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Molecular diagnosis and genetic variation of the Jujube Blue Butterfly (*Tarucus spp*.) in Misan province, southern Iraq

Ayat A. Eidan

Department of Plant Protection, College of Agriculture, University of Misan, Amarah, Misan 62001, Iraq. E-mail: stuom88@uomisan.edu.iq

Ali H. Abu-Ragheef

*Corresponding author. Department of Plant Protection, College of Agriculture, University of Misan, Amarah, Misan 62001, Iraq. E-mail: ali.h.h@uomisan.edu.iq

Zaidon T. Al-Aqbi

Department of Chemistry, College of Science, University of Misan, Amarah 62001, Misan, Iraq. E-mail: zthal@utas.edu.au

Abstract

Objective

The Jujube Blue butterfly, *Tarucus theophrastus*, is one of the significant insect pests affecting *Ziziphus* trees in tropical regions. Traditional identification of these insects has relied on morphological description, focusing on wing spot patterns and genital structures to distinguish species. The current work sought to perform an accurate genetic diagnosis of this species in Misan Province since the phenomena of cryptic species poses difficulties for standard morphological identification.

Materials and methods

Samples of the Jujube Blue butterfly were collected from *Ziziphus* spp, trees distributed across Al-Kahla district, Al-Maimouna district, and the Al-Tabar area in Maysan Province, southern Iraq. The Global Positioning System (GPS) was used to determine the geographic locations for three different areas in Maysan Province in order to spatially document the study's collection stations and guarantee the accuracy of returning to them in the future. The methodology included DNA extraction and amplification of the Cytochrome Oxidase I (*COI*) gene using universal primers (LCO1490 and HCO2198), where gel electrophoresis revealed clear genetic bands at a molecular size of 700 bp.

Results

The Polymerase Chain Reaction (PCR) products' gel electrophoresis showed distinct genetic bands for the Cytochrome Oxidase I (*COI*) gene at the anticipated molecular size of around 700 bp. The results of the genetic sequence analysis and their alignment in the global GenBank revealed a 100% genetic identity with the reference sample (ON436886.1). The local samples were officially registered in the DNA Data Bank of Japan (DDBJ) under accession numbers LC913553, LC913554, and LC913555. Furthermore, the phylogenetic tree designed using the Maximum Likelihood method implemented in MEGA11 software showed complete genetic proximity between the study samples and the documented global strains.

Conclusions

This study is significant because to the best of our knowledge, this is among the first molecular studies of this species in Misan Province. It also adds new strains to the national genetic database, which helps to improve integrated control programs and comprehend the genetic variation of the insect in Iraq.

Keywords: *COI* gene, Cryptic species, Misan Province, *Tarucus theophrastus*

Paper Type: Research Paper.

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Introduction

Butterflies belonging to the genus *Tarucus*, locally known as the "Jujube Blue," are significant insect pests widespread in tropical and subtropical regions, where their larvae cause severe damage to *Ziziphus* trees through voracious feeding on leaves and growing tips (Tamura et al., 2021). A thin white membrane that represents the top epidermis is left behind after the insect larvae scrape the bottom surface of the leaves. As the larvae mature, the infestation usually starts off as narrow strips along the bottom leaf surface and grows longer and wider. As a result, where bigger larvae eat the entire bottom leaf surface, the damage becomes more severe.

According to survey research, these larvae can totally defoliate shrubs during severe infestations, which could worsen the health of young plants (Al Amery et al., 2021). Traditional identification of these insects has relied on morphological description, focusing on wing spot patterns and genital structures to distinguish species (Basu et al., 2019). Although many specialists still rely on morphological description as a primary and rapid tool, this reliance has faced widespread criticism due to the external similarity of species to the point of identity, a phenomenon known as cryptic species. This makes morphological diagnosis alone insufficient and prone to taxonomic errors (Hausberger et al., 2011; Pyrcz et al., 2013). On the other hand, genetic variety is crucial for promoting the development of more sophisticated genes, safeguarding existing populations, advancing evolutionary processes, and enabling adaptation to changing conditions in the natural environment (Mohammadabadi et al., 2021a). Conversely, the molecular identification of organisms is crucial in their studying (Farahvashi, 2026a; Saadatabadi et al., 2023). Moreover, the study of populations and breeds, using molecular techniques is very important and useful for their characterization (Mohammadifar and Mohammadabadi, 2017; Noori et al., 2017). Conservation of genetic diversity requires the proper performance of conservation superiorities and sustainable handling plans that should be based on universal information on population structures, including genetic diversity resources among and between populations and breeds (Mohammadifar & Mohammadabadi, 2018; Mohammadabadi et al., 2024). Genetic variation is an essential element for genetic improvement, preserving populations, evolution and adapting to variable environmental situations (Farahvashi et al., 2026b; Arabpour et al., 2021). On the other hand, determination of variation is important in characterization of various populations (Pakgohar et al., 2026; Khezri et al., 2025) in order to define genotypes of individuals and their associations with different vital aspects of their life (Mohammadabadi et al., 2021b). Moreover, as a result of this scientific debate between proponents of morphological description and supporters of modern techniques, studies have moved toward molecular identification using DNA techniques as a superior and more accurate option for definitive results (Ashfaq et al., 2022). Researchers argue that relying on genes, particularly the Cytochrome Oxidase I (COI) gene, provides a stable "genetic fingerprint" unaffected by environmental variables or minor morphological differences that might mislead taxonomists (Hebert et al., 2003). Therefore, the use of genetic analysis has recently become an indispensable necessity for accurately determining the correct scientific name, especially when morphological traits overlap among sibling species within the genus *Tarucus* (Kanyi et al., 2021). Integrating molecular data with morphological description contributes to building a robust genetic database that ensures the reliability of results when deposited in international data centers such as NCBI (Qraidi et al., 2025). The Iraqi environment possesses distinct climatic conditions and diverse vegetation cover, making it an ideal environment for the

presence and spread of *Ziziphus* pests across various provinces (Hausberger et al., 2011). To the best of our knowledge, this is among the first molecular studies conducted on this species in Misan Province, thus filling the knowledge gap regarding the genetic diversity of this insect and its alignment with globally registered strains. The present study aimed to use DNA methods for molecular diagnosis and genetic analysis of the Jujube Blue butterfly in Misan Province in order to guarantee diagnostic accuracy and ascertain the genetic fingerprint of local samples. Additionally, it seeks to add their sequences to the worldwide GenBank so that they may be used as a reference for upcoming research on biodiversity conservation and pest management.

Materials and methods

Sample collection: Samples of the Jujube Blue butterfly, *Tarucus* spp., were collected from *Ziziphus* spp. trees distributed across Al-Kahla district, Al-Maimouna district, and the Al-Tabar area in Maysan Province, southern Iraq, using hand nets, The samples were morphologically identified using taxonomic keys (Chen et al., 2023; Basu et al., 2019), Morphological characteristics showed a high degree of similarity between the two species, *T. rosacea* and T. theophrastus, as shown in Figure 1, which led to uncertainty in species identification based solely on morphological traits. Therefore, the Global Positioning System (GPS) was used to determine the geographic locations for three different areas in Maysan Province in order to spatially document the study's collection stations and guarantee the accuracy of returning to them in the future. Table 1 shows the geographic details of these stations.

Figure 1. Jujube blue butterfly (*Tarucus* spp.) female and male

Table 1. The geographic coordinates (GPS) of the Jujube Blue butterfly sample collecting locations in Misan province

Station No.	Area Name	Latitude	Longitude
First Station	Al-Tabar Village (Orchard)	31.756772 N	47.123634 E
Second Station	Al-Maimouna District	31.674400 N	46.974400 E
Third Station	Al-Kahla District	31.670123 N	47.164321 E

Consequently, molecular diagnosis was relied upon as the decisive tool to confirm the taxonomic identity of the studied samples. Some insect specimens were preserved directly in clean, sterile nylon bags, labeled with the collection site and date. The samples were then transported to the laboratory and stored in a deep freezer at -20°C to prevent tissue degradation and maintain the integrity of the genetic material until the DNA extraction process began.

DNA extraction: DNA was extracted from insect samples using Gsync™ DNA Extraction Kit (Geneaid, Company), following the specific protocol for insect tissues. The process involved taking 35-50 mg from the head and thorax regions and grinding them with a ceramic mortar while adding liquid nitrogen to ensure the tissues were converted into a fine powder and to protect the genetic material from degradation. The extraction buffer (GST) was added, and the mixture was transferred to 1.5 mL Eppendorf tubes with the addition of 20 µL of Proteinase K enzyme (20 mg/mL). The samples were then incubated in a water bath at 60°C for two hours to ensure complete tissue lysis. For DNA purification, GSB buffer was added with incubation for 10 minutes, followed by the addition of 100% absolute ethanol to precipitate the DNA. The samples were passed through filtration columns (GS column) using a centrifuge at speeds reaching (14,000 rpm), with repeated washes using wash buffers (1 & 2) to remove impurities and suspended proteins. In the final stage, pure DNA was recovered by adding 100 µL of Elution Buffer, pre-incubated at 60°C. To verify extraction efficiency and the integrity of the genetic bands, the samples were examined using gel electrophoresis on a 1% agarose gel.

PCR amplification: The Polymerase Chain Reaction (PCR) method was used to amplify the Cytochrome Oxidase I (COI) gene in order to identify genetic species. Specialized primers were employed in this procedure, such as the reverse primer (HCO2198) and the forward primer (LCO1490), as shown in Table 2.

PCR Master Mix preparation: The Master Mix was prepared with a final volume of 25 µL per sample. The mixture included essential components such as the DNA template, specific primers, and sterile distilled water, according to the volumes specified in Table 3.

Table 2. Universal primer sequences used for COI gene amplification according to (Salis et al., 2024)

Primers	Sequence (5'-3')	Product Size	Annealing Temp (Ta)
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	700 bp	50 °C
LCO1490	GGTCAACAAATCATAAAGATATTGG		

Table 3. PCR Master Mix components and their respective volumes used in the study

Chemical Substance	Master Mix	DNA Template	Primers (Forward / Reverse)	Distilled Water	Final Volume
Volume (µL)	12.5	3	1/1	7.5	25

Thermal cycling program: Using a thermal cycler and a particular thermal program for this gene, the amplification procedure was carried out. To guarantee precise amplification of the target genetic segment, the program comprised denaturation, annealing, and extension steps, as shown in Table 4.

Table 4. Thermal cycler program conditions for COI gene amplification

Stage	Temperature (°C)	Time (min/sec)	No. of Cycles
Initial Denaturation	95	4 min	1
Denaturation	95	0.30 sec	
Annealing	50	0.45 sec	35
Extension	72	1.00 min	
Final Extension	72	7 min	1

DNA sequencing and molecular identification: To verify the efficiency and purity of the extracted DNA, gel electrophoresis was performed on a 1% agarose gel. The results showed clear and high-density genetic bands, confirming the acquisition of sufficient concentration and excellent purity to initiate the PCR amplification. Subsequently, the purified PCR products were sent to Macrogen company in South Korea for DNA sequencing using an automated genetic analyzer.

Sequence analysis and molecular identification: The raw data received from the company had an approximate length of 800 bp. Trimming and editing processes were performed to remove noise and correct readings from the ends using BioEdit software (Version 7.2.5), Consequently, the final length of the sequences used in the study was 513 bp. As after trimming low-quality

ends, a consensus sequence of 513 bp was retained for downstream analyses. These sequences were compared using the BLAST search tool available at the National Center for Biotechnology Information (NCBI). The results showed 100% genetic identity with the reference sample under the accession number (ON436886.1).

Results and discussion

The current study's findings showed that the "Jujube Blue" butterfly samples taken from Misan Province were successfully identified molecularly. The Polymerase Chain Reaction (PCR) products' gel electrophoresis showed distinct genetic bands for the Cytochrome Oxidase I (*COI*) gene at the anticipated molecular size of around 700 bp. This demonstrates the effectiveness of the genetic material extraction process and the accuracy of the primers (LCO1490 and HCO2198) chosen for amplification, as shown in Figure 2.

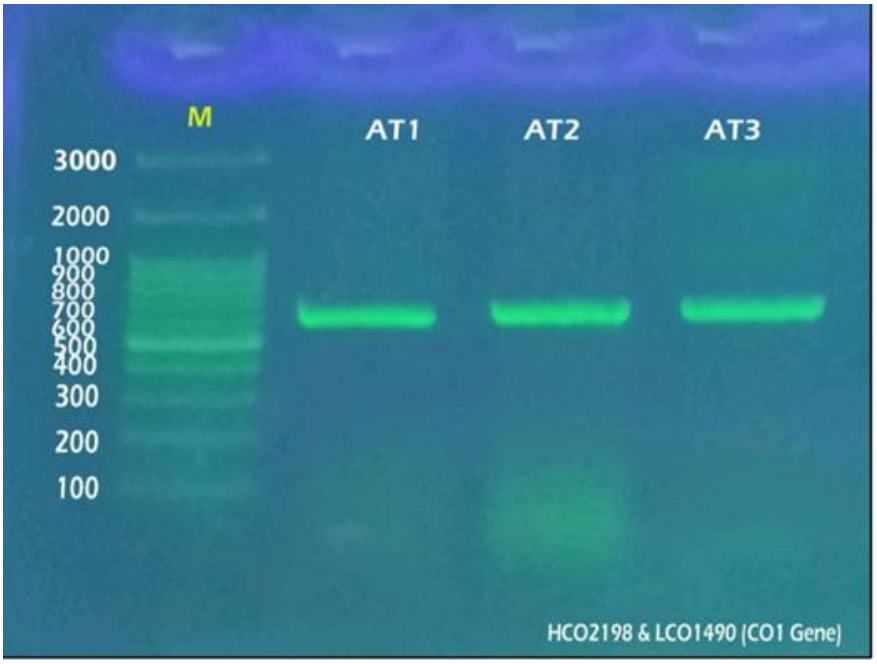

Figure 2. Gel electrophoresis of PCR products for the Cytochrome Oxidase I (COI) gene

DNA sequencing and bioinformatic analysis: DNA sequencing was performed at Macrogen Inc. (Seoul, South Korea) using the ABI 3730XL Genetic Analyzer. The obtained sequences were compared with global reference data deposited in the GenBank database (NCBI) using the Basic Local Alignment Search Tool (BLAST). Regarding the sequence length, the raw data were received with lengths reaching up to 800 bp. Trimming and editing processes were subsequently conducted to remove noise and correct the terminal readings to ensure high-quality data for further analysis.

Sequence analysis and genetic alignment: Using BioEdit software (Version 7.2.5), the final length of the sequences used in the study was trimmed to 513 bp. The alignment results demonstrated 100% genetic identity with the global reference strain registered under accession number (ON436886.1). This complete match reflects significant genetic stability of the species within the region, as shown in Table 5.

Table 5. Genetic alignment results of local samples with global isolates in GenBank, showing identity percentage and query coverage

Sample ID	Organism Used	Global Accession No	Identity Percentage	Query Coverage	DDBJ Accession No. (Our Samples)
AT1	*Tarucus theophrastus*	ON436886.1	100%	100%	LC913553
AT2	*Tarucus theophrastus*	ON436886.1	100%	100%	LC913554
AT3	*Tarucus theophrastus*	ON436886.1	100%	100%	LC913555

Phylogenetic analysis: The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Maximum Likelihood method implemented in MEGA11 software were used to create a phylogenetic tree (Tamura et al., 2021). The results showed the genetic closeness of the local samples, which grouped together in a single genetic clade with strains that have been described worldwide, as shown in Figure 3. When compared to depending only on morphological description, this clustering improves the accuracy of molecular identification.

Conclusions: The three local samples were successfully genetically documented in this investigation, and their sequences were deposited into the DNA Data Bank of Japan (DDBJ) with accession numbers LC913553, LC913554, and LC913555. These findings show that COI-based DNA barcoding is considered a reliable and widely used tool for species identification for settling scientific disputes over the precise identification and characterization of insect pests is the use of the COI gene "DNA Barcoding." Therefore, in order to guarantee the accuracy of comparative identification, we advise researchers to use DNA barcoding techniques in future surveys and environmental investigations.

Author contributions

Conceptualization: A. A. E. and A. H. A. R.; methodology: A. A. E. ; software: A. A. E. ; validation: A. A. E. , A. H. A. R. and Z. T. A. A.; formal analysis: A. A. E. ; investigation: A. A. E. ; resources: A. A. E. ; data curation: A. A. E. ; writing-original draft preparation: A. A. E. ; writing-review and editing: A. A. E. ; visualization: A. A. E. ; supervision: A. A. E. ; project

administration: A. A. E. ; funding acquisition: A. H. A. R. All authors have read and agreed to the published version of the manuscript.

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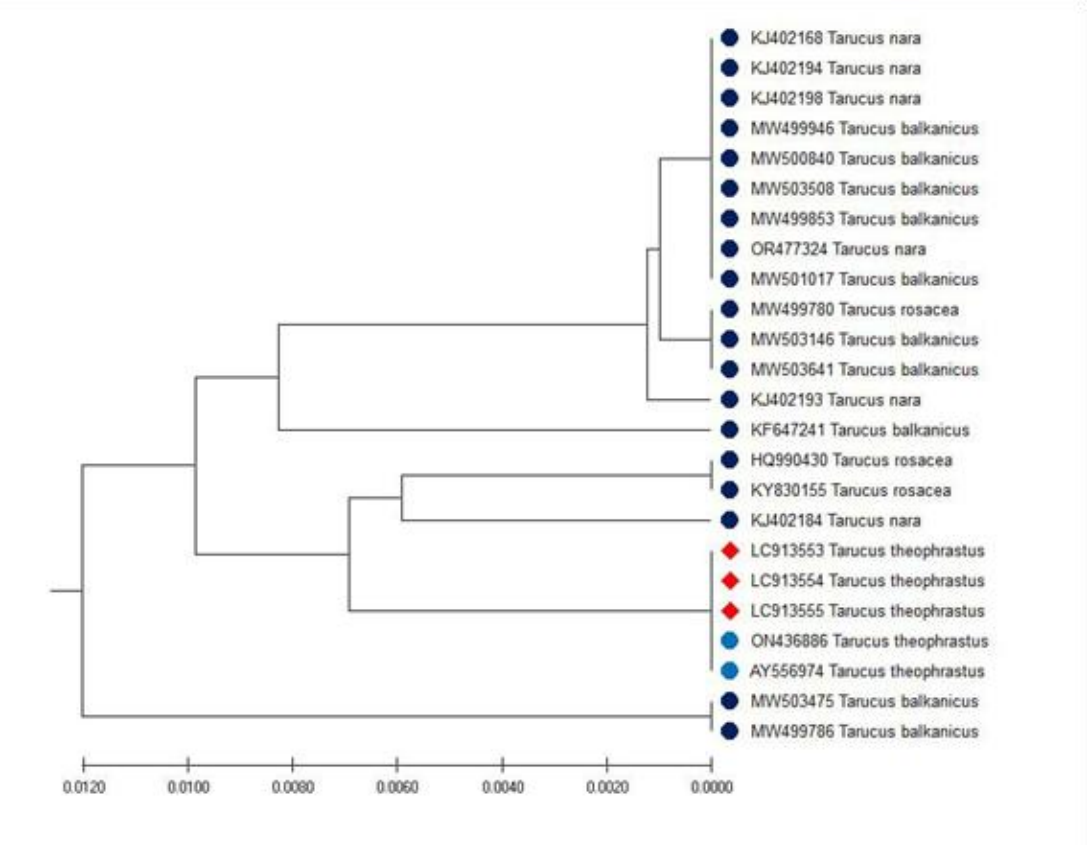

Figure 3. A phylogenetic tree created with the UPGMA technique and MEGA11 software. The correctness of the molecular identification is confirmed by the local samples' apparent clustering in a single genetic clade with the global strain (ON436886.1). The genetic stability of the species *Tarucus theophrastus* within the study region is further demonstrated by the branching from the outgroups. Bootstrap = 1000 replicates. Kimura 2-parameter distance

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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

The authors avoided data fabrication, falsification, plagiarism, and misconduct.

Conflict of interest

The authors declare no conflict of interest regarding the publication of this paper.

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تشخیص مولکولی و تنوع ژنتیکی پروانه آبی کنار (*Tarucus spp.*) در استان میسان، جنوب عراق

آیات ع. عیدان

گروه حفاظت گیاه، دانشکده کشاورزی، دانشگاه میسان، العماره، میسان ۶۲۰۰۱، عراق. ایمیل: stuom88@uomisan.edu.iq

علی ه. ابورغیف

* نویسنده مسئول. گروه حفاظت گیاه، دانشکده کشاورزی، دانشگاه میسان، العماره، میسان ۶۲۰۰۱، عراق. ایمیل: ali.h.h@uomisan.edu.iq

زیدون ط. العقیبی

گروه شیمی، دانشکده علوم، دانشگاه میسان، العماره ۶۲۰۰۱، میسان، عراق. ایمیل: zthal@utas.edu.au

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چکیده

هدف: پروانه آبی کنار (*Tarucus theophrastus*) یکی از آفات مهم درختان کنار (*Ziziphus*) در مناطق گرمسیری به شمار می‌رود. شناسایی سنتی این حشرات بر اساس ویژگی‌های مورفولوژیک، به‌ویژه الگوهای لکه‌های بال و ساختار اندام‌های تناسلی، برای تفکیک گونه‌ها انجام می‌شود. مطالعه حاضر با هدف انجام تشخیص ژنتیکی دقیق این گونه در استان میسان انجام شد، زیرا وجود گونه‌های پنهان (Cryptic species) شناسایی مورفولوژیک استاندارد را با دشواری مواجه می‌سازد.

مواد و روش‌ها: نمونه‌های پرولنه آبی کنار از درختان *Ziziphus spp.* در مناطق الکحلاء، المیمونه و منطقه الطبار در استان میسان، جنوب عراق جمع‌آوری شدند. برای تعیین موقعیت جغرافیایی مناطق نمونه‌برداری و ثبت مکانی ایستگاه‌های جمع‌آوری از سیستم موقعیت‌یاب جهانی (GPS) استفاده شد تا امکان بازگشت دقیق به این مناطق در آینده فراهم گردد. روش تحقیق شامل استخراج DNA و تکثیر ژن سیتوکروم اکسیداز I (COI) با استفاده از آغازگرهای عمومی LCO1490) و HCO2198) بود. نتایج الکتروفورز ژل وجود باندهای ژنتیکی واضح با اندازه مولکولی حدود ۷۰۰ جفت باز را نشان داد.

نتایج: الکتروفورز محصولات واکنش زنجیره‌ای پلیمراز (PCR) باندهای ژنتیکی مشخصی برای ژن COI در اندازه مورد انتظار حدود ۷۰۰ جفت باز نشـــان داد. نتایج تحلیل توالی ژنی و هم‌ترازی آن‌ها با پایگاه جهانی GenBank، تطابق ژنتیکی ۱۰۰٪ با نمونه مرجع (ON436886.1) را آشـکار کرد. نمونه‌های محلی به‌طور رسـمی در بانک اطلاعات DNA ژاپن (DDBJ) تحت شـماره‌های دسـترسـی LC913553، LC913554 و LC913555 ثبت شـدند. همچنین، درخت فیلوژنتیکی طراحی‌شـده با اسـتفاده از روش روش حداکثر احتمال در نرم‌افزار MEGA11 نزدیکی ژنتیکی کامل میان نمونه‌های مورد مطالعه و سـویه‌های جهانی ثبت‌شده را نشان داد.

نتیجه‌گیری: این مطالعه از اهمیت ویژه‌ای برخوردار اسـت، زیرا بر اسـاس دانش ما این پژوهش یکی از نخسـتین مسـتندسـازی مولکولی و اطلاعاتی این گونه در اسـتان میسـان محسـوب می‌شـود. همچنین، افزودن سـویه‌های جدید به پایگاه داده ژنتیکی ملی می‌تواند به بهبود برنامه‌های کنترل تلفیقی و درک بهتر تنوع ژنتیکی این حشره در عراق کمک کند.

کلمات کلیدی: استان میسان، ژن COI، گونه های رمزآلود، *Tarucus theophrastus*

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