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Biological aspects of the Marsh frog *Pelophylax ridibundus* population from the Shadegan Wetland, Khuzestan Province, Iran

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Abstract

The Marsh frog, *Pelophylax ridibundus* (Pallas) is distributed in Central Europe from northeastern France, north to the southern shorelines of the Baltic Sea (and extreme southern Finland), south to northeastern Spain, northern Italy and the Balkans including eastern Greece, east to approximately 81° E in Asiatic Russia, and south to western Iran and Afghanistan. The present study has been conducted on populations of *P. ridibundus* in the northern, eastern and southern regions of the Shadegan Wetland of Khuzestan Province, Iran with the aim of examining some aspects of its morphology and karyotype. Frog specimens were collected from different portions of the Shadegan Wetland during spring and autumn 2016 and transferred to the laboratory. According to the morphological results, three color patterns are identified among the samples in terms of the morphology, morphometry and sex - according to the sex independency test. T-test results between males and females indicate a significant difference in all traits and the results of a T^2 Hotelling test show that there is sexual dimorphism between males and females. However, the results of multivariate analysis of variance (MANOVA) illustrate a separation between males of the northern region and the two other regions, and no differentiation between females in the eastern and southern regions of the wetland. The karyotype of the species in the wetland included 2n= 26, one pair of which had a sex chromosome.

Key words: Marsh frog, morphology, population, sexual dimorphism

Introduction

Amphibians play a decisive role in most aquatic and terrestrial ecosystems (Kiabi, 2007) and among this class, anurans are the largest representative (Vignoli et al., 2016). Lissamphibia which contains modern amphibians, arose during the Carboniferous period and later diversified, probably by the early Triassic period, to produce ancestors of the three major groups of amphibians alive today, frogs, salamanders, and caecilians (Hickman et al., 2017). Currently, 7340 of 8317 known species of amphibians are within the anurans (AmphibiaWeb, 2021).

The Marsh frog, *Pelophylax ridibundus* (Pallas) inhabits Central Europe east of northeastern France, north to the southern shoreline of the Baltic Sea (and extreme southern Finland), south to northeastern Spain, northern Italy and the Balkans including eastern Greece, east to about 81° E in Asiatic Russia, and south to western Iran and Afghanistan. Isolated populations occur in the high Asir and Ha'il regions of western and north-central Saudi Arabia, and some oases of eastern Saudi Arabia. They have been introduced into England and Italy and an introduced hybrid population (with *Pelophylax bedriagae*) occurs in the Kamchatka Peninsula of Russia (Pesarakloo et al., 2017).

The marsh frog is usually found in calm ponds mostly surrounded by beetroot trees of the genus *Elattostachys* (family Sapindaceae) (Martin et al., 2000). The marsh frog has very rapid, zigzag swimming movements in the water and hides under the mud to overcome the adverse conditions during the winter (Rogers and Ralph, 2010; Najibzadeh et al., 2014). The breeding season of *P. ridibundus* begins at the end of April or early May (Najibzadeh et al., 2014) and obvious differences in mating behaviors lead to reproductive separation between adjacent species (Darvish, 2005). Generally, males of *P. ridibundus* arrive at the ponds earlier than females and determine their mating ranges. In addition, mating and egg laying are carried out in the stagnant water and after three weeks, the size of the eggs reaches 5 mm. (Duellman, 1999). During metamorphosis, changes occur in the small intestine, liver and tail (Kaptan et al., 2013) and changes in the size and shape of the oral disk, jaw shape and number of teeth rows are considered as important features to identify this species (Ruff et al., 2014). Investigations into the effects of environmental stress, anthropogenic contaminants and changes in aquatic and non-aquatic ecosystems on the marsh frog have pointed out the significant impact of these parameters on the mating of this species (Lukanov et al., 2014). Protecting this species depends on both habitat and climate variation parameters, given the danger of extinction (Ruff et al., 2014).

Twenty-two international wetlands have been registered in Iran (Eyafat, 2001) and the Shadegan Wetland is unique in having two distinct habitats of fresh and salty waters in the northern and southern regions, respectively. This largest wetland in the country hosts thousands of plant and animal species and has an important role in mediating the climate of the region, preventing the emergence of pathogenic micro-organisms and seasonal flooding (Eyafat, 2001). Unfortunately, climate change, atmospheric emissions, exploitation of resources, and the release of industrial and agricultural wastewater into the wetland have reduced the quality of this habitat (Mohammadi et al., 2015). In addition, the introduction of non-domestic species of Tilapia (Perciformes, Cichlidae) in recent years, has severely threatened the food chain of this wetland (Valikhani et al., 2018).

The study of amphibians in Iran has a relatively long history and has always been a focus of attention. In this regard, many researchers have conducted studies on amphibians in Iran (e.g., Balouch and Kami, 1995). Comparison of the populations of *P. ridibundus* in Mazandaran and Khorasan Provinces based on the measurement of morphological traits indicated that there is a significant difference between the body, thigh, leg and head lengths between males and females of the species (Hojati et al., 2009). Given that previous studies about *P. ridibundus* have not covered all regions of Iran further comprehensive studies are needed in different provinces of Iran on a regional scale (Hojati et al., 2009).

Therefore, the present study aims to investigate the population of *P. ridibundus* in the Shadegan wetland areas of Khuzestan Province in terms of the morphological, morphometric and karyological characteristics.

Material and Methods

The Shadegan Wetland is located 50–150 km south of Ahvaz (Fig. 1). The wetland is considered as a flood plain, the northern part of which has fresh water while its middle and southern parts have brine and salty water, due to its proximity to coastal estuaries. This wetland is a wildlife sanctuary and part of the international wetlands in the Montrob list (Eyafat, 2010). Considering the importance of this natural habitat and the need for further studies on its biodiversity, it has been selected for the current study (Table 1).

Fifty-one specimens of *P. ridibundus* (23 specimens from the northern areas of the Shadegan wetland, 13 specimens from the southern areas and 15 specimens from the eastern areas) were captured during spring and autumn 2016 by handy pack net. The frogs were transferred to the laboratory for morphological and karyological studies. The identification key of Balouch and Kami (1995) was used to identify the species. Different color and pattern morphs were identified and photographs were taken from the available specimens. In addition, nine morphological traits (SVL, snout-vent length; ID, distance between nostrils; LO, eye size; LC, head length; FL, leg length thigh; La, hand arm length; LTYM, size of the tympanic membrane; SP.P, distance between the eyelids, and CINT, internal bulge of the first toe) that are important in identifying the species (Balouch and Kami, 1995) were measured by digital calipers with 0.01 mm precision. The Kolmogorov-Smirnov test was employed for data normality and logarithmic conversion was performed on the abnormal data. Due to the presence of sexual dimorphism in the morphometric traits of this species, MANOVA and T^2 Hotelling tests were performed and they showed no differentiation between males and females in any population.

In order to conduct the karyological studies, colchicine solution was injected intraperitoneally per gram of body weight. After 7 hours, the specimens were killed by chloroform. The bone marrow was emptied by injecting 0.075 M potassium chloride into femur (leg bone). A few drops of fixative (the fresh and cold solution was prepared from 3 parts of methanol and one part of glycolic acetic acid at 4 °C) were added to the bone marrow coming out of the thigh (leg bone) after 20 minutes. The resulting solution was completely homogenized and transferred to a centrifuge tube and centrifuged at 1500 rpm for 10 minutes. After draining the clear supernatant (Sup), fresh and cold fixative solution (acetic-methanol) in a ratio of (3: 1) was added to the resulting pellet precipitate (cc 1), after homogenizing again for 10 minutes at 1500 rpm. The above step was repeated twice more and the precipitate was thoroughly washed. At the end of the wash, 1 ml of cold fixative solution was gradually added dropwise to the precipitate and made uniform by a pasteurizer pipette, and then the resulting solution was applied from a distance of 60–80 cm on the slides. It was cleaned and cooled; the slides were immediately placed on a sloping surface and dried in the air of the laboratory, then coded for future examination. The slides were stained by Geimsa 5% for 10 minutes, then the stained slides were labeled, studied by light microscopy (Olympus-CX31), and the best metaphase plates were observed using a 100 × lens and a BEL microscope equipped with a camera (Jazayeri et al., 2012).

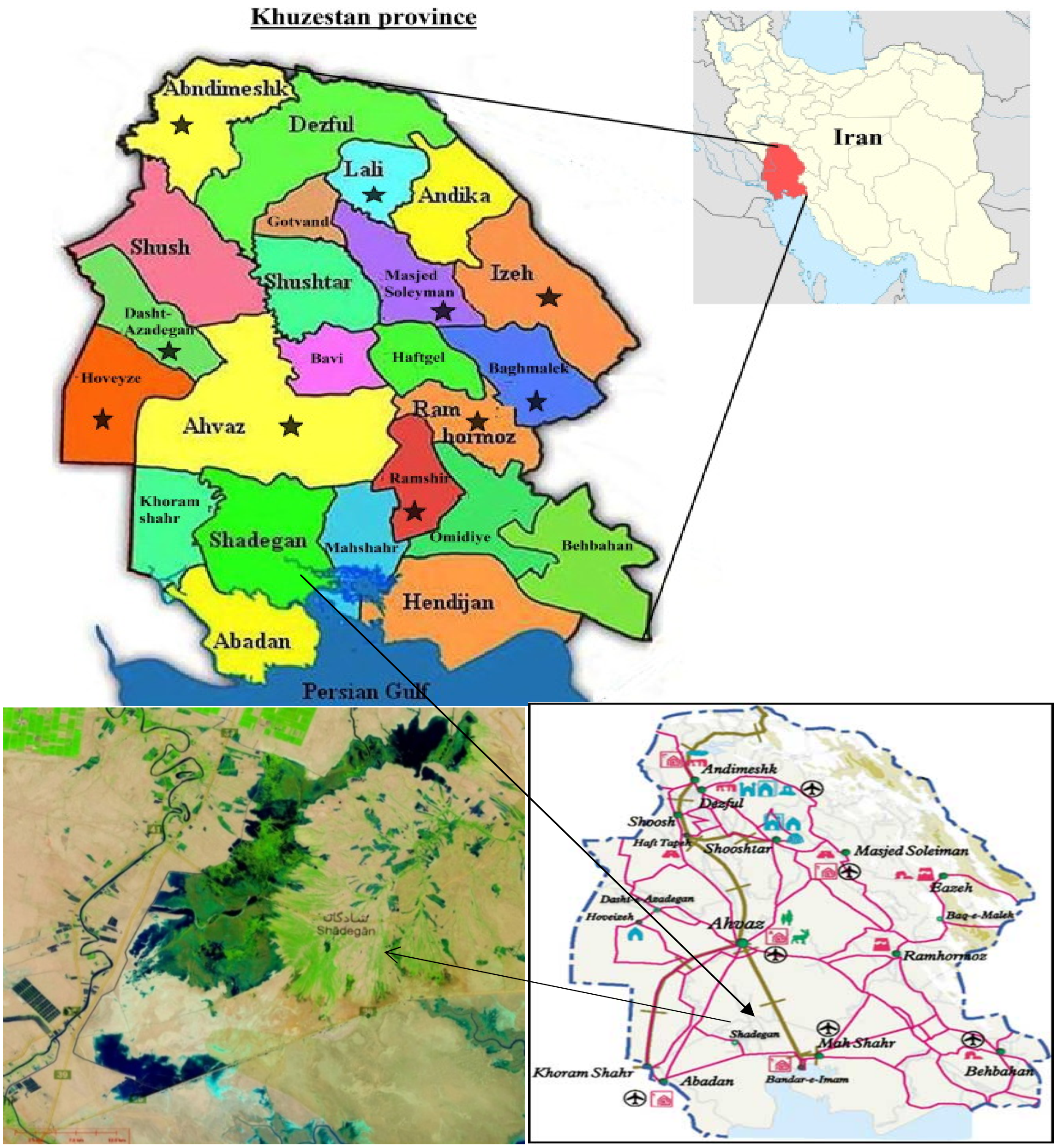

Figure 1: Map of Iran shows the satellite (left) and geographic (right) maps of the Shadegan Wetland, Khuzestan Province, southwest of Iran.

Table 1: Location and characterization of the study areas and number and sex of frog specimens of *Pelophylax ridibundus* collected in the Shadegan Wetland, Khuzestan Province, Iran.

Study area	Number of specimens (divided)		Sex		Geographical location of stations
			Male	Female	
Northern areas of the wetland (freshwater)	22		9	13	30°17′N, 30°58'E
	Mature: 17	Immature: 5			
Eastern areas of the wetland (brine water)	15		6	9	45°17'N, 48°50'E
	Mature: 11	Immature: 4			
Southern areas of the wetland (salty water)	14		5	9	
	Mature: 12	Immature: 2			

Results and Discussion

Three morphs of *P. ridibundus* were observed in the population of Shadegan wetland (Table 2; Figs. 2–4).

Table 2: Sex-independent results of the three morphs of *Pelophylax ridibundus* in the Shadegan Wetland, Khuzestan Province, Iran.

		Morphs		
		1	2	3
Sex	Male	5	14	7
	Female	9	7	9
	Total	14	21	16

Morph 1: This morph has many, different sized, brownish flecks on the dorsal surface of the head, body and limbs, as well as on the sides of the body. The background color of the dorsum is green to olive green. The abdomen is slightly opalescent with no flecks, the legs have dark dorsal stripes and the hands have dark spots (Fig. 2).

Figure 2: The dorsal color of morph 1 in *Pelophylax ridibundus* from the Shadegan Wetland, Khuzestan Province, Iran.

Morph 2: The dark, olive-green flecks are aligned almost parallel on the dorsum of the body and separated into left and right sides by a thin pale stripe extended from the tip of the rostrum to the pelvic region (black arrows). The sides of the body have smaller olive-green spots. The dorsal background color is brighter than in morph 1 and the ventral surface color is pale white with irregular green stripes (black arrows). In addition, green dark stripes are clearly visible on the dorsal surface of the legs and in smaller numbers on the forelimbs (Fig. 3).

Figure 3: The dorsal (right) and ventral color (left) in morph 2 in *Pelophylax ridibundus* from the Shadegan Wetland, Khuzestan Province, Iran.

Morph 3: A distinct bright yellow stripe is observable along the dorsal axis of the body, extending from the tip of the rostrum to behind of the pelvic region (black arrows). The dorsal background color is olive-green containing large brown spots that are almost aligned parallel to the bright longitudinal stripe. The legs have dark stripes from the groin to tip of the toes and dark spots can be clearly seen on the forelimbs, from the humerus to the wrist (Fig. 4).

Figure 4: The dorsal color in morph 3 in *Pelophylax ridibundus* from the Shadegan Wetland, Khuzestan Province, Iran.

Morphometric studies

Statistical studies using the SPSS software (Version 20) on the morphological traits of male and female specimens of *Pelophylax ridibundus* showed the following results.

Univariate analyses

Nine morphological traits were examined to observe the differences between sexes via a T-test ($p < 0.05$). Except for two traits (LO and La), the remaining ones displayed significant differentiation between the sexes (Table 3).

Table 3: Comparison of the mean t-test between males and females for all measured traits of *P. ridibundus* from the Shadegan Wetland, Khuzestan Province, Iran.

Character	t	Sig (2-tailed)	F	Sig (2-tailed)	Std. Error Difference
SVL	-3.54	0.0002	6.32	0.011	1.72
ID	-3.26	0.001	0.03	0.69	1.34
LO	-2.45	0.36	1.82	0.17	0.63
LC	-0.82	0.01	4.14	0.037	0.71
FL	-0.78	0.003	2.12	0.41	0.38
La	-2.34	0.21	1.71	0.02	0.14
LTYM	-2.12	0.008	0.34	0.57	1.49
SP.P	-2.46	0.02	0.056	0.83	0.29
CINT	-2.09	0.003	7.87	0.007	0.11

On the other hand, the T^2 Hotelling test, for investigating the mean vector separation between males and females, pointed to the presence of sexual dimorphism in *P. ridibundus* (P= 0.002, F= 3.21). In addition, the principal component analysis (PCA) associated with 9 morphological traits of the male and female samples led to the extraction of two principal components, the first and second of which explain about 78.6% and 9.3% of the total variance, respectively. As can be observed in Figure 5, the male and female samples of *P. ridibundus* are separate from each other and sexual dimorphism is evident between the members of this species.

Multivariate analyses

A MANOVA was used to examine the mean vector separation associated with each male and female. According to the results, while there was differentiation among males of different populations (P= 0.02, F= 2.65), no significant differentiation was observed among female (P= 1.19, F= 0.12). In addition to that, the PCA for the male population led to the extraction of two significant components

(components 1 and 2). The first and second components indicate 70.92% and 7.81% of the variation, respectively (see Table 4 and Fig. 6).

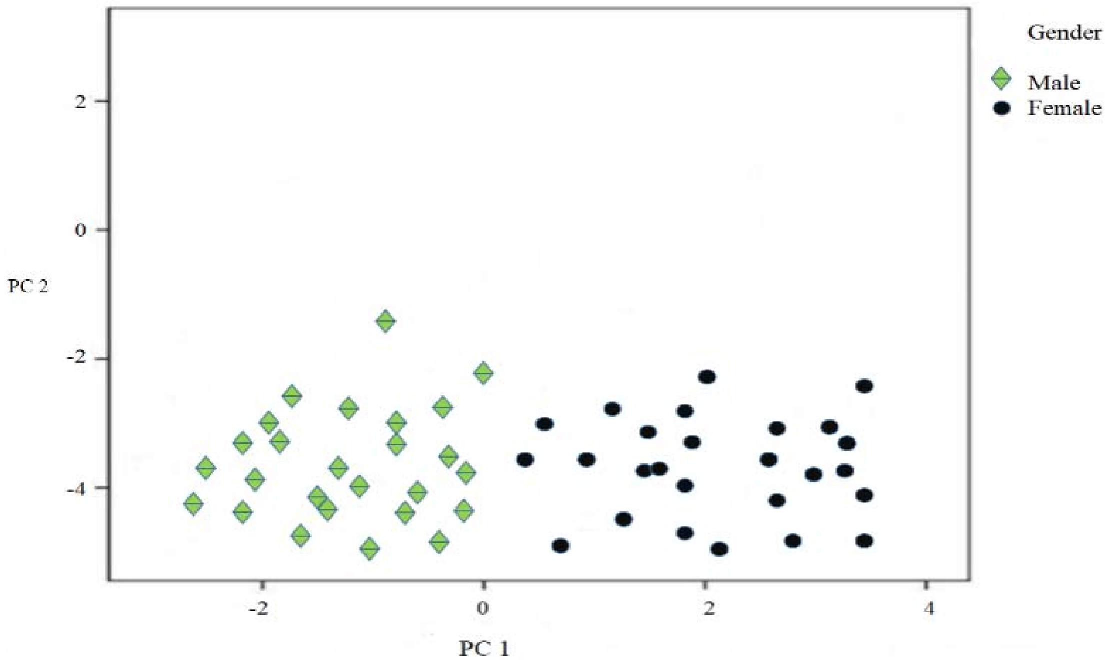

Figure 5: The scatterplot of males and females of *P. ridibundus* from the Shadegan Wetland, Khuzestan Province, around the first and second components.

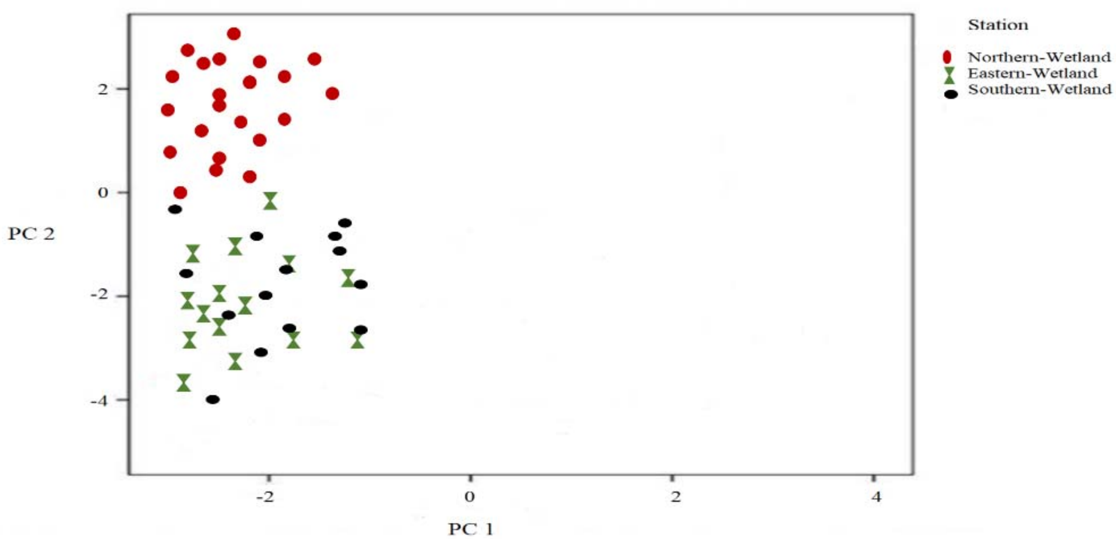

Figure 6: The scatter plot of males around the first and second PCA axes associated with measured morphological traits of *P. ridibundus* from the Shadegan Wetland, Khuzestan Province, Iran.

Table 4: The results of a Principal Component Analysis for the male population of *P. ridibundus* from the Shadegan Wetland, Khuzestan Province, Iran. Extraction method= Principal axis factoring.

Total Variance Explained						
Factor	**Initial Eigen values**			**Rotation Sums of Squared Loadings**		
	Total	**% of Variance**	**Cumulative %**	**Total**	**% of Variance**	**Cumulative %**
1	19.32	69.41	69.104	9.234	32.063	32.98
2	2.605	5.652	74.751	7.032	30.43	63.54

The results of the audit analysis in Table 5 illustrate that the Wilks' Lambda coefficient is only significant for the first component (SVL, ID, LC, FL, LTYM and CINT) while being insignificant for the second one (LO, La and SP.P). In addition, due to the high value of this coefficient (0.116), it can be concluded that the second component has poorly separated the groups (a lower amount of the Wilks' Lambda coefficient indicates the stronger variable performance in separation). According to the classification results, the audit analysis based on nine morphometric traits (Table 6) indicates that 72%, 64% and 100% of the samples associated with the northern, eastern and southern areas of the Wetland are classified into their corresponding groups, respectively. In general, 68% of the samples have been correctly classified into their groups. However, when these nine traits are divided into two components, only 51% of the specimens were correctly classified into their groups. Therefore, according to the above results and the Wilks' Lambda coefficient for the second component, the separation into two components was not performed.

Karyological studies

The chromosome number 2n= 26 was reported for this species in both northern and southern regions of Khuzestan Province. Of these chromosomes, chromosomes 1, 3, 5, 6 and 13 were of a metacentric type and the others were submetacentric. Moreover, the sex chromosomes in females and males were in the forms of XX and XY, respectively, and the number of chromosomal arms (FN) was estimated as 52. The comparison of the karyotypes of the above specimens in the three mentioned regions did not show any difference. In fact, the chromosome number and karyotypic structure of this species were quite similar in the populations of the above-mentioned regions (Fig. 7).

Table 5: The results of the Wilks' Lambda coefficient in *P. ridibundus* from the Shadegan Wetland, Khuzestan Province, Iran.

Test of Function(s)	Wilks' Lambda	Chi-square	df	Sig.
1	0.116	104.432	16	0< 0001

Table 6: Validity of the groups formed using the audit analysis, 98 (97.6) % of the male populations of *P. ridibundus* in the Shadegan Wetland, Khuzestan Province were correctly classified.

		Groups (Cluster Number of CaSP.P)	Predicted Group Membership		Total
			1	2	
Original	**Count**	1	40	1	**41**
		2	0	24	**24**
	%	1	97.6	2.4	**100.0**
		2	0.0	100.0	**100.0**

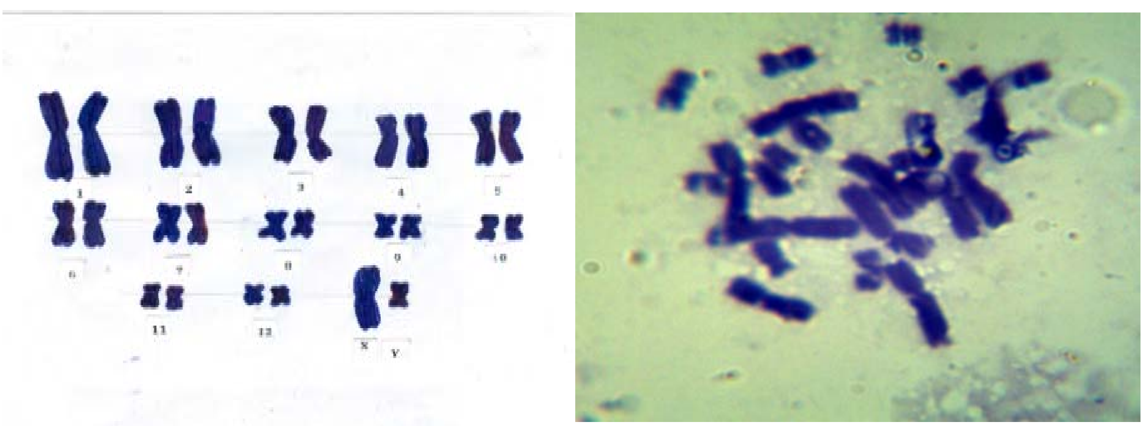

Figure 7: Chromosomes of the *P. ridibundus* species (male sex) from the Shadegan Wetland, Khuzestan Province, Iran.

Conclusion

The environmental factors associated with genetic drift and subsequently change in the morphological traits, contribute to local adaptation in a region's population, its divergence and eventually contribute to the survival of the population within the region through the evolution of groups with flexible phenotypes (Wilbur and Collins, 1973; Kakhki and Darvish, 2015). In the case of an inconsistency between environmental and genetic factors, for example, in aquatic ecosystems at the risk of drying out, the inhabitants have a period of intensive prepuberty or high dispersal in the habitat, and the phenotypic traits in some populations vary in terms of body size and age during the season and fertility is reduced as a consequence (Martin et al, 2000).

The present investigation indicates that morphological traits alone cannot show the correlation between the populations in the three study areas. However, the statistical analyses (univariate and multivariate), based on the measured morphological traits, generally confirm this correlation to a great extent and illustrate that the corresponding populations are not different in terms of most traits.

In the present study, sexual dimorphism was observed among the populations, which is consistent with the results of the study conducted by Hezaveh et al. (2007) on *P. ridibundus*. On the other hand, after measuring 16 morphological traits in similar studies conducted in Khorasan Province, it was found that there is no sexual dimorphism in *P. ridibundus* populations (Hezaveh et al., 2007).

According to the present results, the populations corresponding to the three regions of Khuzestan Province could not be distinguished. It can be inferred then, that the three studied regions are almost similar in terms of the ecological, microclimatic and habitat conditions due to the absence of a physical barrier to their separation. Based on the results of the MANOVA, the population differentiation observed in the males is due to the greater impact of the first category components and eventually demographic separation in this sex.

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Conflict of interest

The authors declare that there are no conflicting issues related to this research article.

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