



Available Online at EScience Press

Plant Protection

ISSN: 2617-1287 (Online), 2617-1279 (Print)
http://esciencepress.net/journals/PP

Research Article

First Molecular Record of *Hydrotaea ignava* and *H. leucostoma* (Diptera: Muscidae) Associated with Palm Rhinoceros Beetle (*Oryctes elegans*) Larvae in Iraq

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ARTICLE INFO

Article history

Received: 24th February, 2026

Revised: 26th May, 2026

Accepted: 29th May, 2026

Keywords

Hydrotaea ignava

Hydrotaea leucostoma

Oryctes elegans

DNA barcoding (COI)

Phylogenetic analysis

ABSTRACT

The palm rhinoceros beetle, *Oryctes elegans* Prell (Coleoptera: Scarabaeidae), is a major pest of date palm in the Middle East. During laboratory rearing of *O. elegans* larvae collected from infested date palms in Salah al-Din Governorate, Iraq, muscid flies emerged from deteriorated beetle larvae, indicating a potential ecological association. Morphological examination and molecular characterization based on mitochondrial cytochrome c oxidase subunit I (COI) sequences identified the emerged flies as *Hydrotaea ignava* and *H. leucostoma*. Sequence analysis revealed 100% identity of the Iraqi *H. ignava* isolate with a Turkish isolate and 100% similarity of the Iraqi *H. leucostoma* isolate with a Romanian reference sequence. The generated sequences were deposited in GenBank under accession numbers PX110999.1 and PX110998.1, respectively. Occurrence data showed that *H. ignava* was more prevalent than *H. leucostoma* across the surveyed agro-ecological zones, with frequencies ranging from 36.33-44.70% and 30.14-33.03%, respectively. Phylogenetic analysis based on COI sequences grouped *Hydrotaea* species into four major clades and demonstrated close genetic relationships among Iraqi, Turkish, and Polish populations of *H. ignava*, whereas *H. leucostoma* showed high genetic conservation despite geographical separation. The association of both species with decaying *O. elegans* larvae suggests a possible ecological role as predators, scavengers, parasitoids, or opportunistic colonizers; however, direct evidence of their trophic interactions was not obtained. This study constitutes the first record of *H. ignava* and *H. leucostoma* in Iraq and provides a basis for future investigations into their ecological significance and potential role in the natural regulation of *O. elegans* populations.

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Introduction

The genus *Hydrotaea* Robineau-Desvoidy (Diptera: Muscidae) includes about 130 species throughout the world, of which several have been the subject of great interest because of their ability as biological control agents in livestock production systems for the control of

filth-breeding flies, especially house flies, *Musca domestica* L. Previously categorized under the genus *Ophyra*, these species are now recognized in the genus *Hydrotaea* as facultative predaceous species during larval stage (Krüger, 2002; De Togni, 2023). They are natural enemies of house flies and play a role in the

suppression of their populations in poultry, swine and cattle facilities which help alleviate nuisance problems and minimize the transmission of pathogens (Tsankova and Luvchiev, 1993).

Hydrotaea species feed on the larvae differently during their development. The 1st instar larvae feed on animal manure and decaying organic matter while the 2nd and 3rd instar larvae remain active predators of other dipteran larvae such as house flies. A single larva can consume 15-20 larvae of house fly each day which proves its high predatory potential (Simon et al., 2011; Paliy et al., 2018).

The species of *Hydrotaea* have, therefore, been extensively studied and used in intensive animal production systems for the biological control of house fly populations. The important species include *H. ignava*, *H. aenescens*, and *H. leucostoma*. Their efficiency is further increased due to some special larval morphological adaptations linked to facultative predation (Krüger, 2002; De Togni, 2023). Biological control programs with *Hydrotaea* generally involve mass multiplication and release of pupae to maintain or enhance predator populations in livestock systems (Hogsette et al., 2002). Soon after emergence, the adults are attracted towards nutrient-rich substrates which are quite suitable for oviposition, facilitating the resulting larval stages to exploit habitats where they can find house fly larvae in abundance (Hinkle and Hogsette, 2021).

The precise identification of natural enemies and understanding their trophozoic interactions are crucial to study their population status and dynamics and effectiveness in biological control programs. In the last few decades, molecular techniques involving polymerase chain reaction (PCR) have proven useful for studying predator-prey and host-parasitoid interactions. Such techniques can be used for the identification and detection of natural enemies at all life stages (predators, endoparasitoids and ectoparasitoids) and also the identification of prey from predator's gut contents or excreta (Agustí et al., 2005; King et al., 2008; Traugott et al., 2008).

Molecular tools have facilitated the host relationship studies and identification of multiple parasitism and hyperparasitism events, which are often hard to detect using traditional morphological approaches. These tools have also allowed to better quantify the levels of parasitism in the field (Dong et al., 2020; Franck et al., 2017). Thus, DNA diagnostic techniques have become

essential to understand ecological relationships and to evaluate the role of natural enemies in integrated pest management programs.

Palm rhinoceros beetle, *Oryctes elegans* Prell (Coleoptera: Scarabaeidae) is one of the most damaging insect pests of date palm (*Phoenix dactylifera* L.) in the Middle East countries such as Iraq, Iran and Arabian Peninsula (Hussain, 1963; Khalaf and Alrubiae, 2016). The larvae are found in decaying organic material, dead palm tissues and decaying trunks, while the adults bore into the crown, leaf bases, fruit stalks, and inflorescences and inflict widespread destruction to palm trees (Hussain, 1963). The badly infected trees become less vigorous, their fruit production and quality deteriorate, and become more prone to secondary pathogens and structural damage (Khalaf and Alrubiae, 2016). Due to its economic importance and large-scale prevalence, a lot of research has been focused on its biology, ecology, microbial associates, natural enemies and sustainable management strategies (Tabein et al., 2025).

Although all species of *Hydrotaea* are known to be predaceous, little is known about their natural host associations and ecological interactions with their hosts under field conditions. Use of molecular diagnostic methods can reliably detect these interactions and advance our understanding of the biological control potential of *Hydrotaea* species.

The aim of the present study was to use molecular tools to identify the species of *Hydrotaea* associated with the palm rhinoceros beetle, *O. elegans* and to evaluate these species as potential natural enemies in date palm agroecosystems.

Materials and Methods

Collection of *O. elegans* larvae and rearing till adult emergence

The insect specimens used in the current study were collected from a previous field survey in the date palm orchards of Salah al-Din Governorate in Iraq performed to ascertain the population density and relative abundance of the palm borer species (Bakr et al., 2022). The larvae of *O. elegans* were collected from naturally infested date palm trees from three locations viz., Tikrit (24 samples), Al-Duluyiyah (27 samples) and Al-Mu'tasim (19 samples). Sampling was done weekly, starting from January 2 to December 30, 2022 to guarantee the representativeness of infestation level in space and time.

Collected larvae were individually transferred to sterilized plastic Petri dishes, wrapped in air tight plastic containers and brought to the Insect Laboratory, Department of Plant Protection, College of Agriculture, Tikrit University. The insect rearing and handling were done following standard entomological laboratory procedures and protocols to maintain coleopteran larvae under controlled conditions (Southwood and Henderson, 2000).

In laboratory rearing, emergence of adults of dipterans was observed when *O. elegans* larvae pupated, indicating the possible presence of predaceous or parasitoidal dipterans. Suspected larvae were collected in 250 mL glass jars covered with muslin cloth with rubber bands. These specimens were kept at $25 \pm 2^\circ\text{C}$ and $80 \pm 5\%$ relative

humidity until they completed their development and emerged as adults, a standard recommendation for the developmental studies of insects (Arunkarthick et al., 2017). Molecular entomology preservation protocol was used to collect and preserve emerging adult flies in 90% ethanol and stored at 4°C (Carvalho and Vieira, 2000). Two morphologically distinct flies resembling muscid were recovered, differing mostly on the basis of their body size. Representative specimens were molecularly identified using the mitochondrial cytochrome c oxidase subunit I (COI) DNA barcoding approach which is generally accepted as a species identification tool for insects (Hebert et al., 2003). The number of flies emerged were counted and the frequency was determined as follows:

$$\text{Fly emergence (\%)} = \frac{\text{Total number of } O. elegans \text{ larvae examined}}{\text{Number of emerging flies}} \times 100$$

Extraction of Genomic DNA

Genomic DNA was isolated from samples of adult fly according to the method of the G-spin™ Total DNA Extraction Kit from Intron Biotechnology, Korea (Cat. No. 23105), as per the guidelines of the manufacturer. The integrity and quality of DNA were verified by electrophoresis on a 1% agarose gel in $1\times$ TBE buffer at 5 V cm^{-1} for 90 min. Nucleic acids were visualized under UV light using the gel documentation system following the standard molecular biology protocols (Sambrook and Russell, 2001).

PCR amplification of the COI gene

For molecular identification, mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified using universal insect primers LC01490 and HC02198 (Folmer et al., 1994), which are commonly used in DNA barcoding studies (Hebert et al., 2003). The expected amplicon size was ~658-720 bp.

Maxime PCR PreMix™ (i-Taq; Intron Biotechnology, Korea; Cat. number 25025) was used to prepare PCR reactions. Briefly, 5 μL of the PCR master mix, 1 μL of each of the primers ($10 \text{ pmol } \mu\text{L}^{-1}$), 1.5 μL of the genomic DNA, and 16.5 μL of nuclease-free water were added together to a total volume of 25 μL .

Thermal cycling was performed with an initial denaturation step at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 45 sec, annealing at 58°C for 45 sec and extension at 72°C for 45 sec; and finally the extension step at 72°C for 7 min. The conditions are similar to those used for standard COI amplifications for insects (Folmer et al., 1994). The PCR

products were run on 2% agarose gel in $1\times$ TBE buffer for 90 min at 5 V cm^{-1} and were subsequently detected by UV visualization.

DNA sequencing and phylogenetic analysis

Successful PCRs products were purified and sent to MacroGen Inc. (Seoul, Republic of Korea) for bidirectional Sanger sequencing reaction, which is extensively used in DNA barcoding studies (Sanger et al., 1977). For each sample, 25 μL of PCR product was supplied together with 17.5 pmol of each primer.

Sequences thus obtained were edited, assembled and compared with known sequences in the National Center for Biotechnology Information (NCBI) databases using BLASTn for species-level identification (Altschul et al., 1990). Identification was made by calculating the percentage sequence similarity with validated reference entries.

Consensus COI sequences were submitted to GenBank and phylogenetic analyses were performed with closely related taxa obtained from NCBI. To infer evolutionary relationships, the multiple sequence alignment and phylogenetic tree were constructed following the molecular systematics approach (Tamura et al., 2021).

Results and Discussion

Emergence of *Hydrotaea* species from *O. elegans* larvae

Adult flies emerged during the laboratory rearing and pupation of larvae, were collected from infested date palms. Representative adults are shown in Figure 1A and 1B. Morphologically, the emerged insects resembled muscid flies and exhibited similar external characteristics, differing primarily in body size. Examination of infested *O. elegans* larvae revealed extensive tissue deterioration and necrosis,

together with the presence of dipteran larvae, pupae, and emergence holes associated with the developing flies (Figure 1C). These observations suggest a close biological association between the muscid flies and the rhinoceros beetle larvae, although the exact nature of this relationship (predatory, parasitoid, saprophagous, or opportunistic colonization) requires further investigation under controlled experimental conditions.

Frequency of occurrence of *H. ignava* and *H. leucostoma*

The occurrence of *H. ignava* and *H. leucostoma* in three

agro-ecological zones of Salah al-Din Governorate of Iraq is given in Figure 2. Of the two species, *H. ignava* was the most abundant species, with 44.70%, 38.28% and 36.33% frequencies in Al-Duluiya, Al-Mu'tasim and Tikrit respectively. On the other hand, *H. leucostoma* was found with frequencies of 33.03%, 30.14%, and 30.34% in the aforementioned locations. *H. ignava* was found more frequently throughout all the sampled areas, suggesting that under local environmental conditions it is more associated with the habitats used by the larvae of *O. elegans*.



Figure 1. Larva of the palm rhinoceros beetle, *O. elegans* (right); adult *H. ignava* (A); adult *H. leucostoma* (B); and necrotic tissue associated with the larval habitat (C).

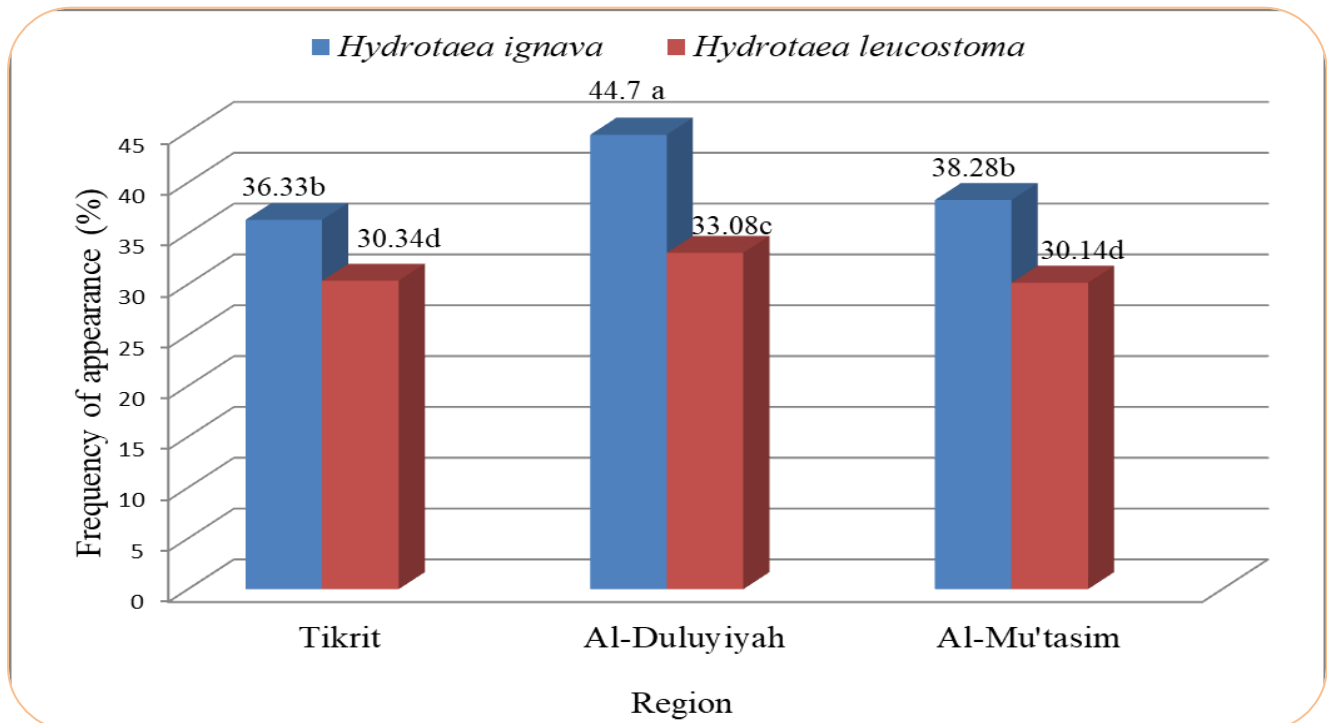


Figure 2. Frequency of occurrence of *H. ignava* and *H. leucostoma* in three regions of Iraq.

Molecular identification of *H. ignava* and *H. leucostoma*

For accurate identification to species level, representative samples were molecularly characterized by sequencing mitochondrial cytochrome c oxidase subunit I (COI) gene. On the basis of molecular analysis, the large sized fly with body length of 6-8 mm was identified as *H. ignava* with a 100% sequence identity with the sequence of *H. ignava* isolate TRHign (GenBank accession OM534563.1) from Turkey. The sequence obtained in the current study was deposited in GenBank as *H. ignava* isolate Has.AA-1 under accession number PX110999.1 (Table 1).

In the same way, the small-sized fly having body length < 6 mm was recognized as *H. leucostoma* which showed 100% sequence similarity to *H. leucostoma* voucher IBIOL15 reported from Romania. The sequence acquired in this research was deposited in GenBank with accession number PX110998.1 (Table 1). To the best of our knowledge, this is the first report of the occurrence

of both *H. ignava* and *H. leucostoma* in Iraq.

DNA barcoding on the basis of mitochondrial COI gene is among the most powerful and extensively used genes for insect identification due to its high discriminatory power and the large number of available reference sequences. The COI sequences have been previously used for accurate species delimitation and molecular diagnosis in various insect species (Asokan et al., 2011; Tyagi et al., 2017; Choudhary et al., 2018; Amad et al., 2019).

Phylogenetic analysis and evolutionary relationships

In order to better understand the relationships of the recovered specimens with the rest of the family Muscidae, a phylogenetic tree was built using the mitochondrial COI gene sequences from GenBank and the sequences generated in this study (Figure 3). The resulting phylogeny showed that the genus *Hydrotaea* comprised four main clades, which represent different divergent evolutionary lineages.

Table 1. Molecular identification of *H. ignava* and *H. leucostoma* based on COX1 gene sequence identity (%) with selected GenBank reference sequences.

Insect Species and Strain	Global Accession Number	Country	Insect Species and Code Registered in Current Study	% Identity	Accession Number for Insects Registered in Current Study
<i>Hydrotaea ignava</i> isolate TRHign	OM534563.1	Turkey	<i>Hydrotaea ignava</i> isolate Has.AA-1	100	PX110999.1
<i>Hydrotaea leucostoma</i> voucher	KY582504.1	Romania	<i>Hydrotaea leucostoma</i> isolate Has.AA-2	100	PX110998.1

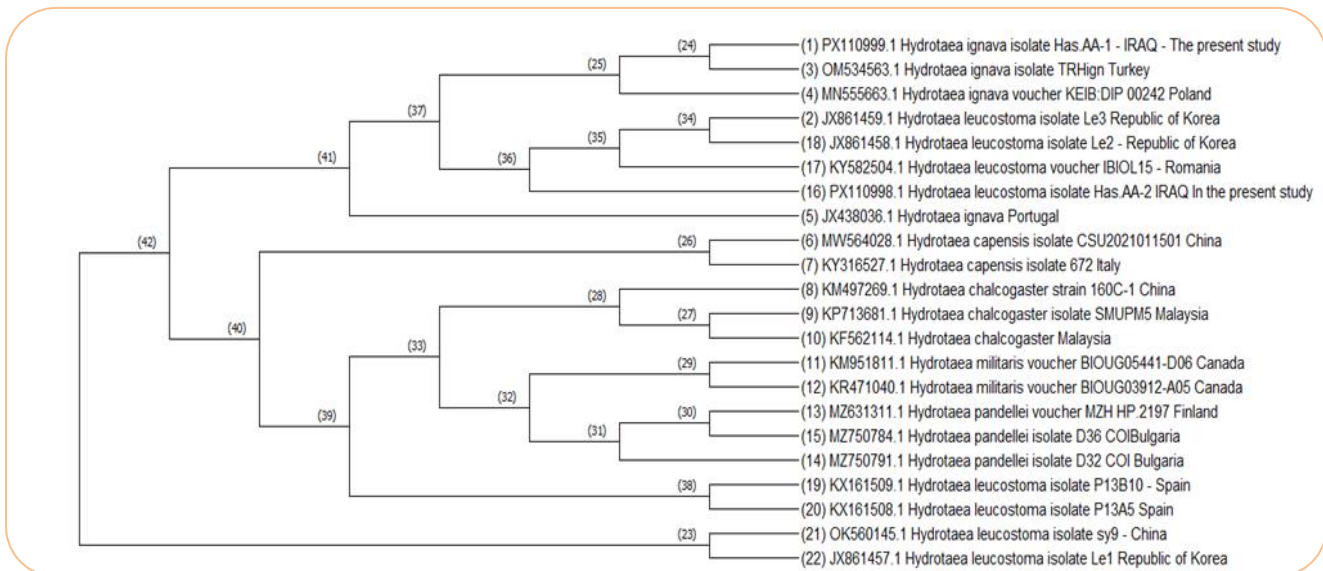


Figure 3. Phylogenetic tree based on mitochondrial cytochrome c oxidase subunit I (COX1) gene sequences showing the genetic relatedness and divergence among *Hydrotaea* isolates from different countries.

Clade A

The Iraqi *H. ignava* isolate (PX110999.1) along with *H. ignava* isolates from Turkey (OM534563.1) and Poland (MN555663.1) were included in clade A. Several isolates of *H. leucostoma* from Iraq, Korea, Portugal, and Romania were also present in this clade. The Iraqi *H. ignava* isolate was 100% identical in sequence with the Turkish isolate, suggesting close genetic relatedness which is probably due to the geographical proximity and ecological conditions of the two countries. Similarly, the Iraqi isolate of *H. leucostoma* was highly similar to the Romanian isolates, with 100% sequence identity. This clustering of isolates is indicative of strong within-species genetic conservation over wide geographical areas.

Clade B

The isolates of *H. capensis* and *H. chalcogaster* from China and Malaysia were grouped into clade B. Although geographically isolated, these species comprised a well-supported cluster, indicating close evolutionary interactions and the comparatively stable genetic divergence among populations from East and Southeast Asia.

Clade C

The Canadian *H. militaris* isolates and the Bulgarian and Finnish *H. pandellei* isolates belonged to clade C. This clade was clearly an evolutionary branch that diverged from the other clades, indicating large genetic differences from the species present in Clades A and B.

Clade D

Other *H. leucostoma* isolates from China, Korea, and Spain were included in clade D. These isolates are separated from the isolates clustered together in Clade A, suggesting geographical structured populations and/or regional genetic variation of the species.

In general, the phylogenetic analysis showed high genetic similarity among the populations of *H. ignava* from Iraq, Turkey and Poland and confirmed the wide distribution of this species, with only a slight divergence in the COI sequences. The high genetic similarity of the Iraqi and Turkish isolates also indicates recent common ancestry, and/or continued regional connectivity. In a similar fashion, the genetic similarity amongst *H. leucostoma* isolates illustrates the considerable conservation found within geographically remote populations and provides evidence for population level differences.

Biological significance and potential ecological role

A number of species of *Hydrotaea* have been found as facultative predators of other dipteran larvae including the house fly. Earlier investigations have shown that the

ratio of predator to prey and the size of the larvae had an effect on the efficiency of predation; the bigger the larvae of *Hydrotaea* the higher the consumption rate of the larvae of prey species (Müller, 1982). These observations are in line with ecological theories which suggest that larger predators have a significant effect on prey populations (Holt and Huxel, 2007).

The mouthparts of the third instar larvae of some members of the *Hydrotaea* group are modified and adapted for predatory feeding, while the serrated spiracular structures assist in the predatory feeding mode. These species are known to prey on house fly larvae and other dipteran species with similar developmental habitats (Duarte et al., 2013; De Togni et al., 2023). In the present study, *H. ignava* and *H. leucostoma* were found in the decaying larvae of *O. elegans*, but did not yield any direct evidence of predation or parasitism. Thus, further laboratory and field research is needed to establish that these species are predators, parasitoids, scavengers and/or secondary colonizers of weakened rhinoceros beetle larvae.

Conclusion

The present study documents the emergence and molecular identification of *H. ignava* and *H. leucostoma* from larvae of the date palm rhinoceros beetle, *O. elegans*. Molecular characterization based on COI gene sequences confirmed species identity and represents the first record of both species in Iraq. Phylogenetic analysis revealed close genetic relationships between Iraqi isolates and previously reported populations from Europe and neighboring regions. Although the biological association between these muscid flies and *O. elegans* larvae remains unclear, their repeated occurrence in association with infested beetle larvae suggests a potentially important ecological interaction. Future studies should investigate their life history, feeding behavior, and possible role as natural enemies or decomposer-associated organisms within date palm ecosystems.

Author Contributions

HARAE and AAKH conceived and designed the study. HARAE conducted field sampling, laboratory rearing, specimen collection, morphological observations, molecular analyses, and data acquisition. AAKH provided overall supervision, coordinated the research activities, and ensured methodological rigor and analytical consistency throughout the study. TSAH and SSAA served

as co-supervisors, providing critical input on study design, molecular identification procedures, data interpretation, and manuscript development. WKA contributed to specimen processing, data validation, and interpretation of the findings. HARAE, AAKH, TSAH, SSAA, and WKA participated in manuscript preparation, including data compilation, literature review, result interpretation, and drafting of the manuscript. All authors critically reviewed the manuscript for intellectual content, verified the accuracy of the data and analyses, approved the final version for publication, and agreed to be accountable for all aspects of the work.

Research Funding

This research did not receive any grant from funding agencies.

Conflict of Interest

The authors declare no conflict of interest.

Sustainable Development Goals Targeted

SDG 2: Zero Hunger

SDG 12: Responsible Consumption and Production

SDG 15: Life on Land

Policy Addressed

This study supports the Convention on Biological Diversity (CBD), FAO Integrated Pest Management (IPM) framework, and International Plant Protection Convention (IPPC) by documenting two previously unrecorded *Hydrotaea* species in Iraq, enhancing biodiversity knowledge, and providing baseline data for sustainable management of *Oryctes elegans* in date palm agroecosystems.

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