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**Review Article**

# Molecular Identification of Five Hormogaster Earthworm Isolates Newly Recorded in Iraq Using COX1 Gene Sequencing

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## Abstract

**Background:** Earthworms are highly valued as key organisms in ecosystems, playing an essential role in maintaining soil health and primary productivity. They are considered ecologically important species with complex and poorly understood genetic diversity. Since morphological methods of classification are often inaccurate, it is necessary to classify species based on molecular approaches using PCR techniques. **Objectives:** This study focused on molecular classification using the mitochondrial *Cytochrome c oxidase I* (COX1) gene as a DNA barcode, due to its genetic stability, low susceptibility to structural variation, and its importance in supporting traditional morphological taxonomy and clarifying differences among species. **Materials and Methods:** Earthworm specimens were collected from grazing areas and aquatic zones in Erbil Governorate, Iraq; genomic DNA was extracted, and the mitochondrial cytochrome oxidase I (COX1) gene was amplified using universal primers targeting a 710 bp fragment. PCR products were verified by agarose gel electrophoresis and sequenced. The obtained sequences were aligned with GenBank references using BLASTn. Phylogenetic trees were constructed in MEGA11 software using the Maximum Likelihood method (Tamura-Nei model) with 1000 bootstrap replicates to support taxonomic placement and to establish evolutionary relationships among earthworm species. **Results:** PCR amplification and sequencing of the mitochondrial COI gene identified two earthworm species in Iraq for the first time: *Hormogaster castillana* and *Hormogaster samnitica*. Five isolates were obtained, two belonging to *H. castillana* (PX257867, PX257868) and three to *H. samnitica* (PX257869, PX257870, PX257871). These sequences were deposited in the NCBI GenBank database under their respective accession numbers. Phylogenetic analysis using the Maximum Likelihood method (Tamura-Nei model, MEGA11, 1000 bootstrap replicates) revealed that the Iraqi isolates clustered within the same clades as their European and Asian reference strains, confirming their taxonomic placement and evolutionary relationships.

**Keywords:** Earthworms, PCR analysis, COX1 gene, Molecular phylogeny, GenBank sequences.

## Introduction

Earthworms (*Annelida: Oligochaeta*) are among the most important soil organisms worldwide, contributing significantly to soil formation, structure, and fertility. They play a vital ecological role by maintaining soil health and primary productivity (1,2). Through their ability to consume and redistribute organic matter, earthworms act as

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natural decomposers and processors of nutrients (3,4). Their burrowing activity reduces soil erosion, creates essential channels and pores, and facilitates gas exchange, water movement, and the transport of dissolved organic compounds, nutrients, signaling molecules, and oxygen within the soil ecosystem (5-7).

The family *Hormogastridae* represents a relatively diverse group of earthworms, with approximately 30 species described and assigned to four genera. A recent systematic revision, integrating multiple sources of information, expanded the classification to nine genera within *Hormogastridae* (8). Research on earthworm taxonomy and evolutionary relationships not only enhances our understanding of their ecological functions but also carries profound importance for biodiversity conservation. Despite their ecological significance, only about 6,000 earthworm species have been formally described, a relatively small number, with many more awaiting discovery and characterization (9,10).

Because earthworms generally lack distinctive morphological traits useful for inferring evolutionary relationships, and often exhibit high variability within species, molecular phylogenetic approaches have been increasingly applied (11). The absence of DNA sequence data for native Iraqi species in GenBank, combined with the lack of molecular identification in national studies, represents a major obstacle. Overcoming this limitation requires comprehensive molecular investigations, accurate morphological identification, and the generation of corresponding sequences to expand the database of local species.

Novo et al. (12) provided a molecular phylogeny of *Hormogastridae*, previously classified based on morphology, highlighting several intriguing evolutionary aspects (13). Mitochondrial DNA has been widely used in earthworm phylogenetics because it evolves more rapidly than nuclear DNA, leading to the accumulation of differences among closely related species (14). Sequence divergence between species is typically much greater than within species, and mitochondrial COX1 lineages often reveal biological discontinuities recognized by taxonomists as species boundaries. The COX1 gene is therefore regarded as a DNA barcode for species identification, being universally present in earthworms and particularly suitable for sequence comparison due to its low incidence of insertions and deletions (15).

This study addresses these gaps by applying COX1-based DNA barcoding to identify and characterize earthworm species in Iraq, providing the first molecular evidence for the occurrence of *Hormogaster castillana* and *Hormogaster samnitica* in Iraq.

## Materials and methods

### Sample collection

Earthworm samples were collected from various locations in Erbil Governorate, including different villages (grazing areas) and the banks of the Greater Zab River (aquatic zones), by digging 30 cm into the soil using a field spade. The worms were fixed on-site using 96% ethanol, stored at 20°C, and transported to the laboratory for molecular identification.

### Polymerase chain reaction (PCR)

- **Extraction of DNA**

Following the manufacturer's instructions, earthworm DNA was extracted using AddPrep Genomic DNA Extraction Kit from Tissue and Blood (Add bio, Korea) as follows:

1. 40 mg of the earthworm tissue was placed in a 1.5 mL Eppendorf tube with 200 µL of Lysis Buffer, then 20 µL of Proteinase K 20 mg/mL) was added to the samples to lyse the protein, and then the sample was mixed and incubated in a water bath at 56 °C for 1 h to completely lyse the tissue.

2. After the incubation period, the tube was placed in a centrifuge for a short spin to remove droplets; then, 20 µL of RNase A 10 mg/mL solution was added to remove RNA.
3. 200 µl of Binding Buffer solution was added and mixed well by pulse mixing with a vortex shaker for 15 s until a homogeneous solution was formed, and all samples were incubated in a water bath at 56 °C for 10 min.
4. To purify DNA, 200 µl of 100% ethyl alcohol was added to the samples and mixed for 15 s.
5. The mixture was carefully placed in the spin column tube in a 2 mL collection tube and centrifuged at 13,000 rpm for 1 min; then the flow-through was discarded.
6. The spin column was placed in a 2 mL collection tube again, and 500 µl of Washing 1 solution was added and centrifuged at 13,000 rpm for 1 min 1 min, then the flow-through was discarded.
7. After centrifugation, the spin column was placed in a 2 mL collection tube, and 500 µl of Washing 2 solution was added and centrifuged at 13,000 rpm for 1 min 1 min, then the flow-through was discarded.
8. The spin column was dried by additional centrifugation at 13,000 rpm for 1 min to remove the remaining ethyl alcohol in the spin column.
9. The spin column was placed in a new 1.5 mL microcentrifuge tube, and 100 µl of Elution solution was added and left for 1 min.
10. Finally, the DNA was eluted by centrifugation at 13,000 rpm for 1 min and then stored at -20°C.
11. The DNA pellet was rehydrated by adding 100 µl of rehydration solution and kept at -20 °C until further assay.

- **Polymerase chain reaction (PCR) assay procedure**

Polymerase chain reaction (PCR) was performed using specific primers for the detection of earthworms. (Table. 1). The primers were obtained from Macrogen Co., Korea.

The PCR reaction mixtures were prepared using AddBio Master Mix (2X) (AddBio, Korea). The PCR cocktail mixtures were prepared in 25 µl containing a final concentration of 1X AddBio Master Mix, 1 µM of each primer, and 2 µl of DNA template Table 2. The PCR was done using a thermal cycler (T100 Bio-Rad, USA). The cycling conditions, including temperature and time, were as shown in Table 3.

**Table 1. Sequences of primers used for amplification of the mitochondrial *COI* gene of the earthworm.**

| No. | Primer Name | Primer Sequence 5’ – 3’ | Annealing Temp °C | Product size (bp) | References |
|---|---|---|---|---|---|
| | LT-F | GGTCAACAAATCATAAAGATATTGG | 55 | 710 | (16) |
| | LT-R | TAAACTTCAGGGTGACCAAAAAATCA | | | |

**Table (2) Reaction mixture for PCR amplification of the mitochondrial *COI* gene of the earthworm.**

| Component | Volume |
|---|---|
| 2X AddBio Master Mix | 12.5 µl |
| PCR-grade water | 8.5 µl |
| LT-F primer (10 µM) | 1 µl |
| LT-R primer (10 µM) | 1 µl |
| DNA (80 ng/µl) | 2 µl |
| **Total** | **25 µl** |

**Table (3) Cycling conditions of PCR for amplification of the mitochondrial *COI* gene of the earthworm.**

| No. | Step | Temp °C | Time | Cycle |
|---|---|---|---|---|
| **1.** | Polymerase activation | 95 | 10 min | 1X |
| **2.** | Denature | 95 | 45 sec | 35X |
| **3.** | Annealing | 55 | 45 sec | |
| **4.** | Extension | 72 | 1 min | |
| **5.** | Final Extension | 72 | 5 min | 1X |
| **6.** | Hold | 4 | 4 °C | ∞ |

### Agarose gel electrophoresis

Agarose gel electrophoresis was done to separate the amplified products using 1.5% agarose (AddBio, Korea) mixed with 3 µl of GelRed dye (AddBio, Korea). 5 µl of each PCR product was loaded into the well of the agarose gel. The electrophoresis was carried out at 75 V for 1 hour using a power supply of 300 mA and an electrophoresis tank (BioRad, USA) containing 1X TBE buffer (GeNetBio, Korea). A 100 bp DNA marker, 6 µl (GeneDirex H3, Korea), was used as a standard molecular weight marker

- **Gel documentation**

The gel was examined under UV light using a gel documentation system (Gel Doc EZ Image, Bio-Rad, USA) for documentation and determination of expected bands.

- **DNA sequencing**

The PCR product with specific primers was sent to Macrogen, Korea, to determine the sequence of the target gene. The genetic sequence files were received as text files and then analyzed using specific bioinformatics software to find the phylogenetic relationship.

### Phylogenetic analysis

Partial COI gene sequences obtained from Iraqi earthworm samples were aligned with reference

sequences retrieved from GenBank using BLASTn. Phylogenetic trees were constructed in MEGA11 software employing the Maximum Likelihood method based on the Tamura-Nei model. Bootstrap resampling with 1000 replicates was performed to assess the robustness of branching patterns. Concatenated partial COI sequences of the query samples and reference isolates were used as input data.

## Results and Discussion

Determining the genus of earthworms based solely on external features such as morphology and coloration is often difficult, as these traits may vary with developmental stage. Consequently, morphological classification is not definitive, and molecular confirmation is required to accurately establish genus and species identity (17,18). In studies of earthworm communities, species-level identification is particularly challenging due to the absence of reliable external diagnostic features (19) and the impossibility of distinguishing juvenile individuals morphologically (20). Moreover, multiple cases of cryptic species variation have recently been reported (21,22). Cryptic variation refers to genetically distinct lineages that are morphologically indistinguishable, making them difficult to separate using traditional taxonomy. For this reason, DNA barcoding based on a fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene has been proposed as a precise tool for species identification (23).

In the present study, *H. castillana* was detected in grazing areas, while *H. samnitica* was recorded along the banks of the Greater Zab River in Erbil Governorate, Iraq. This represents the first report of these species in Iraq, where the commonly documented earthworm is *Aporrectodea trapezoides* (24–29). Out of 30 PCR-amplified products, nine representative samples were selected for sequencing. Amplification yielded specific bands of approximately 710 bp during electrophoresis on a 1.5% agarose gel. Subsequent sequence analysis revealed three isolates belonging to *H. samnitica* and two isolates belonging to *H. castillana*.

Figure 1 shows the PCR amplification of the mitochondrial COI gene from nine samples, of which five produced positive bands. Samples in lanes 4, 6, and 9 were negative controls, representing non-amplified isolates of the genus Hormogaster.

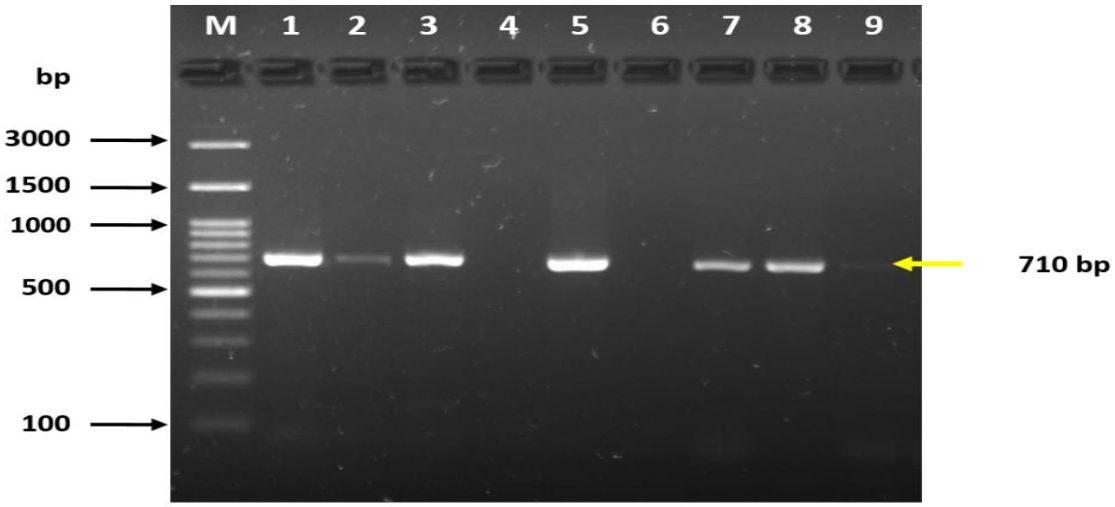


Figure 1. Polymerase chain reaction (PCR) of the mitochondrial COI gene of the earthworm. Lane M: 100 bp DNA ladder. Lanes 1- 3, 5, 7, and 8 are positive samples; lanes 4 and 6 are negative samples. Lane 9 is the negative control.

Table (4) and Figure (2) present the molecular identification of *H. castillana* using amplified COX1 gene sequences. Sequence alignment in BioEdit showed 100% identity with the Spanish reference isolate (GenBank accession KT246393). Additional matches included 99.52% identity with another Spanish isolate (KT246392), 93.70% and 93.54% identity with two further Spanish isolates (KT246376 and KT246377), and 93.86% identity with a U.S. isolate (JN209511). These results confirm that the Iraqi isolates (PX257867 and PX257868) belong to *H. castillana*, clustering closely with European and American reference sequences.

Table (5) and Figure (3) present the molecular identification of *H. samnitica* using amplified COX1 gene sequences. Sequence alignment showed 100% identity with the Italian reference isolate (GenBank accession KF974931). Additional matches included 99.84% identity with another Italian isolate (KF974932) and 99.69% identity with two further Italian isolates (KF974937 and KF974930). These findings confirm that the Iraqi isolates PX257869, PX257870, and PX257871 belong to *H. samnitica*, clustering closely with European reference sequences.

**Table (4) Percentage identity of *H. castillana* isolates based on partial COI gene sequences according to BLAST alignment in GenBank (NCBI).**

| Sample Accession Number | Nematode Identified | Query Cover % | Identic Numbe r % | GenBank Accession Number | Country Identificati on |
|---|---|---|---|---|---|
| PX257867 PX257868 | *Hormogaster castillana* | 100 | 100 | KT246393 | Spain |
| | | 100 | 99.52 | KT246392 | Spain |
| | | 100 | 93.86 | JN209511 | USA |
| | | 100 | 93.70 | KT246376 | Spain |
| | | 100 | 93.54 | KT246377 | Spain |

**Table (5) Percentage identity of *H. samnitica* isolates based on partial COI gene sequences according to BLAST alignment in GenBank (NCBI).**

| Sample Accession Number | Nematode Identified | Query Cover % | Identic Numbe r % | Genbank Accession Number | Country Identificati on |
|---|---|---|---|---|---|
| PX257869 PX257870 PX257871 | *Hormogaster samnitica* | 100 | 100 | KF974931 | Italy |
| | | 100 | 99.84 | KF974932 | Italy |
| | | 100 | 99.69 | KF974937 | Italy |
| | | 100 | 99.69 | KF974930 | Italy |

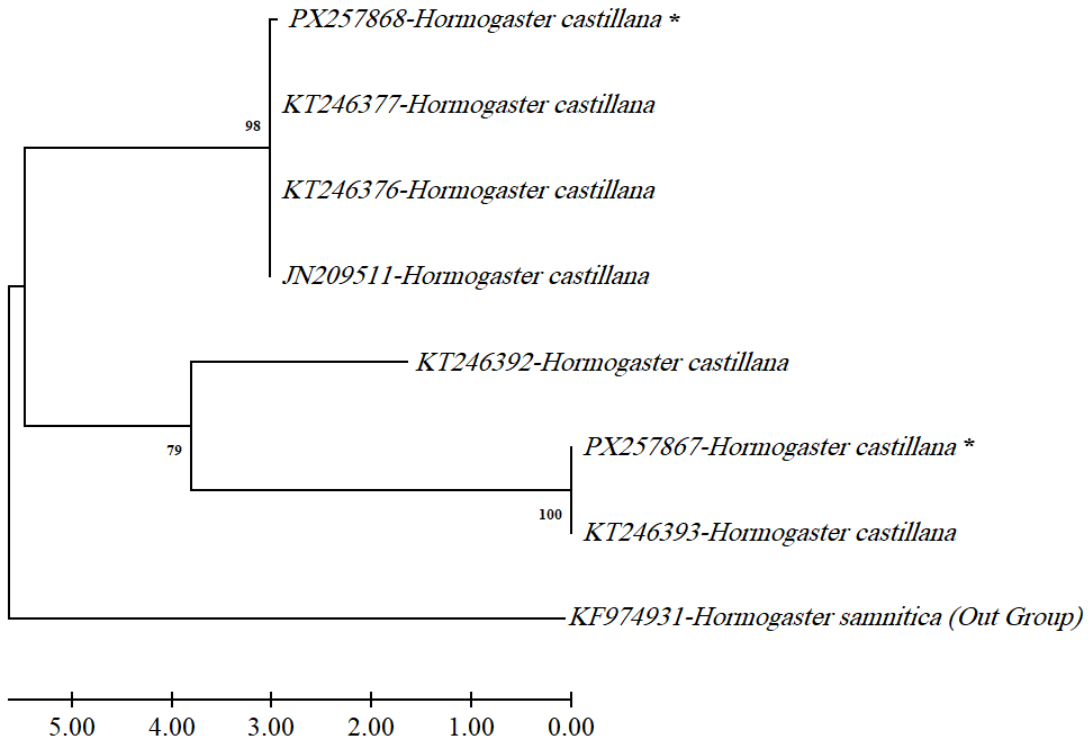


Figure (2) Phylogenetic tree of *H. castillana* from Iraq (*). The phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model in MEGA11 software and bootstrap analysis with 1000 resamplings. Partial DNA sequences of the concatenated partial COI gene were used as input data.

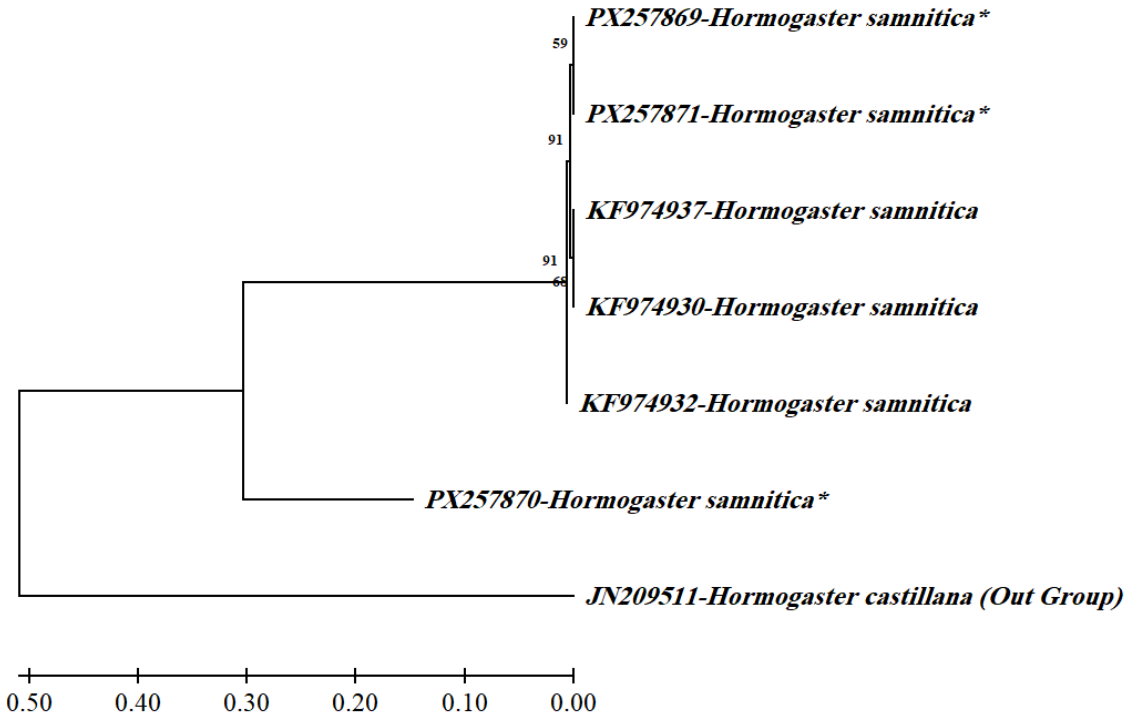


Figure 3. Phylogenetic tree of *H. samnitica* from Iraq (*). The phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model in MEGA11 software and bootstrap analysis with 1000 resamplings. Partial DNA sequences of the concatenated partial COI gene were used as input data.

Genetic distance (p-distance) values among the Iraqi isolates and related Hormogaster species ranged from 0.0% to 0.57%, remaining below 1%. These consistently low values confirm that *H. samnitica* and *H. castillana* from Iraq are genetically very close to their European counterparts, supporting accurate species assignment through COX1 barcoding. The presence of these species in Iraq, despite their native distribution in Spain and Italy, suggests that they were likely introduced via agricultural activities and soil transport, facilitated by irrigation practices and the movement of plants, compost, and soil (30). Such human-mediated dispersal is consistent with global patterns of earthworm spread, where cryptic species often establish beyond their original ranges. Phylogenetic analyses further reinforce these findings, showing Iraqi isolates clustering tightly with European reference strains while displaying moderate divergence from American lineages. Together, these results highlight the reliability of COX1 barcoding, provide strong confirmation of taxonomic identity, and establish a robust baseline for future comparative studies of earthworm populations in Iraq.

## Conclusion

This study provides the first molecular record of *H. castillana* and *H. samnitica* in Iraq, expanding the known distribution of these species beyond Europe. The distinct ecological occurrence of *H. castillana* in grazing areas and *H. samnitica* along riverbanks suggests that human activities and irrigation practices may influence their distribution. DNA barcoding using the COX1 gene proved to be an effective tool for accurate species identification, particularly for morphologically indistinguishable or juvenile earthworms. These findings emphasize the need for further biodiversity surveys to uncover additional cryptic species and enrich the understanding of earthworm diversity in the region.

Conflict of interest: NIL

**Funding: NIL**

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