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First systematic analysis of marsh frog, *Pelophylax ridibundus* sensu lato (Pallas,1771) (Anura: Ranidae) in central Iran based on morphological, morphometric and histometric data

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Abstract
Although marsh frog (*Pelophylax ridibundus*) is spread all over Iran except desert areas, but there is no systematic information about the populations of central Iran. Totally 35 specimens were collected from Aran-Bidgol and Najaf Abad from March 2021 to April 2022. Morphological results revealed high color polymorphism especially on the dorsal surface of their bodies. 15 morphometric characters were measured in this research. The result obtained from the t-test showed that there is no sexual dimorphism. Aran and Bidgol frogs had a larger body size than Najaf Abad. Results of Anova and discriminant analysis represented populations separation between the two regions**.** Histometric study showed the thickness of the skin on the dorsal surface of Najaf Abad frogs was more than Aran-Bidgol frogs**.** The number of serous and mucous glands in the dorsal and ventral skin of male and female *Pelophylax ridibundus* in Aran-Bidgol was significantly higher than that of Najaf Abad. This issue may be related to the desert climate (hot and dry) of Aran- Bidgol. In summary, systematic analysis of marsh frogs based on morphological, morphometric and histometric data showed significant differences between the marsh frogs of the two regions that can be related to the different geographical conditions.

Keywords: Histometry, Isfahan province, Morphology, Morphometry, *Pelophylax ridibundus***.**

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INTRODUCTION

Order Anura is cosmopolitan except for extreme northern latitudes, Antarctica, and most oceanic islands. There are five family (Dicroglossidae, Pelobatidae, Ranidae, Hylidae and Bufonidae) of Anura in Iran. Ranidae family is cosmopolitan except for southern South America, South Africa, Madagascar, and most of Australia (Frost, 2023). There are two genera of the family in Iran, *Rana* (2 species) and *Pelophylax* (1 species). *Pelophylax ridibundus* (Pallas, 1771), was reported from the northern coast of Caspian Sea and Volga River, for the first time. Genus *Pelophylax* is the most problematic anuran in western Palearctic (Dufresnes et al., 2018) and in fact, there is a species complex (*Pelophylax ridibundus* complex) in Iran

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(Pesarakloo et al., 2017). Marsh frog is spread all over Iran except desert areas. The taxonomic status of the species in Iran is unclear and some populations of marsh frogs are not studied, so far.

Many reports are published about the decline of the amphibian population (Zhao et al., 2022) due to the climate change and global warming (Hinderer, 2024). Skin health is strongly affected in the direct contact with the environment and climate changes. Hence, the study of amphibian skin, which has various biological functions including protection, breathing and defense against external factors, is very important. Isfahan province, which is located at the center of Iran, is a suitable area to study amphibians due to its climatic diversity. Since there is no systematic data about marsh frog populations of central Iran, the main purpose of this research is to investigate the marsh frogs of Najaf Abad and Aran-Bidgol regions of Isfahan province based on morphological, morphometric data and histometric structure of their skin that would be helpful to strengthen future taxonomic revision of the species.

MATERIAL AND METHODS

SAMPLES COLLECTION

From March of 2021 to April of 2022, 35 samples of *Pelophylax ridibundus* were collected (Table1). Aran-Bidgol is the northernmost city of Isfahan province. The elevation of this region is 950 m a.s.l and the climate of this region is hot and dry with hot and scorching summers and cold and dry winters. Najaf Abad is located in a plain with a moderate and relatively dry climate. The elevation of this area is1650 m a.s.l.

MORPHOLOGICAL STUDIES

Photos were taken from the dorsal, ventral and lateral surfaces of all collected specimens. Species identification was done using the identification keys (e.g., Baloutch & Kami, 2006). Presence or absence of dorsolateral folds, tympanic membrane, tongue shape, digital disks, jaw teeth, vomerine teeth, vertebral line, head shape, temporal spot, vocal sacs, eyes shape, color variation, teeth and metatarsal tubercles are important characters in species identification. The sex of samples was recognized by observing the presence or absence of nuptial pads associated with forelimbs.

MORPHOMETRIC STUDIES

In this study, 15 morphological traits were measured (Balouch & Kami,2006). These are: snout-vent length (SVL), head length (HL), thigh length (THL), length of tibia (TL), tarsus length (TAL), , distance between tip of snout and eye (ESD),upper eyelids distance (UED), width of upper eyelid (UEW), horizontal diameter of eye (ED), tympanic diameter (DT), foot length (FL), length of first toe (LFT), length of inner metatarsal tubercle (LMT), distance between nostril and anterior corner of eye (NED), and internarial distance (IND). These characters were measured using a digital caliper with an accuracy of 0.01 mm. Only adult specimens were considered.

TISSUE SAMPLING AND PROCESSING

The samples were scarified and the dorsal and ventral parts of the skin of each frog were peeled off and immersed in Bouin's solution for 48 hours before routine paraffin embedding process. The specimens were dehydrated in alcohol, cleared in xylene, and sections with 5 µm thickness were prepared using rotary microtome (Leica RM2145, Germany) and stained with hematoxylin-eosin. Microscopic images were captured using light microscopy (Olympus BH, Japan) equipped with camera (Olympus DP71, Japan).

HISTOMETRIC ANALYSIS

The thickness of the skin, epidermis and dermis as well as the diameters and number of serous, mucous and granular glands were evaluated by Axiovision 4.5 LE software (*Zeiss*, Oberkochem) on digital images. The skin, epidermis and dermis thickness and diameters of serous, mucous and granular glands were analyzed in 10 microscopic fields in each sample. Mean number of different glands was evaluated in 1mm length of skin in each sample.

TABLE 1. Information of sampling areas of *P.ridibundus* in this study.

No	Sampling areas	N	Coordinates	Elevation
1	Khazagh village, Aran-bidgol, Esfahan Provine	18	34°03'38"N 51°18'52"E	951 m
2	Fooladshahr, Najaf abad, Esfahan Provine	17	32°30'06"N 51°23'44"E	1640 m

TABLE 2. T-test result of *Pelophylax ridibundus.*

Characters	(Sig)	Sig(2tailed)
SVL	0.00	0. 84
HL	0.00	0.12
ESD	0.96	0.36
IND	0.26	0.00
UED	0.10	0. 33
UEW	0.14	0.24
ED	0.00	0.85
DT	0.38	0.08
THL	0.00	0.05
TL	0.00	0.05
TAL	0.00	0.14
FL	0.01	0.13
LFT	0.05	0.12
LMT	0.00	0.13
NED	0.19	0. 84

STATISTICAL ANALYSIS

The data were analyzed using SPSS software, version 16.0 (SPSS Corp., Chicago, IL, USA). An independent sample t-test was used to compare the histometric parameters of the skin between two areas. Statistical differences were considered significant at P-values less than 0.05. For morphometric analysis, univariate and multivariate analyses were used. Univariate analysis includes t test, Anova and descriptive statistics. Discriminant analysis was used to describe functions that maximize the possibility of correct classification of samples to their original population.

RESULTS

MORPHOLOGY AND SPECIES IDENTIFICATION

Samples were carefully examined using the mentioned morphological characteristics and identification keys. Characteristics such as the presence of dorsal folds on the sides, the presence of a bright vertebral stripe on the dorsal surface of most samples and the fact that the digits of hind limbs were not webbed completely so that the last joint of the fourth finger had no web (Fig. 1), indicate that all collected frogs based on the morphological characters belong to *Pelophylax ridibundus* species. The frogs examined in this research show high color polymorphism (Fig. 2), especially on the dorsal surface of their bodies.

TABLE 3. Morphometric measurements (Mean±SD; range) and ANOVA values of *P. ridibundus.*

Characters	Aran - Bidgol (n=16)		Najaf Abad (n=13)		P
	Mean ± SD	Min / Max	Mean ± SD	Min / Max	
SVL	68.84 ± 10.7	52.68-92.26	61.96 ± 4.05	52.32-67.59	0.03
HL	23.25 ± 3.04	19.41-30.79	20.54 ± 1.10	18.60-22.52	0.00
ESD	11.33 ± 2.03	8.33-15.78	8.66 ± 0.61	7.63-9.50	0.000
IND	4.36 ± 0.63	3.23-5.95	3.94 ± 0.56	2.98-5.24	0.07
UED	4.08 ± 0.80	2.51-5.42	3.25 ± 0.59	2.28-4.29	0.00
UEW	4.90 ± 0.66	3.61-6.39	3.77 ± 0.33	3.10-4.19	0.000
ED	8.34 ± 0.98	7.10-10.33	6.49 ± 1.13	3.13-7.78	0.000
DT	4.59 ± 0.61	3.61-5.93	4.39 ± 0.37	3.54-4.91	0.30
THL	33.05 ± 5.1	23.98-44.51	28.03 ± 2.21	22.92-30.80	0.00
TL	34.21 ± 4.5	28.01-44.20	32.03 ± 1.8	28.22-34.94	0.12
TAL	17.53 ± 3.2	14.10-25.51	14.87 ± 1.2	13.01-17.08	0.01
FL	35.10 ± 5.005	28.91-48.07	32.07 ± 2.89	27.18-36.94	0.06
LFT	8.73 ± 1.2	6.92-11.22	8.10 ± 0.87	6.51-10.16	0.14
LMT	3.74 ± 0.59	3.05-5.43	3.24 ± 0.42	2.59-4.04	0.02
NED	4.82 ± 0.59	3.54-5.67	4.21 ± 0.47	3.52-5.09	0.00

TABLE 4. Classification results of discriminate analysis with fifteen morphometric characters.

	Groups	Aran - Bidgol	Najaf Abad	Total
Count	Aran - Bidgol	15	1	16
	Najaf Abad	2	11	13
%	Aran - Bidgol	93.8	6.3	100.0
	Najaf Abad	15.4	84.6	100.0

MORPHOMETRY

The result obtained from the T-Test (Table2) showed that no significant difference (P>0.05) was observed between male and female frogs in all measured characters (except IND). Therefore, there is no sexual dimorphism.

Descriptive statistics revealed that Aran-Bidgol frogs have a larger body size (SVL =68.84 ± 10.7) than Najaf Abad frogs. According to Table 3, there are significant differences in 10 characters, snout-vent length (SVL), head length (HL), distance between tip of snout and eye (ESD), upper eyelids distance (UED), width of upper eyelid (UEW),), horizontal diameter of eye (ED), thigh length (THL), tarsus length (TAL), length of inner metatarsal tubercle (LMT), distance between nostril and anterior

corner of eye (NED) between the populations (Aran-Bidgol and Najaf Abad), (P<0.05). Therefore, separation between populations is observed in many characters.

In discriminant analysis, single function was considered, which represented 100% of the changes and was significant (P<0.05). About 93.8% of the samples from Aran-Bidgol region are in their own group and 6.3% are in the Najaf Abad group. Moreover, 84.6% of the samples from Najaf Abad region are in their own group and 15.4% are in the Aran-Bidgol group. Totally, 89.7% of samples were classified correctly (Table 4).

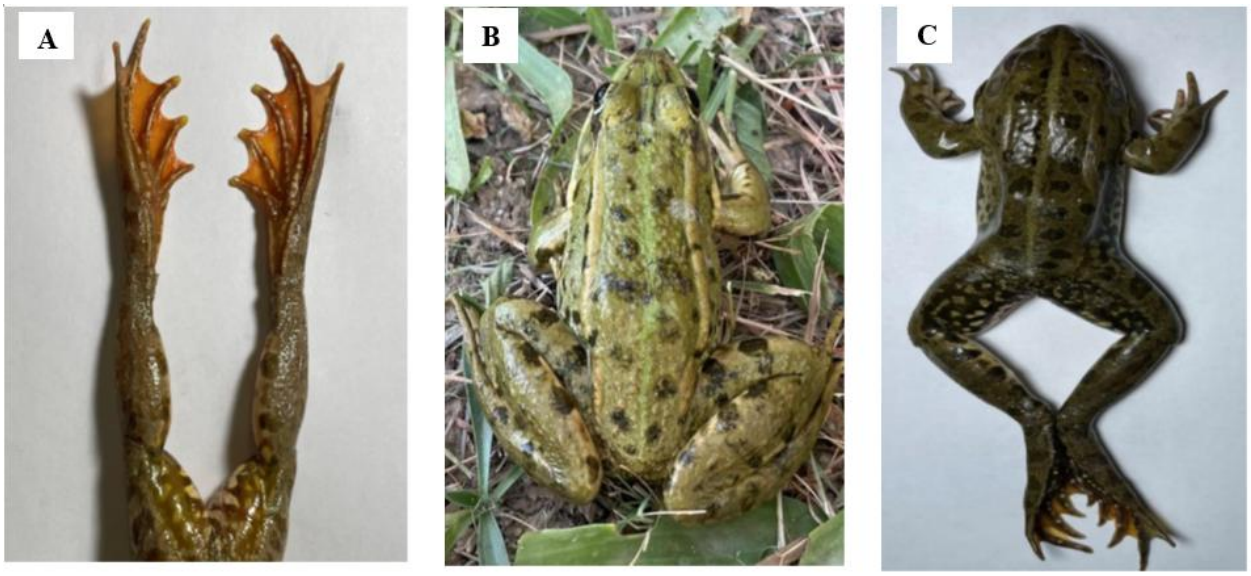

FIGURE 1. Characters used in the identification of *Pelophylaxis ridibundus*. A) web of hindlimbs; B) dorsal folds; C) vertebral strip.

HISTOMETRIC ANALYSIS

The dorsal and ventral skin in male and female of *Pelophylax ridibundus* of Aran-Bidgol and Najaf Abad regions consist of epidermis and dermis. There were two types of small skin glands, including mucous glands and serous glands, and large skin glands, including granular (poisonous) glands, in the spongy layer of the dorsal skin in male and female *Pelophylax ridibundus* of different regions (Fig. 3).

Significant differences were observed in the dorsal dermis ($P<0.001$) and skin ($P<0.001$) thickness of females *Pelophylax ridibundus* between Aran-Bidgol and Najaf Abad (Table 5). There are no significant differences in the ventral skin of male and female *Pelophylax ridibundus* between Aran-Bidgol and Najaf Abad (Table 5). Significant differences ($P<0.001$) were observed in the dorsal skin thickness in the female *Pelophylax ridibundus*, in Najaf Abad and Aran-Bidgol, respectively (Table 5).

There are significant differences ($P<0.001$) in the dorsal dermis and skin thickness ($P<0.001$) between male and female *Pelophylax ridibundus* in Aran-Bidgol (Table 6). The ventral dermis thickness was significantly different ($P<0.001$) between male and female *Pelophylax ridibundus* in Najaf Abad (Table 6). A statistically significant difference ($P<0.001$) was observed in the dorsal skin thickness between male and female *Pelophylax ridibundus* in Aran-Bidgol (Table 6). There is no significant difference in the diameters of serous and mucous glands in the dorsal and ventral skin of male and female *Pelophylax ridibundus* from Aran-Bidgol and Najaf Abad (Table 7). The number of serous and mucous glands in the dorsal and ventral skin of male and female *Pelophylax ridibundus* in Aran-Bidgol was significantly ($P<0.05$) higher than those in Najaf Abad (Table 8).

FIGURE 2. Color polymorphism of *P. ridibundus* in studied areas.

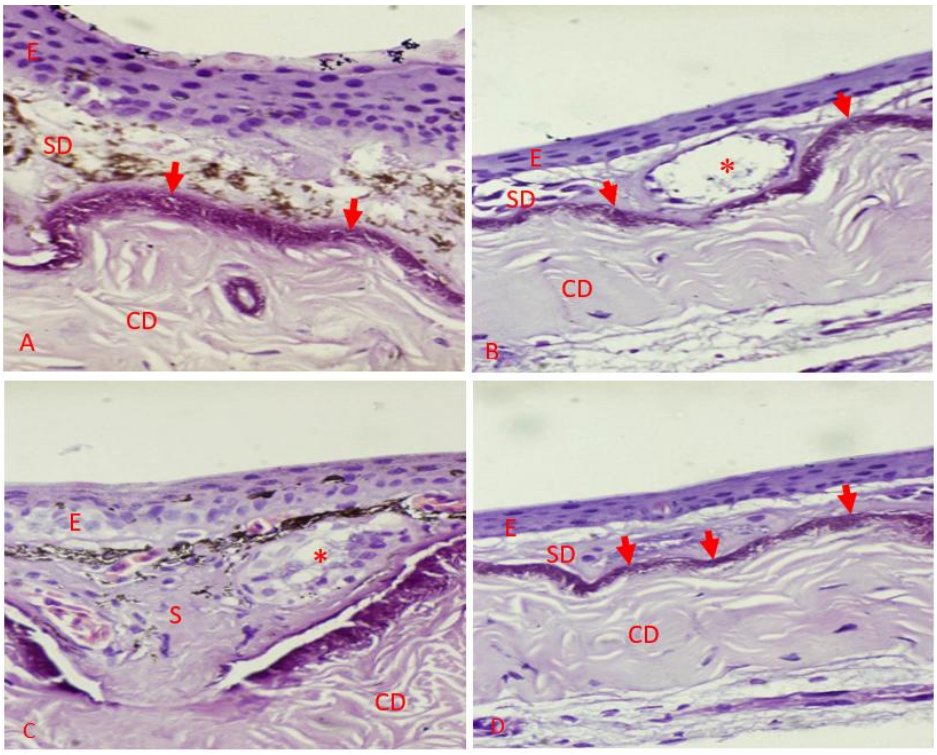

FIGURE 3. Microscopic sections of the skin in *Pelophylax ridibundus* of Aran-Bidgol and Najaf Abad regions (Hematoxylin-Eosin staining, ×40). (A): Dorsal skin in male frog (Najaf Abad), (B): Ventral skin in male frog (Najaf Abad), (C): Dorsal skin in male frog (Aran-Bidgol), (D): Ventral skin in male frog (Aran-Bidgol). Epidermis (E), Spongy dermis (SD), Compact dermis (CD), Mucous gland (Red star), Ebert-Kastchenko layer (Red arrow).

TABLE 5. Comparison of epidermis, dermis and total skin thickness (μm) between male and female *Pelophylax ridibundus* in Najaf Abad and Aran-Bidgol regions.

Parameter	Dorsal surface of body			Ventral surface of body		
	Skin	**Epidermis**	**Dermis**	**Skin**	**Epidermis**	**Dermis**
			Najaf Abad			
Males	41.03±11.611	4.23±2.910	41.10±5.807	32.60±2.143	3.83±0.648	31.10±3.916
Females	45.47±9.333	2.77±0.935	21.47±1.676	28.30±5.240	3.73±0.740	19.00±3.787
***P* value**	0.010	0.001	0.010	0.182	1.000	0.000
			Aran-Bidgol			
Males	40.63±10.601	4.33±1.900	42.20±5.857	3.77±2.046	4.43±0.774	27.77±2.661
Females	32.23±3.390	3.30±0.651	28.43±2.861	27.40±4.940	3.68±0.740	18.91±3.787
***P* value**	0.000	0.001	0.000	0.080	1.000	0.010

TABLE 6. Comparison of epidermis, dermis and total skin thickness (μm) in male and female *Pelophylax ridibundus* between Najaf Abad and Aran-Bidgol regions.

Parameter	Dorsal surface of body			Ventral surface of body		
	Skin	**Epidermal**	**Dermis**	**Skin**	**Epidermis**	**Dermis**
			Males			
Najaf Abad	41.03±11.611	4.23±2.910	41.10±5.807	32.60±2.143	3.83±0.648	31.10±3.916
Aran-Bidgol	40.63±10.601	4.43±1.900	42.20±5.857	32.77±2.046	4.43±0.774	27.77±2.661
***P* value**	0.089	0.234	0.457	1.000	0.236	0.090
			Females			
Najaf Abad	45.47±9.333	2.77±0.935	21.47±1.676	28.30±5.240	3.73±0.740	19.00±3.878
Aran-Bidgol	33.23±3.390	3.30±0.651	28.43±2.861	27.40±4.940	3.68±0.740	18.91±3.787
***P* value**	0.000	0.384	0.000	1.000	0.245	0.412

TABLE 7. Comparison of skin glands diameters (μm) in male and female *Pelophylax ridibundus* between Najaf Abad and Aran-Bidgol regions.

Parameter	Dorsal surface of body		Ventral surface of body	
	Serous glands	**Mucous glands**	**Serous glands**	**Mucous glands**
		Males		
Najaf Abad	182.07±2.07	241.10±3.58	183.55±1.91	237.88±2.85
Aran-Bidgol	187.25±2.35	243.8±3.56	187.68±1.85	241.79±3.06
***P* value**	0.105	0.593	0.127	0.353
		Females		
Najaf Abad	187.57±2.04	240.12±3.41	185.42±2.02	237.88±2.83
Aran-Bidgol	196.04±4.54	238.70±2.85	187.42±2.27	241.79±3.06
***P* value**	0.094	0.750	0.514	0.353

TABLE 8. Comparison of skin glands numbers in male and female *Pelophylax ridibundus* between Najaf Abad and Aran -Bidgol regions.

Parameter	Dorsal surface of body		Ventral surface of body	
	Serous glands	Mucous glands	Serous glands	Mucous glands
		Males		
Najaf Abad	2.86±0.73	2.63±0.13	2.90±0.13	2.90±0.13
Aran-Bidgol	3.26±0.63	3.20±0.12	3.20±0.10	3.13±0.10
***P* value**	0.028	0.002	0.085	0.184
		Females		
Najaf Abad	2.96±0.12	2.90±0.12	2.83±0.13	2.93±0.12
Aran-Bidgol	3.36±0.11	3.33±0.12	3.16±0.15	3.03±0.14
***P* value**	0.019	0.022	0.108	0.609

DISCUSSION

Our morphological results show the collected samples are *P.ridibundus.* This species demonstrates high color polymorphism among sample areas, which is consistent with the results of previous studies of the species in Iran (Poorjafary & Fakharzadeh, 2022; Pesarakloo et al., 2011; Delavar et al., 2017). Marsh frogs of central Iran did not show sexual dimorphism. Although this result is consistent with some of the previous studies (Seydi and Fakharzadeh, 2016; Nemati,1998), most examined populations of the frogs in Iran show sexual dimorphism (e.g. Poorjafary & Fakharzadeh, 2022; Fathinia et al.,2012; Hezaveh et al., 2007). Various reasons have been proposed for the emergence of sexual dimorphism in amphibians. Some researchers believe it is due to sexual selection (Shine, 1979). But some scientists indicate sexual size dimorphism in the Anura can be explained by differences in the age structure between the sexes in breeding populations. If sexual selection has an effect on size dimorphism in anurans, this is probably to be only a secondary one (Monnet and Cherry., 2002). Results of ANOVA and discriminant analysis represent populations separation between the two regions that would be related to different geographical conditions of the studied areas. Such interpopulation variation for this frog from different areas of Iran has been reported in previous studies (e.g. Fakharzadeh et al., 2023; Hezaveh et al., 2007; Seydi and Fakharzadeh, 2016)

According to classical taxonomy, Iranian water frog has been considered as *P. ridibundus.* Recently a molecular study (Pesarakloo et al., 2017) based on mitochondrial gene (Cytb) showed that two major clades of water frog can be recognized in Iran. One of these clades includes samples from the West, the northwest and southwest of Iran called *Pelophylax bedriagae* and the second one (*Pelophylax* sp.) includes North and Northeast populations. Some populations of this frog, including the populations of central Iran (present study), have not been investigated in the mentioned molecular study. Due to lack of sufficient information, definitely cannot be talked about the taxonomic status of this species in Iran. Although the comparison of our results in central Iran with previous papers on marsh frogs in the west of Iran shows some differences in populations, it seems necessary to conduct comprehensive molecular studies on Iranian marsh frogs.

Present study showed that the dorsal and ventral skin of male and female *Pelophylax ridibundus* in Najaf Abad and Aran-Bidgol consist of epidermis and dermis. Our microscopic analysis showed that the dorsal and ventral dermis of the male and female *Pelophylax ridibundus* in the different regions are composed of two distinct layers structurally, which respectively includes the spongy layer, consisting of loose connective tissue containing blood vessels and skin glands, and the dense layer, consisting of dense connective tissue contains thick bundles of collagen fibers that extend parallel to the skin surface. Our histologic findings are in accordance with the results of Felsemburgh (2009), and Saberi et al., (2020). Bingol-Ozakpinar & Murathanoglu (2011) showed that the ventral and dorsal skin in *Triturus karelinii*

have similar morphological structure. However, Azevedo (2006) reported that there is water in the spongy layer in *Rana catesbeiana*, which has turned this layer into a water reservoir.

Furthermore, this study showed that two types of small skin glands, including mucous glands and serous glands, and large skin glands, including granular (poisonous) glands were observed in the spongy layer of the dorsal skin of male and female of *Pelophylax ridibundus* in Najaf Abad and Aran-Bidgol. However, only mucous and serous glands exist in the spongy layer of the ventral skin of male and female frogs in both regions. This result is in accordance with the findings of Haslam (2014), Brunetti (2016) and Papahn et al., (2017).

Histometrical analysis showed that the thickness of the dorsal skin of *Pelophylax ridibundus* in Najaf Abad is greater than Aran-Bidgol. This issue may be related to the desert climate (hot and dry) of Aran-Bidgol, because the skin must be moist to be able to breathe through it. So, the dorsal skin of *Pelophylax ridibundus* in Aran-Bidgol is thinner. The result of a study on the skin of water frogs' populations in Golestan and Mazandaran provinces showed that populations live in higher altitudes have thicker skin, and the authors stated that this observed difference in the dorsal skin between populations in different regions may have an adaptive role (Taziki et al., 2024).

Moreover, our study showed that the number of mucous and serous glands of male and female *Pelophylax ridibundus* in Aran-Bidgol is higher than that in Najaf Abad. Duellman and Trueb (1994) reported that there are intraspecific variations in Ranids in relation to habitat differences.

Sinsch & Lehr (2010) reported that the number of epidermal mucous glands in the *Telmatobius carrillae* is not related to gender and skin thickness. Papahn et al., (2017) found that the number of the epidermal mucous glands of *Pelophylax ridibundus* in cold region is higher than that in warm region.

Mills & Prum (1984) found that the number of granular glands in the dorsal skin of *Pelophylax ridibundus* is more than that of the ventral skin. In addition, Duellman & Trueb (1994) reported that the mucous glands in the dorsal skin of *Pelophylax ridibundus* are more than the ventral skin, and they are more numerous and widespread than granular glands. Thiner skin of dorsal surface in Aran - Bidgol samples comparing to Najafabad samples can be related to their adaptation to hot and dry climate. As we know subcutaneous respiration is a principal phenomenon in amphibia and thinness of the epidermis plays an important role in such respiration (Kardong, 2019). The thickness of the epidermal layer and the distribution of glands differ between the dorsal and ventral areas, implying an underlying strategy of adaptation (Sulaiman et al., 2025). So having thinner skin with more mucous and serous glands for Aran-bidgol marsh frogs that live in such climate conditions seems to be an appropriate strategy for them.

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