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The importance of molecular diagnosis in controlling the old-world screwworm fly *Chrysomya bezziana* (Vill.) (Diptera: Calliphoriadae) using the sterile insect technique in Iraq

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Abstract

Larvae of the fly *Chrysomya bezziana* (Vill.) (Diptera: Calliphoridae), responsible for myiasis, were collected from 60 infected cattle in three Iraqi governorates (Diyala, Baghdad, and Maysan). A total of 600 adult flies were subjected to mitochondrial cytochrome b gene sequencing, targeting a 317 bp fragment of the 3' terminal region. The analysis revealed the presence of two distinct haplotypes: one occurring in Diyala and Baghdad, and the other in Maysan, southern Iraq. The two haplotypes differed in the number of nucleotide deletions and substitutions, with a total of 189 genetic mutations identified—79 in samples from Baghdad and Diyala combined, and 110 in those from Maysan. Minor differences in morphological traits, such as size and shape, were also observed. These findings indicate genetic diversity among *Ch. bezziana* populations across Iraq. Such diversity suggests that a single laboratory colony could be sufficient for implementing the Sterile Insect Technique (SIT) within an integrated control program.

Keywords: *Chrysomya bezziana*, Genetic diversity, Cytochrome b gene, Sterile Insect Technique (SIT)

Introduction

The Old-World screwworm fly (Diptera: Calliphoridae), *Chrysomya bezziana*, is an obligate ectoparasite of mammals, causing significant economic damage to various ruminant livestock species in sub-Saharan Africa, parts of the Middle East (Arabian Gulf), and Southeast Asia [1]. Understanding the genetic diversity of screwworm fly populations is essential for implementing two control methods for this insect pest, namely Integrated Pest Management IPM and Sterile Insect Technique SIT [2]. This helps identify and isolate the most susceptible populations, the environmental conditions for rearing, and the populations of these populations. In addition to determining the distance and direction of potential invasion operations by this type of pest through certain means, it can also be used to determine the source of new infestations [3]. Hall *et al.* [4] presented a comparative nucleotide sequence analysis of a short part of the gene (CB3) and the mitochondrial Cytochrome (Cyt b) gene, to be considered the first evidence of the relative geographic

variation of the Old World Screwworm Fly (OWSF), where it showed that the populations of this insect in Africa and south of the Sahara constitute different proportions than those in Asia, as they are characterized by two individual Cyt b haplotypes extending from the Arabian Gulf to New Guinea. Ready *et al.* [5] further demonstrated low genetic diversity across much of Asia by analyzing geographic variation between Sumba and Sulawesi Islands in Indonesia, based on a longer fragment of the cyt b gene and exon regions of the nuclear elongation factor 1-alpha (EF-1α) and white-eye genes. These genetic markers were suggested as useful tools for applying advanced control strategies against the pest.

Molecular characterization of *C. bezziana* thus provides valuable insights into dispersal mechanisms across regions. Building on this, Wardhana *et al*. [6] expanded the sampling geographic scope to include 10 Indonesian Islands. A large dataset was analyzed: 754 samples for cyt b, 256 for EF-1α, and 242 for the white gene. Cyt b was identified as the most suitable marker for studying myiasis prevalence in infested regions. The study revealed four sublineages and 37 haplotypes (designated CB-bezz01–37) across Asia.

Clear genetic differentiation among *C. bezziana* populations in Iraq has also been observed. The initial documented case of myiasis occurred in September 1996. Between 1996 and 1997, a total of 54,704 cases were documented in humans and animals, leading to economic losses estimated at 8.5 million USD [7]. This prompted international organizations to implement a myiasis eradication program in Iraq [8]. As a result, case numbers decreased drastically, reaching zero in several provinces [7]. However, it remains unclear whether recent infestations are linked to newly emerging genetic variants, since the latest molecular survey confirmed the presence of *C. bezziana* in Iraq about 12 years ago [6].

Therefore, the objective of this study is to demonstrate the importance of molecular approaches in identifying and controlling *C. bezziana* by characterizing the genetic diversity within its populations through analysis of the Cytb gene.

Materials and Methods

Sample collection and study area

Fully mature third-instar larvae of the Old World screw-worm fly *Chrysomya bezziana* were collected between early March and mid-April 2025. Fully mature third-instar larvae were recognized by examining larvae that had left food, which were light pink in color with a black head. The larvae were identified by sending samples to the Natural History Research Center and Museum / University of Baghdad, and were identified by observing the presence of 5 spiracles. A total of 90 larval samples were obtained from 60 infested livestock heads (cattle and sheep), with 15 larvae taken from each animal. Sampling was evenly distributed across three Iraqi provinces: Diyala (20 heads), Baghdad (20 heads), and Maysan (20 heads).

Insect larvae rearing

After collection, the larvae were transferred to the laboratory and reared under controlled environmental conditions favorable for the insect's life cycle: 37 °C, 70–80% relative humidity, and a photoperiod of 16 hours light and 8 hours darkness (Al-Saray, 2002) [9]. The humidity was controlled using an outdoor thermometer instrument by spraying the sawdust inside the breeding boxes with water using a 760 ml plastic sprayer. The larvae were placed in transparent acrylic (Perspex) rearing cages (50 × 50 × 50 cm^3, 1.5

mm). Covered with a fine white cloth, the opening is positioned at the top to allow ventilation. One side of each cage was altered to incorporate a tube crafted from dense white fabric (measuring 35 cm in length and 18 cm in diameter) for introducing food. The cages were secured within aluminum frames measuring 51 × 51 × 51 cm^3, as described by Al-Juwari [10]. Following pupation, which lasted 5–7 days, the adult flies emerged and were collected for subsequent analysis and continuation of the stud

DNA extraction from samples

Adult flies were placed individually in Eppendorf tubes, to which 200 µL of GST buffer was added. Each specimen was thoroughly homogenized using a micropestle. Subsequently, 20 microliters of Proteinase K was added, and the solution was mixed using a vortexer for 30 seconds. The tubes were incubated in a 60 °C water bath for 30 minutes.

Following incubation, Samples were spun at 14,000 revolutions per minute (rpm) for 2 minutes. The liquid above the sediment was moved to a fresh tube, followed by the addition of 200 µL of GSB buffer. The combination was agitated using a vortex for 10 seconds to aid in DNA precipitation. The solution was then transferred into a GD Column containing a filter membrane and centrifuged again for 1 minute.

After centrifugation, the column was removed and transferred to a fresh GD Column tube. A volume of 400 µL W1 buffer was added, followed by centrifugation for 1 minute. The filtrate was disposed of., and after that, 600 microliters of Wash Buffer were added to it and it was placed in the centrifuge again for two minutes for the purpose of drying.

After the drying process is completed, the tube is discarded, and the filter is taken and placed in a new Eppendorf tube, and 50 microliters of Elution Buffer are added to it. After that, it is placed in the centrifuge at a speed of 14,000 revolutions per minute for only one minute. The extracted DNA appeared as a colorless, gelatinous material. The quality and presence of DNA were confirmed through agarose gel electrophoresis.

Performing the PCR reaction for cytochrome b gene sequencing

The PCR reaction was performed using a specialized primer PDR-WR04 (ATT TCA CGC TCA TTA ACT): Cb) (CB1 –SE (TAT GTA CTA CCA TGA GGA CAA ATA TC) which arranges the nitrogenous bases in a specific sequence, manufactured by Bioneer – Korea. The Master Mix reaction mixture was prepared by adding the components (D. Water, Acc power PCR PrmiX, Primer – F (10 PMpol), Primer – R (10 Pmol)) into a sterile tube, and these components were mixed using a glass pipette. Then it was placed in a Vortex shaker to mix the components well, and the mixture was distributed into 9 sterile tubes of 0.5 ml volume, labeled according to the samples from the three studied regions, with 15 µL of the mixture for each tube. Then, 5 µL of DNA was added for each sample. The tubes were placed in a Thermo cycler, and the reaction was carried out based on [11]. After that, the electrophoresis process for the samples was performed on an agarose gel by adding 5 µL of Ethidium bromide dye to the gel contained in the glass beaker, and placing DNA Ladder in the first and last wells of the gel for comparison purposes, with a volume of 7 µL. Then the samples were loaded into the remaining wells that were made in the gel, according to the sequence given for each sample. Afterwards, the electrophoresis device was run at 100 volts for 90 minutes. After the specified time for the electrophoresis process was completed, the gel was removed from the electrophoresis

device and placed on the surface of the Gel documentation device to confirm the presence of the amplified gene bands [12].

Results and Discussion

The genetic analysis of the studied samples relied on 317 base pairs from the 3' end of Cyt b, amplified by PCR as two overlapping DNA fragments. Three lineages of *Chrysomya bezziana* were identified. Two lineages, obtained from Diyala and Baghdad, were highly similar in most characteristics, while the third lineage, collected from Maysan, showed slight differences in morphological traits such as body size and shape. In addition, this lineage displayed distinct patterns of nucleotide substitutions and deletions within its DNA sequence.

Comparative analysis of the nucleotide sequences from nine samples (three from each province) and reference sequences from GenBank revealed noticeable sequence variation, primarily in the form of point mutations (deletions and substitutions). Across all samples from the three provinces, a total of 153 deletion mutations and 36 substitution mutations were recorded within the mtDNA cyt b gene, spanning nucleotide positions 3–300.

Specifically, deletion mutations in Maysan samples occurred at a single site, whereas Diyala and Baghdad samples exhibited deletions at six distinct sites each. The total number of deletions and substitutions was higher in Maysan (91 and 19, respectively) compared with Diyala and Baghdad (62 deletions and 17 substitutions, respectively). These findings indicate a closer genetic relationship between populations from Diyala and Baghdad, while the Maysan population appears genetically more distinct. The geographic isolation (geographic race), barriers impeding the gene flow, and the ecological differences, such as nutritive availability, temperature, humidity, and precipitation, can lead to genetic variation found among the populations studied in this work. The results of this paper are in agreement with those found by Chong *et al*. [13], who studied the amplification of mitochondrial DNA COI and COII gene products from *C. megacephala* collected at five Malaysian locations: Penang, Selangor, Pahang, Johor, and Sabah. The sequence lengths of the two genes in their study were 1240 bp and 1140 bp, respectively. The authors reported that polymorphisms were detected on two sites by multiple sequence alignment; one of them was a transitional substitution between cytosine (C) and thymine (T) and between guanine (G) and adenine (A). In addition, Penang and Selangor populations had the highest substitutional polymorphism in COI and COII, suggesting that higher genetic diversity occurred at these regions.

Different lineages of *Chrysomya megacephala* have been reported in several countries; some of these have a range originating from southern Europe, throughout the Middle East, through Iraq, and to the north Arabian Gulf states, as well as to Iran. A recent analysis has shown that all samples from the Iraqi Provinces belong to the eastern clade [14]. This indicates that the distribution of this pest in Iraq seems to be introduced from outside the country, and particularly through the importation of infected meat or livestock containing mature larvae. This is corroborated by what farmers and veterinarians said that they had never seen this fly before 1996.

Furthermore, studies conducted in countries neighboring Iraq confirmed the hypothesis that the increase in cases of myiasis infection in livestock caused by the *Ch. megacephala* fly is due to the emergence of historically endemic populations in the country and not solely its entry from neighboring countries or through the importation of infected livestock or materials carrying eggs or larvae [15].

The analysis showed that the studied samples belong to one of the haplotypes (genetic patterns). Genetic diversity was high across all areas studied. If there had been a recent entry of *Ch. megacephala* eggs or larvae, it would have come from a single source, such as a shipment of infected animals with only one or two haplotypes. The presence of genetic differences between the samples from Diyala and Baghdad on one hand, and the samples from Maysan on the other, indicates the presence of barriers to the fly's spread, the most important of which are long distance, difference in environmental conditions, and food scarcity. The presence of more than one species of *Ch. megacephala* in Iraq compared to other areas of the Arabian Gulf and Iran explains how this species has spread. Through this study, it becomes clear that the degree of genetic diversity in any region may be linked to several factors, including animal trade, which can overcome natural barriers to the spread of pests. The success of the Sterile Insect Technique experiment in Iraq will lead to a reduction in *Ch. megacephala* numbers and will also constitute a barrier preventing its spread to the rest of the country and neighboring countries.

A high genetic diversity of the mitochondrial cytochrome b gene of populations of *Ch. bezziana* collected from Diyala, Baghdad, and Maysan areas of Iraq was found in the present study. Two main haplotypes were detected, one of which (mean divergence 1.14%) is more variable in Maysan population than in the northern populations and exhibited only minimal morphological differentiation. These results indicate that geographic restriction and environmental parameters may be responsible for this diversity. Crucially, the results suggest that a single colony of laboratory-reared insects will be suitable for using Sterile Insect Technique (SIT) in an area-wide integrated pest management programme against the Old World screwworm fly within Iraq.

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