA extraction

DNA molecules were extracted from the whole body of mosquitoes using a DNA extraction kit (Sinaclon, Iran). Then, the extracted DNA molecules were treated with RNaseI (Roche Germany) according to the manufacturer's instructions and were stored at − 20 °C.

Primer design

The nucleotide acid sequence of the CO1 Ae.aegypti gene, which had already been deposited and recorded in the Gene Bank (NCBI) by other researchers was used as the primer design for identifying Aedes aegypti species. The following four Aedes aegypti CO1 nucleic acid sequences were extracted from NCBI: MN019006.1, LC482636.1, LC482632.1, and LC482631.1. The sequences were aligned by MEGA 6.0 software. Then, the primers were designed based on the conserved points. DNA alignment was done by MEGA6 software based on ClustalW. In addition, a pair of primers was designed based on the conserved points using Oligo.7 software, with an expected size band of about 370 base pairs (Table 1). The primers were synthesized by the Bioneer Company (Republic of Korea).

Table 1
The primers designed and used to identify Aedes aegypti mosquitoes

	Primers	Sequences	Expected size
1	Fage	5'-AACAGTTTATCCTCCTCTCTCTTCAG-3'	370 bp
2	Rage	5-AATCCGGGTAAAATTAAAATATAAACTTCTGG-3'	

Polymerase chain reaction

All Polymerase Chain Reactions (PCRs) were performed in a total volume of 20 µl for 35 cycles, using 100–200 ng of genomic DNA in each reaction as templates. The reaction mixture contained 400 nM of each primer, 1.5 mM of MgCl2, 1 unit of Taq DNA polymerase, 0.2 mM of dNTPs, and 2 µl of 10 X reaction buffer, with the final volume being adjusted to 20 µl with Double Distilled Water (DDW). The amplification program was set as follows: 5 min at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 80 s, and an additional final extension at 72 °C for 10 min. The amplified amplicons were purified using the DNA gel purification kit (GF-1 Vivantis, Malaysia). In order to confirm the expected size band, PCR assay was sequenced by the primers introduced by Pishgam Biotech Company, Iran.

Sequencing

The expected size bands after gel purification were sequenced and subsequently analyzed and assembled via Chromas (version 2.31, 2005), DNA star (version 7.10, 2006), and BLAST by NCBI online. The amplicons with sizes close to the predicted range were sequenced using forward and reverse Gene Specific Primers (GSPs) in two sequencing readings.

Results

Entomological checks were conducted in Hormozgan province on a monthly basis from August 2017 to January 2020. The entomological survey was done across 60 villages in five selected cities. A total of 2500 houses were surveyed for the presence of Aedes aegypti. In this study, 4560 adults and 3520 larvae of mosquitoes were collected and identified morphologically. After the morphological identification of all specimens, 31 samples (three adults from Kishi village in Bandar Khamir and 25 larvae and three adults from Bandar Lengeh) were identified as Aedes aegypti. A part of these specimens was selected for molecular confirmation.

All three collected adult mosquitoes in Bandar Khamir (Kishi village) were captured in a sheep stable in March 2017. Kishi had an average temperature of 32 °C in March, with the lowest temperature being 12 °C during the same period. Moreover, the lowest and highest levels of relative humidity were 32% and 72%, respectively. Besides, the average rainfall was 6.2 mm throughout the mentioned time period. In this region, Aedes aegypti was collected only in March. Although all old tires and other artificial breeding places around the houses were checked weekly, no larvae of Aedes aegypti was detected in this area.

Larvae and adults of Ae. aegypti were also collected in Bandar Lengeh (around a carting track that was full of scrap tires) by dipping and hand catch methods, respectively in December 2019.

Females of Aedes aegypti mosquitoes have a moderate size, with a pattern of scales on the head, chest, legs, and abdomen. This species can be easily distinguished from other members of the genus due to the white lyre shape on the dorsal side of the thorax [9, 14]. All major morphological characteristics of this species have been shown in Figs. 4 and 5.

The whole body of mosquitoes, or a part of the body, can be useful for molecular studies [16]. Using the PCR assay and gene replication of the COI portion ~ 370 bp for Ae. Aegypti, it was found that this method was useful in providing a reliable source of the DNA genome in the molecular identification of mosquitoes. In this study, the band was implied by conventional PCR assay and the expected size was 370 bp (Fig. 6).

The tested specimens were identified as Ae. aegypti (Fig. 7). The amplified partial COI sequence of Ae. eagypti characterized in this research was submitted to the Gene Bank (Gene Bank accession no. MT122745). The DNA of Aedes eagypti COI sequence was aligned by Nucleotide BLAST. The sequence showed 100% similarity to Aedes aegypti mitochondrial subunit 1 sequence (MN019006).

A phylogenetic tree of COI was constructed on the basis of the COI nucleic acid sequence of different mosquitoes by using the maximum likelihood method. The results of phylogenetic analysis showed that the most similar physiological function and evolutionary relatedness were associated with Aedes eagypti subunit 1 (Fig. 8)

Discussion

In the present study, larvae and adults of Aedes agypti were collected from two different parts of the oriental area of Iran for the first time. Surprisingly, all Aedes agypti mosquitoes were collected from the coastal areas of the province. This crucial point reinforced the theory that this invasive species has entered the country through boats and ships from neighboring countries.

This anthropophilic invasive Aedes mosquito was identified morphologically, which was confirmed by molecular assay for the first time in the country. Given that this species has been collected from two different locations in two periods of time in this study, this vector has been able to adapt to the new region’s climatic conditions rapidly after entering the country. At the end of the hot season and at the beginning of the rainy season, Aedes aegypti mosquitoes began to grow and increase their population. The study results showed that scrap tires in disposal sites (both rural and urban areas) were ideal breeding places for Aedes aegypti adults to rest and lay their eggs. This has been reported in many other studies around the world [17–18].

Mitochondrial genes, especially the Culicidae family, are a good source for entomological studies [19–23]. Mitochondrial genes exist in several versions, are easily reproducible, and can be read and studied

more easily compared to the genes of nuclear chromosomes. Another advantage of mitochondrial genes is that they are directly inherited from the mother [24]. Furthermore, the study findings demonstrated that COI genes could be appropriately used for the detection of this invasive species. In general, the molecular identification of mosquito species has been proved to be very useful for identifying vector-borne diseases, particularly when it is hard to identify mosquitoes morphologically. This method is capable of identifying vector species throughout the life cycle from egg to adult and can even detect the pathogenic agents in these vectors.

In the recent years, there have been numerous reports of dengue fever from such countries as Pakistan, Sudan, Yemen, Saudi Arabia, India, Somalia, and Egypt. Some of these countries share a common border with Iran, and the risk of getting the disease is imminent. In most of these countries, Ae. aegypti is the main vector of dengue fever [25–29]. The detection of Ae. aegypti in Iran for the first time warns us that it may increase the risk of transmission of chikungunya, dengue, yellow fever, and Zika viruses from the endemic areas of the diseases in neighboring countries like Pakistan. In addition to significant health hazards, this phenomenon will have negative effects on the economy and ecotourism of the region. Therefore, it is crucial to extend and strengthen the surveillance of invasive Aedes mosquitoes and to consider the need for the rapid suppression of newly introduced Ae. aegypti populations in this tropical region of Iran. This dangerous species has the capacity to act as a potential vector for the transmission of a variety of arboviral diseases and, consequently, poses a real risk to public health and veterinary medicine. Hence, along with emphasis on vector control strategies, regular and more accurate inspections of seaports, particularly cargoes arriving from endemic countries of Ae. aegypti, are needed.

Conclusions

Aedes aegypti is an important vector for the transmission of arboviral diseases. The correct diagnosis of this vector represents one of the most important steps towards controlling vector-borne diseases. This study provided the first comprehensive detective report of this species in Iran (both molecular and morphological identification). Given the confirmation of the presence of this invasive vector in the studied area, the risk of local transmission is really high through the introduced cases of diseases from neighboring countries. Therefore, implementation of vector control programs with a precise and immediate surveillance system should be considered as the priority of the local health system in order to prevent the epidemic of arboviral diseases, such as dengue fever and chikungunya.

Declarations

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Authors' contributions

KA, HD, and **AS** designed and performed the experiments, analyzed the data, and co-wrote the paper. **KA, HD, AS, SAJH, MSN and HA** performed the morphological and molecular identification of the specimens and co-wrote the paper. **RS** helped collect the samples and co-wrote the paper. **KA and HA** supervised the research.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Figures

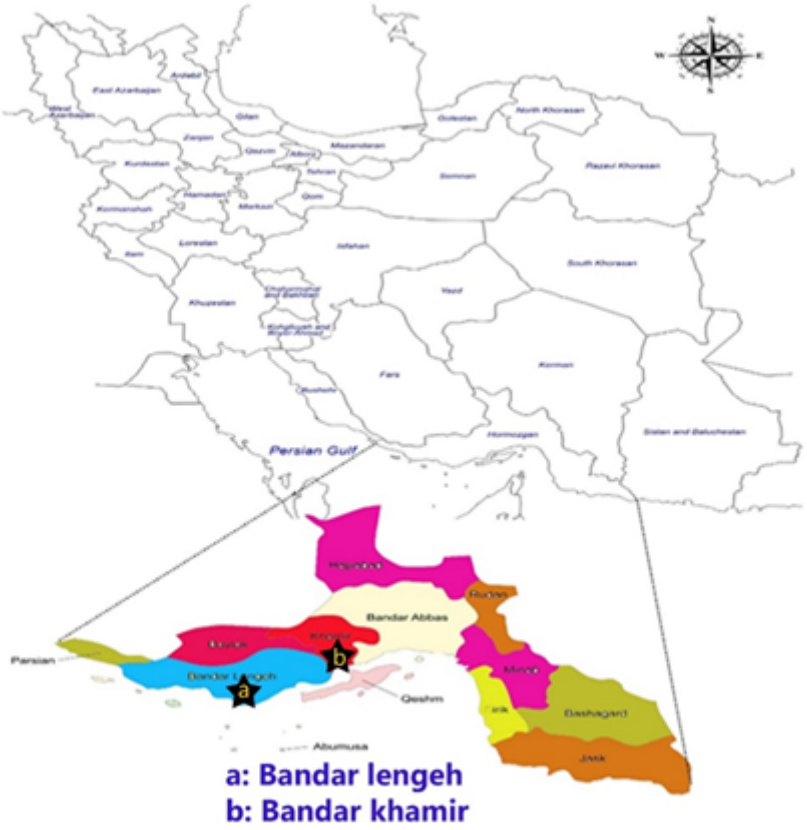

Figure 1

Map of Hormozgan province and its cities, south of Iran and the two points where Ae. aegypti were caught for the first time in the country

Figure 2

Various water reservoirs and sampling sites. a: a traditional water jug, b: a plastic water tank as a mosquito breeding place, c: a rainwater-filled hole, d: a rural scrap tire site, e: used tire disposal sites in an urban area, f: a small cargo ship, g: tires attached to a small ship, h: Abanbar (traditional water storages in arid areas of Iran), f: inside view of a water storage.

Figure 3

a and b: larval sampling from scrap tires as mosquito breeding places

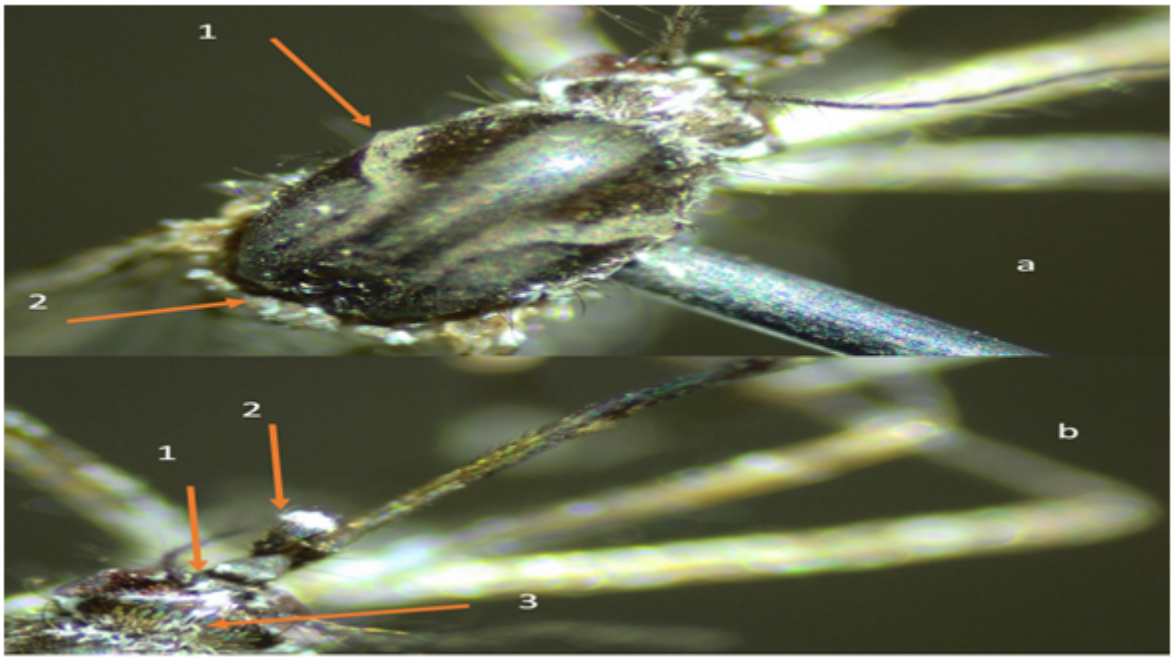

Figure 4

Dorsal view of Ae. Aegypti. a1: The scutum is predominantly covered with narrow dark brown scales, with a distinctive pattern of light scales (lyre shape). a2: The postpronotum has a patch of broad white scales and some dark and pale narrow scales on the upper part. b1: The clypeus with lateral white scales and the pedicel with large patches of white scales on the sides. b2: The palps 1/5 of the length of the proboscis with white scales on the apical half. b3: Erect scales are restricted to the occiput and are all pale.

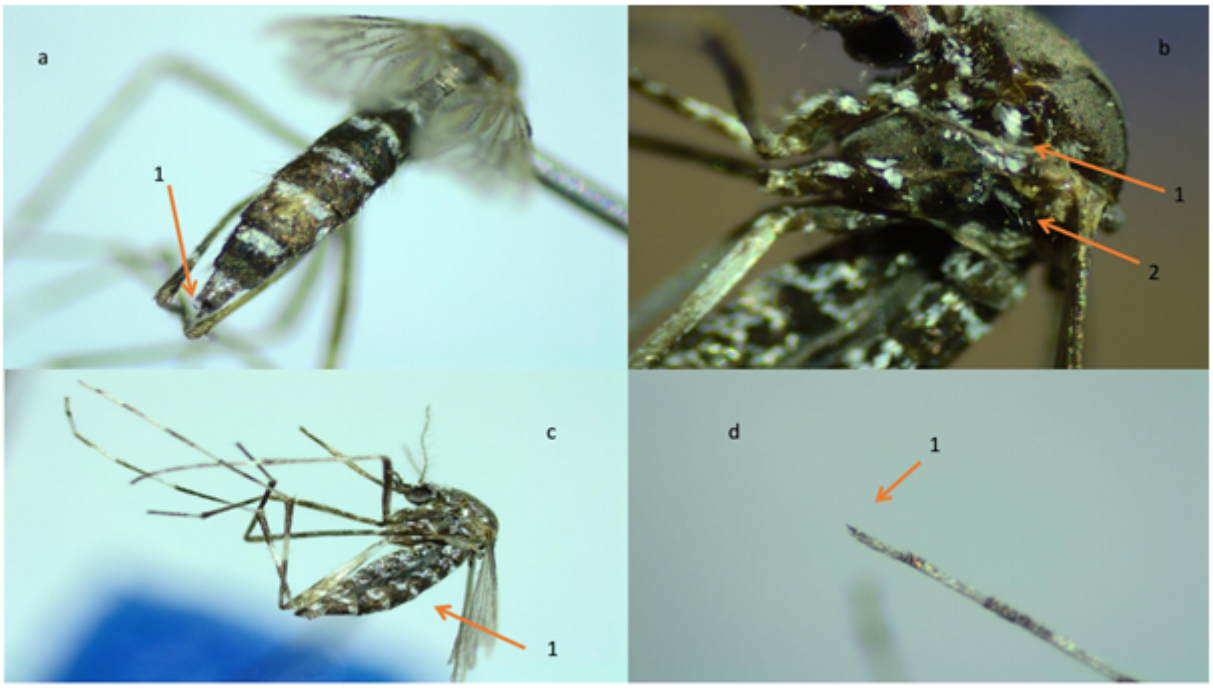

Figure 5

a1: The terminal part of the abdomen is needle-shaped. b1 and b2: The postspiracular area is without scales, but there are patches of broad white scales on the propleuron and subspiracular and hypostigmal areas. c1: All the tibiae are dark anteriorly, the fore and mid tarsi have a white basal band on tarsomeres I and II, the hind tarsus has a broad basal white band on tarsomeres I–IV, and tarsomere V is all white. d1: The claws of the fore and mid tarsi have a subbasal tooth, and the claws of the hind tarsi are simple.

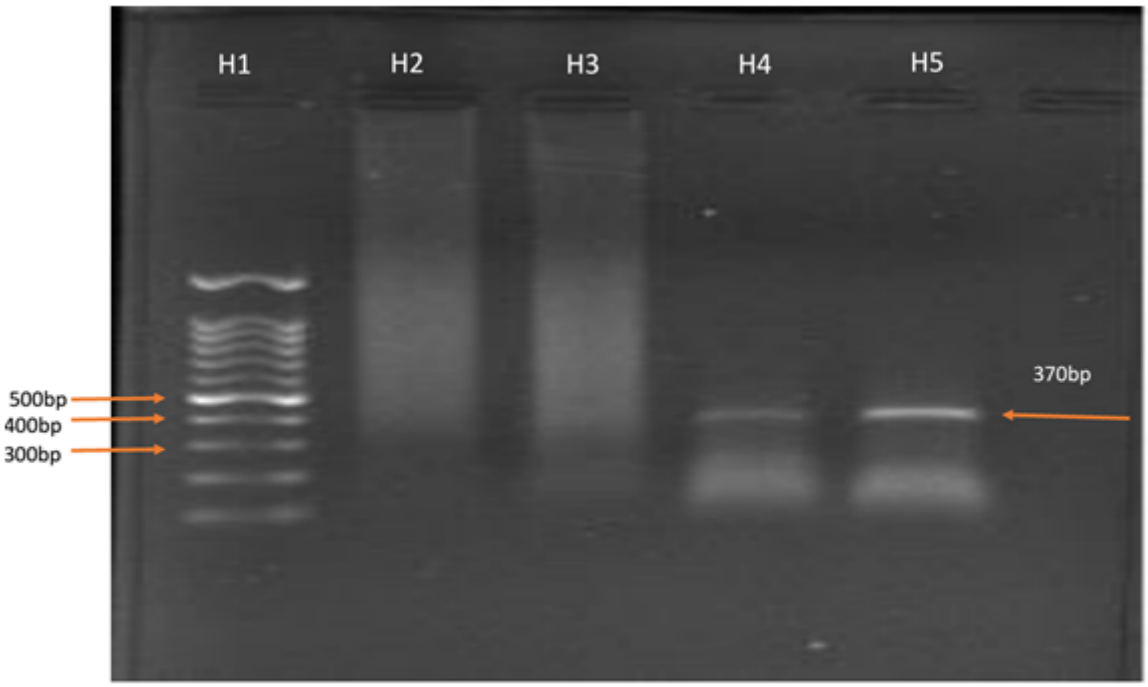

Figure 6

The electrophoresis image showing the amplification of the CO1 region of the collected Aedes aegypti mosquitoes. 0.1% agarose gel; H1: ladder 100 bp (sina clon), H2: negative control, H3: DNA from adult Culex pipiens as the negative control, H4: adult Aedes aegypti mosquitoes, H5: larvae of Aedes aegypti mosquitoes.

Score	Expect	Identities	Gaps	Strand
535 bits(593)	6e-148	298/299(99%)	0/299(0%)	Plus/Plus

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Query  1    GCTATTTTTTCTCTTCATTTAGCTGGAATTTCCTCAATTTTAGGGGCAGTAAATTTTATT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  187  GCTATTTTTTCTCTTCATTTAGCTGGAATTTCCTCAATTTTAGGGGCAGTAAATTTTATT  246

Query  61   ACAACTGTAATTAATATACGATCGTCAGGAATTACTTTAGATCGACTACCTTTATTTGTT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  247  ACAACTGTAATTAATATACGATCGTCAGGAATTACTTTAGATCGACTACCTTTATTTGTT  306

Query  121  TGATCTGTAGTTATTACAGCTATCTTATTACTTCTTTCTCTTCCTGTTTTAGCTGGAGCT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  307  TGATCTGTAGTTATTACAGCTATCTTATTACTTCTTTCTCTTCCTGTTTTAGCTGGAGCT  366

Query  181  ATTACTATATTATTAACAGACCGAAACTTAAATACATCTTTCTTTGATCCAATCGGAGGA  240
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  367  ATTACTATGTTATTAACAGACCGAAACTTAAATACATCTTTCTTTGATCCAATCGGAGGA  426

Query  241  GGAGATCCTATTTTATACCAACACTTATTCTGATTCTTTGGACACCCAGAAGTTTATAT  299
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427  GGAGATCCTATTTTATACCAACACTTATTCTGATTCTTTGGACACCCAGAAGTTTATAT  485
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Figure 7

The result of alignment sequencing (COI gene) of the captured Aedes aegypti in south and southwest of Iran by NCBI

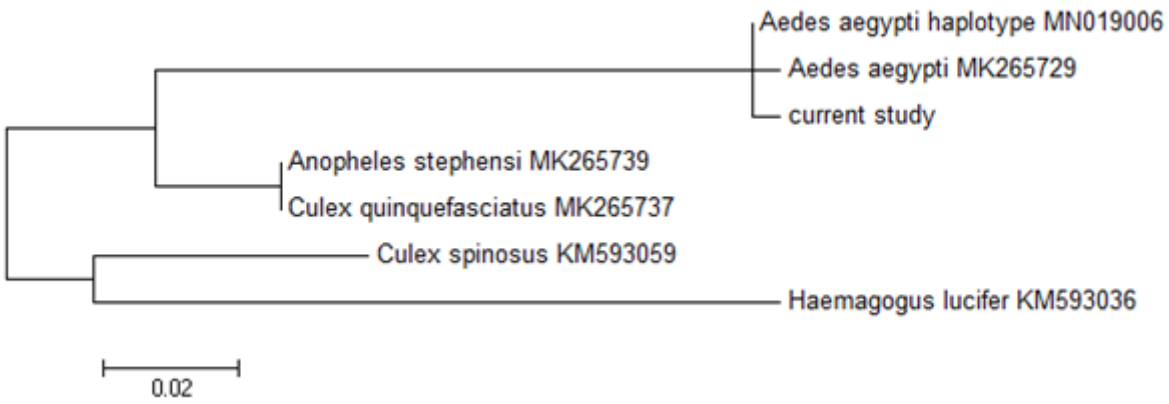

Figure 8

Phylogenetic tree (COI gene) of the captured Aedes aegypti. The COI nucleotides were aligned using MEGA 6.0 software based on the ClustalW method by using the sequences characterized from Aedes, Anopheles, and Culex. The phylogenetic tree revealed that the current sequence was highly similar to Ae. eagypti.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GraphicalAbstract.png