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New recorder with Morphological and molecular identifications of the Tropical leather leaf slug *Eleutherocaulis alte* (Ferussac,1822) from Basrah Province, Iraq

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Abstract

Land slugs have been able to spread all over the world due to their possession of a set of basic characteristics and qualities such as the leathery dorsal surface and the narrow foot that reduces evaporation during the dry period. Given the lack of any previous studies in Iraq on these slugs, we conducted this study to record a new species of land slug *Eleutherocaulis alte* (ferussac, 1822). In this study, bioinformatics analysis was used to identify the species *E. alte* and determine the phenotypic characteristics of this species, which was collected from Karmat Ali area, Basrah Governorate, for a period of three months, March, April and May 2023. The results of the study showed a variation in the phenotypic measurements (animal length, animal width) for the size categories (A, B, C), as the highest measurements in the average length and width of the animal were observed in category A and the lowest measurements in category C. Bioinformatic analysis results based on the mitochondrial cytochrome c oxidase subunit I gene revealed five unique Haplotypes (H1 to H5) of *E. alte*. The largest haplotype 1 (H1) contained twenty-five sequences (24 sequences from Japan, one from the United Kingdom and one from India), followed by H3 (containing only four sequences from Japan) and H4 (containing three sequences from India and one sequence from our current study). H2 and H5 were from India and Egypt, respectively. In total, the Iraqi sequences were grouped into the common HaplotypeH4 between Iraq and India. In short, based on the nucleotide level, H1 to H3 were far removed from H4 and H5. Finally, the Iraqi *E. alte* belonged to H4 and was more closely related to H5 than the other Haplotypes. Although these five Haplotypes had many changes at the nucleotide levels, they shared almost similar amino acids throughout the protein lengths. H1 and H3 had 100% amino acid identity, while H2, H4 and H5 had 98.08, 96.79 and 94.23% identity.

Keywords: Land slugs, Haplotypes, Cytochrome C,Veronicellidae, biodiversity indicators

Introduction

Land slugs are serious agricultural pests due to their lack of host specificity. They are multi-feeding animals that feed on many different plants. They can also feed on other materials such as decomposing organic matter, and thus they are capable of causing severe damage not only to plants, but also to slugs. It transmit pathogens to humans indirectly by eating fruits and vegetables contaminated with mucus and feces (USDA-APHIS, 2010; Herbert and Kiburn, 2004)

In contrast, it was found that these animals have an important role in terrestrial ecosystems as they contribute to the decomposition process by breaking down organic matter into smaller particles through their feeding activities, thus accelerating decomposition rates in the soil. The presence of these slugs also greatly helps in the decomposition of plant waste, which leads to an increase in the availability of nutrients (Barker, 2016). These animals also enhance local biodiversity by digging in the soil or hiding under rocks and leaves, thus providing these microhabitats with shelter for other organisms, including invertebrates, insects and small mammals. Their excavation activities also enhance soil aeration and moisture levels, which helps in microbial decomposition processes (Kourtev *etal*., 2020; Jones *et al*., 2018). These slugs also serve as indicators of ecosystem health and biodiversity because Its sensitivity to environmental changes (Grimm *et al*., 2019) The Land slug *Eleutherocaulis alte* (Ferussac, 1822) belongs to the order Systellommato phora of the Veronicellidae family, which includes 500 species of tropical and semi-tropical slugs (Mollusca Bace, 2021). The *E. alte* slug is of African origin (Cowie, 2000) and is found in central and eastern Africa southward to KwaZulu-Natal (Herbert, 2010). It has no common name, but it is usually referred to as Tropical leather leaf slug. This species has been introduced into many tropical regions (Herbert and Kiburn, 2004). Since previous Iraqi studies did not mention any information about ground slugs, the current study aimed to add new information about ground slugs and also use bioinformatics analysis to identify the species *E. alte* in Basrah Governorate, Iraq.

Material and Methods

Study area and sample collection: The Land slug *Eleutherocaulis alte* was obtained from some gardens in Karmat Ali district, Basrah Governorate during the months of March, April and May 2023

To make the necessary measurements and diagnosis, and based on the manual collection method (Hlavac and pletanova,2010) samples of the Land slug *E. alte* were collected from among the fallen leaves of plants or above the soil surface and also in humid places, then brought to the laboratory using a tightly closed plastic box with perforated covers to ensure air entry.

Morphological characteristics: Based on the taxonomic key of Neubert (1998), the Land slug *Eleu therocaulis alte* was diagnosed morphologically by measuring two dimensions of the slug for each of the three categories by means of a Vernier caliper/mm (Ali and Robinson, 2022). The dimensions measured were length (total length of the body when extended) and width (width at the widest part of the body). Ten replicates were used for each category. To confirm the morphological diagnosis, bioinformatic analysis was used to identify the species.

DNA extraction and PCR: DNA samples (20) were extracted from the *E. alte* land snails using the tissue protocol of a commercial kit (Geneaid). The extraction was performed according to the manufacturer's instructions. The concentration of DNA was determined using Implen NanoPhotometer, and then DNA was stored at -20°C until used in the molecular study. PCR was applied to amplify the partial mitochondrial cytochrome c oxidase subunit I (cox1) gene using a universal set of primers. These primers were forward (LCO1490) 5'-GGTCAACAAATCATAAAGATATTGG-3' and reverse (HC02198) 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'. A PCR reaction contained 25 µl of Promega Master Mix(GoTaq® Green), 1 µl of each primer (10 µM, Macrogen), 18 µl of nuclease-free water (Promega) and 5 µl of genomic DNA. The thermal conditions were 95°C for 5 min and 35 cycles of 95°C for 1 minute, 40°C for 1 minute and 72°C for 90 sec followed by72°C for 7 minutes in a thermocycler. PCR products were loaded into the appropriate wells of the TAE agarose gel (1.5% (w/v)) stained with 1 – 3 µl ethidium bromide dye. The agarose gel was run at 85 V for 50 minutes. The DNA within agarose gel was visualized using UV transilluminator. The PCR products were purified using the Promega PCR Clean-up System.

DNA Sequencing and bioinformatics analysis : The purified PCR products were sequenced in both the upstream and downstream directions using the universal primers. Sequencing was performed by the Macrogen company (South Korea). For each sample, both directions sequences were lined up, treated and then assembled. The assembled sequences were lined up and compared with other GenBank sequences. The current study sequence was deposited in the GenBank database accessed via the NCBI website. The sequence of cox1 was translated using a BLASTX program. A phylogenetic analysis and a multiple sequence alignment were done using Molecular Evolutionary Genetic Analysis version 11 and PopART.

Statistical analysis : One-way analysis of variance (ANOVA) was used to assess the data in statistical studies, which were conducted using SPSS version 22. When the *P* value was less than 0.05, significant differences were counted.

Results

Morphological characteristics

The ground slug Eleutherocaulis alte. It does not have a shell and is dark brown to black in color. The dorsal surface is granular and is characterized by a thin line of cream to light brown color extending along the dorsal side. The mantle is leathery and covers the entire back and the head. The ventral side Is broad and yellowish in color. The lower pair of tentacles Is bilobed. The results of the current study showed the main characteristics of the ground slug E. alte through the morphological measurements of the slug classes (C, A, B) during three months (Table 1). It was found that there were differences between the different sizes of the three classes. The highest morphological measurements were recorded for class A in the average length of the slug (5.67 cm) and the width of the slug (1.79 cm). While the study recorded the lowest morphological measurements for class C in the average length of the slug (1.95 cm) and the width of the slug (0.43 cm). While category B was of medium size, the results of the statistical analysis showed significant differences in the length and width of the spit, as the different letters between the length and width of the animal for the three categories indicate statistical differences at the probability level of 0.05 P. The current study also showed the presence of E. alte spit in different sizes during the study months, as it was found that there were differences between the different sizes at the level of most measurements for the three spit categories (A, B, C). The average length of the animal ranged between (5.67-1.95 cm), while the average width of the animal ranged between (1.79-0.43 cm).

Plate 1.A-*Eleutherocaulis alte* B and C -*E.alte* found among the plants

Table 1. Morphological measurements of *Eleutherocaulis alte* snails. The data are the mean and standard deviation of the mean (n =30).

Size	Length	Width
A	$5.67^{a} \pm 0.48$	$1.79^{a} \pm 0.33$
B	$4.63^{b} \pm 0.61$	$1.12^{b} \pm 0.34$
C	$1.95^{c} \pm 0.92$	$0.43^{c} \pm 0.18$
P value	0.0001	0.0001

Detection of the partial *COX1* gene

PCR was employed to amplify the partial *COX1* gene of *E. alte* using a universal primer set and the results are shown in Figure 1. In all samples, the size of amplified PCR products was approximately 700 bp. Selected samples (10) were sequenced in both directions using the same primer set, and the consensus sequence was deposited in GenBank under the accession numbers (PP528209.1). Based on the nucleotide sequences, the current study samples shared 100% identity with each other and with the Indian sequences (KX514443.1, OR234676.1 and PP716841.1).

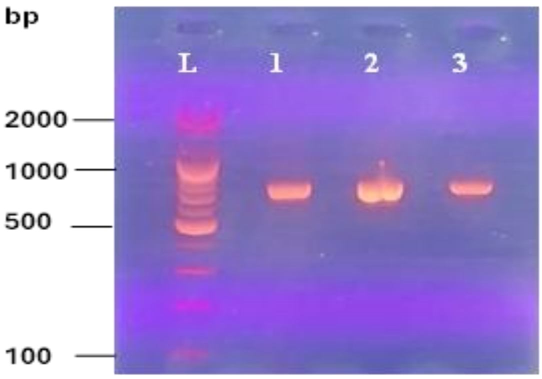


Figure 1. An agarose gel shows PCR products of the *E. alte COX1* gene. L represents the DNA ladder and 1 – 3 represents the current study snails' samples.

Comparison of *Eleutherocaulis alte* DNA sequences

An evolutionary network was built to gain more understanding of the relation of the current study sequences with their counterparts from GenBank samples (Figure 2). All available GenBank sequences were from four countries (India, Egypt, Japan and the UK). Twenty-one sequences were from Japan (LC636089.1 to LC636112.1 and LC636219.1 to LC636221.1), five sequences from India (OQ029440.1, KX514443.1, KY774830.1, OR234676.1 and PP716841.1), and one sequence each for Iraq, Egypt and the UK were used in this comparison. The network results showed that there were five haplotypes. The largest haplotype 1 (H1) contained twenty-five sequences (24 sequences from Japan, one from the UK and one from India), followed by H3 (contained only four sequences from Japan) and H4 (contained three sequences from India and one sequence from the current study). H2 and H5 were restricted to India and Eygpt, respectively. In total, the Iraqi sequence was clustered in haplotype 4 which is shared between Iraq and India.

The network also showed there were 81 variable sites, distributed across haplotypes (Figure 2). H1 had five and seven variable sites with H2 and H3, respectively. However, it had 68 and 76 variable sites with H4 and H5, respectively. Similarly, H2 and H3 had a few variable sites (12) compared with 72 and 78 for H4 and 67 and 75 for H5, respectively. In contrast, H4 and H5 had only 10 variables between them. To sum up, based on nucleotide levels, H1 to H3 were far away from H4 and H5.

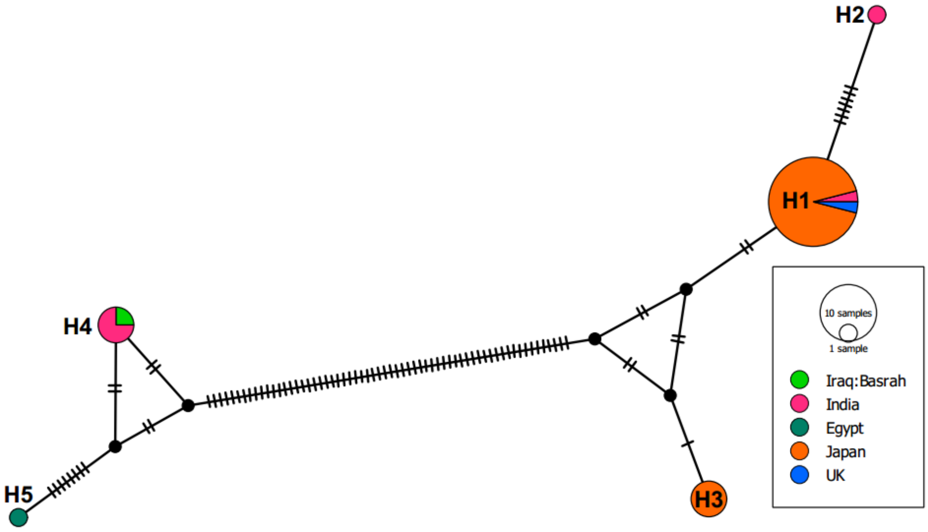


Figure 2. An evolutionary network of the current study samples and the GenBank samples. H represents the haplotype. A dash represents a mutation and a black circle represents a median vector.

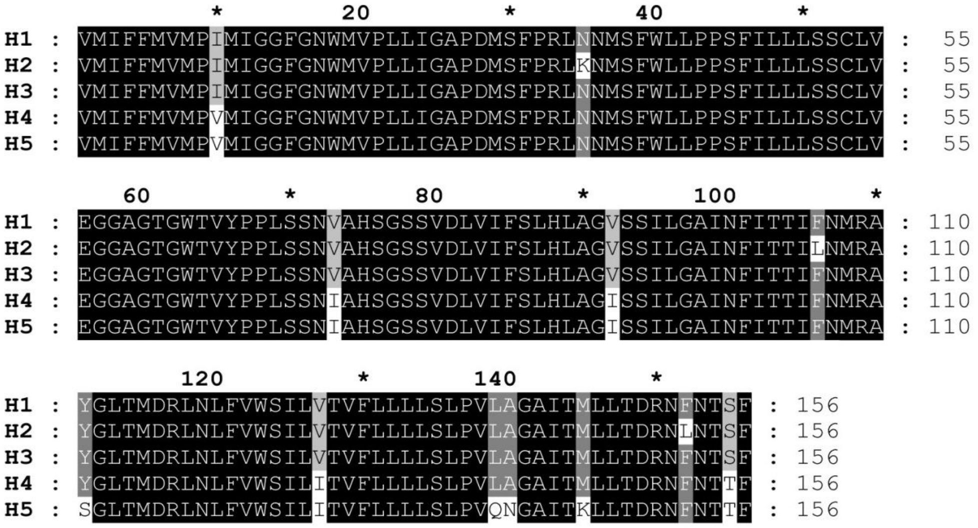
Furthermore, the percent identity of these five haplotypes is shown in Table 2. Haplotypes (1-3) shared higher identities which ranged from 97.45 to 98.94% compared with H4 and H5. The later haplotypes shared 97.87% identity compared with the rest of the haplotypes that had less than 86% identity with H4 and H5. As mentioned above, the Iraqi *E. alte* belonged to H4 and it was more closely related to H5 than other haplotypes. These two haplotypes had only 10 variable sites at different positions (297, 332, 384, 416, 418, 419, 420, 432, 434 and 438).

Table 2. DNA percent identity matrix of the five haplotypes

	H1	H2	H3	H4	H5
H1	100	98.51	98.94	85.53	83.83
H2		100	97.45	84.68	83.4
H3			100	85.74	84.04
H4				100	97.87
H5					100

Comparison of haplotypes' amino acids

An alignment of amino acid sequences of the five haplotypes is shown in Figure 3. H1 and H3 had 100% amino acid identities whereas they shared 98.08, 96.79 and 94.23% identities with H2, H4 and H5, respectively. The percentage of differences among these haplotypes ranged from 1.92 to 5.77%. Although these five haplotypes had many changes at nucleotide levels, they almost shared similar amino acids over all protein lengths, except for 12 sites. These exception sites were distributed among three haplotypes (H2, H4 and H5). In H2, there were 3 amino acid replacements compared with the rest of the haplotypes. These replacemnts were lysine^{35} (K^{35}), leucine^{106} (L^{106}) and L^{152} residues inteaded of asparagine (N^{35}), phenyalanine (F^{106}) and F^{152} residues found in H1 and H3 to H5, respectively. In H4 and H5, the results showed five identical amino acid replacements and these identical replacements were valine^{10} (V^{10}), isoleucine^{73} (I^{73}), I^{92}, I^{127} and theronine^{155} (T^{155}) residues instead of I^{10}, V^{73}, V^{92}, V^{127} and Serine^{155} (S^{155}) residues found in other haplotypes, respectively. In addition to this, H5 had other four extra replacments those are S^{111}, glutamine^{139} (Q^{139}), N^{140} and K^{145} residues insteaded of tyrosine^{111} (Y^{111}), L^{139} , Alanine^{140} (A^{140}) and methionine^{145} (M^{145}) residues, respectively.



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                   *        20         *        40         *
H1 : VMIFFMVMPIMIGGFGNWMVPLLIGAPDMSFPRLNNMSFWLLPPSFILLLSSCLV :  55
H2 : VMIFFMVMPIMIGGFGNWMVPLLIGAPDMSFPRLKNMSFWLLPPSFILLLSSCLV :  55
H3 : VMIFFMVMPIMIGGFGNWMVPLLIGAPDMSFPRLNNMSFWLLPPSFILLLSSCLV :  55
H4 : VMIFFMVMPVMIGGFGNWMVPLLIGAPDMSFPRLNNMSFWLLPPSFILLLSSCLV :  55
H5 : VMIFFMVMPVMIGGFGNWMVPLLIGAPDMSFPRLNNMSFWLLPPSFILLLSSCLV :  55

        60         *        80         *       100         *
H1 : EGGAGTGWTVYPPLSSNVAHSGSSVDLVIFSLHLAGVSSILGAINFITTIFNMRA : 110
H2 : EGGAGTGWTVYPPLSSNVAHSGSSVDLVIFSLHLAGVSSILGAINFITTILNMRA : 110
H3 : EGGAGTGWTVYPPLSSNVAHSGSSVDLVIFSLHLAGVSSILGAINFITTIFNMRA : 110
H4 : EGGAGTGWTVYPPLSSNIAHSGSSVDLVIFSLHLAGISSILGAINFITTIFNMRA : 110
H5 : EGGAGTGWTVYPPLSSNIAHSGSSVDLVIFSLHLAGISSILGAINFITTIFNMRA : 110

            120         *       140         *
H1 : YGLTMDRLNLFVWSILVTVFLLLLSLPVLAGAITMLLTDRNFNTSF : 156
H2 : YGLTMDRLNLFVWSILVTVFLLLLSLPVLAGAITMLLTDRNLNTSF : 156
H3 : YGLTMDRLNLFVWSILVTVFLLLLSLPVLAGAITMLLTDRNFNTSF : 156
H4 : YGLTMDRLNLFVWSILITVFLLLLSLPVLAGAITMLLTDRNFNTTF : 156
H5 : SGLTMDRLNLFVWSILITVFLLLLSLPVQNGAITKLLTDRNFNTTF : 156
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Figure 3. An alignment of haplotypes' amino acid sequences.

Discussion

Invasive Mollusca once introduced into new environments becomes difficult to eradicate (Cowie, 2001) and are non-native animals that adapt easily to new environments and negatively affect them, and are the second largest cause of extinction of native species (Bellard, 2016). These invasive species may affect. On native species through direct or indirect competition (Holland *etal*., 2012; Rawlings *etal*., 2007) The veronicellidae family was included by (Cowie *etal*., 2009) among the eight largest families of invasive soft-bodied slugs that are likely to have the greatest negative impact on the global economy and ecosystems. The slugs belonging to this family are often found in human-related environments such as gardens (Daglio *et al*., 2020; Brodie and Barker 2012). The results of the current study showed the presence of *E. alte* slugs of different sizes during the study months. The study confirmed the presence of significant differences between the different sizes of the three slugs (A, B, C) at the probability level of p 0.05. The average length of the animal ranged between 5.67 cm - 1.95 cm, while the average width of the animal ranged between 1.79 - 0.43 cm. Accordingly, the A category obtained the highest phenotypic measurements, while the C on the lowest measurements and the B category was of medium sizes.

E. alte was recorded from Karmat Ali, Basrah Governorate for the first time in Iraq. The spread of these slugs is may be due to human activities including trade in ornamental and horticultural plants (USDA - Aphis, 2010)

For these sluges, using phenotypic charactersistics is may not sufficient to identification species. This is because morphological characters are almost indistinguishable between species and the large morphological variation within the species between slugs belonging to the family veronicellidae makes species identification difficult, which requires the use of molecular techniques that rely on nucleic acid sequences (Kim *et al*., 2016)

The development that occurred in molecular genetics has a large and important role in eliminating errors, ambiguity and confusion that occurs in the classification of imported species. And DNA barcoding has the potential. In interpreting the level of lineage diversity, especially in species with complex classifications. Because they have a similar external appearance, many studies have confirmed the importance of DNA barcoding technology. As In the study of invasive ground slugs (Hutchinson *et al.*, 2020; Zemanova *etal*., 20.18, Dorler *et al*., 2018; Zemanova, Knop and Heckel, 2016; Barretal., 2009)

The species *E. alte* was diagnosed in the current study using PCR technique to amplify the gene in order to confirm the phenotypic diagnosis. A database for the species was also established in the NCBI GenBank And the studies have used the mitochondrial gene mtcoxI to detect the evolution and identifying of species. It is a genetic gene characterized by its ease of isolation and abundance (Lahay *etal*., 2008). The samples of the current study showed, based on nucleotide sequences, a 100% identity with each other and with the Indian sequences (pp716841.1, OR234676.1, KX514443.1). To better understand the relationship between the sequences of the current study and their counterparts from GenBank samples, an evolutionary network was built (Figure (2) if all Genbank sequences were available from four countries: India, Egypt, Japan and the United Kingdom). The results of the evolutionary network also showed the presence of five genetic patterns. The largest genotype 1 (H1) contained twenty-five sequences, followed by H3 and H4, and H2 and H5 were restricted to India and Egypt respectively. Overall, the Iraqi sequence was grouped into genotype H4, which is common to Iraq and India. As for the alignment of amino acid sequences of the five genotypes, the current study showed that genotypes H1 and H3 had 100% amino acid identity, while the identity was 98.08, 96.79, and 94.23 for genotypes H2, H4, and H5. Several studies have indicated the use of the common coxI primer used in the current study to distinguish between species, including the study of Jena and Mishra (2019) by on the ground slug *E. alte* using the mitochondrial coxI gene, which focused on the molecular convergence of molecular evolutionary relationships and concluded that the species *E. alte* is more closely related to individuals of the same species belonging to the family Veronicellidae across the Indian subcontinent.

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