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Morphological and Molecular Identification of Some Terrestrial Snails: First Report

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ABSTRACT

Land snail identification researches in Iraq is limited. In Erbil-Iraq, a study was carried out to explore the identification of various land snails. In earlier studies, most species determination was based on shell morphology, anatomy of genitalia, and mantle color, these criteria are continuously applied to describe new species/new records. Species of similar morphology have ambiguous identities, especially juvenile specimens, due to the great level of morphological variation observed in some snail species. Five species of land snails belong to four families were found in different locations of Erbil City, Iraq, during November 2020 to April 2021, when average temperature was arranged (4-25 C°). Morphology and Cytochrome c oxidase subunit I (*COI*) gene were used to identify specimens, *Rumina decollate* (Linnaeus, 1758), found for the first time in Iraq. In addition to four species: *Eobania vermiculata* (Müller, 1774), *Cornu aspersum* (Müller, 1774), *Cochlicella barbara* (Linnaeus, 1758) and *Polygyra cereolus* (Megerle von Mühlfeldt, 1818) were previously registered in Iraq and have never been recorded in Erbil City, Kurdistan Region, Iraq. This study highlights the impotence of genetic analyses along with morphological analyses in identification of species. This finding suggests, for precise evaluation of species diversity, molecular tools should be included in future biodiversity researches.

1. Introduction

The gastropods are the largest and most diverse class of the Mollusca phylum, with the most remarkable diversity in morphology and ecology (Ponder et al., 2019). Land snails have been considered destructive pests capable of inflicting excessive agricultural and horticultural damage by breeding in large numbers (Kumar, 2020).

There have been limited studies done in Iraq on land snails. Mousson (1874) described four species from different parts of Iraq, and described *Xeropicta mesopotamica* as a new species from Mesopotamia. The most significant contribution to the land snails of Mesopotamia by Germain (1921) and Pallary (1939), who referred to some species of land snails in Iraq. Biggs (1959) listed many land snails from north Iraq and reported *Xeropicta derbentina*. (Najim, 1959, 1961) revealed eight species of terrestrial snails throughout Iraq. Since these publications, the scientific names and taxonomic positions of the numerous forms described in the past have been altered or synonymized. Also, collections since that time have indicated that many of those species might be threatened in the area (Harris, 1978).

Recent studies have documented three species in Basra City (South Iraq), which were already recorded in previous researches: *Monacha obstructa* (Abdul-Sahib, 2006), Xeropicta mesopotamica (Al-Khafaji, 2009) and *Allopeas gracile* (Naser, 2010).

New records of seven land snail species in Iraq were registered; six of them were in Baghdad, namely: *Cornu aspersum* by Kamal and Almuktar (2010), Monacha cantiana by Khan and Almuktar (2013), *Paropeas achatinaceum* by Al-Doori et al. (2017), *Cochlicella barbara* by Al-Doori et al. (2018), *Eobania vermiculata* by Al-Doori (2019), *Polygyra cereolus* by Jihad and Ali (2021). In addition, *Monacha cartusiana* was described in Babylon City by Hadi (2018).

In earlier studies, species determination was based on shell morphology, anatomy of genitalia, and mantle color, criteria are continuously applied to describe new species (Huňová et al., 2012). However, species of similar morphology have ambiguous identities, especially juvenile specimens, due to the great level of morphological variation observed in some snail species (Blacket et al., 2016). Potentially, DNA barcoding enables a fast and accurate approach to the routine diagnosis of species for phylogenetic studies (Johanson et al., 2009).

Currently, there is no published information about the distribution and molecular study of terrestrial snails in Erbil City- Kurdistan Region-Iraq. However, the present study was conducted to report one first record of land snail species in Iraq, and the molecular evidence to confirm its existence.

2. Material and Methods

2.1. Study Area

The terrestrial snails were collected during November 2020-April 2021 from the soil surface and grasses from different locations (Azadi Quarter, Mantikawa Q., Shawis Q., Turaq Q. Shanadar Park, Minara P., and Sami Abdul-Rahman P., …. etc.) in Erbil City, Iraq (Fig. 1). The snails were kept alive in a container with optimal temperature and O_2 aerated and transferred immediately to the Advance Invertebrate Laboratory of Biology Department, Education College, Salahaddin University-Erbil, Iraq.

2.2. Specimens' identification

Morphologically, all these characters were used to determine the species or subspecies identification, which includes: the height, width, aperture width and aperture height of the shell and the number of whorls were counted. The shells were measured with a caliper (accurate to 0.1 mm). The classification using (Welter-Schultes, 2012), also by comparing to literature and to material in the Auffenberg et al. (2023) and MolluscaBase (2023).

2.3. DNA extraction, COI gene amplification, sequencing and phylogenetic analysis

DNA was extracted from fresh soft tissues of the snails after removing them from the shells. The

tissues (mostly feet) were sliced into 2-3 mm thick pieces and squashed between two sheets of clean Aluminum foil, except for small-sized snails where the entire specimen was used (Carmona et al., 2013).

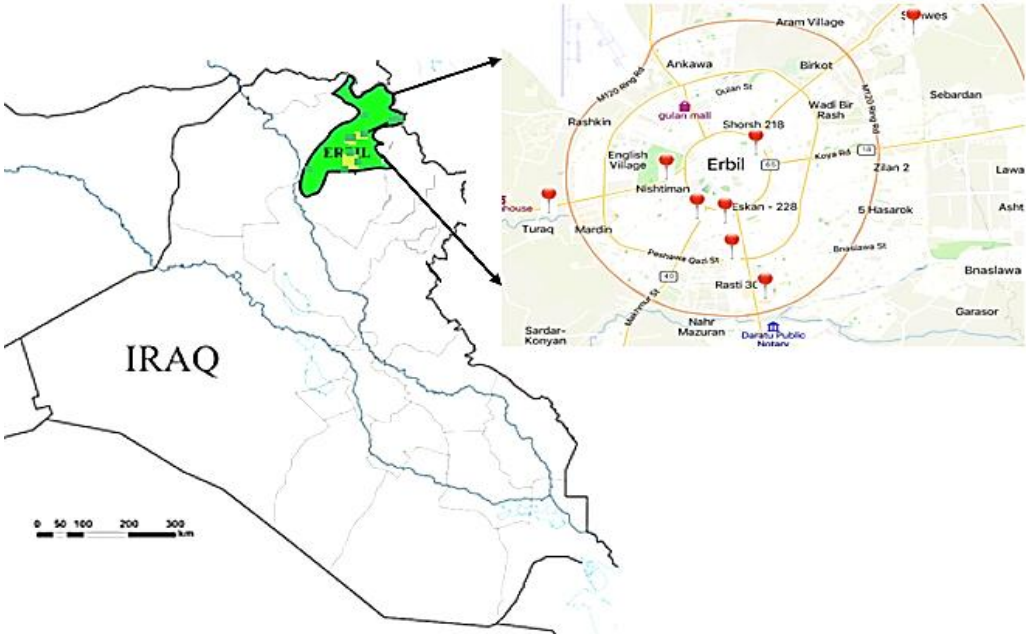

FIGURE 1: Map of Iraq, Erbil City showing samples locations

The DNA was extracted using a commercially available kit (GeneAll® Exgene™ Clinic SV, Korea) following the manufacturer's protocol.

The DNA barcoding for terrestrial snails (five species) 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`).

Master mix (Ampliqon PCR Enzymes & Reagents, Denmark) was used to amplify the partial sequences of (COI) gene by (PCR): the amplification of the locus was conducted in a total volume of (20 µl) containing: 10 µl master mix, 0.2 µM for each primer, 2 µl DNA and 5 µl ddH2O. PCR was carried out in (PCRmax Alpha thermal cycler, UK) as follows: (2 min at 94 °C, 35 cycles of 30-sec step: 94 °C, 50 °C and 72 °C and a final extension step 7 min at 72 °C). PCR reactions were verified by 1.3% agarose gel. PCR products were purified by applying a gel purification kit (QIAquick Gel Extraction, Qiagen, Germany) following the manufacturer's instructions.

DNA sequencing was performed utilizing an ABI 3730XLs nucleotide sequence analyzer through Macrogen Inc. (Korea). All obtained DNA sequences were edited 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) was implemented in the NCBI GenBank database to evaluate all sequences. Further estimates of COI gene variation and relationships among species (applying Maximum Likelihood and Maximum Parsimony Methods) were conducted using MEGA X (Kumar et al., 2018), including a sequence representative from many species currently present available on GenBank. The consistency index was (0.871395), the retention index was (0.864917), and the composite index was 0.894144 (0.880701) for all sites and parsimony-informative sites (in parentheses). The bootstrap consensus tree

inferred from 100 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1992). Branches corresponding to partitions were reproduced in fewer than 0% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). The final dataset included a total of 654 positions.

3. Results

In the present study, five terrestrial snail species belonging to four families, Order Stylommatophora, were collected from various locations in Erbil City, Iraq. The average temperatures from November 2020 to April 2021 less than 50% bootstrap replicates are collapsed. Twenty nucleotide sequences were involved in this analysis. All positions with a site coverage of less than 100% were eliminated; i.e., were arranged (4-25 °C). The species *Rumina decollata* (Linnaeus, 1758) recorded for the first time in Iraq. The other four species, *Eobania vermiculata*, *Cornu aspersum*, *Cochlicella barbara*, and *Polygyra cereolus* already been recorded in Iraq. However, these five species have never been documented in Kurdistan region, Iraq (Figure 2).

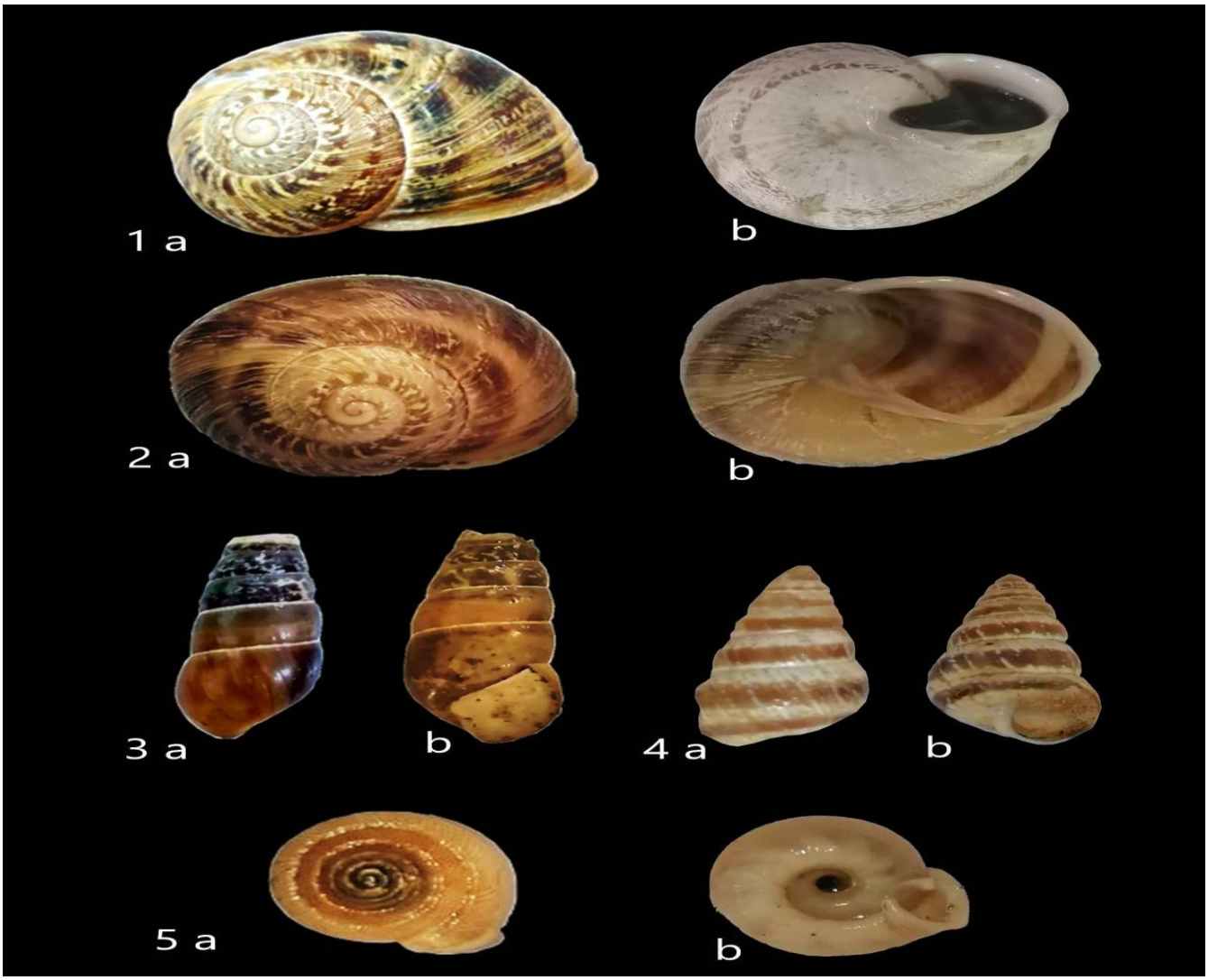

FIGURE 2:1. Eobania vermiculata; 2. Cornu aspersum; 3. Rumina decollata; 4. Cochlicella barbara and 5. Polygyra cereolus. (a=dorsal view; b=ventral view)

3.1. Morphological description

Eobania vermiculata (Müller, 1774) (Stylommatophora, Helicidae)
Shell height (13-25) mm and width (21-33) mm. Shell with (3-5) whorls expand uniformly and has a conical-compressed shape, having whitish along with spiral brown bands, and spotted with white strips. Spire raised; umbilicus covered; aperture lip white and reflected (Figure 2-1 a, b).

Cornu aspersum (Müller, 1774) (Stylommatophora, Helicidae)
The shell has a globose shape with a raised spire. It is wrinkled with yellow-brown color in addition to dark brown spiral strips. The height is (18-34) mm, and the width is (25-37) mm. The number of whorls (3-5) with a closed umbilicus. The aperture is large and rounded with a thick, white and strongly reflected lip (Figure 2-2 a, b).

Rumina decollata (Linnaeus, 1758) (Stylommatophora, Achatinidae)
Shell is long cone-shaped with decollate blunt end (only in adulthood). It is a tan to dark brown color, glossy, translucent, and without bands. It grows to roughly (40-48) mm in length and (12-13) mm in width. The number of whorls in adulthood retains (4-7) whorls, while in juvenile are (8-10). The umbilicus is broadly open, about 1/3 shell diameter (Figure 2-3 a, b).

Cochlicella barbara (Linnaeus, 1758) (Stylommatophora, Geometridae)
The shell has a small conical-narrow and pointed end shaped with a brownish or greyish color and white bands. The height of the shell (8-9) mm and the width (4.5-5) mm. The number of whorls (7-8) is slightly convex with shallow sutures. The umbilicus is very narrow (Figure 2-4 a, b).

Polygyra cereolus (Mühlfeld, 1816) (Stylommatophora, Polygyridae)
The shell has a discoidal shape. It is light grey with grey or brown radial streaks or spots. The upper side of the shell is flattened or slightly convex, while the lower side is flattened or slightly concave. The umbilicus is vortex shaped, while the aperture is heart-shaped with a thickened margin. The height is (3-4.5) mm, the width is (11-18) mm. The whorls varied from (7-9) (Figure 2-5 a, b).

3.2. Molecular Identification

Mitochondrial DNA COI from five different species of land snails was sequenced. The sequences available in GenBank were used to compare the results, which confirmed the identities of these species and corresponded with morphological characters. The obtained sequencing results were deposited in GenBank database under the following accession numbers (ON045123 - ON045127) for *Eobania vermiculata*, *Cornu aspersum*, *Rumina decollata*, *Cochlicella barbara*, and *Polygyra cereolus* respectively (Diagram 1).

4. Discussion

Researches about terrestrial snails generally in Kurdistan region and in Erbil city specifically were inattentive has been ignored, and we think a lot of gaps in this field. The current work was the first to analyze the biogeographic and phylogenetic of five species of land snails belong to different genera, collected from various locations in Erbil City, Kurdistan region, Iraq. All differ considerably in both morphologically and genetically.
Since there are many morphologically similar potential exotic land snail species worldwide and, in this study, many morphological features detected, including; size, color, and shell pattern differences, were distinctive among land snail adults. The accurate identification of species at all life stages is crucial for biosecurity (Blacket et al., 2016). DNA barcoding provides a remarkably valuable approach to precise species identification (Davison et al., 2009). The sequence data presented in the current study are derived from *COI* gene, and currently this method considered one of the most widely used technology for phylogeny, systematics, and

species identification. There are many reference DNA barcode sequences that belong to Gastropod species available in GenBank which can be used for identification purposes (Ran et al., 2020). Till now, there was no study in Iraq has been used molecular and phylogenetics to identify land snail specimens except Hussein et al. (2018), who used *COI* gene in classification of *Paropeas achatinaceum*.

Our results showed that Helicidae is monophyletic, but this topology might change with the addition of more loci. In the current research, diagram (1), clade (A) of *Eobania vermiculata* sequence data obtained showed a 100% similarity to the partial *COI* gene recorded in Croatia (JF277391.1) by Rada et al. (2012), and was sister group with clade (B). The morphometric characteristics of the different shell sizes (Fig. 1-1 a and b) revealed a multivariate intrapopulation variation existed among populations exposed to anthropogenic activity.

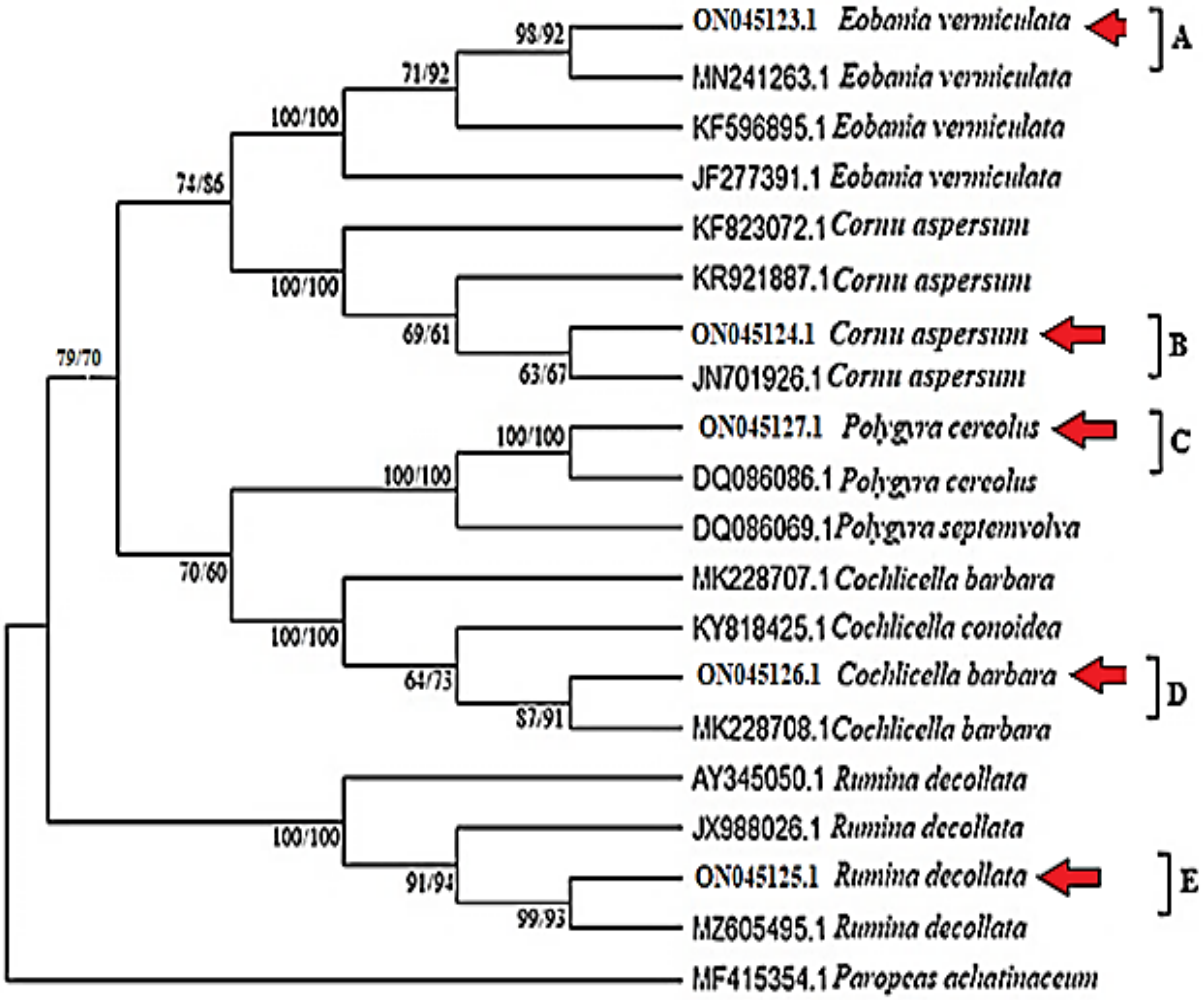

DIAGRAM 1

Both the Maximum Likelihood (Kimura 2-parameter model) and Maximum Parsimony methods were applied to infer the evolutionary history, all nodes supported by high bootstrap (ML/MP).

In clade (B), *Cornu aspersum* (Fig. 1-2 a and b) in monophyletic (98% similarity) to JN701926.1 recorded by Madec (1991) from France and other localities. Multiple lineages of *C. aspersum* are known to have spread worldwide (Madec et al., 2003). Several COI haplotypes have been identified among *Cornu aspersum* and *Cantareus apertus* (sequence divergence of <1.5% within each species) (Blacket et al., 2016). In clade (C), the sequence of *Polygyra cereolus* (Fig. 1-5 a

and b) monophyletic and matched to DQ086086.1 with similarity (99%) described by Perez (2011) from northeastern Mexico. Also, sister group to clade (D). Distinguishing *Polygyra cereolus* from *P. septemvolva* is difficult, and misidentification is likely common, as suggested by Perez et al. (2014). *Polygyra cereolus* is characterized by having a well-developed parietal lamella located within the first half of the last whorl wall (Pilsbry, 1939). Our specimen matches the morphology, shell size, spire height variability, and parietal lamellae display (Charles and Lenoble, 2020).

In Clade (D), *Cochlicella barbara* (Fig. 1-4 a and b) is monophyletic to MK228708.1 with similarity (%99) recorded by Jourdan et al. (2019) in France, and it is paraphyletic to other species with fluctuated bootstraps among them. This snail originated in Europe and Australia and has spread to several other countries (Khalaf et al., 2020). Morphologically, *C. barbara* can be distinguished from *C. conoidea* by shell heightless than 2x width, whorls more flattened, and sutures shallow with *C. barbara*, while in other species shell conical, whorls convex and shallow sutures (Ali and Ramdane, 2020), which represent a molecular difference from the results of the current study.

In Clade (E), *Rumina decollata* (Fig. 1-3 a and b) is monophyletic with MZ605495.1 (with similarity %98) described by Rau et al. (2021) from Argentina. In the other way, its paraphyletic with other clades with various bootstraps. Morphological differentiation in this genus has been interpreted as reflecting different nominal species (Mienis, 2002), varieties or ecophenotypes within a single species referring to differences in shell shape, color and size (Prevot et al., 2013). *Rumina decollata* occur all over the western Mediterranean region and have also been introduced in many other areas in the world (Matsukuma and Takeda, 2009, Prevot et al., 2013).

People on the inhabited islands alter the natural habitat through farming, reforestation and horticulture in their populated areas. In this manner, ecological conditions of terrestrial snails are improved (larger quantities of food and higher humidity), and greater variations are displayed within populations than individuals. As a result, the color and pattern of garden snail shells are highly variable, associated with geographical and/or ecological factors (Blacket et al., 2016).

Finally, *Paropeas achatinaceum* (MF415354.1) was recorded by Fontanilla et al. (2017) distributed in Indo-Pacific region used as outgroup; this species was recorded in Iraq by Hussein et al. (2018). Regarding the phylogeny, the tree topology obtained on the *COI* gene can be interpreted as a preliminary phylogenetic framework for the various families. However, most of the deeper nodes are well-supported (Neiber et al., 2017). Overall, molecular data were added to clarify the taxonomic validity and phylogenetic relationships of some land snail species.

Climate change might contribute to the extension or invasion of several land snail species around the world (Proćków et al., 2019). Terrestrial snails (such as *Monacha cartusiana* and *Helix lucorum*) with Atlantic or Mediterranean distributions rapidly spread northwards throughout Europe (Peltanová et al., 2012). The recent presence of these five exotic terrestrial snail species in Iraq and their establishment may be attributed to human mediated transportation; this could be the reason for not mentioning these species in the old literature. Although some of the highly invasive species can cause a substantial impact on ecosystem functioning, biodiversity, and alterations in food webs, the extinction of native species may be contributed to some of these species (Simberloff et al., 2013). Land snail biodiversity and distribution are influenced by a variety of conditions, including soil properties, climatic factors, and anthropogenic disturbances (Zaidi et al., 2021).

International Union for Conservation of Nature [IUCN] Red List revealed that *Euglandina rosea*, a non-native land snail, was introduced as a biological control agent against the giant African land snail *Achatina fulica* across the South Pacific. This species is thought to be the direct cause of the extinction of at least 134 native snail species (Blackburn et al., 2019).

Up to date, the land snails (37) species which are revealed in Iraq, includes (Tab.1). Current

accepted (valid) names of these taxa were updated according to Auffenberg et al. (2023), MolluscaBase (2023) and World Register of Marine Species (Mees et al., 2015).

5. Conclusions

Five species of land snails were recorded, with one species found as a first record to the Iraqi fauna. Species identification was based on morphology and molecular techniques. Phylogenetic relationships were analyzed among the current species and with other closely related species.

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Table (1): Accepted (valid) name and original name of land snails recorded in Iraq

Accepted (Valid) name	Original name
Allopeas gracile Hutton, 1834	*Bulimus gracilis* (Hutton, 1834)
Buliminus alepensis Pfeiffer, 1841	*Petraeus halepensis* (Pfeiffer, 1841)
Buliminus alepensis var. *elongata* Pallary, 1939	*Petraeus halepensis* var. *elongata* (Pallary, 1939)
Buliminus damascensis Pallary, 1929	*Petraeus damascensis* (Pallary, 1929)
Buliminus egregius Nägele, 1902	*Buliminus egregius* Nägele, 1902
Buliminus macfadyeni Pallary, 1939	*Petraeus macfadyeni* (Pallary, 1939)
Buliminus samavaensis Mousson, 1874	*Buliminus samavaensis* Mousson, 1874
Bulimus iraqensis Pallary, 1939	*Bulimus iraqensis* Pallary, 1939
Cecilioides minuta Mousson, 1874	*Acicula* (*Caecilianella*) *minuta* (Mousson, 1874)
Ena macfadyeni Pallary, 1939	*Ena* (*Cirna*) *macfadyeni* (Pallary, 1939)
Geminula isseliana Bourguignat, 1865	*Bulimus isselianus* (Bourguignat, 1865)
Helix figulina Rossmässler, 1839	*Helix figulina* Rossmässler, 1839
Helix lucorum Linnaeus, 1758	*Helix lucorum* Linnaeus, 1758
Helix salomonica Nägele, 1899 (fossil snail)	*Helix* (*Pomatia*) *salomonica* Nägele, 1899 (fossil snail)
Lauria cylindracea da Costa, 1778	*Turbo cylindraceus* (da Costa, 1778)
Leucomastus kindermanni mesopotamica von Martens, 1874	*Bulimus (Petraeus) mesopotamicus* (von Martens, 1874)
Levantina bellardi Mousson, 1854	*Helix bellardii* (Mousson, 1854)
Levantina spiriplana caesareana Mousson, 1854	*Helix caesareana* (Mousson, 1854)
Levantina escheriana Bourguignat, 1864	*Helix escheriana* (Bourguignat, 1864)
Levantina guttata Olivier, 1804	*Helix guttata* (Olivier, 1804)
Levantina kurdistana Pfeiffer, 1861	*Helix kurdistana* (Pfeiffer, 1861)
Levantina macfadyeni Pallary, 1939	*Assyriella macfadyeni* (Pallary, 1939)
Levantina macfadyeni var. *minor* Pallary, 1939	*Assyriella macfadyeni* var. *minor* (Pallary, 1939)
Metodontia houaiensis Crosse, 1882	*Helix houaiensis* (Crosse, 1882)
Monacha obstructa Pfeiffer, 1842	*Helix obstructa* (Pfeiffer, 1842)
Orculella sirianocoriensis libanotica Tristram, 1865	*Orcula* (*Orculella*) *iraqensis* (Pallary, 1939), *Orcula* (*Orculella*) *iraqensis* var. *minor* (Pallary, 1939) and *Orcula* (*Orculella*) *iraqensis* var. *porrecta* (Pallary, 1939)
Pene bulimoides Pfeiffer, 1842	*Buliminus sidoniensis* (Charpentier, 1847)
Succinea urensis Pallary, 1939 (fossil snail)	*Succinea urensis* Pallary, 1939 (fossil snail)
Xerocrassa commeata Mousson, 1874	*Helix* (*Xerophila*) *commeata* (Mousson, 1874)
Xerocrassa seetzeni Pfeiffer, 1847	*Helix seetzeni* (Pfeiffer, 1847)
Xeroleuca macfadyeni Pallary, 1939	*Xeroleuca macfadyeni* Pallary, 1939
Xerophila connollyi Pallary, 1939	*Xerophila connollyi* Pallary, 1939
Xeropicta derbentina Krynicki, 1836	*Helix derbentina* (Krynicki, 1836)

Xeropicta krynickii Krynicki, 1833	*Xerophila vestalis* (Pfeiffer, 1841) *Xerophila vestalis* var. *joppensis* Roth, 1855
Xeropicta mesopotamica Mousson, 1874	*Helix mesopotamica* Mousson, 1874
Xeropicta mesopotamica var. *ghaesiana* Mousson, 1874	*Helix mesopotamica* var. *ghaesiana* Mousson, 1874
Zebrina detrita ver. *tumida* Mousoon 1874	*Zebrinus detritus* ver. *tumida* ((Parueyss) Mousoon, 1874)

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