زانکۆی سەلاحەدین - هەولێر
Salahaddin University-Erbil

Morphological and Molecular Identification of terrestrial Isopod Porcellionides pruinosus *(Brandt, 1833) in Erbil city - Iraq*

Research Project

Submitted to the Department of Biology in partial fulfillment of the requirements for the degree of BSc. in Biology

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﴿ يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنكُمْ وَالَّذِينَ أُوتُوا
العِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ ﴾

صدق الله العظيم

SUPERVISOR CERTIFICATE

This research project has been written under my supervision and has been submitted for the award of the degree of BSc. in Biology with my approval as a supervisor.

Signature :

Name: Dr. Sarwat Ekram Al-Qassab

Date: /04/2024

I confirm that all the requirements have been fulfilled.

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Date: /04/2024

I confirm that all the requirements have been fulfilled.

DEDICATION

I dedicate this work to:

- My dear parent, who always prayed for me and supported me in everything, and my sisters and brothers, who are beside me.
- My supervisor, Dr. Sarwat Ekram Al-Qassab.
- My best friend who helped me.

Rayan

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ABSTACT

The terrestrial isopod, *Porcellionides pruinosus* (Brandt, 1833) (Porcellionidae), is one of the most widespread woodfly species. This species was collected from a house garden in Erbil city - Iraq and is regarded as the first record in Iraq. This species is identified based on morphology and molecular analysis of *COI* gene.

Keywords: Isopod, Morphology, DNA barcoding, *Porcellionides pruinosus*.

1. INTRODUCTION

Order Isopoda, which belongs to the phylum Arthropoda, refers to the crustaceans group, which mainly includes the woodlice and their relatives (Martin and Tiunov, 2023). The word Isopoda is derived from the Greek words iso- (meaning "equal") and -pod (meaning "foot") (Scarborough, 1992). Isopods dwell in the sea, in freshwater, or on land. Over 10,000 species of the Order Isopod are found worldwide, out of which around 4,500 species are found in marine environments, i.e., on the seabed, and 500 species in freshwater. The remaining 5,000 species are found to dwell on land (Boyko et al., 2023).

All the isopod species have rigid and segmented exoskeletons. They also consist of two pairs of antennae along with seven pairs of jointed limbs on the thorax. There are a total of five pairs of branching appendages on the abdomen, which are used by the isopods during respiration. The female isopods brood their young ones in a pouch under their thorax (Heeley, 1941).

The isopods are known for their varied feeding habits; some survive on dead or decaying plant and animal matter, while others are grazers or filter feeders. Few of the isopod species are predators and sometimes are internal or external parasites (mostly of fish) (Smit and Davies, 2004). The terrestrial species move around by crawling, mostly found in cool and moist places. It has also been found that some of the species have the ability to roll themselves into a ball as a source of defense mechanism or for conserving moisture (Vincent, 2005).

In the wild, isopods feed on decaying plant and animal material. They search for dead leaves, wood, decaying fruits, and insects as their main sources of nutrition. For added nutrition, they'll even eat the feces of other animals (Harris, 2012). Most terrestrial isopod species conceal/ hide

under objects in crevices or under the bark and are often slow-moving (Eaton and Kaufman, 2007).

Isopods are hermaphroditic, which means they have both male and female reproductive organs. They mate by facing each other and exchanging sperm packets. Females will carry the fertilized eggs in a pouch on their underside until they hatch (Legendre and Billard, 1996).

DNA barcode is one or more short gene sequences (generally 200–900 base pairs) taken from a standardized portion of the genome to aid species identification and discovery by employing sequence divergence based on nucleotide alignment (Emerson et al. 2011; Hebert et al. 2003, 2004).

In this study, morphological and molecular identification was achieved to identify an Isopod species in Erbil city – Iraq.

1. MATERIALS OF METHODS

2.1 Samples collection

Terrestrial Isopod specimens were collected by hand from a house garden located in Zanco-1 Quarter within Erbil city - Iraq. The samples were preserved in 96% ethanol and kept in (-20° C) freezer. Photos of the dorsal and ventral sides of the samples were taken using a Celestron Handheld Digital Microscope Pro (China).

2.2 Molecular Techniques

2.2.1 DNA extraction by using a Qiagen DNA extraction kit

DNA extraction from Isopod tissue was done by using a DNaesy Blood and Tissue kit (Qiagen, Germany) as follows:

1. About ≤ 25 mg of tissue was cut into small pieces and placed in a 1.5 ml microcentrifuge tube.
2. Add 180 µl Buffer ATL.
3. Add 20 µl proteinase K, mix by vortexing and incubate at 56°C until completely lysed. Vortex occasionally during incubation. Vortex 15 s directly before proceeding to step 2.
4. Add 200 µl Buffer AL. Mix thoroughly by vortexing.
5. Add 200 µl ethanol (96–100%). Mix thoroughly by vortexing.
6. Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at (8000 rpm) for 1 min. Discard the flow-through and collection tube.
7. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at (8000 rpm). Discard the flow-through and collection tube.
8. Place the spin column in a new 2 ml collection tube, add 500 µl Buffer AW2 and centrifuge for 3 min at (14,000 rpm). Discard the flow-through and collection tube.
9. Transfer the spin column to a new 1.5 ml microcentrifuge tube.

10. Elute the DNA by adding 200 µl Buffer AE to the center of the spin column membrane. Incubate for 5 min at room temperature (15–25°C). Centrifuge for 1 min at (8000 rpm).

11. Optional: Repeat step 8 for increased DNA yield

1.2.2. PCR preparation

The DNA barcoding for terrestrial Isopod was achieved based on the amplification and sequencing of mitochondrial DNA Cytochrome Oxidase subunit I (*COI*) gene (680-720 bp long) (Folmer et al., 1994) by using primer pairs: LCO1490: (**5`-GGTCAACAAATCATAAAGATATTGG-3`**) and HCO2198: (**5`-TAAACTTCAGGGTGACCAAAAAATCA-3`**) (Macogen, Korea). Master mix (Ampliqon PCR Enzymes & Reagents, Denmark) was used to amplify the partial sequences of (*COI*). The amplification was done in a total volume of (25 µl) as in Table (2.1):

Table (2.1) PCR material mixture.

Master Mix	12.5 µl
Primer F	1.5 µl
Primer R	1.5 µl
Templete DNA	2 µl
ddH_20	7.5 µl
Total volume	25 µl

PCR was carried out in (PCRmax Alpha thermal cycler, UK) and PCR thermal reaction applied according to (Hajibabaei et al., 2006) as shown in Table (2.2)

Table (2.2) shows PCR reaction

94 °C	2 min	
94 °C	30 sec	5 cycles
45 °C	40 sec	
72 °C	1 min	
94 °C	30 sec	35 cycles
51 °C	40 sec	
72 °C	1 min	
72 °C	10 min	

1.2.3. Agarose gel electrophoresis

PCR samples were run in (1.5%) of agarose gel electrophoresis as following:

1. **Prepare Gel**: Mix agarose powder with TAE buffer, heat to dissolve, pour into mold, and let it solidify.
2. **Load Gel**: Set up gel in electrophoresis tank, insert comb to create wells, pour in TAE buffer, and load samples into wells.
3. **Run electrophoresis**: Apply (80) voltage across the gel, allowing DNA fragments to migrate through the gel based on size using GeneRuler 50 bp DNA Lader marker (Thermo Inc., USA).

4. **Visualize DNA**: After electrophoresis, visualize DNA bands under UV light.
5. **Analyze results**: Determine size and quantity of DNA fragments by comparing to size of marker

1.2.4. Gel Extraction Kit

For sequencing, QIAquick Gel Extraction Kit (Qiagen, Germany) was used as following:

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg gel ~ 100 pl). The maximum amount of gel per spin column is 400 mg. For 2% agarose gels, add 6 volumes Buffer QG.
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2-3 min to help dissolve gel. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 ul 3 M sodium acetate, pH 5.0, and mix. The mixture turns yellow.
4. Add I gel volume isopropanol to the sample and mix.
5. Place a QIAquick spin column in a provided 2 ml collection tube or into a vacuum manifold. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min or apply vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 pl, load and spin/apply vacuum again.

6. If DNA will subsequently be used for sequencing, in vitro transcription, or microinjection, add 500 ul Buffer QG to the QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.

7. To wash, add 750 pl Buffer PE to QlAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube. Note: If the DNA will be used for salt-sensitive applications (e.g., sequencing, blunt-ended ligation), let the column stand 2-5 min after addition of Buffer PE. Centrifuge the QlAquick column in the provided 2 ml collection tube for 1 min to remove residual wash buffer.

8. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

9. To elute DNA, add 50 pl Buffer EB (10 mM Tris-CI, pH 8.5) or water to the center of the QlAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 pl Buffer EB to the center of the QlAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min. After the addition of Buffer EB to the QlAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA.

10. If purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

2.2.5 DNA sequencing

DNA sequencing was performed utilizing an ABI 3730XLs nucleotide sequence analyzer through Macrogen Inc. (Korea). All obtained DNA sequences were edited by using Chromas software and aligned with (ClustalW algorithm), available in MUSCLE program within EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/muscle/).

To verify the closest species match for DNA sequences obtained in this research, Basic Local Alignment Search Tool for nucleotides (Blastn) implemented in the NCBI GenBank database was used to evaluate all sequences.

3. Results

Morphological and DNA analyses revealed that present Isopod belongs to *Porcellionides pruinosus* (Brandt, 1833).

3.1 Taxonomy of termite genus of *Porcellionides pruinosus*

Table (3.1) shows the taxonomy of Isopod:

Scientific classification	
Domain	**Eukaryota**
Kingdom	**Animalia**
Phylum	**Arthropoda**
Class	**Malacostraca**
Order	**Isopoda**
Suborder	**Oniscidea**
Family	**Porcellionidae**
Genus	***Porcellionides***
Species	***P. pruinosus*** **(Brandt, 1833)**

3.2.Morphological description

The Isopode species *Porcellionides pruinosus* is a medium sized woodlouse of distinctive appearance (and habitat) (Figure 3.1 and 3.2). It

is typically a purplish-brown in colour with a characteristic blue-grey bloom (but rarely it may be orange). The body colour contrasts against the long whitish legs and the pale annulations on the antennae. The body has an obviously stepped outline allowing rapid movement, the antennal flagella comprising two segments and with two pairs of pleopodal lungs (Gregory, 2021). Common name Plum Woodlouse diet leaf litter and decomposing softwoods like cork bark (Theobald, 1903). Body oblong-ovate, twice as long as wide, 6 mm X 13.5 mm. Abdomen abruptly narrower than thorax. Head twice as wide as long, 1 mm.: 2 mm., with the anterior margin slightly convex; antero-lateral lobes small. The eyes are small, composite, and situated at the base of the antero-lateral lobes. The first pair of antennae are small and inconspicuous. The abdomen is abruptly narrower than the thorax. All six segments are distinct. The first two have the lateral parts covered by the seventh thoracic segment. The third, fourth, and fifth segments have small lateral parts and are not greatly expanded. The sixth or terminal segment is triangular in shape. Telson with angulated margins and sharp tip. (Richardson, 1910).

Fig. (3.1) shows the dorsal view of the Isopod.

Fig. (3.2) shows the ventral view of the Isopod.

3.3. Molecular identification

3.3.1. Gel electrophoresis

PCR successfully generated a 658 bp a target *COI* gene for Isopod as shown in Figuure (3.3).

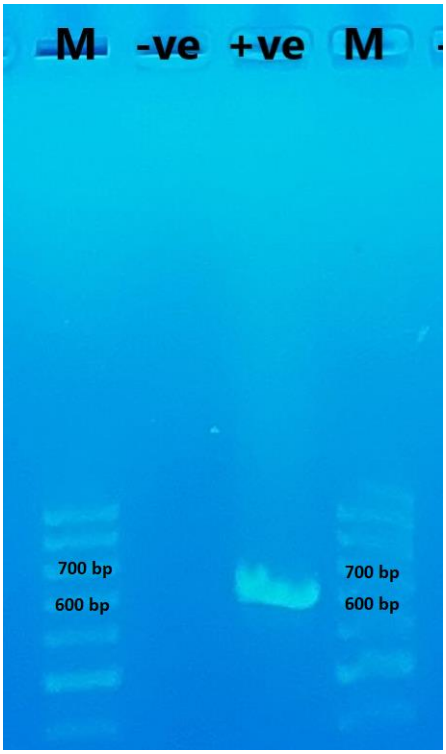

Fig. 3.3 shows gel electrophoresis run of the target *COI* gene (658 bp). M = GeneRuler 50 bp DNA ladder marker, (-ve) = ddH_2O, (+ve) = Isopod DNA.

3.3.2 Sequencing analysis

A chromatogram of sequencing (Figure 3.4) belongs to mitochondrial DNA of *COI* gene obtained from Macrogen Inc. by applying Chromas software. The nucleotides of sequencing of *COI* gene belong to the present Isopod reveals that this Isopod belongs to *Porcellionides pruinosus* with identities of (100%) to Accession number (**MG887934.1**) by applying **Blastn** tool which available in NCBI webpage (Figure 3.5).

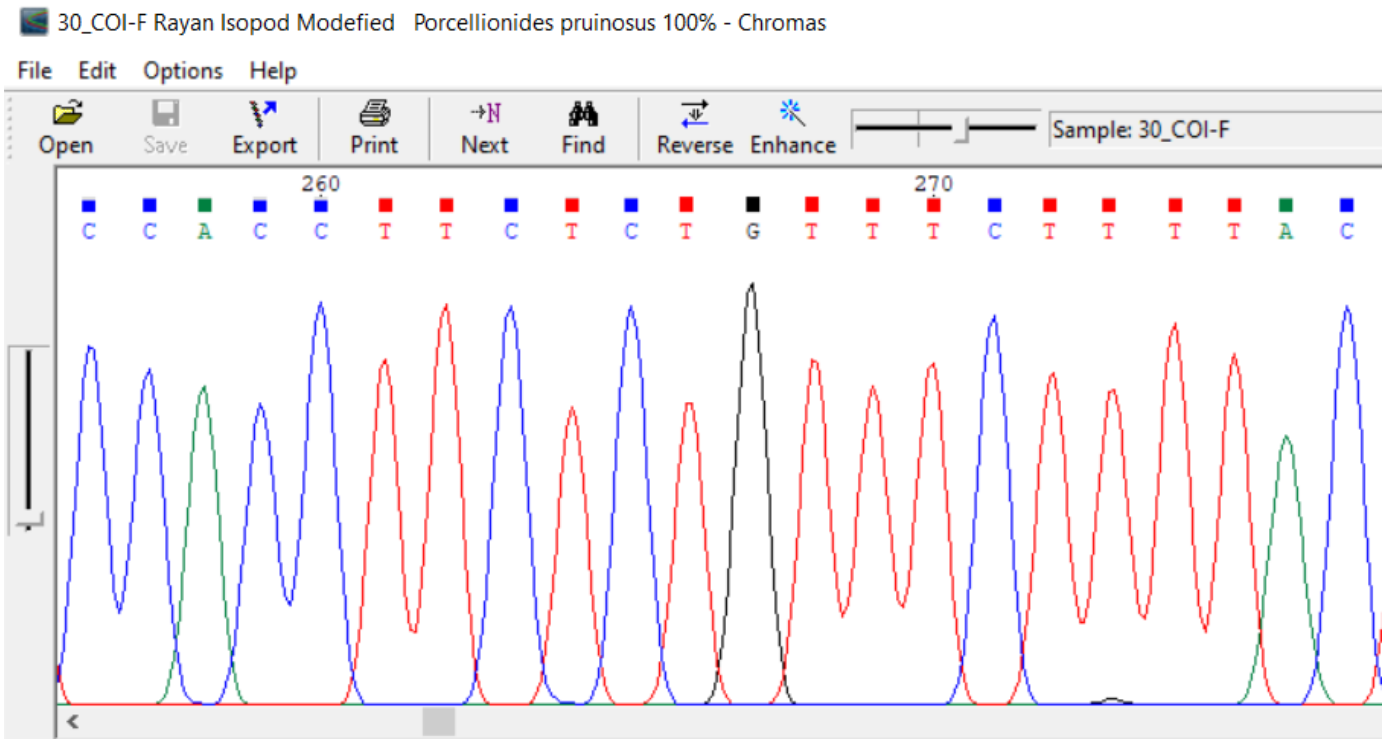

Fig. (3.4) shows the chromatogram of the sequence of the Isopod

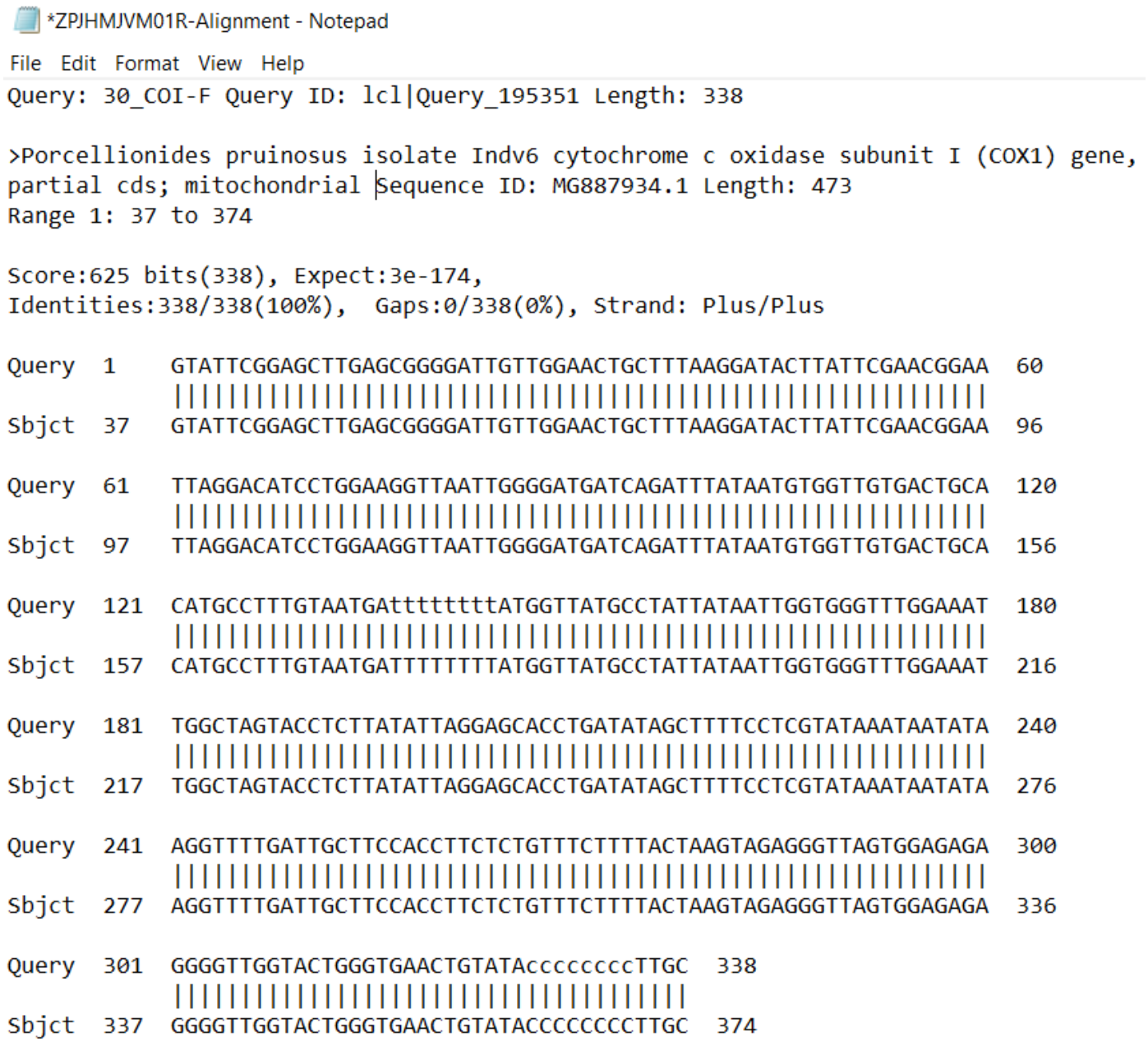

Fig. (3.5) Two sequence alignment between present (Query) and MG887934.1 (subject)

4. DISCUSSION

Terrestrial isopods (Oniscidea) are one of the most diverse groups within Isopoda due to their morphophysiological, ecological, and behavioral adaptations, which allowed successful colonization of almost all terrestrial habitats since ancient geological periods (Sfenthourakis and Hornung, 2018).

Up to date four species of isopods were recorded in Iraq and mainly distributed in middle and south (Table 4.1). The current species *Porcellionides pruinosus* is recoded for the first time in Iraq. This species of Mediterranean origin, now with a synanthropic cosmopolitan distribution (Schmalfuss, 2003). Using molecular anlysis combined with morphological characters confirmed the identity of this species.

Table (4.1) shows species of terrestrial isopods recorded in Iraq.

Species	Location	References
Procellio scaber **(Latreille, 1804)**	**Baghdad & Al-Najaf**	**Abound and Al-Doori 2019; Mizhir 2022**
Procellio leavis **(Laterllile, 1804)**	**Al-Najaf**	**Mizhir, 2022**
Procellio spinicornis **(Say, 1818)**	**Al-Najaf**	**Mizhir, 2022**
Procellio evansi **(Omer-Cooper, 1923)**	**Al-Basrah**	**Ahmad, 1974**

5. CONCLUSIONS

The terrestrial Isopode *Porcellionides pruenosus* was recored for the first time in Iraq using morphology and DNA barcoding of *COI* gene.

6. REFERENCES

BOYKO, C. B., BRUCE, N. L., HADFIELD, K. A., MERRIN, K. L., OTA, Y., POORE, G. C. B. and TAITI, S. (Eds) 2023. World Marine, Freshwater and Terrestrial Isopod Crustaceans database. Accessed at https://www.marinespecies.org/isopoda on 2023-11-25. doi:10.14284/365.

EATON, E. R. and KAUFMAN, K., 2007. *Kaufman field guide to insects of North America*. Houghton Mifflin Harcourt.

EMERSON, B. C., CICCONARDI, F., FANCIULLI, P. P. and SHAW, P. J., 2011. Phylogeny, phylogeography, phylobetadiversity and the molecular analysis of biological communities. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *366*(1576) 2391-2402.

FOLMER, O., BLACK, M., HOEH, W., LUTZ, R. and VRIJENHOEK, R., 1994. DNA primers for amplification of mitochondrial Cytochrome Oxidase Subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*(5) 294-299.

GREGORY, S., 2021. An Introduction to the Identification of the Woodlice (Isopoda: Oniscidea) occurring in Berkshire, Buckinghamshire and Oxfordshire.

Hajibabaei, M., Janzen, D.H., Burns, J.M., Hallwachs, W. and Hebert, P.D., 2006. DNA barcodes distinguish species of tropical Lepidoptera. *Proceedings of the National Academy of Sciences*, *103*(4) 968-971.

HARRIS, M., 2012. *The abominable pig. In Food and Culture.* Routledge. pp. 73-85.

HEBERT, P. D., CYWINSKA, A., BALL, S. L. AND DEWAARD, J. R., 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270* (1512) 313-321.

HEBERT, P. D., RATNASINGHAM, S. and DE WAARD, J. R., 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(suppl-1) S96-S99.

HEELEY, W., 1941. Observations on the life-histories of some terrestrial isopods. In *Proceedings of the Zoological Society of London. 111*(1-2) 79-149.

LEGENDRE, M., LINHART, O. and BILLARD, R., 1996. Spawning and management of gametes, fertilized eggs and embryos in Siluroidei. *Aquatic Living Resources*, *9*(S1) 59-80.

MARIN, I. N. and TIUNOV, A. V., 2023. Terrestrial crustaceans (Arthropoda, Crustacea): taxonomic diversity, terrestrial adaptations, and ecological functions. *ZooKeys*, 1169, 95-162.

Richardson, H., 1910. Marine Isopods Collected in the Philippines: By the US Fisheries Steamer Albatross in 1907-08 (No. 736). US Government Printing Office.

SALE, K., 2000. *Dwellers in the land: The bioregional vision*. University of Georgia Press.

SCARBOROUGH, J., 1992. *Medical and biological terminologies: classical origins* (Vol. 13). University of Oklahoma Press.

SCHMALFUSS, H., 2003. World catalog of terrestrial isopods (Isopoda: Oniscidea). *Stuttgarter Beiträge zur Naturkunde*, *654*, 1–341.

SCHMIDT, C., 2003. Contribution to the phylogenetic system of the Crinocheta (Crustacea, Isopoda). Part 2.(Oniscoidea to Armadillidiidae). *Zoosystematics and Evolution*, *79*(1) 3-179.

SFENTHOURAKIS, S. and HORNUNG, E., 2018. Isopod distribution and climate change. *ZooKeys*, *801*, 25-61.

SMIT, N. J. and DAVIES, A. J., 2004. The curious life-style of the parasitic stages of gnathiid isopods. *Advances in Parasitology*, *58*, 289-391.

SHRIMPS, F.D.S.P., Uropods distinctly exceeding telson; protopodite with distoventral angel rounded; exopod 2.0 times longer than broad, lateral margin convex, glabrous, distolateral angles damaged, probably with feeble or obsolete angle and larger mobile spine, diaeresis distinct; endopod about 0.9 of exopod length, 2.7 times longer than broad. Ova numerous, small..

THEOBALD, F.V., 1903. *Report on Economic Zoology* (Vol. 2). order of the trustees.

VINCENT, J. F., 2005. Defense and attack strategies and mechanisms in biology. In *Biomimetics.* CRC Press. pp. 359-382.