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Molecular identification and phylogenetic characterization of the fig bud mite *Aceria ficus* (Acari: Eriophyidae) from Iraq using mitochondrial COI sequences

Z. A. AL-Tamimi

* Corresponding author. Department of Environment, College of Environmental Sciences, University Al-Qasim Green, Babylon, Iraq. E-mail: zaynabaltimi7@environ.uoqasim.edu.iq

Feryal H. Sadaq

Department of Plant Protection, College of Agricultural Engineering Sciences, University of Baghdad, Baghdad, Iraq. E-mail: feryal.alhasony@coagri.uobaghdad.edu.iq

Abstract

Objective

Aceria ficus (Acari: Eriophyidae), is one of the minute microscopic pests that feed on plant tissues and are characterized by their high specialization and environmental sensitivity. This group is considered among the mite types whose classification is regarded as a challenge because of the great morphological similarity among closely related species, which makes traditional diagnosis ineffective when using morphological traits alone. This study aimed to molecularly identify *Aceria ficus* collected from Iraqi fig orchards using the mitochondrial COI gene, compare the obtained sequences with available GenBank records, and infer their phylogenetic relationships

Materials and methods

During the 2024-2025 seasons, a survey of several fig orchards was conducted in Baghdad (Abu Ghraib) and Babil Province (Al-Kifl and Al-Siyahi) to collect mite samples. Following rigorous morphological identification of the specimens as *Aceria ficus*, genetic and phylogenetic analyses were performed using the Cytochrome c oxidase subunit I (COI) marker.

Results

BLASTn analysis of the amplified COI fragments, utilizing LCO1490/HCO2198 primers, revealed a maximum sequence identity of 82% with Aceria microcis. The relatively low sequence identity (82%) does not provide definitive species-level identification. Instead, it reflects the absence of authenticated COI sequences of *Aceria ficus* in GenBank. Therefore, species identification relied on the combination of morphological examination and molecular

phylogenetic analysis. Consequently, the nucleotide sequences obtained in this study represent the first publicly available COI sequences of *Aceria ficus* deposited in GenBank under accession numbers PV123164-PV123173. Genetic relatedness analysis using MEGA 12 and the Neighbor-Joining (NJ) method placed all nucleotide sequences of the CO1 region from the identified *Aceria ficus* samples in a single cluster, separate from other species, confirming their belonging to the same species

Conclusion

Neighbor-Joining (NJ) phylogenetic analysis conducted in MEGA12 grouped all sequenced samples into a single, highly supported clade, formed a distinct clade separated from the reference sequences included in the present analysis, further corroborating the molecular distinctiveness of the identified populations.

Keywords: *Aceria ficus*, COI gene, fig, molecular diagnosis, phylogenetic analysis

Paper Type: Research Paper.

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Introduction

Fig (*Ficus carica* L.) is considered among the oldest cultivated fruit trees in the world in tropical and subtropical regions, particularly in Iraq and the Middle East, where fig productivity is exposed to multiple pressures from pests and diseases. Among the most important pests is the fig mite *Aceria ficus* (Acari: Eriophyidae), which is one of the minute microscopic pests that feed on plant tissues and are characterized by their high specialization and environmental sensitivity (Daneshnia et al., 2025; Diekmann et al., 2025). This group is considered among the mite types whose classification is regarded as a challenge because of the great morphological similarity among closely related species, which makes traditional diagnosis ineffective when using morphological traits alone (de Lillo et al., 2010; Skoracka et al., 2015). A. ficus causes

deformation of leaves in the form of curling and physiological changes in the vegetative tissues, and this affects plant growth and photosynthesis. It also transmits fig mosaic disease, which belongs to a complex group of viruses whose spread has been recorded in many regions of the Mediterranean basin and West Asia. Many studies indicate the potential role of the eriophyid mite in transmitting these viruses (Elbeaino et al., 2009; Martelli et al., 2022). The European and Mediterranean Plant Protection Organization (EPPO, 2023) has shown the economic importance of this pest, and it has been included among the recorded pests of risk to the health of fig trees. With the rapid development in molecular diagnostic techniques, it has become possible to overcome the morphological difficulties in the classification of eriophyid mites through the use of molecular techniques that depend on the analysis of DNA sequences. Studies have demonstrated that the use of molecular diagnosis together with morphological diagnosis has a role in improving classification accuracy and reducing cases of confusion among closely related species (Barone et al., 2024; Cruickshank, 2002). Among the available genetic markers, the cytochrome oxidase first subunit (COI) gene is the best and most effective choice for studying evolutionary relationships and accurate diagnosis because it is a highly variable genetic marker. It possesses a superior ability to distinguish closely related species and separate genetic lineages that are difficult to differentiate phenotypically, making it the ideal tool for precise molecular analyses at the species level (Dai et al., 2012; Daneshnia et al., 2025; Diekmann et al., 2025). Many previous studies have confirmed the significant effectiveness of cytochrome oxidase subunit (COI) gene sequencing as a genetic barcode in accurately identifying species and reconstructing the evolutionary relationships of closely related lineages within moth populations, thus enabling their reliable and precise placement within the evolutionary tree (Barone et al., 2024). Molecular techniques were applied to assess genetic diversity and taxonomic relationships in mites and ticks, with a focus on the extensive use of specific genomic regions such as mitochondrial DNA and rDNA. Researchers reviewed various tools, including AFLP, RAPD, and microsatellites, along with their respective advantages and disadvantages, to aid in species identification, population structure analysis, and understanding evolutionary relationships. The results demonstrated that these techniques are effective in exploring genetic diversity, particularly in morphologically challenging species. They also helped identify dispersal sources, analyze interspecies relationships, and facilitate classification and identification of new species (Navajas & Fenton, 2000). At the local level, Iraqi studies have addressed many aspects related to fig trees, including the genetic diversity of cultivars using molecular markers (Hussein & Jubrael, 2021), as well as the dynamics of pest spread on fig. However, most of these studies were limited to morphological diagnosis only or inaccurate markers and did not introduce the molecular diagnosis of *Aceria ficus* using ribosomal gene sequencing with phylogenetic analysis and linking it with

the global GenBank database. Many current studies indicate a clear lack of published studies that rely on molecular analysis techniques to confirm the species identity of Iraqi isolates of A. ficus and link them with isolates in the rest of the world (Skoracka et al., 2015). Therefore, this study aimed to provide a precise molecular identification of *Aceria ficus* collected from fig orchards in Iraq using mitochondrial cytochrome c oxidase subunit I (COI) gene sequencing and to investigate its phylogenetic relationship with worldwide isolates available in GenBank.

Materials and methods

During the 2024-2025 seasons, a targeted field survey was conducted in three fig orchards: one located in the Hameed Shaaban area of Abu Ghraib (Baghdad Province), a second in Al-Kifl, and a third in the Al-Siyahi area (Babil Province). In each orchard, three trees exhibiting the highest levels of infestation were specifically selected for sampling. From each selected tree, 20 leaves displaying characteristic symptoms of eriophyid mite infestation-such as leaf curling and physiological deformation-were randomly collected from the four cardinal directions of the canopy. The collected leaf samples were immediately placed in plastic bags, properly labeled with the collection date and specific location, and transported to the laboratory in cooled containers to preserve the specimens. In the laboratory, the leaves were examined under a stereomicroscope. A pooled sample of approximately 30 adult mites per experimental replicate was meticulously isolated using a fine needle for both morphological and molecular diagnosis. The precise morphological identification of the specimens was confirmed by Dr. Parisa Lotfollah (Shahid Madani University of Azerbaijan). Following identification, the isolated mites designated for molecular analysis were immediately preserved in Eppendorf tubes containing 99% absolute ethanol to prevent DNA degradation and stored at -20°C until DNA extraction.

Extraction of genomic DNA: Total genomic DNA was extracted using a modified CTAB protocol based on Folmer et al. (1994) with making some modifications suitable to the nature of the sample. Thirty adult mites were placed in a 1.5 mL centrifuge tube having 100 µL absolute ethanol. It was then centrifuged at 8000 rpm for 15 minutes, and the ethanol was allowed to evaporate completely. Afterwards, 2-3 glass beads were added to each tube, then the samples were exposed to liquid nitrogen for 2-3 minutes, and then ground using a mechanical grinder. 500 µL of CTAB buffer solution pre-heating was added for to the samples, then incubated at 65°C in a water bath for 60 minutes. Centrifugation was then done at 12,000 rpm for 10 minutes and the clear phase was carefully removed to a new sterile tube. An equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged for 10 minutes at the same speed. The aqueous phase was gently transferred to a clean tube and 200 µL of isopropanol added. Incubation at −20 °C for 1 hour was performed to precipitate the DNA. The top phase was discarded, the pellet washed

with 70% ethanol and air dried. The pellet was suspended in 15-20 µL of sterile distilled water and kept at −20°C until use. Given the extremely minute size of the specimens (30 mites) and the resulting low DNA yields, successful PCR amplification and subsequent high-quality Sanger sequencing were utilized as the primary and most reliable indicators of adequate DNA quality and purity for downstream analyses. The primers specific for amplification of the mitochondrial DNA region were selected depending on Hebert et al. (2003) (Table 1).

Table 1. PCR primers used for amplification of the mitochondrial cytochrome c oxidase subunit I (COI) gene

Primer name	Primer sequence (5′-3′)	Target gene
LCO1490	GGTCAACAAATCATAAAGATATTGG	Mitochondrial cytochrome c oxidase subunit I (COI)
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Mitochondrial cytochrome c oxidase subunit I (COI)

Polymerase chain reaction (PCR): The PCR reaction mixture was prepared according to the concentrations and volumes shown in Table 2. All reaction components were mixed except for the template DNA, then a brief centrifugation was performed for 2-3 minutes. Subsequently, 24 µL of the mixture was distributed into sterile PCR tubes, and 1 µL of template DNA was added to each tube, bringing the final reaction volume to 25 microliters. Conventional polymerase chain reaction (PCR) was performed to amplify the genomic barcode region of the cytochrome oxidase subunit 1 (COI) gene using specific primers. The reaction was carried out in a thermal cycler according to the described thermal program shown in Table 3. The reaction products were detected using the electrophoresis technique on a 1.2% agarose gel, prepared using TAE buffer solution (Tris-Acetic acid-EDTA). TAE solution was prepared at a 10× concentration, then diluted to a 1× concentration before use. For a 1.2% agarose gel preparation, 0.6 g of agarose powder and 50 mL of 1× TAE buffer solution was placed in a conical flask, and heated until completely dissolved, then left to cool to a suitable temperature before adding 15 microliters of Safe stain dye, mixed well before pouring the gel into the mold. After the gel had solidified, it was placed in the electrophoresis tank, and 5 microliters of the PCR product mixed with 2 microliters of loading dye were loaded, along with a standard DNA ladder (100 bp DNA ladder) to estimate molecular sizes. Electrophoresis was carried out at a constant voltage of 100 volts for one hour. Following the completion of the run, the bands were recorded using a Gel Documentation System.

Nucleotide sequencing: The PCR products were sent to a specialized company in Korea (Bioneer) for nucleotide sequencing. Sequencing was performed in both directions (forward and reverse) using the same primers employed in the amplification process.

Table 2. Composition of the PCR reaction mixture

PCR reaction component	Volume (µL)
Sterile distilled water	16.0
DNA template	1.0
10× PCR buffer	2.5
dNTP mix (10 mM each)	1.0
Forward primer (10 µM)	1.5
Reverse primer (10 µM)	1.5
$MgCl_2$	1.5
Taq DNA polymerase	0.2
Total reaction volume	25.0

Table 3. Thermal cycling conditions for polymerase chain reaction (PCR) amplification

Step	Temperature (°C)	Time	Number of Cycles
Initial denaturation	95	3 min	1
Denaturation	94	30 s	35
Annealing	55	30 s	35
Extension	72	1 min	35
Final extension	72	5 min	1

Genetic and phylogenetic analysis: Genetic and phylogenetic analysis was carried out based on CO1gene sequences. The study included the sequences obtained in this study as well as reference sequences retrieved from the public GenBank database.

Results and discussion

The results of the nucleotide analysis using the Blastn software tool within the NCBI website showed that the nucleotide sequences under study (ZF1-ZF10) resulting from the amplified DNA fragments from the mite samples using the primer set (LCO1490/HCO2198) are the closest to the genus of the eriophyid mite Aceria, achieving the highest nucleotide match percentage of 82% with the gene cytochrome c oxidase subunit I (CO1) in species belonging to this genus. The closest species was *A. microcis*, which confirms the morphological diagnosis. The used primer set was able to amplify the target fragment (Folmer et al., 1994; Chetverikov et al., 2015). Figure 1 shows the results of agarose gel electrophoresis of approximately 600-bp PCR amplicons

corresponding to the mitochondrial cytochrome c oxidase subunit I (COI) gene, amplified using the primer pair LCO1490/HCO2198 from *Aceria ficus* eriophyid mite samples collected from fig plants.

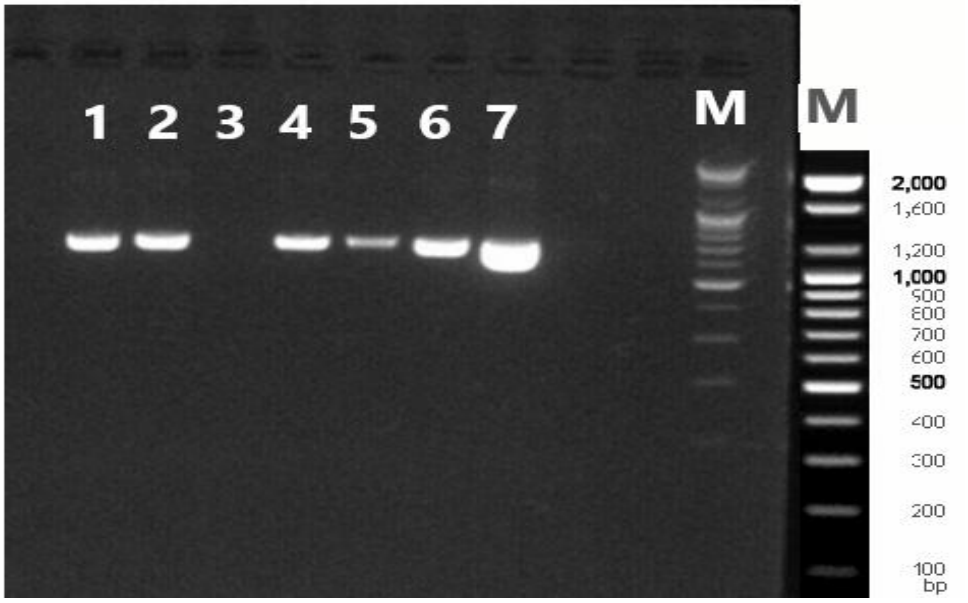

Figure 1. Agarose gel electrophoresis of PCR products (~600 bp) amplified from *Aceria ficus* eriophyid mite samples collected from fig plants using the primer pair LCO1490/HCO2198 targeting the mitochondrial cytochrome c oxidase subunit I (COI) gene. Lanes 1-7: *A. ficus* samples; lane M: 100-bp DNA ladder (Bioneer, South Korea)

Cytochrome c oxidase subunit I (CO1) gene region of eriophyid mite species infecting various economically important plants in many countries of the world (Inak et al., 2021). The deposition of the nucleotide sequences of the diagnosed samples for the cytochrome c oxidase subunit gene region in NCBI under accession numbers (PV123164-PV123173) within the species *Aceria ficus* was accepted. Analysis of the nucleotide sequence data using the SDT v.1.3 program for the DNA fragments amplified from the cytochrome c oxidase subunit gene region by the primer set (LCO1490/HCO2198). It also proved that all mite samples under study belong to the same species, with a nucleotide match percentage of 100% (Figure 2) (Muhire et al., 2014; Muhire et al., 2025). Genetic relatedness analysis using MEGA 12 and the Neighbor-Joining (NJ) method placed all nucleotide sequences of the CO1 region from the identified *Aceria ficus* samples in a single cluster, separate from other species, confirming that all sampled individuals belong to a single species (Figure 3). *A. microcis* was the closest to the isolated nucleotide sequences, indicating that the studied species belongs to the genus Aceria but represents a distinct species.

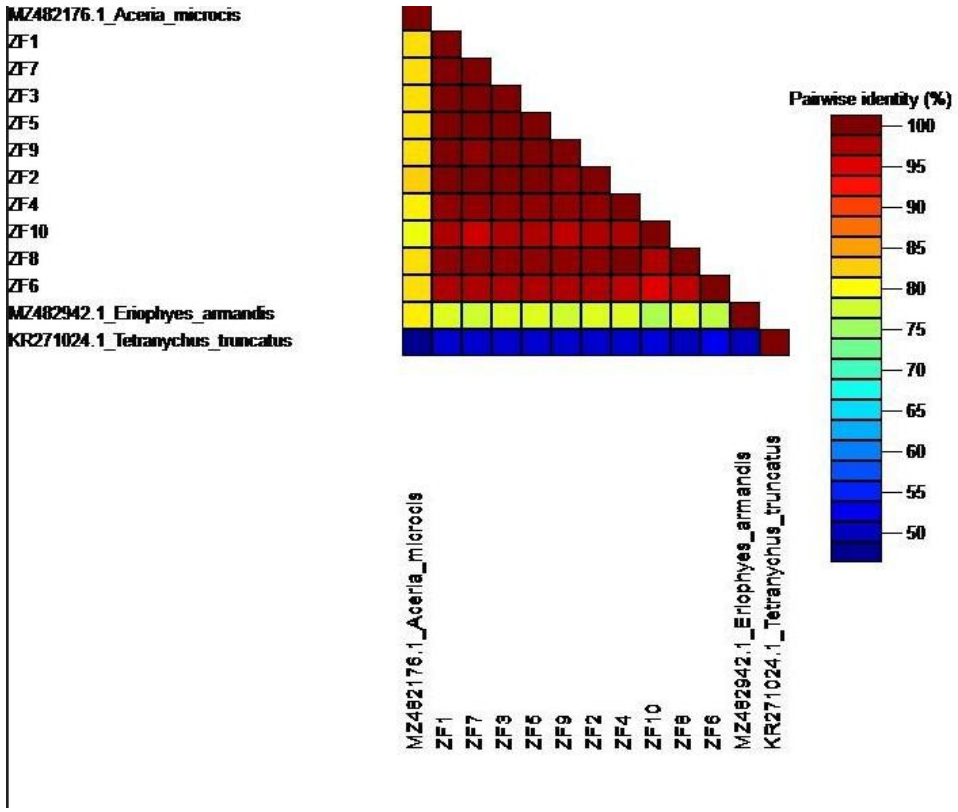

Figure 2. Color-coded alignment of partial mitochondrial cytochrome c oxidase subunit I (COI) gene sequences from Iraqi *Aceria ficus* samples collected from fig orchards (ZF1-ZF10). The sequence of *Aceria microcis* (GenBank accession no. MZ482176), representing the closest match retrieved from NCBI, was included for comparison. *Eriophyes armandis* (MZ482942) and *Tetranychus truncatus* (KR271024) were used as outgroup species

The nucleotide sequence of the CO1 region allows differentiation between species of the Eriophyidae family and determination of their genetic origins and relatedness (Stecher et al., 2026; Zhang et al., 2024).

Conclusion: The present study is the first study on Iraqi populations of *Aceria ficus* to use mitochondrial COI sequences for their molecular characterization. The sequences reported by this study are the first publicly available COI records for this Aceria ficus. These results and the sequences provided will provide a valuable reference for future molecular identification and phylogenetic studies of eriophyid mites.

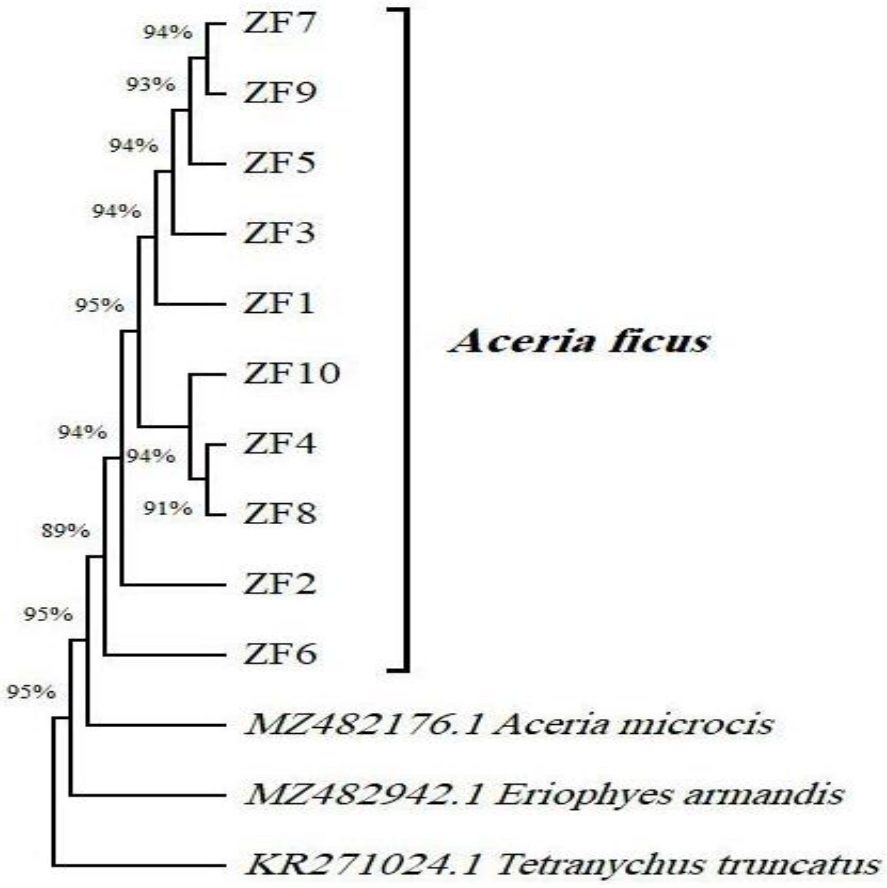

Figure 3. Neighbor-Joining (NJ) phylogenetic tree inferred from partial mitochondrial cytochrome c oxidase subunit I (COI) gene sequences of Iraqi *Aceria ficus* eriophyid mite samples collected from fig orchards (ZF1-ZF10). The sequence of *Aceria microcis* (GenBank accession no. MZ482176), the closest matching species retrieved from NCBI, was included for comparison. *Eriophyes armandis* (MZ482942) and *Tetranychus truncatus* (KR271024) were used as outgroup taxa. Bootstrap values (1,000 replicates) are shown at the nodes

Author contribution

Zaynab A. AL - Tamimi: Conceptualization, Methodology and Writing-Original Draft Preparation. Sadaq H. Feryal: Software, Data Curation and Supervision, Investigation, Writing-Review & Editing.

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Ethical considerations

Not applicable. This study focused on agricultural pests (eriophyid mites) and did not involve human subjects or animal experiments that require ethical approval.

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Conflict of interests

The authors declare no conflict of interest.

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شناسایی مولکولی و ویژگی‌یابی فیلوژنتیکی کنه جوانه انجیر *Aceria ficus* (Acari: Eriophyidae) از عراق با استفاده از توالی‌های میتوکندریایی ژن COI

ز. ا. التمیمی

* نویسنده مسئول. گروه محیط‌زیست، دانشکده علوم محیط‌زیست، دانشگاه سبز القاسم، بابل، عراق. ایمیل: zaynabaltimi7@environ.uoqasim.edu.iq

فریال ح. صداق

گروه حفاظت گیاه، دانشکده علوم مهندسی کشاورزی، دانشگاه بغداد، بغداد، عراق. ایمیل: feryal.alhasony@coagri.uobaghdad.edu.iq

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چکیده

هدف: کنه *Aceria ficus* (Acari: Eriophyidae) یکی از آفات میکروسکوپی بسیار ریز است که از بافت‌های گیاهی تغذیه می‌کند و به دلیل اختصاصی بودن میزبان و حساسیت زیاد به شرایط محیطی شناخته می‌شود. این گروه از کنه‌ها به دلیل شباهت ریخت‌شناختی بسیار زیاد میان گونه‌های نزدیک به هم، از دشوارترین گروه‌ها برای رده‌بندی محسوب می‌شوند؛ به‌گونه‌ای که تشخیص سنتی بر پایه ویژگی‌های مورفولوژیک به‌تنهایی اغلب قابل اعتماد نیست. هدف این مطالعه، شناسایی مولکولی *Aceria ficus* جمع‌آوری‌شده از باغ‌های انجیر عراق با استفاده از ژن میتوکندریایی سیتوکروم c اکسیداز زیرواحد I (COI)، مقایسه توالی‌های به‌دست‌آمده با داده‌های موجود در پایگاه GenBank و بررسی روابط فیلوژنتیکی آن‌ها بود.

مواد و روش‌ها: در طی فصل‌های زراعی ۲۰۲۴-۲۰۲۵، از چندین باغ انجیر در بغداد (ابوغریب) و استان بابل (الکفل و السیاحی) نمونه‌برداری انجام شد و نمونه‌های کنه جمع‌آوری گردید. پس از شناسایی دقیق ریخت‌شناختی نمونه‌ها به‌عنوان *Aceria ficus*، مطالعات ژنتیکی و فیلوژنتیکی با استفاده از نشانگر سیتوکروم c اکسیداز زیرواحد I (COI) انجام شد.

نتایج: نتایج تحلیل BLASTn بر روی قطعات تکثیرشده ژن COI با استفاده از آغازگرهای LCO1490/HCO2198 نشان داد که بیشترین میزان همانندی توالی (۸۲ درصد) با گونه *Aceria microcis* وجود دارد. این میزان نسبتاً پایین همانندی، امکان شناسایی قطعی گونه را تنها بر اساس داده‌های توالی فراهم نمی‌کند و عمدتاً ناشی از نبود توالی‌های تأییدشده ژن COI مربوط به

Aceria ficus در پایگاه GenBank است. ازاین‌رو، شناسایی گونه بر اساس تلفیق ویژگی‌های ریخت‌شناختی و تحلیل فیلوژنتیکی مولکولی انجام شد. در نتیجه، توالی‌های نوکلئوتیدی به‌دست‌آمده در این پژوهش به‌عنوان نخستین توالی‌های عمومی ژن COI مربوط به Aceria ficus در پایگاه GenBank با شماره‌های دسترسی PV123164 تا PV123173 ثبت شدند. تحلیل خویشاوندی ژنتیکی با استفاده از نرم‌افزار MEGA 12 و روش Neighbor-Joining (NJ) نشان داد که تمامی توالی‌های ناحیه COI مربوط به نمونه‌های شناسایی‌شده *Aceria ficus* در یک خوشه (Clade) واحد و مجزا از سایر گونه‌ها قرار گرفتند که تعلق همه نمونه‌ها به یک گونه را تأیید می‌کند.

نتیجه‌گیری: تحلیل فیلوژنتیکی با روش Neighbor-Joining (NJ) در نرم‌افزار MEGA 12 تمامی نمونه‌های تعیین توالی‌شده را در یک شاخه تکاملی (Clade) واحد با پشتیبانی آماری بالا گروه‌بندی کرد. این شاخه به‌طور مشخص از توالی‌های مرجع موجود در تحلیل حاضر متمایز بود و بدین ترتیب، تمایز مولکولی جمعیت‌های شناسایی‌شده *Aceria ficus* را به‌خوبی تأیید کرد.

کلمات کلیدی: انجیر، تحلیل فیلوژنتیکی، تشخیص مولکولی، ژن COI، *Aceria ficus*

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