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Molecular analysis of Old World screwworm fly (Chrysomya bezziana) in Iraq

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ABSTRACT

Chrysomya bezziana is a parasite that causes myiasis in sheep. In order to discriminate, the parasite can causes morbidity and death to animals and humans, and also causes economic losses in the livestock industries. Because of the aggressive and destructive nature of this disease in hosts *Chrysomya bezziana* larvae eat vigorously on the host's live tissues and fluids. Female flies are attracted to wounds, at the edges of which each female lays an average of 175 eggs. The larvae emerge within 12–24 hours and immediately begin to feed, burrowing head-downwards into the wound. Identify, and clarify the phylogenesis of *Ch. bezziana*, this work evaluate the efficacy of mitochondrial 16S ribosomal RNA (mt16S rRNA) gene sequences as a novel study for genomic analysis. Fly larvae were obtained from sheep suffering from excruciating myiasis in the Al-Diwaniyah Governorate of Iraq. Following the isolation of the mtDNA, the hypervariable region of the *16S rRNA* sequence encoding for domains IV and V was amplified by PCR using a set of primers (LR-N-13398 and LR-J-12887), resulting in a 548 bp product. The global reference strains of *Ch. bezziana* listed in the Gen Bank were compared with the *16S rRNA* gene of the isolates from the area. The local strains shared 100% of their identity with isolates from Australia, but they also shared 99% and 98% of their nucleotide sequences with sequences from Brazil. Our local strains may have close ties to some global strains according to the data from the current inspection work that may show genetic evolution in the local strains with others listed in the GeneBank.

INTRODUCTION

A form of parasitism known as myiasis occurs when dipterous larvae infest the live tissues of a vertebrate host (1). Around the world, this phenomenon is common, particularly in tropical areas. Usually, it affects domestic and wild animals as well as, in certain cases, people (2, 3). The Old World screwworm fly, *Ch. bezziana* Villeneuve (Diptera: Calliphoridae), is one of the most significant myiasis-causing flies and a highly dangerous pest for all warm-blooded animals, especially field animals like sheep, cows, goats, and buffalo (4), as well as wild animals, such field rabbits, foxes, stray dogs, and deer (5). The danger of this insect lies in the completion of its life cycle, particularly when its larvae reach the third stage in the living tissues of humans and other animals. It also attacks wounds as an obligatory parasite, depending on the host where the adult insect lays its eggs. Especially those that occur in the umbilical cord of newborn animals and the wounds of surgical operations performed on animals such as castration and removal of horns (6). Once inside the living tissue, the maggots cause severe damage and in severe cases, even death, if left untreated (7). Because of this feeding behavior, *Ch. bezziana* is regarded as a major medically significant pest and the source of financial losses for domesticated animals (3, 8). FAO calculated that the OWSF invasion caused the livestock industry in the Eastern Hemisphere, especially in Iraq in the 1990s, to suffer an economic loss of 8,555,000$US (9). Iraq reported the first cases of myiasis brought on by OWS in 1996 (10) .The number of myiases in Iraq increased quickly in the years that followed (almost to 50,000 in 1997) (4), prompting requests for an area-wide to control the species in the Arabic Gulf (11). According to Almansour and his coworkers (12), there were 22 human instances of OWS and 120789 cases of OWS in animals reported in all of Iraq up until 2007. Identification of flies is necessary for control methods as well as epidemiological research (13-18), as effective pest identification is the foundation of pest management programs. Identification can be difficult when females of a particular species complex of insects, such as flies, share similar morphology (19). Nucleotide sequence analysis and other molecular approaches were presented as solutions to the taxonomy identification issues (20). The identification of insect pests presents certain obstacles that could be mitigated by a breakthrough technology called DNA barcoding (21, 22). As the primary technique for species identification in integrated pest management (IPM) programs, DNA barcoding is being more widely used to compare unknown genes with known (23).

Genes' conserved and variable areas can be found in mtDNA, which reflects the molecule's varying rate of evolution (24). Consequently, sequencing the DNA is no prior sequence information is made possible by the capacity to be amplified using universal primers. Apart from their role in protein coding, mitochondrial genes are generally conserved across taxa, which make it easier to gather information on taxa that haven't been researched before (25). Since there are just two mtrRNA subunit genes (12S and 16S), ribosomal genes are used for make the phylogenetic studies (26). The *16S rRNA* is used in flys'

detection. *16S rRNA* is molecular and valuable tool in the phylogenetic studies (24-30). Aims of the present work are evaluate sequences of the *16S rRNA* gene on detection of *Ch. bezziana*.

Materials and methods

Ch. bezziana larval instars

Larvae from sheep wounds were sampled in Diwaniyah Governorate, Iraq, either by visiting farms or by gathering from animals whose owners brought them to veterinary clinics .After traumatic myiasis were detected in an animal, the larvae were extracted with the use of forceps. Samples were gathered in accordance with a routine procedure, Characterization of different instars larvae of *Ch. bezziana* was done according to Zumpt (31).

DNA extraction

We used of proteinase k (20) µl and lysis solution (200) µl to lyse individual worms within Eppendorf tubes. By periodically introducing a pestle into these tubes, they were physically disrupted. These were incubated for three hours at 56°C, or until the tissues dissolved completely. The tubes were rapidly centrifuged for two minutes at 5000 rpm, after which the supernatant was moved to a fresh collecting tube (Coaster) so that the DNA isolation process (32) with some modifications .

Oligonucleotides primers design

For the *16S rRNA* amplification, the universal primers 16Sbr and 16Sar were created in accordance with (33),(Table 1). Primers sets were synthesized by (Operon Technologies, Germany).

Table 1: Primers used to amplify *16S rRNA* genes partial sequences.

Name	Sequence	Product size	Target gene
16Sbr-F	CCGGTCTGAACTCAGATCACGT	548bp	16S rRNA
16Sar-R	CGCCTGTTTAACAAAAACAT		

Amplification of 16S rRNA mtDNA genes partial sequences

With the right primer sets, PCR was used to precisely amplify the sequence hyper variable portion of the mitochondrial *16S rRNA* gene sequence that codes for domains IV and V. A single set of primers was used for the amplification of the *16S rRNA* gene in a total amount of 50µl. the amplifications were done in the thermal cycler (PTC-100™.MJ Research Inc., USA) (28,29). With each PCR, a reagent blank was run concurrently as a control. The products are emigrate in the electrophoresis (Tris-borate (45) mM, EDTA (1mM), Sigma Aldrich) with ethidium bromide (Sigma Aldrich). The ladder have 100 bp (Alliance Bio, USA) are used, the lab Image software was used for evaluation of the gel photo.

Sequencing DNA and phylogenetic analysis

Ten positive samples were chosen for DNA sequencing and phylogenetic analysis, These were compared to other strains from around the world after being placed in the gene bank with accession numbers provided as following: by DHL shipped the PCR products in an ice bag to Macrogen Company in Korea so they could be sequenced for DNA using a sanger sequencing system. Accession numbers of GenBank were provided from submitting the sequences to NCBI-GenBank. Mega x and multiple sequence alignment analysis are used to make DNA sequencing analysis (34).

Results and Discussion

Amplified partial 16S rRNA gene sequences by PCR assays

The *16S rRNA* gene PCR findings according to primer combination in every sample that was analyzed, the amplified *16S rRNA* gene fragment was found at 548 bp (Figure 1).

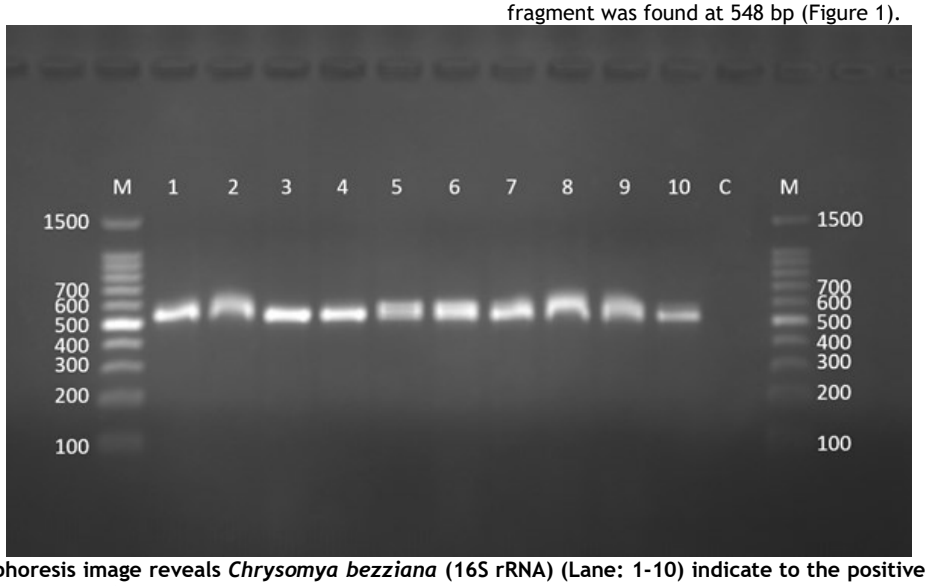

Figure 1: Gel electrophoresis image reveals *Chrysomya bezziana* (16S rRNA) (Lane: 1-10) indicate to the positive band of the samples (548 bp). M is ladder. C is control negative

In addition to the enormous financial losses that myiasis-causing larvae inflict to cattle globally, scientists are interested in these larvae because some of them parasitize people and they are of interest in a number of nations for monitoring control tactics and early incursion detection. Because it is hard to detect larvae to the species level just based on morphology, a growing amount of DNA sequence data has been gathered in an effort to use molecular phylogenetic approaches to identify species (24). Numerous researches have use of DNA sequences to identify insects, with the majority using mtDNA as the starting point for sequencing (35).

Using the primer set (LR-N-13398 and LR-J-12887), the present study found that the *Ch. bezziana* mitochondrial *16S rRNA* gene amplicon was consistently identified at 548 bp. This outcome was consistent with earlier research on the closely related Oestridae family, in which the *16S rRNA* gene was amplified specifically , producing amplicons of 550 bp for Rhinoestrus species and 549–551 bp for Gasterophilus species, respectively (28,29). Sixteen species of flies were detected by using COI and COII of *Ch. Bizana* in Thailand (36). RT-PCR is used for detection of *Ch. Bezziana* in Australia. RT-PCR used ribosomal DNA (rDNA ITS1) as a target for detection of *Ch. bezziana*, it has high sensitivity for detection screwworm fly (37).

Sequencing and phylogenetic tree construction of 16S rRNA gene

The Table 1 displays the percentage of homology sequence identity (NCBI-BLAST) between the local *Ch. bezziana* sequences (OR821777, OR821778, OR821779, OR821780, OR821781, OR821782, OR821783, OR821784, OR821785, OR821786) and the NCBI-BLAST sequences deposited from other nations.

Table (1): NCBI-BLAST Homology Sequence Identity between local Ch. bezziana sequences and other nations in the gene bank

Access No.	NCBI-BLAST Homology Sequence identity (%)			
	Species	Access No.	Country	Identity (%)
OR821777	*Chrysomya bezziana*	JX913737	Australia	100
OR821778		JX913737		100
OR821779		JX913737		100
OR821780		JQ246710	Brazil	98.95
OR821781		JX913737		99.58
OR821782		AF352790		98.95
OR821783		JQ246710		99.16
OR821784		JQ246710		99.16
OR821785		JQ246710		99.16
OR821786		JQ246710		98.95

The *16S rRNA* gene of local *Ch. bezziana* isolates were compared with reference *Ch. bezziana* in (the Gen Bank). Our strains have similarity at (100) % with isolates from Australia as in isolates no (1, 2, 3). It have 99% and 98% like isolates sequences from Brazil, as in isolates no (4, 5, 6, 7, 8, 9, 10).

Figure 2 displays phylogenetic tree analysis of *Ch. bezziana* targeting partial sequences within the *16S rRNA* gene after obtaining NCBI accession numbers represented as red circles while the other global sequences referred as blue circles. These were analysed by Mega X.

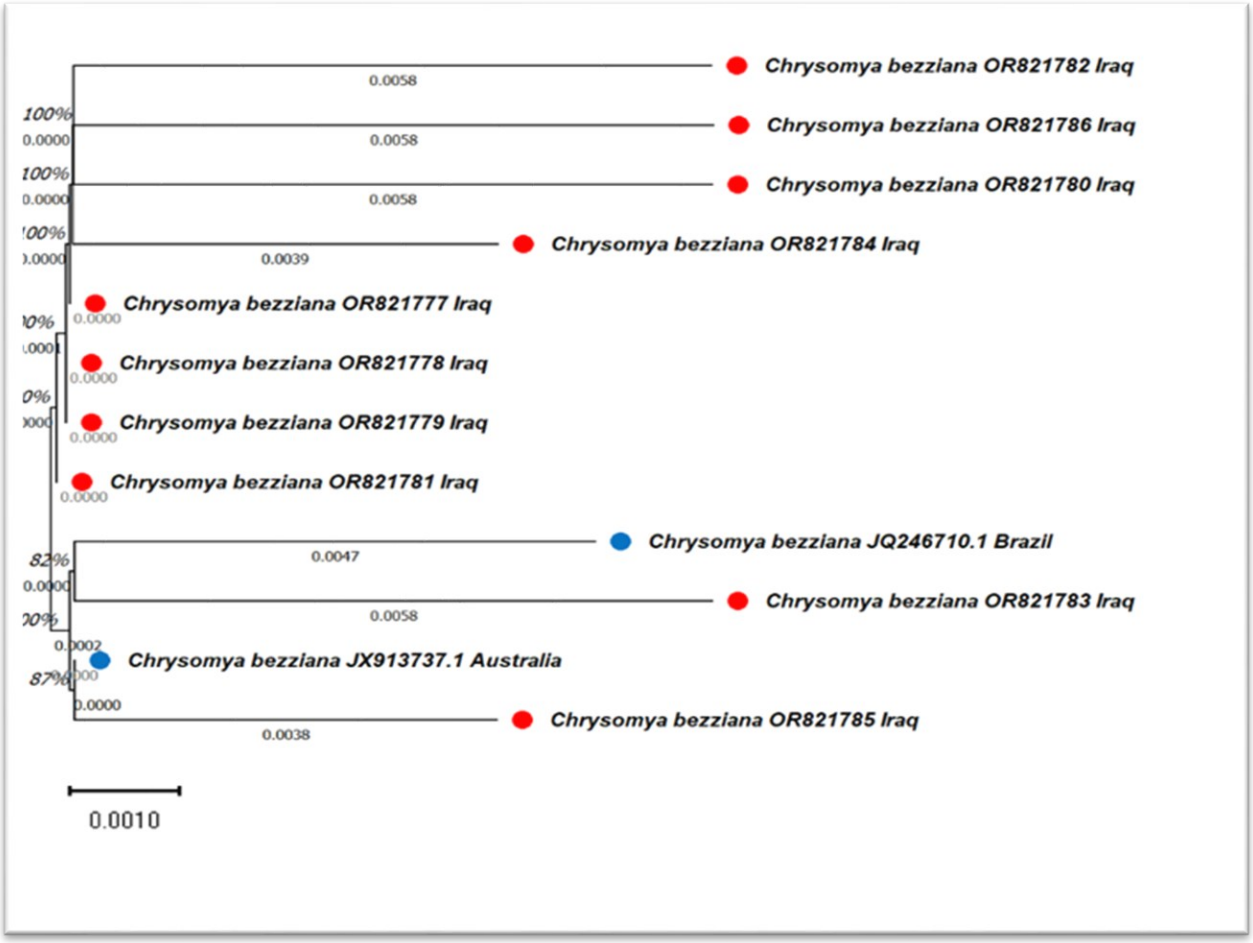

Figure (2): Phylogenetic tree analysis of local *Ch. bezziana* (red circles). UPGMA technique in MEGA v6.0 was used to the evolutionary distances calculation

With greater certainty, the data from the current inspection effort may demonstrate the genetic evolution of the local strains, raising the possibility that they were sisters or cousins of the global strains.

While the figure 3 depicts the similarity and differences of the nucleotide sequences of this study that deposited in the NCBI (OR821777, OR821778, OR821779, OR821780, OR821781, OR821782, OR821783, OR821784, OR821785, and OR821786) in comparison with the isolate from Brazil (accession number: JQ246710.1) and with the isolate from Australia(accession number: JQ913737.1) . This analysed by MEGA x software.

The *16S rRNA* gene of *Ch. bezziana* and its discovered nucleotide sequence aligned numerous times of identified samples compared with homologues global sequence. There were mutations in different sites.


Chrysomya_bezziana_JQ246710.1_Brazil
Chrysomya_bezziana_JX913737.1_Australia
Chrysomya_bezziana_OR821777_Iraq
Chrysomya_bezziana_OR821778_Iraq
Chrysomya_bezziana_OR821779_Iraq
Chrysomya_bezziana_OR821780_Iraq
Chrysomya_bezziana_OR821781_Iraq
Chrysomya_bezziana_OR821782_Iraq
Chrysomya_bezziana_OR821783_Iraq
Chrysomya_bezziana_OR821784_Iraq
Chrysomya_bezziana_OR821785_Iraq
Chrysomya_bezziana_OR821786_Iraq

Figure (3): Nucleotide Sequence alignment of *Ch. bezziana* with the global sequences the differences and similarity

CONCLUSION

We have proven that the *16S rRNA* gene of local *Ch. bezziana* isolates was closely related phylogenetically to Australia and Brazil strains infecting sheep by analyzing the phylogenetic tree of *Ch. bezziana*.

Ethical approval

The study was approved by University of Al-Qadisiyah, College of Veterinary Medicine (IRAS0822019) and consent was obtained from the farm owners before animal examination and sampling.

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