

MORPHOLOGICAL AND BIOINFORMATICS STUDY FOR *RADIX AURICULARIA* SNAILS IN FRESHWATER IN BASRAH PROVINCE, IRAQ

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ABSTRACT

Radix auricularia (*Lymnaea auricularia*) snails are the main intermediate host of *Fasciola gigantica* in the middle and South of Iraq. Recently, some studies suggest these snails are not present in Iraq, in particular Basrah province. Thus, the current study was aimed to use the bioinformatics analysis in the determination of *Radix* sp. in Basrah and also aimed to identify morphological characteristics of this species in the AL-Sewaib river, Basrah. Four hundred *R. auricularia* snails were collected from the AL-Sewaib river during May 2018 to March 2019. The results of the bioinformatics analysis showed according to the mitochondrial cytochrome c oxidase subunit I, the presence of *R. auricularia* snails in Basrah province and these snails are more closely related to Iranian *R. auricularia* snails than those for European counties and Russia. This study also displayed the existence of the polymorphism phenomenon in *R. auricularia* snails collected from the AL-Sewaib river which there were six different forms (A, B, C, D, E and F). These forms had variations in the colour and form of shells along with a variation in the number of eggs (30-56) per an egg mass. In addition to this, these *R. auricularia* forms had variations in their distributions. Form E had the highest spread percentage (33%) followed by 17, 15, 14, 11 and 10% for forms B, D, C, A and F, respectively. These forms had also variations in their morphological characteristics. The largest form was the form D whereas the smallest form was the form B but other forms A, C, E and F were similar and had moderate sizes. Thus, the current study concludes the existence of *R. auricularia* snails in Basrah as a representative city for the southern part of Iraq and also confirmed the presence of the polymorphism phenomenon in the members of *R. auricularia* obtained from the AL-Sewaib river.

Keywords: *Fasciola gigantica*, AL-Sewaib river, Cytochrome c oxidase subunit I, Polymorphisms

الاسدي

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دراسة جزيئية وشكلية لقواقع *Radix auricularia* في المياه العذبة في محافظة البصرة، العراق

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المستخلص

تعد قواقع المياه العذبة من نوع *Radix auricularia* المضيف المتوسط الرئيسي لديدان الكبد العملاقة في وسط وجنوب العراق. بعض الدراسات اشارت مؤخرًا الى عدم وجود هذه القواقع في العراق وبالأخص في محافظة البصرة. لهذا هدفت الدراسة الحالية الى استخدام تحليل المعلومات الحيوية في تحديد نوع *Radix* وتحديد الصفات المظهرية لهذا النوع الذي جمع من نهر السويب في محافظة البصرة. جمع 400 قواقع من *R. auricularia* من نهر السويب خلال الفترة من ايار 2018 - آذار 2019. اظهرت نتائج تحليل المعلومات الحيوية بالاعتماد على الجين المايستوكوندرى سايتوكروم سي اوكسديز 1 بان قواقع *R. auricularia* متواجده في محافظة البصرة وانها قريبة جدا الى قواقع *R. auricularia* المتواجده في ايران من تلك الموجودة في اوربا وروسيا. كما بينت الدراسة الحالية وجود ظاهرة تعدد الاشكال في قواقع *R. auricularia* التي جمعت من نهر السويب اذ كان هناك ستة اشكال من القواقع (A, B, C, D, E, F) تمتاز بانها مختلفة في الوانها وكذلك مختلفة في اعداد البيوض (30-56) في كتلة البيض الوحده. بالاضافة الى هذا تمتاز هذه القواقع بان نسبة انتشارها في نهر السويب متباينة من شكل الى اخر فالقواقع E اظهر اعلى نسبة انتشار (33%) تبع بعد ذلك بنسب الانتشار 17 و 15 و 14 و 11 و 10% للقواقع B و D و C و A و F على التوالي. كما امتازت هذه القواقع الستة بانها مختلفة في صفاتها المظهرية اذ سجل اعلى قياسات مظهرية للقواقع D بينما اقل القياسات للقواقع B بينما الاشكال الاخرى كانت ذات احجام متوسطة. تستنتج الدراسة الحالية وجود قواقع *R. auricularia* في محافظة البصرة كمدينة ممثلة لجنوب العراق كما اكدت وجود ظاهرة تعدد الاشكال في افراد قواقع *R. auricularia* التي جمعت من نهر السويب.

الكلمات المفتاحية: ديوان الكبد العملاقة، نهر السويب، سايتوكروم سي اوكسديز 1، تعدد الاشكال

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INTRODUCTION

Lymnaeidae has many freshwater snails that involve in the different helminth life cycles (9, 13). These snails gain the large parasitological significance as they are mainly worked as first intermediate hosts for several trematodes. The transmission of trematode parasites by a specific lymnaeid species typically depends on the parasite-host specificity as well as depend on the geographical region considered and local ecological characteristics (12). Plant-borne trematodes, in particular liver flukes, cause fascioliasis in cattle, sheep, buffaloes and occasionally, these trematodes can infect human (20). *Galba truncatula*, known as *Lymnaea truncatula*, is the most important intermediate host for *Fasciola hepatica* whereas *Radix auricularia*, known as *Lymnaea auricularia*, is the most important intermediate host for *F. gigantica* (15, 21). Globally, *F. hepatica* is usually found in temperate regions while *F. gigantica* is mainly found in tropical regions. In Iraq, the *F. hepatica* parasite and its intermediate host *G. truncatula* are found in the northern and north-eastern parts while the *F. gigantica* parasite and its intermediate host *R. auricularia* are found in the middle and southern parts (6). In the last two decades, the majority of studies have been reported the presence of *F. gigantica* and its intermediate host in the southern part of Iraq (1-3, 7, 8). In contrast to this, only two studies reported that there is no *L. auricularia* (*R. auricularia*) in the southern part of Iraq, in particular in Basrah province (11, 19). This needs to be investigated further. Thus, the present study aimed to use the morphometric and bioinformatics analyses in the determination of *Radix* sp. serving as an intermediate host of some trematode parasites in Basrah province.

MATERIALS AND METHODS

Bioinformatics

A search was done of the Barcode of Life Data System (BOLD) (22) to find all data regarding *Radix* spp. collected from Basrah province, Iraq. In the BOLD identification system, the essential barcode sequence for individuals from the animal kingdom is the mitochondrial cytochrome c oxidase subunit I (COI) (5, 18). In the search results, there were seven different COI sequences under the name *Radix* sp. that obtained from Basrah province. In order to

determine the species of *Radix* in Basrah province, the seven different COI sequences from Basrah in addition to other COI sequences for *Radix* species from different countries were obtained from the “Nucleotide Database” on the National Center for Biotechnology Information (NCBI) website. The sequences obtained in this way were aligned using Clustal X version 2.1 (17). Then the Neighbor-joining method (23), accessed through Molecular Evolutionary Genetic Analysis (MEGA) version X (26), was utilised to produce a phylogenetic tree. The identity matrix of nucleotide sequences was calculated using Clustal Omega (25).

Sample collection and breeding

Four hundred *R. auricularia* snails were collected from the AL-Sewaib river, Basrah province during May 2018 to March 2019. Collected snails were distributed depending on the color and form of shells onto six plastic tanks (20 x 50 x 10 cm). These tanks were supplied with dechlorinated water and aerated for 24 h. The snails were fed with dried celery and incubated at room temperature for three weeks (3). Egg masses were collected from each tank after 1-2 weeks.

Morphometric analysis of the *R. auricularia* shells

Shell distances for each *R. auricularia* form measured using Vernier Caliper as previously described (3). These shell distances included shell length, shell width, spire length and body whorl length. Twenty replicates were used to each *R. auricularia* form.

Statistical analyses

SPSS version 25 was employed in statistical analyses and data were analyzed utilizing one-way analysis of variance (ANOVA). Significant differences were counted when the *P* value was ≤ 0.05 .

RESULTS AND DISCUSSION

Phylogenetic analysis

Figure 1 shows a phylogenetic analysis of relationships between the COI sequences of *Radix* sp. from Basrah and other COI sequences of *R. auricularia*, *R. dolgini*, *R. labiate*, *R. natalensis*, *R. balthica*, *R. lagotis*, *R. zazurnensis*, *R. ampla*, *R. relicta* and *R. pinteri*. A *Chilina santiagoi* COI sequence was utilized as the out-group to root the tree. The phylogenetic analysis generated two clearly

separate clusters, one for the COI sequences from *R. auricularia* and another one for the COI sequences from other *Radix* species. Within the *R. auricularia* cluster, the seven COI sequences from Basrah were only grouped together with six Iranian COI sequences (KT280423.1, KT280421.1,

KT280424.1, KT280422.1, KT280426.1 and KT280425.1).

DNA identity analysis

Table 1 shows the percentage of the DNA identity in COI sequences among *Radix* sp. from Basrah (Iraq) and selected *R. auricularia* from

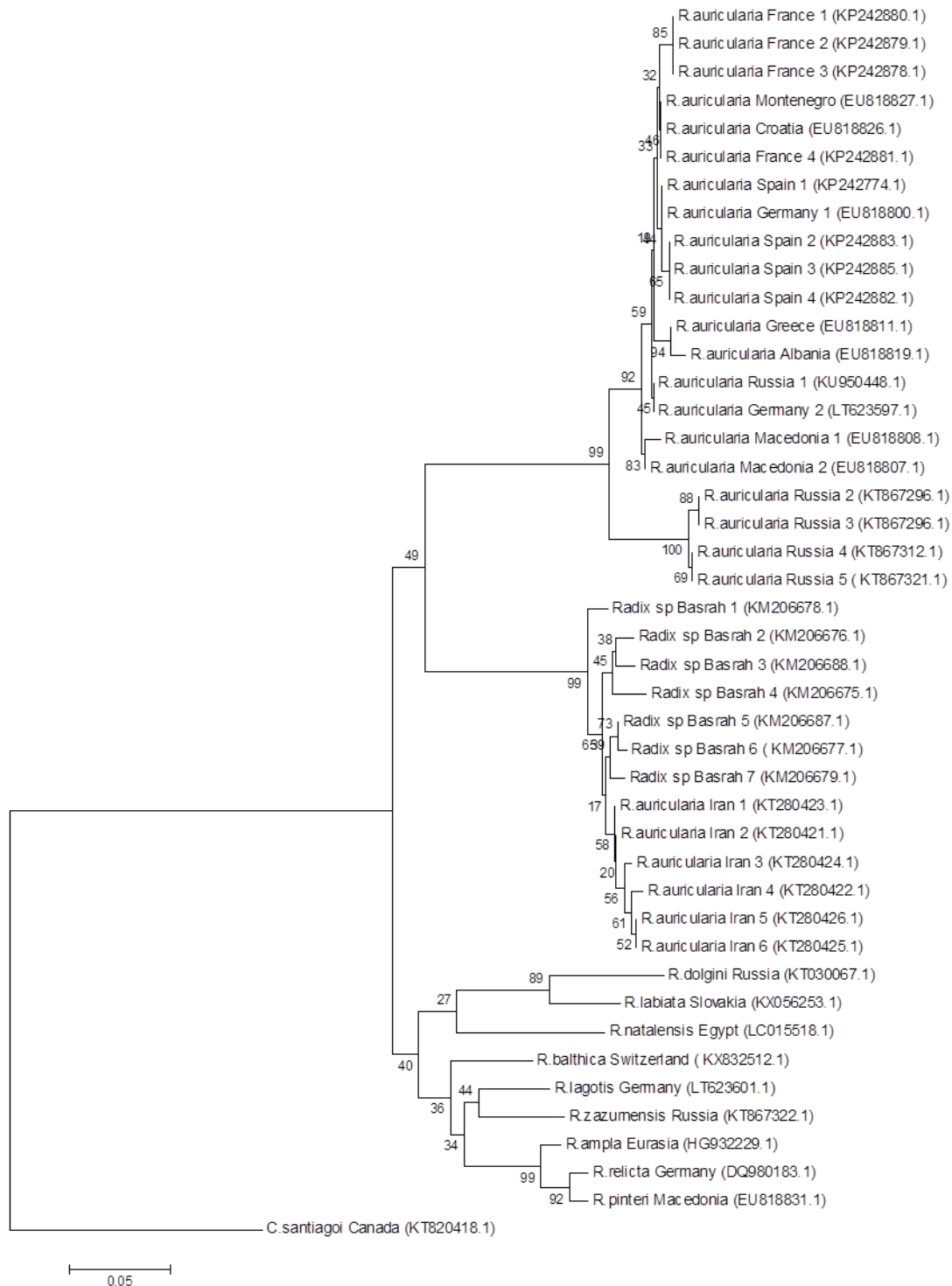


Figure 1. A phylogenetic tree showing the relationships between the COI sequences of *Radix* sp. from Basrah and the COI sequences of *R. auricularia*, *R. dolgini*, *R. labiate*, *R. natalensis*, *R. balthica*, *R. lagotis*, *R. zazurnensis*, *R. ampla*, *R. relictata* and *R. pinteri* from different countries

different countries. The results revealed that *Radix* sequences from Basrah shared an identity value of 96 - 99% with Iranian COI sequences. In contrast to this, *Radix* sequences from Basrah shared an identity value of 86 – 88% with COI sequences from France, Greece

and Spain and 87 – 88% and 86 – 87 with COI sequences from Germany and Russia, respectively. Again, *Radix* sp. from Basrah is more closely related to *R. auricularia* from Iran than those from European countries and Russia.

Table 1. DNA identity matrix for COI sequences of *R. auricularia* from different countries and *Radix* sp. sequences from Basrah, Iraq.

	<i>Radix</i> sp.							<i>R. auricularia</i>											
	Basrah_1	Basrah_2	Basrah_3	Basrah_4	Basrah_5	Basrah_6	Basrah_7	Iran_1	Iran_2	Iran_3	Iran_4	Iran_5	Iran_6	France_1	Greece	Spain_1	Germany_1	Russia_1	
<i>Radix</i> sp_Basrah_1	100	96	97	97	98	96	96	97	97	97	97	97	97	87	88	88	88	87	
<i>Radix</i> sp_Basrah_2		100	98	98	97	97	97	98	98	97	97	97	97	87	87	86	87	86	
<i>Radix</i> sp_Basrah_3			100	98	97	97	97	98	98	98	97	98	98	86	86	86	87	86	
<i>Radix</i> sp_Basrah_4				100	97	96	96	97	97	97	96	97	97	86	87	86	87	86	
<i>Radix</i> sp_Basrah_5					100	99	99	99	99	99	98	99	99	87	87	87	87	87	
<i>Radix</i> sp_Basrah_6						100	99	99	99	98	98	98	98	88	87	87	87	87	
<i>Radix</i> sp_Basrah_7							100	98	98	98	97	98	98	88	88	87	87	87	
<i>R. auricularia</i> _Iran_1								100	100	99.5	99	99.5	99.5	88	88	89	88	88	
<i>R. auricularia</i> _Iran_2									100	99.5	99	99.5	99.5	88	88	89	88	88	
<i>R. auricularia</i> _Iran_3										100	99	99	99	88	88	89	88	88	
<i>R. auricularia</i> _Iran_4											100	99.5	99.5	87	87	88	87	88	
<i>R. auricularia</i> _Iran_5												100	100	88	87	88	87	88	
<i>R. auricularia</i> _Iran_6													100	88	87	88	87	88	
<i>R. auricularia</i> _France_1														100	99	99	99	99	
<i>R. auricularia</i> _Greece															100	99	99	99	
<i>R. auricularia</i> _Spain_1																100	99.5	99	
<i>R.auricularia</i> _Germany_1																	100	99.7	
<i>R. auricularia</i> _Russia_1																			100

Amino acid sequence comparison of the COI proteins from *Radix* sp. (Basrah, Iraq) and *R. auricularia* (Iran): An amino acid sequence analysis revealed that the *Radix* COI proteins from Basrah were highly similar to the *R. auricularia* COI proteins from Iran (Fig. 2). However, seven differences were noted in the COI amino acid sequences of *Radix* sp_Basrah. These included the RLE motif which conserves in *R. auricularia* COI proteins at residues 1-3 was replaced with the SFK motif in the COI sequence of *Radix* sp_Basrah_3. The M residue conserved

in *R. auricularia* sequences at the position 62 was only replaced with the L residue in the COI sequence of *Radix* sp_Basrah_2 and *Radix* sp_Basrah_4. In addition to this, the I residue conserved in *R. auricularia* sequences at the position 8 was replaced with the T residue in the COI sequence of *Radix* sp_Basrah_6 and the residue P conserved in *R. auricularia* sequences at the place 136 was substituted with the S residue in the COI sequence of *Radix* sp_Basrah_3. Lastly, the L residue conserved in *R. auricularia* sequences at the position 159 was

only replaced with the M residue in the COI sequence of *Radix*_sp_Basrah_2. These results indicated that *Radix* snails in Basrah province

belong to *R. auricularia*. Thus, the *Radix* sp. from Basrah is hereafter referred to as *R. auricularia*.

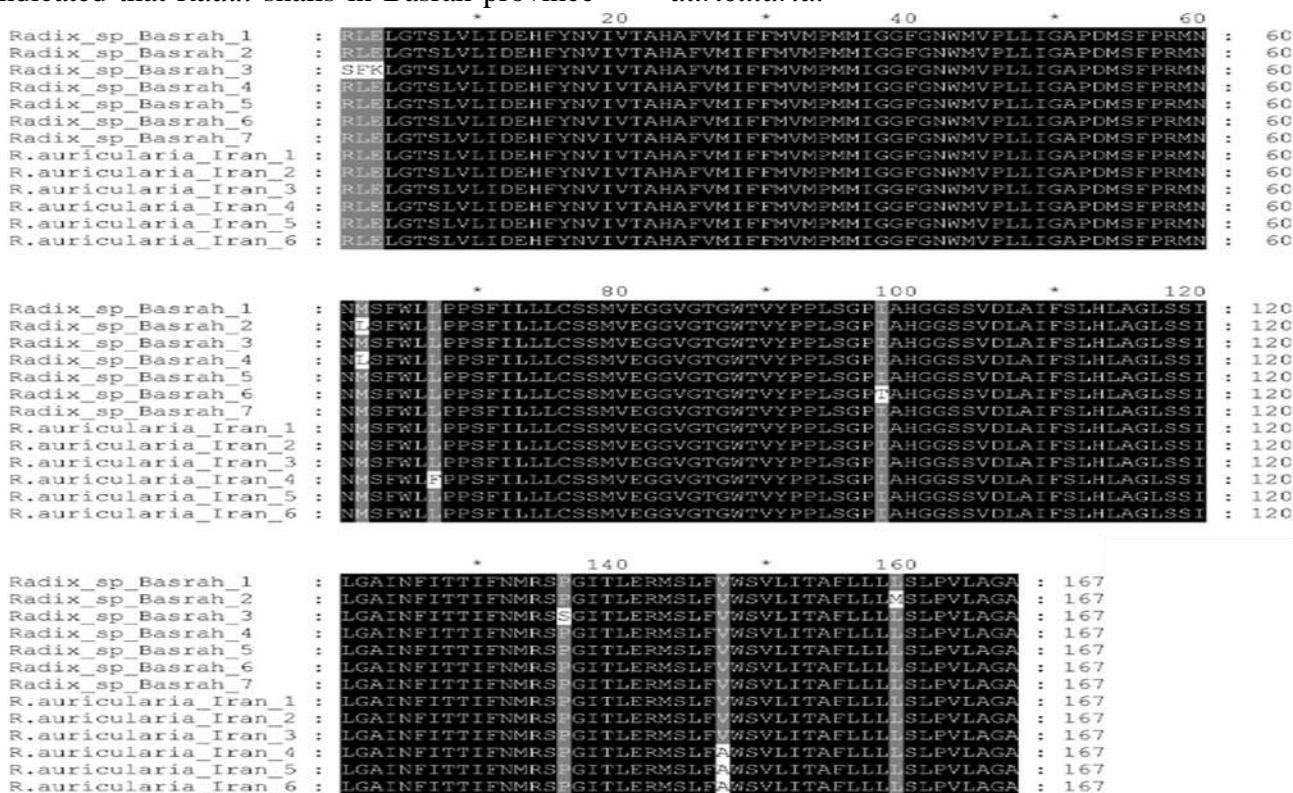


Figure 2. An amino acid sequence alignment of COI proteins from *Radix* sp. (Basrah, Iraq) and *R. auricularia* (Iran).

***R. auricularia* forms and their egg masses:**
 The current study showed that there were six different forms of *R. auricularia* snails collected from the AL-Sewaib river, Basrah and these forms were separate based on the colour and form of shells (Fig. 3). The colours of these forms were dark red (A), dark moderate red (B), very dark desaturated red (C), brown (D), dark moderate orange (E) and black (F). Figure 3 also revealed the rate of eggs in the egg mass of *R. auricularia* forms. The *R. auricularia* form A had the highest rate of eggs in egg masses which reach about 56

eggs per an egg mass compared with other *R. auricularia* forms. This rate of eggs per an egg mass in the Form A was somewhat higher than that for the form B (48 eggs per an egg mass) but it was not statistically significant. However, it was statistically significantly higher than that for either forms C, D, E or F. In contrast to this, the form F had the lowest rate of eggs in egg masses which reached about 30 eggs per an egg mass and it was similar to the form E in the number of eggs per an egg mass (34). Moreover, there were no significant differences between forms E and F.

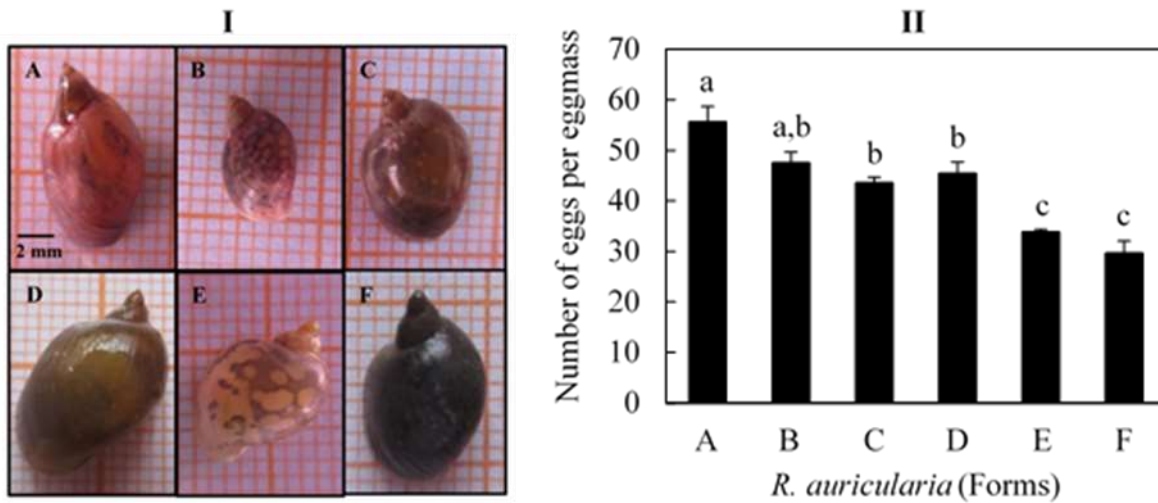


Figure 3. *R. auricularia* forms collected from AL-Sewaib river. I, Variation in the color and form of shells of *R. auricularia* snails. The colors are dark red (A), dark moderate red (B), very dark desaturated red (C), brown (D), dark moderate orange (E) and black (F). II, Number of eggs in the egg mass of *Radix auricularia* forms.

Distribution percentages of *R. auricularia* forms: The distribution percentages of *R. auricularia* snails are shown in Figure 4. These percentages were varied between forms. The highest distribution percentage was observed in the form E (dark moderate orange) which reached up to 33% compared with 11, 17, 14, 15 and 10% for the form A, B, C, D and form F, respectively. In addition to this, the statistical analysis revealed the distribution percentage of form E was statistically

significantly higher than that for other forms. In contrast to this, the lowest distribution percentage was noted in the form F (black) which reached up to 10%. The distribution percentage of form F was statistically similar to either forms A, C or D but was statistically significantly lower than the distribution percentages of either forms B or C. These results indicated that the *R. auricularia* form E possessed the highest spread percentage in the AL-Sewaib river compared with other forms.

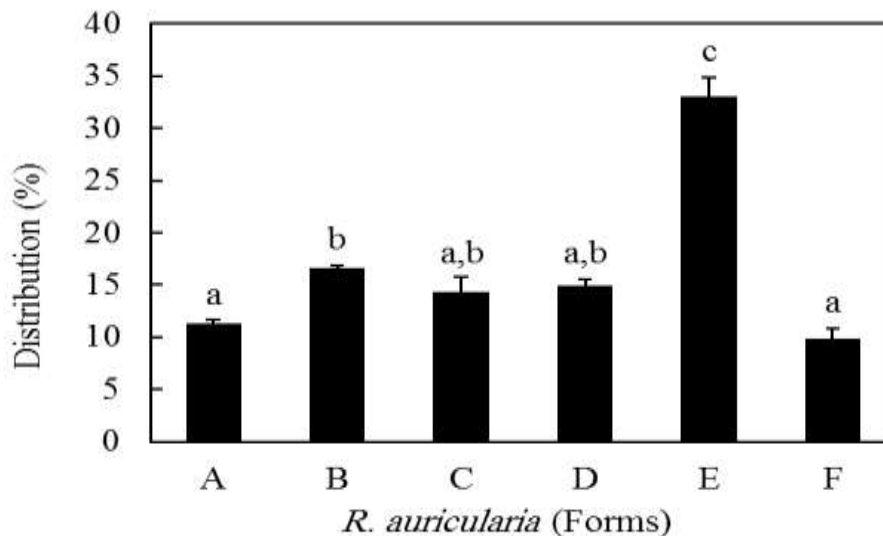


Figure 4. Distribution percentage of *R. auricularia* forms in the AL-Sewaib river. The data are the mean ± the standard error of the mean (n = 5). Significant differences ($P < 0.05$) among *R. auricularia* forms are indicated by different letters.

Morphological characteristics of *R. auricularia* shells

Table 2 shows morphological characteristics for the *R. auricularia* forms (A, B, C, D, E and

F). Results of the present study showed that the six *R. auricularia* forms had some variances in the shell length, shell width, spire length and body whorl length. The *R.*

auricularia form D had the highest values in the shell length (14.52 mm), shell width (9.45 mm), spire length (3.80 mm) and body whorl length (10.72 mm) compared with other *R. auricularia* forms. Moreover, these values were statistically significantly higher than those for other *R. auricularia* forms. In contrast to this, the *R. auricularia* form B had the lowest values in the shell length (8.31 mm), shell width (5.12 mm), spire length (2.79 mm) and body whorl length (5.52 mm)

compared with other *R. auricularia* forms and these values were statistically significantly lower than those for other *R. auricularia* forms. Unlike *R. auricularia* forms B and D, the forms A, C, E and F had almost similar shell measurements (moderate) with some statistical differences. These results indicated that the largest form was the form D whereas the smallest form was the form B but other forms A, C, E and F were similar and had moderate sizes.

Table 2. Morphological characteristics of *R. auricularia* shells collected from the AL-Sewaib river

<i>R. auricularia</i>	Shell colour	Shell length	Shell width	Spire length	Body whorl length
Form A	Dark red	10.70±0.44 ^{b,d}	6.26±0.17 ^a	3.04±0.07 ^a	7.66±0.46 ^{d,b}
Form B	Dark moderate red	8.31±1.10 ^a	5.12±0.75 ^b	2.79±0.23 ^{a,c}	5.52±0.91 ^a
Form C	Very dark desaturated red	10.26±0.37 ^b	7.05±0.40 ^a	2.96±0.39 ^a	7.3±0.34 ^b
Form D	Brown	14.52±1.15 ^c	9.45±0.43 ^c	3.80±0.51 ^b	10.72±0.97 ^c
Form E	Dark moderate orange	10.18±1.02 ^b	6.63±0.98 ^{a,d}	2.85±0.60 ^c	7.97±0.73 ^{d,b}
Form F	Black	11.59±0.74 ^d	7.39±0.53 ^d	3.33±0.45 ^{a,b}	8.26±0.49 ^d

The data are the mean ± the standard division of the mean (n = 20). Within each individual column, significant differences ($P < 0.05$) among *R. auricularia* forms are indicated by different letters.

In the South of Iraq, there are too many infections with *F. gigantica* transmitted to water buffalos, cattle and sheep by an intermediate host from *R. auricularia* known as *L. auricularia* (2, 4, 7, 8). Surprisingly, Al-Waaly (10) and Naser *et al.* (19) reported that *R. auricularia* snails are not present in the Southern regions of Iraq including Basrah province. If this situation is true, infections with fascioliasis in these regions of Iraq cannot happen due to the absence of the most important intermediate host. Thus, the current study was performed to answer this and to identify the morphometric characteristics of *Radix* species in Basrah as representative species for the Southern part of Iraq. Based on the phylogenetic analysis, the *Radix* COI sequences from Basrah were more closely related to *R. auricularia* COI nucleotide sequences than that for other *Radix* species. In the present study, the *Radix* COI nucleotide sequences from Basrah were highly identity to *R. auricularia* COI nucleotide sequences from Iran than those *R. auricularia* COI nucleotide sequences from European countries and Russia. Al-Waaly (10) reported the *Radix* COI nucleotide sequences from Basrah share 87% identify with *R. auricularia* (Croatia (EU818826.1) and France (JN614403.1)), *R. nataleinsis* and *R. relicta*. According to this, Al-Waaly (10) recorded *Radix* samples from

Basrah as a new species called *Radix* sp. This was not correspondent with the results obtained in the present study which confirms the presence of *R. auricularia* in Basrah province. In addition to this, an alignment of COI amino acid sequences indicated that *Radix* COI sequences from Basrah were highly similar to the *R. auricularia* sequences from Iran. In total, the results of present study completely disagree with Al-Waaly (10) study and suggest that the *Radix* sp. in Basrah belongs to *R. auricularia*. *Radix* spp. snails have been used in many studies including parasitology, biodiversity and ecotoxicology (24). Nevertheless, a few studies have been focused on the polymorphism phenomenon in these snails (10, 16, 19). The current study revealed the presence of six different forms of *R. auricularia* in the AL-Sewaib river that had variations in the colour and form of shells as well as variations in the number of eggs per an egg mass in each form. This was consistent with previous studies that showed the presences of the polymorphism phenomenon in *Radix* sp. (*R. auricularia*) snails (10, 14, 19). In Iraqi, Al-Waaly (10) recorded three different forms of *Radix* sp. (*R. auricularia*) in Basrah and two forms of *Radix* sp. in Maysan and one form of *Radix* sp. for each city in the middle and South of Iraq. The difference in the number of *R. auricularia* forms [6] obtained in

the present study from the number of *R. auricularia* forms [3] obtained in the Al-Waaly (10) study could be due the geographic area that collected snails from. The polymorphisms phenomenon in *R. auricularia* snails reflected on the distribution of these snails and the number of eggs per an egg mass in each *R. auricularia* form. For instant, unlike other *R. auricularia* forms recorded in the present study, the *R. auricularia* form E had the largest distribution percentage (33%) compared with other forms (10 -17%) collected from the AL-Sewaib river but it shared the similar rate of eggs per an egg mass with the *R. auricularia* form F. Thus, the distribution percentage of the *R. auricularia* form E was consistent with the distribution percentage (27.4 – 37.1%) of the *R. auricularia* forms collected from Basrah in another study (10). The variation in *R. auricularia* forms in the AL-Sewaib river also extended to include the variation in the length, width, spire length and body whorl length of shells. The *R. auricularia* form D, had the highest rate in the all morphological characteristics compared with the rest of *R. auricularia* forms obtained in the present study. Al-Waaly (10) reported that there were variations in morphological characteristics of *Radix* sp. (*R. auricularia*) obtained from three cities (Basrah, Kerbala and Diwaniya) in Iraq. For the *R. auricularia* obtained from Basrah, these morphological characteristics were 10.24 mm for shell length, 6.07 mm for shell width and 3.07 mm for spire length (10). These morphological characteristics were partially similar to *R. auricularia* forms A, C and E obtained in the present study. The current study shows based on the bioinformatics analysis the presences of the main vector of fascioliasis, *R. auricularia*, in Basrah province. This study also revealed the occurrence of the polymorphism phenomenon in *R. auricularia* snails collected from the AL-Sewaib river. As a result, there were six different forms of *R. auricularia* in the AL-Sewaib river that had variations in the colour and form of shells as well as variations in the number of eggs per an egg mass in each form. In addition to this, these *R. auricularia* forms had variations in their distributions and morphological characteristics.

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