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Original Article

Molecular, Morphological, and Spatial Study of *Galba schirazensis* (Pulmonata, Lymnaeidae) from Southeastern Iran

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Abstract

Background: Snails of the genus *Galba* are the intermediate hosts of *Fasciola* species, the etiological agents of liver fluke disease, fascioliasis. A genetically different but morphologically very similar species in the genus, *G. schirazensis,* is sympatrically distributed with *G. truncatula* in some regions of the world. We aimed to investigate the occurrence of *G. schirazensis* in Kerman province, Iran and to characterize genetically *G. schirazensis* specimens from southeast Iran.

Methods: Field-collected snails from four localities in Jiroft, Bam and Faryab, Kerman province, southeastern Iran were studied. Hydrological variables including temperature and pH were recorded for each habitat. Each specimen was identified using morphological as well as conchological characteristics. Genetic characterization was performed using PCR-sequencing followed by phylogenetic analyses on nuclear *ITS2* as well as mitochondrial *cox1* gene fragments. MaxEnt software was used to predict the most appropriate ecological niches for the targeted species.

Results: *G. schirazensis* was found in 4 out of 28 locations. One *ITS2* and two *cox1* haplotypes were detected among *G. schirazensis* populations from the four localities. Habitat study showed that *G. schirazensis* thrives in habitats with alkaline pH. *G.schirazensis* from South America were clustered with specimens from Bam, Kerman, Iran; however, north Iranian isolates of *G. schirazensis* were strongly correlated with specimens from Jiroft and Faryab. MaxEnt model for the most appropriate ecological niches of the targeted species predicted environmental suitability for this species in western Africa as well as coastal areas in north and southwestern Africa.

Conclusion: *G. schirazensis* is frequently present in southern areas of Kerman Province. At least two genetically different haplotypes are present in southeastern Iran.

Introduction

Lymnaeidae is a large family of molluscs of which some members are known intermediate hosts of diseases affecting humans and animals. One medically important genus in the family is the genus *Galba* (Schrank, 1803), of which some species are potentially capable of transmitting fascioliasis, a food-borne trematodiasis. Many species of *Galba* including *G. truncatula, G. schirazensis, G. neotropica, G. cubensis, G. viatrix, G. humilis and G. cousini* have been described and are believed to share a very close relationship, however it is remained to be determined that how many of them are valid.

While *G. truncatula* is known to carry *Fasciola* parasites, not much is known about *G. schirazensis* (1–3). Recently *G. schirazensis* has been described in different geographical regions throughout Europe, America, and the Middle East including Iran (4). To date, six species of Lymnaeidae: *Radix auricularia* (Linnaeus,1758), *Stagnicola palustris* (O. F. Müller,1774), *Galba truncatula* (O. F. Müller,1774), *Lymnaea stagnalis* (Linnaeus,1758), *Radix rufescens* (Gray,1822) and *Galba schirazensis* (Kuster,1863) have been reported from different provinces of Iran (4–6). Among six valid species of *Galba* only adults of *G. cousini* could be differentiated from other species by using shell and internal anatomical features (7). High phenotypic similarity and genotypic differences between *G. schirazensis* and *G. truncatula* is an important issue in the molecular epidemiology of fascioliasis (4).

The spread of snail-borne diseases depends on the spatial distribution of intermediate snail hosts (8). A complex of ethological agents covering biological, physical, chemical, environmental and climatic factors are determinative factors affecting the occurrence and density of snail populations (9). Climate simulation models predict the mean global temperature will increase in the coming years and this change will affect the biology and ecology of freshwater snails (10,11). As a result, knowing the occurrence and relative abundance of snail populations will be helpful in understanding the impact of global climate change on the transmission of soil-transmitted helminths.

Iran is a large country (1,648,000 km^2) with a diverse climate and various weather conditions. Distribution and molecular characterization of *G. schirazensis* has been carried out in the Caspian areas in the northern province of Gilan where at least 15,000 infections were reported in two human fascioliasis outbreaks over the last 10 years (12–15). However, *G. truncatula* is known as the intermediate host of *F. hepatica* in this region, and our knowledge on the distribution of *G. schirazensis* in other parts of the country is very limited. Kerman in the south-eastern Iran (54°27' to 59°29' East) and (26°05' to 32°03' North) is a province with a mainly warm and dry climate with arid / semi-arid regions.

We aimed to characterize *G. schirazensis* by using molecular and conchological tools as well as the spatial analysis of this species in south-eastern Iran.

Materials and Methods

Snail collection and habitats

This study was approved by the Research Review Committee of Kerman University of Medical Sciences, approval No. 92-134.

Snails were collected by hand from four localities in southeast Iran. Specimens were placed in 0.1 L plastic containers with fresh water, transferred to the laboratory for analyses, and stored in absolute ethanol. All sampling localities were geo-referenced using a hand-held global positioning system (GPS) (Garmin Oregon 550, Garmin Ltd, USA).

Snail specimens were collected from water bodies in southern parts of Kerman Province, i.e. Enayat-Abad, Jiroft (28.686389 N, 57.663528 E), Narjoo, Jiroft (28.681083 N,

57.692194 E), Bagh-Dasht, Bam (29.091444 N, 58.327778 E) and Faryab (28.077833 N, 57.216333 E), Kerman, Iran. Simultaneously to determine optimal environmental settings needed for *G. schirazensis* at each sampling site, water parameters included temperature (°C) and pH were measured with a multimeter (MIC, Taiwan). Furthermore, we also noted habitat characteristics, including bottom type (rocky, sandy or muddy), water flow (standing, fast running or low running), periodicity (temporary or permanent), and origin (natural or man-made).

Morphological assessments

According to taxonomic keys each specimen was identified using morphological as well as conchological characteristics, with the following abbreviations: **AL**, aperture length, **SAW**, shell aperture width, **LSL**, last spire length, **SW,** shell width, **SL,** shell length. The measurements were made with a digital caliper ±0.01 mm (Mitutoyo, Japan).

Molecular and phylogenetic analysis

Following morphological examinations, three snails per location were randomly selected and a peace of foot tissue was dissected and frozen at −20 °C. DNA extraction was carried out according to the instructions recommended by High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany).

The rDNA spacer *ITS2* was PCR-sequenced using NEWS2 5'tgtgtcgatgaagaacgcag 3' and ITS2-RIXO 5'ttctatgcttaaattcagggg 3' as forward and reverse primers respectively (16). In addition the mitochondrial *cox1* gene fragment was amplified using the universal primer set LCO1490 5'ggtcaacaaatcataaagatattgg 3' (forward) and HC02198 5'taaacttcagggtgaccaaaaaatca3' (reverse) (17). Sequences were aligned using BioEdit (ver.7.0.9.0) and MEGA 6.0 and homologies were evaluated using the BLASTN program from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). GenBank / EMBL sequences were used for phylogenetic analysis.

Phylogenetic analysis of *ITS2* and *cox1* combined haplotypes were inferred from DNA sequences using Maximum Likelihood (ML) estimates with Mega 6.0 based on Kimura 2-parameter model with 1000 bootstrap replicates (18). The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated.

Modeling the global environmental suitability for Galba schirazensis

Considering all studies that were conducted earlier in the world (Table 1) (1,2,4). Records of *G. schirazensis* were collected and entered an Excel database and then changed to a Comma-separated values (CSV) format for using in the model.

Modeling

MaxEnt software Version 3.4.1 was used to predict the most appropriate ecological niches for the targeted species (19). The contributions of the environmental variables were calculated by Jackknife analysis. Table 1 shows coordinates of the collection sites and estimates of the environmental variables for this snail. Eighty percent of collection points were used in random by MaxEnt for model training and 20% kept for testing the results.

Model variables

Five bioclimatic variables and altitude layers were retreated from the WorldClim global climate database (http://www.worldclim.org/current) at a spatial resolution of 1 km^2. These variables were derived from the long-term (1950–2000) monthly temperature and rainfall values to generate more biologically meaningful variables.

Table 1: Collection sites for *G. schirazensis* in the World and some environmental variables derived from WorldClim database

Collection site	*Longitude*	*Latitude*	*Annual Mean Temperature °C*	*Mean Diurnal Range (max - min temp)*	*Temperature Annual Range (Max- min in Warmest- Coldest Month)*	*Annual precipitation (mm)*	*Altitude*
Iran	49.61	37.46	15.8	8.2	26.3	1434	-26
Spain	-0.344	39.29	17.7	9.2	23.1	452	-2
Egypt	30.18	31.17	20.4	9.8	22.4	155	0
Spain	-0.10	39.83	17.3	8.3	22.3	435	0
Egypt	30.27	30.84	20.5	11.7	24.6	88	1
Egypt	30.48	30.84	20.5	12.6	25.7	72	4
Spain	-6.47	37.13	18	9.1	24.2	519	5
USA	-90.25	30.43	19.3	12.5	29.3	1582	11
Iran	49.63	37.19	15.8	9.5	27.2	1293	29
Ecuador	-76.86	-12.20	19.2	8.7	16.2	23	127
France	55.41	-21.24	21.1	6.2	12	1372	460
La Reunion Island	55.41	-21.24	21.1	6.2	12	1372	460
Iran	57.21	28.07	23.7	11.8	30.7	114	655
Iran	57.69	28.68	23.2	12.8	33.5	83	727
Iran	57.66	28.68	22.9	12.8	33.7	89	776
Venezuela	-71.44	8.735	22.7	10.5	12.7	1027	836
Iran	58.32	29.09	21.3	13.6	36	76	1103
Dominican Republic	-70.74	18.90	18.5	13.1	17.1	1049	1155
Venezuela	-71.76	8.36	20.3	9.8	12	873	1245
Colombia	-73.08	7.10	21.4	8.4	9.1	1170	1251
Dominican Republic	-70.72	18.87	17.4	13	17	1166	1305
Mexico	-99.18	18.88	22.2	15.3	21.6	960	1384
Ecuador	-78.29	-1.40	18.9	9.4	11.2	2698	1462
Venezuela	-71.81	8.27	18.8	9.4	11.8	786	1529
Peru	-71.81	-16.48	18.8	16.7	19	12	1569
Colombia	-75.37	6.43	20.8	10.6	11.9	2271	1659
Venezuela	-71.45	8.55	17.5	10.4	12.7	997	1755
Venezuela	-71.45	8.55	17.4	10.3	12.7	994	1771
Mexico	-98.44	18.86	20.1	16.8	22.1	878	1780
Venezuela	-71.14	8.62	17.3	11	13.3	1200	1819
Venezuela	-71.84	8.23	16.8	9.6	12.1	780	1875
Mexico	-98.43	18.93	19	16.6	22.2	872	1893
Mexico	-98.39	18.94	18.5	16.4	22.4	846	1951
Venezuela	-71.46	8.60	15.8	10.8	13.2	1208	2065
Venezuela	-71.10	8.67	15.3	11.9	14.3	1377	2157
Venezuela	-71.39	8.64	14.7	11.2	13.5	1409	2254
Ecuador	-78.57	-0.05	14.6	13.4	15.4	1132	2651
Venezuela	-71.07	8.461	12.2	11.5	14.3	1076	2743
Ecuador	-78.56	-0.45	13.5	13.3	14.4	1330	2787
Ecuador	-78.14	0.06	13.3	12	13.3	890	2828
Ecuador	-78.61	-0.77	11.9	11.7	13	671	2988
Ecuador	-78.64	-0.29	10.8	11.4	12.6	1315	3192
Ecuador	-79.27	-2.79	6.7	8.4	9.9	1018	3747
Mean			**17.97**	**11.3**	**18.46**	**910.79**	**1348.3**
SD			**3.56**	**2.56**	**7.16**	**581.00**	**1018.0**

These bioclimatic variables were Bio1: annual mean temperature (°C), Bio2: monthly mean diurnal range (°C), Bio3: isothermality (BIO2/Bio7) (×100), Bio7: annual temperature range (°C) and Bio12: annual precipitation (mm). Altitude (m), was also used at the same spatial resolution for modeling.

Results

Environmental and habitat data

Fig. 1 presents the map of localities where *G. schirazensis* was found. Our results showed that *G. schirazensis* is more likely to live in water with temperature range of 23 °C in Bam to 25.5 °C in Jiroft and Faryab.

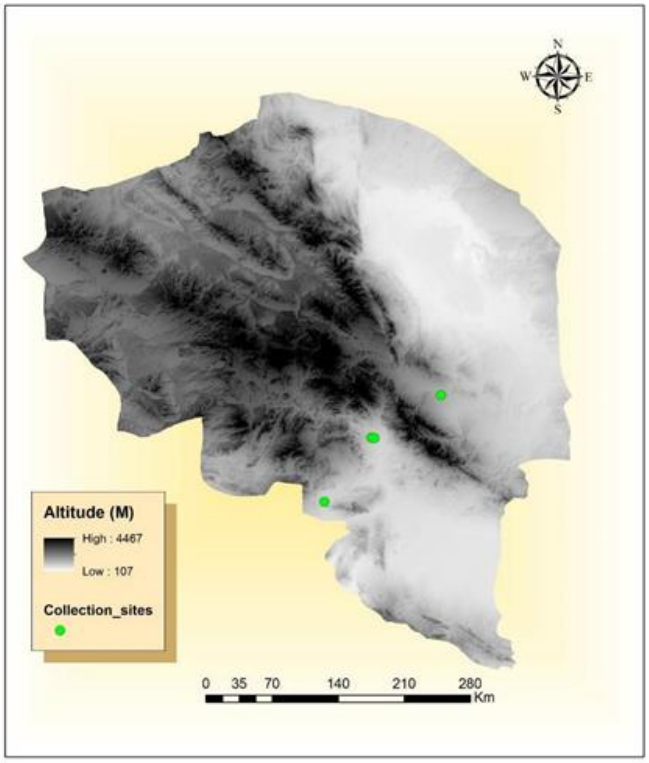

Fig. 1: Map of Kerman province showing localities where *Galba schirazensis* were collected

In Enayatabad, *G. schirazensis* was found on a rocky bottom in permanent streams with a depth of less than one meter. The water was shallow and fast running (Fig. 2A). In Narjoo, another habitat of *G. schirazensis*, downstream of Jiroft dam, the snails were found in muddy surfaces among grass in the stream (Fig. 2B). In this location, water flow was very slow and in both habitats pH values were alkaline (pH=8). In Faryab the snails were found along in a roadside, close to the edges of a small spring on sandy soil under vegetation with the trees protecting the snails from direct sunlight (Fig. 2C). Bagh-Dasht, located in suburbs of Bam city, has a Qanat (aqueduct) with approximately 100 L/sec water flow that runs in man-made cement canals (Fig. 2D). In this habitat, *G. schirazensis* was found on rocks near the water and on cane stems with the same water alkalinity pattern, with a pH value of 8.2.

Fig. 2: Type of habitats where *Galba schirazensis* specimens were collected: A: Enayatabad, Jiroft B: Narjoo, Jiroft C: Faryab D: Bagh-Dasht, Bam. Scale bar = 1m

Morphological and Conchological analysis

Figure 3 shows the morphological characters of the live sample as well as the shells of *G. schirazensis* in comparison with those of *G. truncatula*. As shown in the figure, regularly convex whorls with a straight columella, a greyish cephalopedal mass, large black eyes, and dark brown to blackish mantle roof were found in all *G. schirazensis* isolates. We collected an average of 20 snails with 4-5 whorl from each location. The smallest shell length was found in Faryab (3.02-3.88 mm, $\ddot{x}$ = 3.45) whereas the largest occurred in Bam (4.2-6.81 mm, $\ddot{x}$ = 5.50).

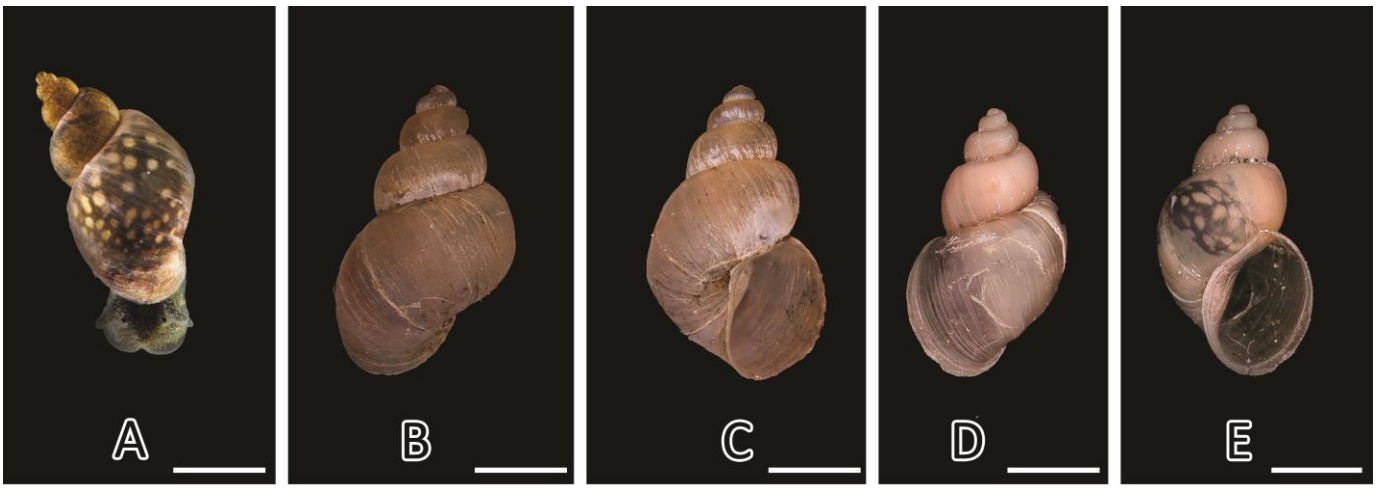

Fig. 3: Gross morphology of Lymnaeid snails: A: *G. schirazensis* live specimen B-C: *G. schirazensis* shells (ventral and dorsal view) D-E: *G. truncatula* shells (ventral and dorsal view) Scale bar = 2 mm

Molecular study and phylogenetic analysis

PCR sequencing showed one *ITS2* haplotype (KT365868-KT365871), 430 bp, among *G. schirazensis* populations from four localities in the southern regions of Kerman province. Two *cox1* haplotypes (KT267209-KT267212), 611 bp, were detected among the specimens. *G. schirazensis* specimens from Bam were different at five transversions (T/C or A/G) in positions 271-287-345-360-417 compared with those snails isolated from Jiroft and Faryab regions. Molecular investigation of *F. hepatica* DNA has not detected the parasite in any of *G. schirazensis* specimens.

Phylogenetic analysis of *ITS2* produced a single uniform cluster consisting of all the snails in the present study (Fig. 4). However, *cox1* analysis presented two different sister clades including specimens from Bam together with *G. schirazensis* isolates from Peru, and Faryab and Jiroft samples located in the same clade with the isolates from north Iran (Fig. 5).

Distribution model

Figure 6 shows the distribution map for *G. schirazensis* in the world. Findings of this study have expanded the geographical distribution of *G. schirazensis* further to the east in the southeastern province of Kerman.

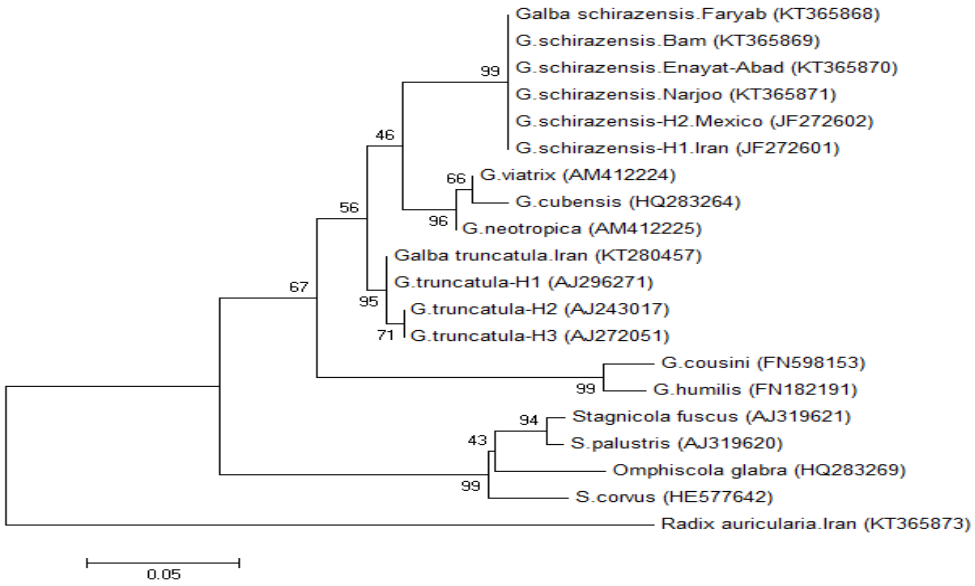

Fig. 4: Molecular phylogenetic analysis of *G. schirazensis* isolates and other Lymnaeid species based on *ITS2* sequences. Phylogenetic tree was inferred by Maximum Likelihood method and using highest log likelihood (-1612.1600) based on the Kimura 2-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 283 positions in the final dataset. Evolutionary analyses were conducted in MEGA6

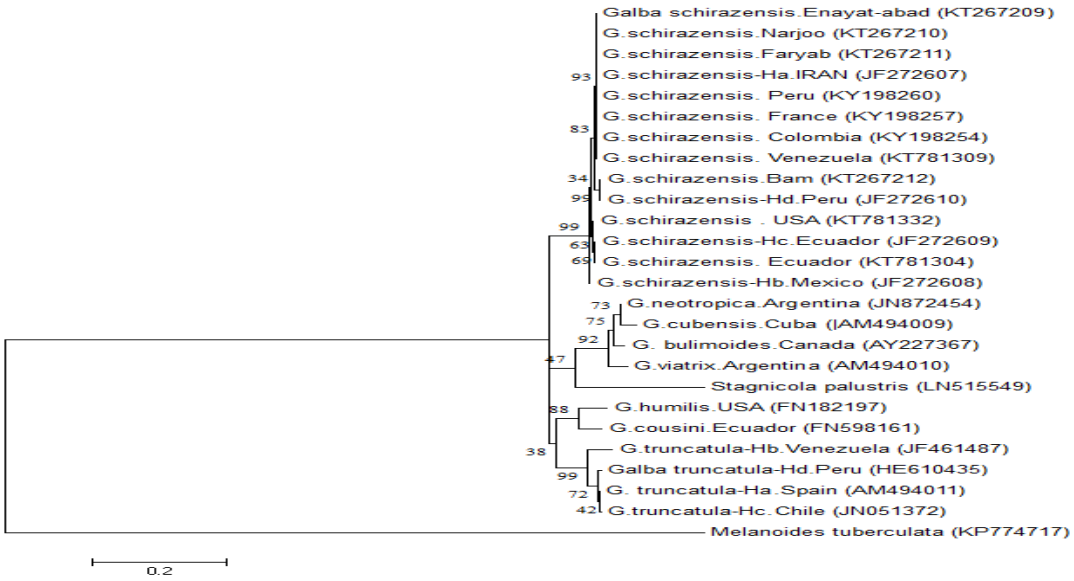

Fig. 5: Molecular phylogenetic analysis of *G. schirazensis* isolates and other Lymnaeid species based on *cox1* sequences. Phylogenetic tree was inferred by Maximum Likelihood method and using highest log likelihood (-2725.1824) based on the Kimura 2-parameter model (Kimura 1980). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 25 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 563 positions in the final dataset. Evolutionary analyses were conducted in MEGA6

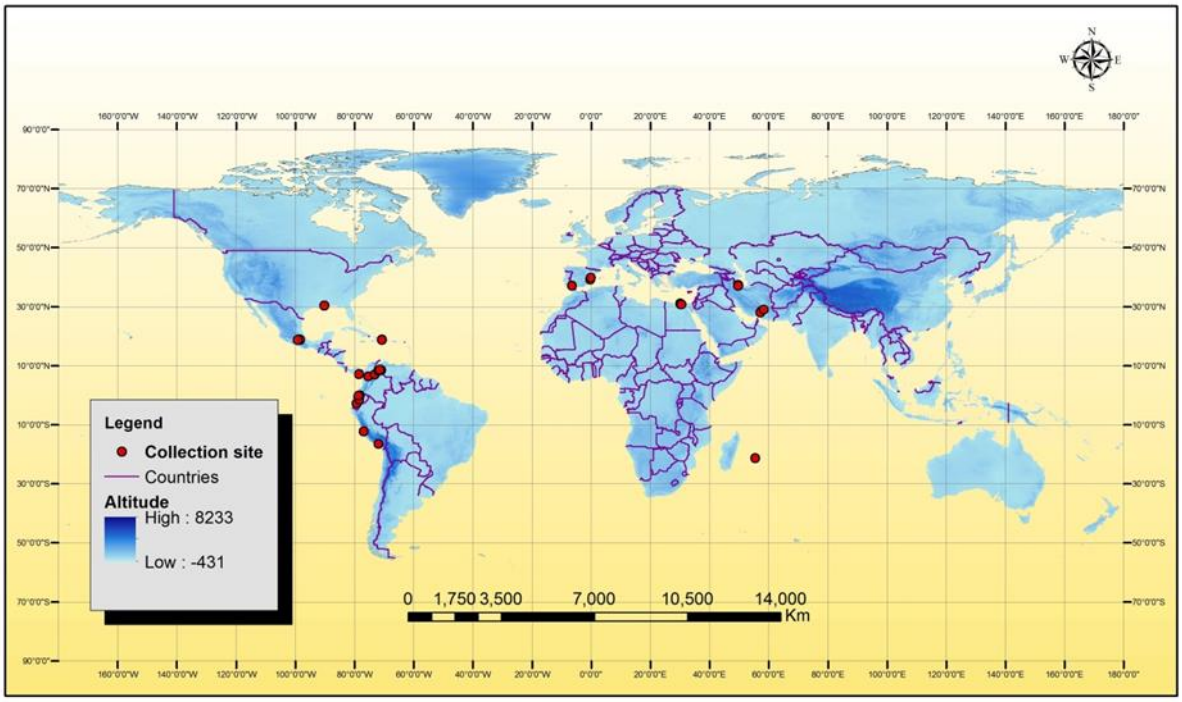

Fig. 6: The distribution map for *G. schirazensis* in the world

Table 2 shows estimates of relative contributions of the climatic and environmental variables to the MaxEnt model for *G. schirazensis*. As shown, the variables with the most contribution are bio3 (46.5%) and bio1 (22.2%).

Area under curve (AUC) was calculated as 0.981 for training and 0.979 for test data (Fig. 7). The results of the jackknife test of variable importance showed that the environmental variable with highest gain, when used in isolation, was bio3. This variable appears to have the most useful information by itself (Fig. 7). Fig. 8 is a representation of the MaxEnt model for *G. schirazensis*. Warmer colors (Red) show areas with better environmental suitability for this snail.

Table 2: Estimates of relative contributions of the environmental variables to the MaxEnt model for *Galba schirazensis*

Variable	*Percent contribution*	*Permutation importance*
Bio3	46.5	4.6
Bio1	22.2	64.5
Altitude	13.9	7.1
Bio12	9.5	14.8
Bio7	5	5
Bio2	2.9	4

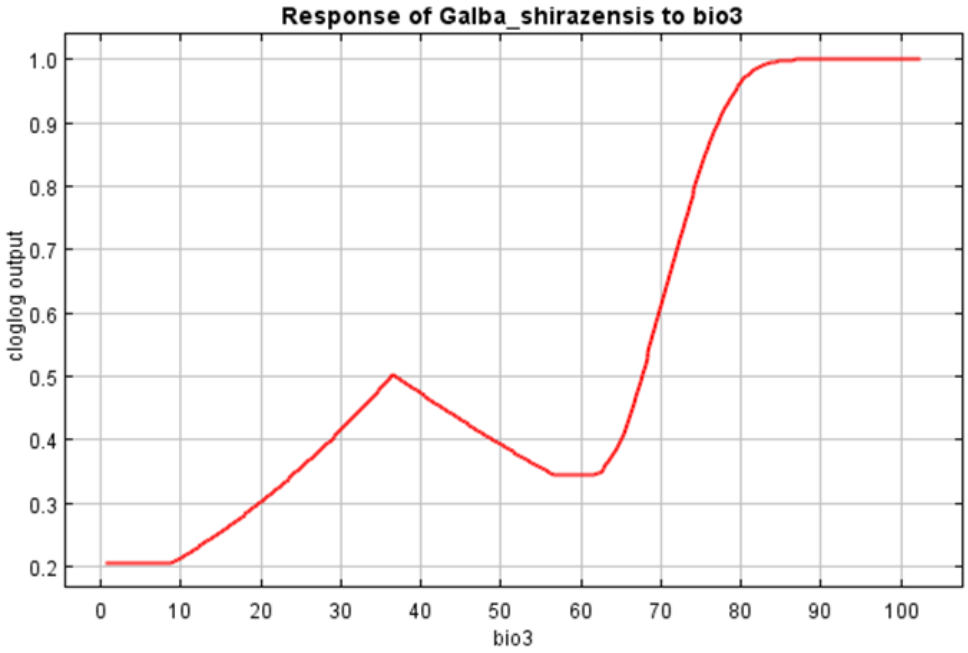

Fig. 7: Area under curve, jackknife analysis and response curve for the most important variable in MaxEnt model for *G. schirazensis* in the world

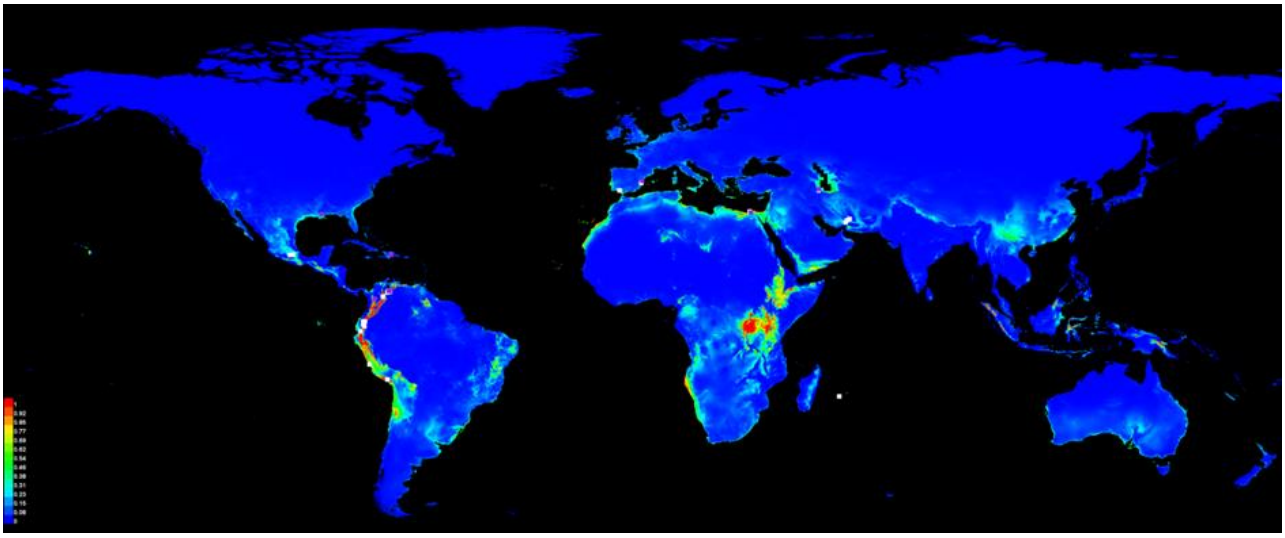

Fig. 8: Environmental suitability map produced by MaxEnt model for *Galba schirazensis*

Discussion

Snails of the family Lymnaeidae are the focus of many biological, ecological, and parasitological studies. One of the key features of freshwater snail faunas is their biological diversity. Geographical factors, such as latitude and altitude, are the most important factors in this diversity (20,21). In the present study *G. schirazensis* was investigated in four localities in the southern parts of southeast province of Kerman, Iran.

Our results have expanded the geographical distribution of *G. schirazensis* further to the east

in Kerman province. The localities where *G. schirazensis* specimens have been collected are the southernmost known habitats of *G. schirazensis* in the old world (N 28° 4.67'). Conchological data were in accordance with other data obtained from Iran, Egypt, Spain, the Dominican Republic, Mexico, Venezuela, Ecuador and Peru (4). The shell lengths of the specimens from Enayat-Abad, Faryab, and Bam was in range of 3.0 to 6.8 mm, which is consistent with the corresponding values of specimens from Trinidad Tepango, Mexico, Baños del Inca Peru, Alexandria, Egypt and 'La Fontana, Ecuador (1–4).

This study reiterated the fact that *G. schirazensis* is capable of living in diverse habitats from very low altitudes in Bandar-e-Anzali, Iran (-26 m) to highlands of Huagrahuma, in Ecuador (3747 m) and in very humid areas of northern province of Gilan in Bandar-e Anzali, Iran (annual rainfall of 1853 mm which is 4-6 times more than mean annual rainfall in Iran) to arid regions of Bam, Iran (59 mm) and Lima, Peru (13 mm).

Our results showed that *G. schirazensis* prefers to live in water temperature of 23-25.5 °C. The direct effect of temperature on the biological indicators e.g., growth, maturity, fecundity, and metabolism of freshwater pulmonates including *Lymnaea* snails has been demonstrated in West Azerbaijan Province, Iran (24). Little is known about the physicochemical characteristics of water habitats of *G. schirazensis*. For the first time, this study presented water temperature and pH of four habitats of *G. schirazensis* in Iran. Results indicated that physicochemical characters of water habitats for *G. schirazensis* and *G. truncatula* are not significantly different (22–26). This confirms the observations indicating the sympatric distribution of *G. schirazensis* and *G. truncatula* in several localities e.g. in Spain (4). However, in this study the two species were not found in the same location. Highest value of conductivity is effective on density of snail species (27). In the present study the highest number of *G. schirazensis* was collected in Narjoo (No=27) with high value of EC. The minimum number of *G. schirazensis* was recorded in Bam with the lowest pH. Morphological characters of the living specimens of *G. schirazensis* isolates showed a dark-coloured mantle roof, large black eyes, and a greyish cephalopedal mass which is in agreement with previous records published for this species (4).

In this study one haplotype was found in *ITS2* region; while for *cox1* sequences, two different haplotypes were found, indicating greater genetic diversity in mitochondrial than nuclear regions. Significant sequence differences in *ITS2* and *cox1* were detected in *G. schirazensis* (this study) and *G. truncatula* (Fig. 4 and 5).

G. schirazensis is reported from Africa (Egypt, La Reunion Island), Europe (Spain, France), Americas (Mexico, Ecuador, Colombia, Peru, USA, Venezuela and Dominican Republic) and Asia (Iran). Furthermore, according to the distribution map, most parts of Africa are suitable for the presence of *G. schirazensis*. The model predicted environmental suitability for this species in West Africa in the countries like Kenya, Tanzania, Uganda as well as coastal areas in North and South-West of Africa. So far, no study has been performed on *G. schirazensis* in this continent and studies on this species will improve our knowledge of *G. schirazensis* distribution in the world. The phylogenetic data presented here indicate that *G. schirazensis* has a systematic affinity to *Galba / Fossaria* group in southeastern Iran. Phylogenetic analysis of *ITS2* produced a single uniform cluster consisting of all the snails from the present study together with other *G. schirazensis* specimens from northern Iran and Pueblo in the eastern central Mexico as well as Rio Lurin near Lima, Peru. A more heterogeneous picture was observed in *cox1* phylogeny. *G. schirazensis* from Peru, Mexico, and Ecuador were clustered with specimens from Bam; however, north Iranian isolates of *G. schirazensis* were strongly correlated with specimens from Jiroft and Faryab.

Conclusion

This study presented new information on the distribution and genetic characteristics of *G. schirazensis* from southeast of Iran. *G. schirazensis* is frequently present in southern areas of Kerman Province. This species is not a genetically uniform population in Iran and at least two genetically different haplotypes are present in southeastern Iran. Studies that are more extensive are required on larger number of specimens to cover vast geographical areas. It will provide a clear picture of the ecology and geographical distribution of *Galba* species in Iran and neighbouring countries.

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Conflict of interest

The authors declare no conflict of interest.

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