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Molecular characterization of Chrysomya bezziana isolated from sheep and goats in Al Muthanna Governorate, Iraq

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Abstract

Objective

The Old World screwworm fly (*Chrysomya bezziana*) is an obligate parasitic fly that causes myiasis in livestock and occasionally in humans. It causes obligate myiasis in livestock and can infest humans in some cases. The aim of the current study was to identify *Chrysomya bezziana* larvae collected from sheep and goats in Al-Muthanna Governorate, Iraq using molecular techniques for the mitochondrial cytochrome c oxidase subunit I (COI) gene and investigate the phylogenetic relationship between Iraqi isolates and global reference strains.

Materials and methods

In this study, A total of 100 larvae were collected from infected sheep and goats (n = 150 examined animals) from different regions of Al-Muthani Governorate, Iraq. Genomic DNA was extracted from the tissues of the collected larvae using a standard method. Species-specific primers were designed to study the mitochondrial cytochrome c oxidase subunit I (COI) gene. PCR was used to amplify the 670 bp fragment expected for the designed primers. PCR products from selected positive samples were subjected to Sanger sequencing. Sequences were analyzed using BLAST (NCBI) and phylogenetic analysis was performed using MEGA11 software to determine their genetic relationships with global isolates.

Results

Genetic analysis showed that a 670-base-pair segment of the COI gene accurately identified the screwworm fly. Subsequent genetic analysis confirmed this identification by matching the DNA sequence with adult fly samples. The phylogenetic tree shows a degree of separation between Iraqi and global isolates. The Iraqi samples cluster together in their own specific cluster within the tree, while the global isolates are distributed in separate clusters. This indicates that the *C.*

bezziana isolates from Iraq belong to the same species at the global level, but they exhibit slight genetic variation compared to some strains recorded in other countries.

Conclusion

According to the results obtained in this study, it can be concluded that the COI gene can be used as an effective molecular marker for the accurate identification of Chrysomya bezziana. The genetic similarity obtained between the Iraqi isolates in this study, although slightly different from global strains, could indicate the presence of regional genetic characteristics of this species in Iraq.

Keywords: Chrysomya bezziana**,** COI, Molecular identification, Phylogeny, sheep

Paper type: Research paper.

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Introduction

Myiasis is a type of parasitism that happens when dipterous larvae infest a vertebrate host's living tissues (Francesconi et al., 2012). It typically affects both domestic and wild animals and occasionally humans as well (Marrana, 2022). One of the most important flies that cause myiasis is the Old-World screwworm fly, *C. bezziana Villeneuve* (Diptera: Calliphoridae). Facultative myiasis flies can develop on decaying organic matter. *Chrysomya bezziana* is an obligate parasite whose larvae develop exclusively in the living tissues of warm-blooded hosts. It is a very dangerous pest for all warm-blooded animals, particularly field animals like sheep, cows, goats, and buffalo (Al-Helfi, 2008), as well as wild animals. Infestation commonly occurs in newborn animals, particularly in the umbilical cord and postoperative wounds such as castration and dehorning sites (Chowdary et al., 2025). Once inside the live tissue, the maggots can cause serious harm and, in extreme situations, even death if treatment is not received (Francesconi et al., 2012). Differentiating larvae of C. bezziana through using external traits alone is difficult as a result of its strong resemblance to other closely related species, such as Chrysomya megacephala. This similarity is evident in color, body size, and bristle shape. Besides, the lack of reliable

classification keys can lead to errors in identification, as reported Sontigun et al. (2018), the situation is further complicated when specimens are damaged, as the absence of key morphological features makes identification based solely on morphological characteristics even more challenging. Consequently, DNA-based identification methods have been extensively used for accurate identification of medically and veterinary important myiasis-causing fly species due to their speed and accuracy in identifying different stages of their life cycle. Furthermore, the ability of extracted DNA sequences to estimate the evolutionary history and relationships between different myiasis-causing fly species through genetic analysis adds further value (Sontigun et al., 2018). Mitochondrial DNA (mtDNA) is maternally inherited, does not undergo recombination, and has a relatively high evolutionary rate compared to nuclear DNA. Therefore, it is widely used in molecular classification. Genetic diversity in mtDNA is region-dependent. Its non-coding regions, such as the control region (D loop), show high levels of diversity. In contrast, protein-coding genes, such as the cytochrome c oxidase subunit I (COI), are relatively conserved across species. This means that the COI gene has a sufficient balance of conservation within species and sufficient divergence between species. Therefore, this gene can be used as a reliable marker for DNA barcoding and species-level identification (Salem et al., 2015). The cytochrome oxidase 1 (COX1 or COI) gene is one of the most useful mitochondrial DNA genes for taxonomic identification and evolutionary analysis, due to its conserved and highly diverse regions that allow for the investigation of different classifications in humans and animals (Ghaffar et al., 2018). Small ruminants, particularly native breeds, play a crucial role in the livelihoods of a significant portion of the human population in tropical regions from socio-economic perspectives (Molaei Moghbeli et al., 2013; Alhasoon et al., 2026; Saadatabadi et al., 2023; Mohammadabadi et al., 2024). These animals are essential sources of meat, milk, wool, and hides, contributing to food security and rural incomes. Furthermore, they are well-adapted to harsh environmental conditions, making them vital for pastoral and small-scale farming systems (Hajalizadeh et al., 2021). Given their importance, combined efforts that focus on both effective management strategies and genetic improvement are crucial to enhancing animal productivity and ensuring sustainable development (Mohammadipour Saadatabadi et al., 2022; Vahabzadeh et al., 2020; Amirteymoori et al., 2021; Mohammadabadi et al., 2022). Genetic improvement programs, such as selective breeding, molecular marker-assisted selection, and genomic approaches, can significantly boost desirable traits like growth rate, milk yield, and resistance to diseases (Nejad et al., 2024). The economic and biological efficiency of small ruminant production enterprises generally improves by increasing both productivity and reproductive performance in these animals (Zamani et al., 2011; Safaei et al., 2022; Barazandeh et al., 2016; Mohammadinejad, 2016; Shokri et al., 2023). Enhanced reproductive performance can be achieved through improved nutrition, strategic breeding practices, and advanced reproductive technologies such as artificial insemination and embryo transfer (Noori et al., 2017). By integrating these approaches, small ruminant breeders can improve flock productivity, ensure food security, and contribute to the economic well-being

of rural populations (Mohammadabadi et al., 2022). Al-Muthanna Governorate is one of the important livestock-rearing regions in southern Iraq. In this region sheep and goat farming are widely breed under semi-arid climatic conditions. It has the high animal density, extensive grazing system, and warm environmental conditions. This situation made it favorite for the occurrence and spread of myiasis-causing flies, including *Chrysomya bezziana*. Moreover, molecular epidemiological data regarding the genetic characteristics and distribution of the parasite are limited. Therefore, the aim of this study was to identify *Chrysomya bezziana* larvae collected from sheep and goats in Al-Muthanna Governorate, Iraq using molecular techniques for the mitochondrial cytochrome c oxidase subunit I (COI) gene. The investigation of the phylogenetic relationship between Iraqi isolates and global reference strains was also other goal of the study.

Materials and methods

Study area: In the current cross-sectional study, a total of 150 animals (75 sheep and 75 goat) were collected from Al-Muthanna Governorate, Iraq, from October 2025 to March 2026. districts including Al-Samawah center, Al-Najmi, Al-Hilal, Al-Suwair, Al-Warkaa, and Al-Khidhir were clinically examined for myiasis infestation. Animals that showed visible wound myiasis and had larvae were applied for sampling. Sterile forceps were used to collect samples purposively from infected animals. From the examined animals, 100 larval specimens morphologically identified as *Chrysomya bezziana* were selected for molecular analysis. In cases where multiple larvae were collected from the same animal, only representative larvae were used to avoid duplication bias. Each larval specimen was preserved separately in 70% ethanol and transported to the laboratory of the College of Veterinary Medicine, Al-Qasim Green University, for morphological and molecular identification.

Primer design: The primers used in this study were designed based on the mitochondrial cytochrome c oxidase subunit I (COI) gene sequence of *Chrysomya bezziana*. This sequence is available in GenBank database (Accession No. AF295548). In order to identify conserved regions specific to *C. bezziana*, we used multiple COI gene sequences of *Chrysomya* species and aligned them using ClustalW implemented in MEGA11 software. Primer3 software was used to design primers. For this purpose, standard criteria including melting temperature (Tm), primer length, GC content, and absence of secondary structures such as hairpins and primer dimers were considered. The NCBI BLASTn tool against nucleotide database was used to evaluate the specificity of the designed primers. This process helped to confirm that the primers specifically matched *C. bezziana* sequences without significant similarity to other closely related species. The expected amplicon size was 670 bp (Table 1). Amplification of the target fragment in positive samples and absence of amplification in the negative control confirmed experimentally validity of designed primers. Moreover, sequencing results of PCR products showed high identity with *C. bezziana* sequences deposited in GenBank also confirmed the specificity and accuracy of our designed primers

Table 1. Characteristics of designed primers were based on the mitochondrial cytochrome c oxidase subunit I (COI) gene sequence of *Chrysomya bezziana* designed in the current study

Primer name	Sequence	Target gene	GenBank Accession number	Start loci	End loci	Amplified fragment size
Forward	TCGCGACAATGGTTATTTTCTACT	COI	AF295548	7	30	670 bp
Reverse	CTCCTCCTGCTGGGTCAAAG			657	676	

DNA extraction from samples: The DNA samples were extracted from the parasite larvae of *Chrysomya bezziana* according to the instructions of (Addbio, South Korea) (Al-Musawi et al., 2022).

Molecular study: Polymerase chain reactions (PCR) with 50 µL final volume were performed for *cytochrome oxidase subunit 1* (*COI*) gene in standard microtubes. Each tube contained 4 µL (50-100 ng/mL) of DNA (template), 2.5 mM $MgCl_2$, 25 pmol of each primer, 250 µM each of dNTPs, and 2 U Taq polymerase. The thermal profile included step 1 (initial denaturation for 5 min at 94 °C), step 2 (included 35 cycles with denaturation for 30 s at 94 °C, annealing for 45 s at 50 °C, and synthesis for 35 s at 72 °C), and step 3 (final extension for 10 min at 72 °C). PCR products were electrophoresed on 1% (w/v) agarose gel. PCR products of positive samples (10 samples) were sent for sequencing to Macrogen company (Korea) by DHL service. Sanger sequencing technique was used for DNA sequencing. After receiving the sequences, the generated sequences were trimmed from noisy signals and submitted to NCBI for accession numbers.

Phylogenetic tree: The Molecular Evolutionary Genetics Analysis version 11 (MEGA11) software was used to create the phylogram. Clustal W was used to perform the alignments. Reference COI gene sequences of *Chrysomya bezziana* from different geographical regions (India, Malaysia, Thailand, Australia, and the USA) were derived from the GenBank database. These sequences were included in the phylogenetic analysis to compare with the Iraqi isolates. Reference sequences were selected based on sequence completeness, species confirmation, and geographic diversity.

Results and discussion

Optimization: The optimization results of the thermal condition of Conventional PCR (universal primer) showed that the optimal temperature (50 °C), was employed in the thermal conditions of the PCR to detect all of positive samples (Figures 1).

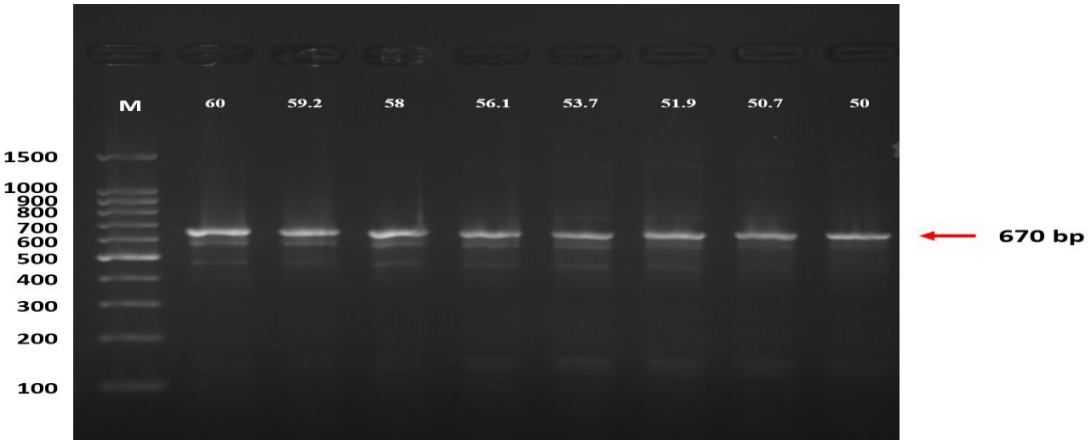

Figure 1. Agarose gel electrophoresis image (1.5 %) shows the amplicons of COI gene (size= 670 bp) by gradient protocol in which similar PCR conditions were used except the annealing temperature (60-50 °C). This shows that 50 °C was the optimal annealing temperature. NC is negative control in which H2O was added instead of template DNA. M is molecular marker (100-1500 bp) from GeneDirex (South Korea)

Conventional PCR: Conventional polymerase chain reaction (PCR) was carried out to amplify the mitochondrial cytochrome c oxidase subunit I (COI) gene. Clear amplification bands were detected in the majority of samples, with only a small proportion showing no detectable amplification. The PCR products were visualized using agarose gel electrophoresis and showed 670 bp fragment which is the expected size for this gene. This indicates the efficiency of the primers and reaction conditions used to amplify the target gene segment. The absence of any band in the negative control (NC) sample indicates the absence of contamination in the reaction components. These results confirmed successful amplification of the COI gene (Figure 2). The mitochondrial *cytochrome c oxidase subunit I* (*COI*) gene is one of the most important molecular markers used in DNA barcoding systems, as it is highly efficient at distinguishing between different vertebrate and invertebrate species. Other studies conducted in Egypt, such as those by Rashed et al. (2025), have demonstrated the use of the cytochrome c oxidase I (COI) gene in molecular diagnostics. This gene is among the most widely used molecular markers for species identification because it exhibits a reasonable degree of genetic variation between species while maintaining relatively conservative sequence within a single species, making it a more effective and accurate tool for molecular diagnosis compared to some other genes. This is due to its suitable degree of genetic variation between species and its relative conservation within a single species, making it a reliable tool in species identification and molecular taxonomic analysis, as confirmed by several previous studies, such as that of Rodrigues et al. (2017).

The phylogenetic tree helps to know how close or distant species are from each other on the genetic level, understanding evolutionary relationships and supports a more perfect classification

of organisms based on evolutionary history, not just physical similarities (Al-Khafaji, Z. K., & Al-Musawi, 2025).

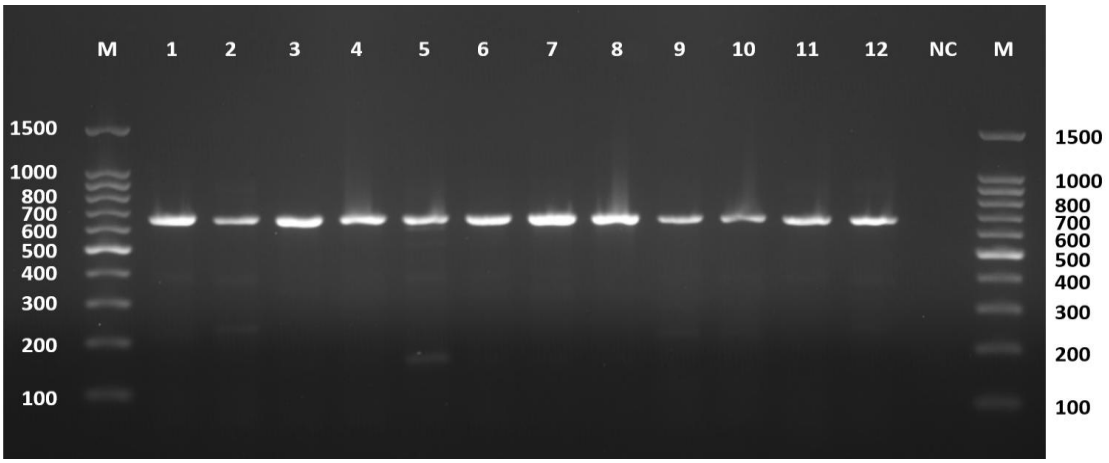

Figure 2. Agarose gel electrophoresis image (1.5 %) shows the amplicons (1- 12) of COI gene (size= 670 bp). NC is negative control in which H2O was added instead of template DNA. M is molecular marker (100-1500 bp) from GeneDirex (South Korea)

Phylogenetic analysis based on the COI gene showed that all Iraqi isolates formed a strongly supported monophyletic clade with a bootstrap value of 100%. It demonstrates the high confidence in the genetic relatedness among the local isolates. High bootstrap values (>70%) observed in the major branches support the reliability and stability of the inferred phylogenetic relationships. The Iraqi sequences were clearly separated from isolates originating from India, Malaysia, Thailand, Australia, and the USA, suggesting regional genetic differentiation (Figures 3). This indicates that the isolates are genetically similar and likely originate from a common local population. This may be attributed to the adaptation of the *C. bezziana* parasite to the Iraqi environmental conditions over a long period of time. This suggests: Geographic genetic structure and Iraqi strains may represent a regional haplotype group. The Iraqi samples show low genetic divergence among the them. Isolates PZ098408, PZ098417, and PZ098412, as well as PZ098410 and PZ098414, along with PZ098409, PZ098413, and PZ098416, are grouped together, while isolates show low genetic divergence PZ098411 and PZ098415 are grouped individually. This may be attributed to the mutation between Iraqi samples. The phylogenetic tree drawn in this study showed a clear geographical clustering pattern among Iraqi C. bezziana isolates. The Iraqi isolates were grouped into a distinct monophyletic clade. This could indicate that this studied sample could be a relatively conserved local population with limited intra-population genetic diversity. On the other hand, the placement of isolates from other countries in separate clusters could indicate possible geographical differentiation and evolutionary divergence related to their environmental adaptation and regional isolation. However, all isolates remained in a single species complex, confirming the taxonomic identity of the Iraqi isolates as C. bezziana. The minor sequence variations observed among some Iraqi isolates could be due to natural mutations or local

haplotype diversity within the studied population. The *Cytochrome c oxidase subunit* I (*COI*) gene is an important mitochondrial gene in evolutionary studies. It possesses a superior phylogenetic signal compared to many other mitochondrial genes, making it more efficient for analyzing evolutionary relationships between different species, as noted by Strüder-Kypke & Lynn (2010).

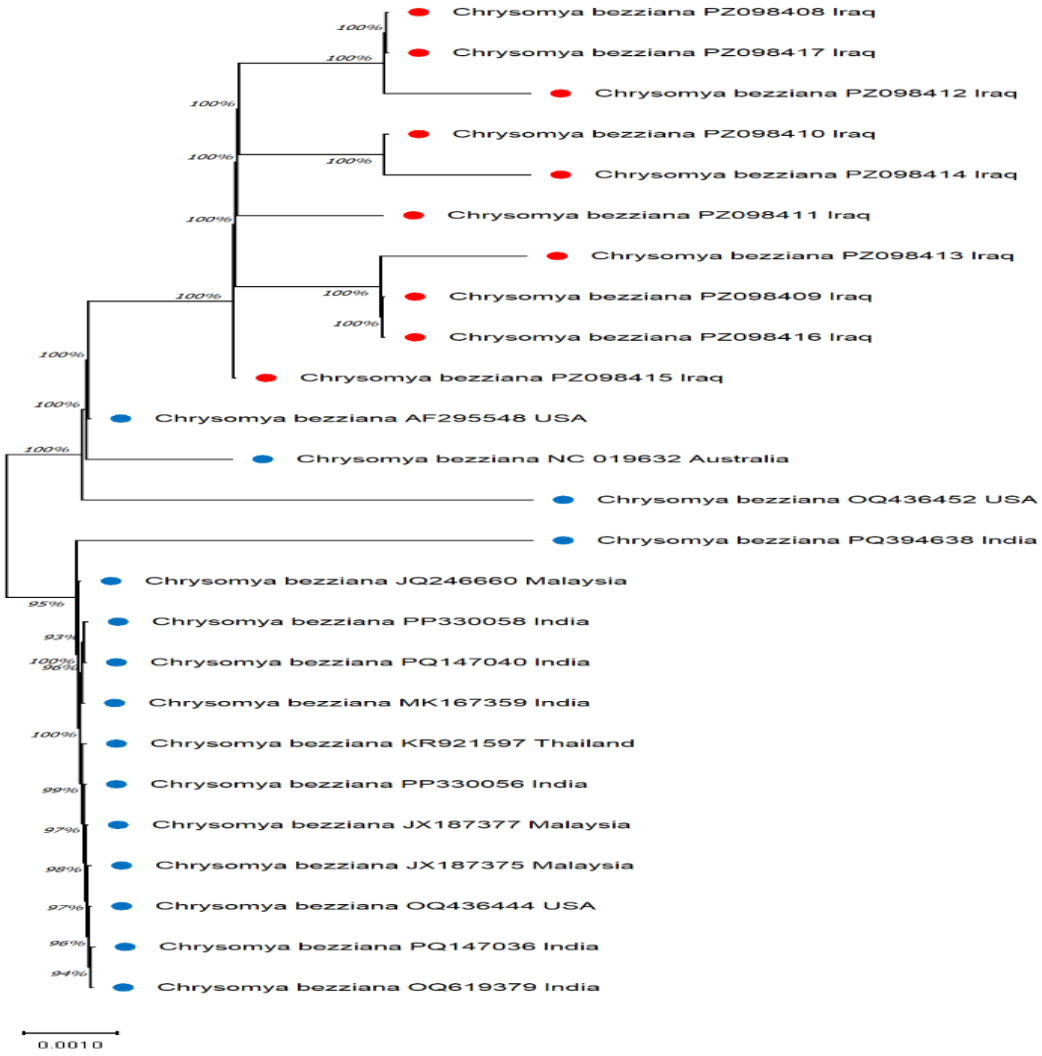

Figure 3. Evolutionary analysis tree of *Chrysomya bezziana* within COI gene by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 25 nucleotide sequences. There were total of 645 positions in the final dataset. Evolutionary analyses were conducted in MEGA11

The COI gene is characterized by both conserved and variable regions; the variable regions allow for genetic variation between species, while the conserved regions remain relatively stable. However, many mutations in this gene are often silent mutations that do not affect the resulting

protein structure, a finding confirmed by (Pentinsaari *et al* 2016; Makouloutou et al. 2015). The mitochondrial COI gene has a relatively conserved structure within species. It also has sufficient nucleotide diversity among species. For these reasons, it is widely used in phylogenetic studies and DNA barcoding. Because of these properties, it is considered an effective marker for species differentiation and evolutionary analysis.

Conclusion: This study provides molecular evidence supporting the identification of the *Chrysomya bezziana* fly isolated from sheep and goats in Al-Muthanna Governorate using the mitochondrial *COI* gene. The results demonstrate that molecular analysis provides a precise and reliable method for species identification compared to traditional morphological methods. Furthermore, genetic analysis revealed a high degree of genetic similarity among Iraqi isolates, with only slight variation compared to some global isolates, suggesting the possibility of regional genetic differentiation. These findings highlight the importance of molecular studies in understanding the genetic diversity and distribution of this species in Iraq, underscoring the need for further research to cover broader geographic areas.

Author Contributions

Z.Q.F. designed the study, conducted laboratory work, analyzed the data, and drafted the manuscript. A.M.K.A. supervised the research, contributed to data interpretation, and reviewed and edited the manuscript.

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Data availability statement

The data contributing to the findings of this study are available from the investigating researcher upon request.

Ethical Considerations

All procedures involving animal samples were conducted in accordance with institutional and national ethical guidelines for animal research. Sample collection was performed with minimal harm to animals and under appropriate veterinary supervision.

Conflict of Interest

The authors declare no conflict of interest.

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شناسایی مولکولی *Chrysomya bezziana* جدا شده از گوسفند و بز در استان المثنی، عراق

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چکیده

هدف: مگس کرم‌خوار دنیای قدیم (*Chrysomya bezziana*) یک مگس انگل اجباری است که موجب مایازیس (آلودگی با لارو مگس) در دام‌ها و گاهی انسان می‌شود. هدف این مطالعه شناسایی لاروهای *Chrysomya bezziana* جمع‌آوری‌شده از گوسفند و بز در استان المثنی عراق با استفاده از روش‌های مولکولی مبتنی بر ژن میتوکندریایی سیتوکروم اکسیداز زیرواحد I (COI) و بررسی روابط فیلوژنتیکی میان ایزوله‌های عراقی و سویه‌های مرجع جهانی بود.

مواد و روش‌ها: در این مطالعه، تعداد ۱۰۰ لارو از حیوانات آلوده (۱۵۰ رأس گوسفند و بز بررسی‌شده) از مناطق مختلف استان المثنی عراق جمع‌آوری شد. DNA ژنومی از بافت لاروها با استفاده از روش استاندارد استخراج گردید. پرایمرهای اختصاصی برای بررسی ژن COI (mitochondrial cytochrome c oxidase subunit I) میتوکندریایی طراحی شدند. PCR برای تکثیر قطعه ۶۷۰ جفت‌بازی مورد انتظار انجام شد. محصولات PCR از نمونه‌های مثبت منتخب برای توالی‌یابی Sanger ارسال شدند. توالی‌ها با استفاده از پایگاه BLAST (NCBI) تحلیل و سپس تحلیل فیلوژنتیکی با نرم‌افزار MEGA11 برای تعیین روابط ژنتیکی با ایزوله‌های جهانی انجام شد.

نتایج: تحلیل ژنتیکی نشان داد که قطعه ۶۷۰ جفت‌بازی ژن COI به‌طور دقیق قادر به شناسایی مگس کرم‌خوار است. تطبیق توالی DNA با نمونه‌های بالغ نیز این شناسایی را تأیید کرد. درخت فیلوژنتیکی نشان داد که ایزوله‌های عراقی در یک خوشه جداگانه

قرار گرفته‌اند، در حالی که ایزوله‌های جهانی در خوشه‌های متفاوت پراکنده شده‌اند. این نتایج نشان می‌دهد که اگرچه ایزوله‌های عراقی متعلق به همان گونه در سطح جهانی هستند، اما تفاوت‌های ژنتیکی جزئی نسبت به برخی سویه‌های ثبت‌شده در کشورهای دیگر دارند.

نتیجه‌گیری: بر اساس نتایج این مطالعه، ژن COI می‌تواند به‌عنوان یک نشانگر مولکولی مؤثر برای شناسایی دقیق *Chrysomya bezziana* مورد استفاده قرار گیرد. شباهت ژنتیکی ایزوله‌های عراقی در کنار تفاوت‌های جزئی با سویه‌های جهانی ممکن است نشان‌دهنده ویژگی‌های ژنتیکی منطقه‌ای این گونه در عراق باشد.

کلمات کلیدی: شناسایی مولکولی، فیلوژنی، گوسفند، *Chrysomya bezziana*، COI

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