Herpetology Notes, volume 7: 569-574 (2014) (published online on 3 October 2014)

Genetic diversity and differentiation of the moor frog (*Rana arvalis*) in Western Siberia

Oxana Zhigileva[1*], Irina Kirina[1] and Anna Burakova[2]

Abstract. Genetic features of populations of the moor frog *Rana arvalis* in Western Siberia, Russia, were studied. Allele frequencies of allozyme loci in the moor frog were uniform, the percentage of polymorphic loci was 56.2%, the mean heterozygosity – 0.167 ± 0.009. The level of genetic variability of moor frog populations in Western Siberia was high, but lower than in populations of Central Europe, and higher than in populations of Northern European countries in the same latitude. The two groups of moor frog populations (the northern and southern) can be distinguished in both Western Siberia and Europe. These differences may be associated with the adaptation of frogs to different environmental conditions.

Keywords. Brown frogs, genetic variability, allozyme, population differentiation, Russia.

Introduction

Studies of genetic variability of the genus *Rana* (Amphibia: Ranidae) attracted the attention of researchers due to the ability amphibian to occupy modified environments (Hitchings and Beebee 1997; Merilä et al., 2004; Zhigileva and Burakova, 2005), where they are vulnerable, endangered components of biological diversity (Ishchenko, 1996; Sas et al., 2006; Kuzmin, 2010). Although there are several publications on genetics of the moor frog, they are distributed unevenly throughout the range of this species in Europe (e.g., Sjogren-Gulve and Berg, 1999; Rafiski and Babik, 2000; Babik et al., 2004; Roček and Šandera 2008; Knopp and Merilä, 2009). However, populations in vast areas of Siberia (Northern Asia) are practically not sampled.

The high level of genetic differentiation of species (Li, Green and Sharbel, 1989; Kim et al., 2002; Che et al., 2007), subspecies (Song et al., 2006; Litvinchuk, Borkin and Rosanov, 2008) and populations (Palo et al., 2004; Zhang et al., 2010) is observed in brown frogs. Genetic differentiation of amphibian populations is even more pronounced in altered areas, where there is also a reduction in the level of allelic diversity and heterozygosity (Hitchings and Beebee, 1997). Since there is a relationship between genetic diversity and fitness components, the global trend of impoverishment of amphibian gene pools causes concern among specialists (Allentoft and O'Brien, 2010).

The aim of this paper is to study the genetic variability and population differentiation of the moor frog in Western Siberia.

Materials and Methods

Frogs were collected during the period from July to August in 1995–2012 in 9 localities of Western Siberia, Russia (Fig. 1). The studied areas are located in climatic subzones of middle and southern taiga, subtaiga and northern steppe. A total number of 718 individuals of the moor frog were sampled (Table 1).

Allozyme analysis was used to describe the population genetic structure of moor frog. Muscular tissue, liver and blood samples were stored frozen at –40 °C. Proteins were extracted in a standard way using Tris-HCl buffer (pH 8.0). Vertical electrophoresis in 7.5 % polyacrylamide gel (Maurer, 1971) and continuous system of buffers (Tris-borate-EDTA, pH 8.3) (Peacock and Dingman, 1967) were used for protein separation. Electrophoresis

[1] Department of Ecology and Genetics, Tyumen State University, Semakova 10, 625003 Tyumen, Russia.
[2] Institute of Plant and Animal Ecology, Ural Centre Academy of Sciences, 8 March Street 202, 620144 Ekaterinburg, Russia.
* Corresponding author: zhigileva@mail.ru

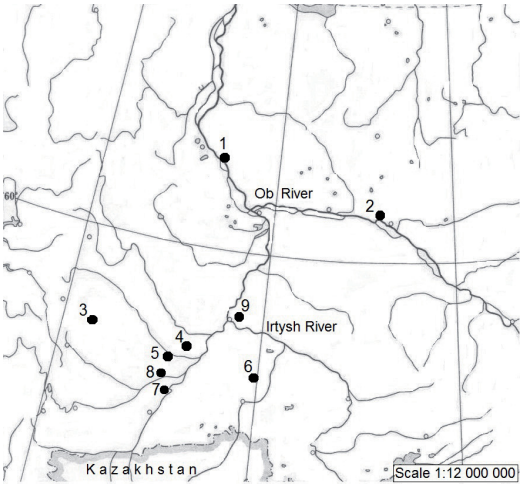

Figure 1. Places of samples collection: 1 – Octjabrsky, 2 – Megion , 3 – Irbit, 4 – Kuchak, 5 – Tyumen, 6 – Aromashevsky, 7 – Isetsky, 8 – Chervishevsky area, 9 – Uvatsky area.

was performed in Helicon electrophoretic chamber with current rate of 80 mA, voltage of 200 V for 2.5 hours. Histochemical identification of proteins was carried out in accordance with the guidelines (Korochkin et al., 1977; Richardson, Baverstock and Adams, 1986).

Table 1. Locations and amount of animals investigated.

Locations	Coordinates	Year	Sample size
Octjabrsky	62°27′N, 66°2′E	1999	3
Megion	61°05′N, 76°10′E	1999	25
Uvatsky area	58°20′N, 68°25′E	2011	7
		2012	20
Irbit	57°67′N, 63°06′E	1996	79
Kuchak	57°20′N, 66°3′E	1995	54
		1996	5
		1997	45
		2003	40
		2004	43
Tyumen	57°14′N, 65°26′E	1997	22
		1998	63
		2003	20
		2006	48
		2007	40
Aromashevsky area	56°86′N, 68°64′E	1999	147
Chervishevsky area	56°51'N, 65°22'E	2005	7
		2007	14
Isetsky area	56°39′N, 65°38′E	2007	22
		2008	14

Six enzyme systems: malate dehydrogenase (MDH, 1.1.1.37), lactate dehydrogenase (LDH, 1.1.1.27), aspartate aminotransferase (AAT, 2.6.1.1), superoxide dismutase (SOD, 1.15.1.1), nonspecific esterases (EST, 3.1.1.1, 3.1.1.2) and myogene system were studied.

In addition, an ISSR-PCR (polymerase chain reaction of inter simple sequences repeats) was used to genotype individuals of two frog populations. ISSR-PCR method identifies polymorphisms between microsatellites sequence and has more high sensitivity for differentiation (Zhigileva et al., 2013). Total genomic DNA was extracted from cardiac muscle fixed in 70% ethanol using the technique of alkaline lysis (Bender, Pierre and Hognes, 1983). Six primers: $(AG)_8C$ (*P1*), $(AG)_8G$ (*P2*), $(AG)_8T$ (*P3*), $(CA)_8G$ (*P4*), $(AC)_8T$ (*P6*) and $(TC)_8C$ (*P7*), were used for ISSR-PCR analysis. Amplification was carried out in 25 µl of reaction mixture containing PCR buffer (0.01 M Tris-HCl, 0.05 M KCl, 0.1 % triton X-100), 4 mM $MgCl_2$, 0.2 mM of each dNTPs, 1 µl of total DNA solution, 2.5 mM of primer and 0.2 unit/µL of Taq-polymerase (Fermentas®), using Chromo-4 thermal cycler (Bio-Rad®), in the following mode: 94 °C – 7 min; then 94 °C – 30 sec, 52(56) °C – 45 sec, 72 °C – 2 min (40 cycles); 72 °C – 7 min. PCR-fragments were analyzed by 2% agarose gel electrophoresis with Tris-EDTA-Borate buffer. The sizes of the fragments were determined using 100 *bp* DNA molecular weight markers (Fermentas®). Electrophoretic gels were documented using VersaDoc system (Bio-Rad®). Electrophoretic results were combined into binary matrices, where the presence of the band in gels was designated as «1» and was considered as a dominant allele; absence of the band was designated as «0» and considered as a recessive allele.

Standard population genetic characteristics – allele frequencies, Chi-square test for Hardy-Weinberg equilibrium, the percentage of polymorphic loci ($P_{95\%}$), expected (H_E) and observed heterozygosity (*Ho*), observed number of alleles (*na*), effective number of alleles (*ne*), Nei's gene diversity (*h*), Nei's original measures of genetic identity (*I*) and genetic distance (*D*) (Nei, 1972), gene flow (*N*m), F-statistics (F_{ST}, G_{ST}), were computed using POPGEN program (Yeh, Yang and Boyle, 1999). Dendrogram was plotted based on Nei's genetic distances (Nei, 1972) according to the allele frequency of all loci studied. The UPGMA algorithm was used as a clustering method. A Mantel test (Mantel, 1967) was used to estimate the correlation between geographic and genetic distance matrices within species.

Table 2. Allele frequencies and indicators of allozyme polymorphism in *R. arvalis* populations.

Locus	Allele	Octjabrsky	Megion	Irbit	Kuchak	Tyumen	Aromashevsky	Chervishevsky	Isetsky
Es-1	105	0.167	0.040	0.139	0.166	0.184	0.265	0.071	0.208
	100	0.833	0.620	0.222	0.412	0.269	0.354	0.071	0.056
	95	0	0.340	0.639	0.422	0.547	0.381	0.857	0.694
	89	0	0	0	0	0	0	0	0.041
Es-2	105	0	0	0.019	0	0	0	0	0
	100	0.333	0.560	0.475	0.443	0.573	0.568	0.500	0.611
	94	0.667	0.440	0.506	0.556	0.427	0.432	0.500	0.389
Es-3	105	0	0	0	0.005	0	0	0	0
	100	0	0.400	1.000	0.933	0.666	1.000	0.476	0.861
	89	1.000	0.600	0	0.062	0.334	0	0.524	0.139
Aat-1	140	0.500	0.500	0.278	0.385	0.430	0.320	0.405	0.583
	100	0.500	0.500	0.722	0.615	0.570	0.680	0.595	0.417
My-5	100	1.000	1.000	0.886	0.711	0.863	0.867	0.404	0
	73	0	0	0.114	0.289	0.137	0.133	0.595	1.000
Sod-2	100	1.000	1.000	1.000	0.963	0.915	1.000	1.000	0.639
	105	0	0	0	0.037	0.085	0	0	0.361
Sample size		3	25	79	187	193	147	21	36
$P_{95\%}$		0.188	0.250	0.312	0.438	0.500	0.250	0.430	0.375
Ho		0.083	0.100	0.099	0.095	0.079	0.077	0.066	0.073
H_E		0.092	0.126	0.116	0.167	0.179	0.114	0.179	0.152

Results

According to results of allozyme analysis, 12 loci of moor frog from the 20 loci studied were completely invariant: *Mdh*-1, 2, *Ldh*-1, 2, *Sod*-1, *Aat*-2, *Es*-4, 6, *My*-1, 2, 4, *Hb.* Loci *Aat*-1, *Est*-1, 2, 3, 5, *Sod*-2, *My*-3, 5 were polymorphic between populations or within populations. A relative uniformity of allele frequencies of polymorphic loci was observed on investigated part of the moor frog area (Table 2). The similarity of allele frequencies in moor frog is mainly determined by the value of gene flow that depends on the distance between the studied localities (Shapovalov and Zhigileva, 2002).

Expected and observed genotype frequencies in two samples (Octjabrsky and Megion) were in Hardy-Weinberg equilibrium in all loci. Hardy-Weinberg disequilibrium ($P < 0.001$) in locus *Est*-1 was observed in samples Irbit and Isetsky (in 2007), in locus *Est*-2 – in sample Chervishevsky (in 2007), in locus *Aat*-1 – in sample Aromashevsky. The highest disequilibrium (in four loci: *Est*-1, *Est*-2, *Est*-3, *Sod*-2) was observed in some years in certain habitats of the urbanized area (samples Tyumen). In a majority of cases, there was heterozygote deficit that may be due to inbreeding or the Wahlund effect caused by subdivision of frog population (Zhigileva, 2010). The percentage of polymorphic allozyme loci ($P_{95\%}$) in moor frog was 56%, the mean values of observed (*Ho*) and expected heterozygosity (H_E) were 0.086 ± 0.007 and 0.167 ± 0.009, respectively.

The cluster analysis indicated the presence of two population groups of the moor frog in Western Siberia (Fig. 2). Northern and southern population groups most clearly distinguished. Genetic distances between the northern and southern populations ranged from 0.067 to 0.113, but they were 0.005–0.062 between different southern populations. The values of genetic differentiation (F_{ST} 0.179 and *N*m 1.15) indicated a high degree of isolation between populations of *R. arvalis*. The value of Mantel correlation coefficient (R_M) was low – 0.058.

According to ISSR-PCR data, Nei's genetic identity (*I*) among moor frog populations was 0.829, distance

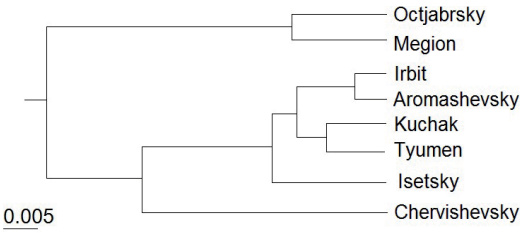

Figure 2. UPGMA dendrogram based on Nei's genetic distances (*D*) at allozyme data among moor frog populations.

(*D*) – 0.187. The G_{ST} value as the measures of genetic subdivision was 0.26, this means that about 70% of the genetic variability is accounted for intrapopulation one. The gene flow (*N*m) was equal to 1.36, indicating a high degree of reproductive isolation between the studied populations.

The percentage of polymorphic ISSR-bands in the moor frog in Uvatsky area was 93% (Table 3). This index, as well as the observed number of alleles per locus were lower in the moor frog from Tyumen, the territory with high levels of anthropogenic transformation. Reducing of the number of alleles and measures of polymorphism are typical for frog populations dwelling anthropogenically transformed landscapes (Hitchings and Beebee, 1997).

Discussion

Currently moor frog is very widespread in Western Siberia, it can be found in many habitats, including anthropogenically transformed landscapes. This species is 92–95% of the abundance of all species of amphibians that inhabit the taiga zone of Western Siberia. Values of genetic variability of moor frog populations in Western Siberian are included in the range of variability typical for the moor frog populations in Central Europe, where the percentage of polymorphic loci reaches 53% and the mean heterozygosity varies from 0.075 to 0.167 (Rafiski and Babik, 2000).

It was shown previously, that the maximum value of genetic diversity of the moor frog was in the middle latitudes and decreased toward the north (Knopp and Merilä, 2009). In populations of northern Europe, which were on the same latitude as our study area, the lower level of genetic variability was revealed. In east-central Sweden (60 degrees N), average heterozygosity (*H*) and percentage of polymorphic loci (*P*) in the moor frog were 0.099 and 33%, respectively (24 allozyme loci) (Sjogren-Gulve and Berg, 1999).

High level of genetic variability and differentiation are typical for amphibians in general and exceeds that for other groups of vertebrates (Mezhzherin, 1992). This is explained by the fact that amphibians are conservative when choosing spawning ponds and have low migration activity (Chernyshov and Truveller, 2007). Mountains can be a barrier in mountain areas (Toda et al., 1997; Zhang et al., 2010), and isolation-by-distance factor applies to the plains populations (Palo et al., 2003). In our case, low value of Mantel correlation coefficient means, that isolation-by-distance factor do not play a main role in differentiation of *R. arvalis* populations. Heterozygote deficit and *F*-statistics (F_{IS} = 0.387 > F_{ST} = 0.179) point, that the inbreeding and high degree of population subdivision are observed.

Significant differences in the values of genetic variability and measures of divergence between northern and southern populations of frogs have also been described in Europe (Sinsch and Eblenkamp, 1994; Rafiski and Babik, 2000) and in Asia (Toda et al., 1997; Trakimas et al., 2003). These authors explain the differences with the Pleistocene history of species – fast settling of vast northern territories from several southern refugia (Veith, Kosuch and Vences 2003; Palo et al., 2004). This is true for animal populations of Western Siberia that was completely covered with ice during the Pleistocene.

Table 3. Level of genetic variability in populations of *R. arvalis* according to ISSR-PCR-data.

Indices of genetic variability	Tyumen (*n* = 19)	Uvatsky (*n* = 28)
The percentage of polymorphic loci	63.3	93.3
Observed number of alleles (*na*)	1.6	1.9
Effective number of alleles (*ne*)	1.3	1.3
Index of Nei's gene diversity (*h*)	0.202	0.176

Note: *n* – sample size

Acknowledgements. We express our appreciation to Volkova E., Surel O. for help in catching of frogs. The animals were treated according to the regulations of Ministry of Health Order 755 of August 12, 1977. The Ministry of Education of Russian Federation (Grant PD02-1.4-8) financially supported this research.

References

Allentoft, M.E., O'Brien, J. (2010): Global amphibian declines, loss of genetic diversity and fitness: a review. Diversity **2**: 47–71.

Babik, W., Branicki, W., Sandera, M., Litvinchuk, S., Borkin, L.J., Irwin, J.T., Rafinski, J. (2004): Mitochondrial phylogeography of the moor frog, *Rana arvalis*. Molecular Ecology **13**: 1469–1480.

Bender, W., Pierre, S., Hognes, D.S. (1983): Chromosomal walking and jumping to isolate DNA from Ace and rosy loci of bithorax complex in *Drosophila melanogaster.* Journal of Molecular Biology **168**: 17–33.

Che, J., Pang, J., Zhao, E.M., Matsui, M., Zhang, Y.P. (2007): Phylogenetic relationships of the Chinese brown frogs (genus *Rana*) inferred from partial mitochondrial 12S and 16S rRNA gene sequences. Zoological Science **1**: 71–80.

Chernyshov, K.I., Truveller, K.A. (2007): Quantitative evaluation of the genetic divergence of amphibian taxa of different rank by the spectra of total proteins and nonspecific esterases. Zhurnal Obshchei Biologii **2**: 149–160.

Hitchings, S.P., Beebee, T.J.C. (1997): Genetic substructuring as a result of barriers to gene flow in urban *Rana temporaria* (common frog) populations: implications for biodiversity conservation. Heredity **79**: 117–127.

Ishchenko, V.G. (1996): Problems of demography and declining populations of some euroasiatic brown frogs. Russian Journal of Herpetology **2**: 143–151.

Kim, J.B., Min, M.S., Yang, S.Y., Matsui, M. (2002): Genetic relationships among Korean brown frog species (Anura, Ranidae), with special reference to evolutionary divergences between two allied species *Rana dybowskii* and *R. huanrenensis*. Zoological Science **3**: 369–382.

Knopp, T., Merilä, J. (2009): Microsatellite variation and population structure of the moor frog (*Rana arvalis*) in Scandinavia. Molecular Ecology **14**: 2996–3005.

Korochkin, L.I., Serov, O.A., Pudovkin, A.I., Aronshtam, A.A., Borkin, L.J., Malecki, I., Polyakova, E., Manchenko, G.P. (1977): Genetics of isoenzymes. Moscow, Nauka.

Kuzmin, S.L. (2010): Declines of amphibian populations in north and central Mongolia. Russian Journal of Herpetology **4**: 259–268.

Li, F., Green, D.M., Sharbel, T.F. (1989): Allozyme relationships of some frogs (genus *Rana*) from Yunnan, China. Amphibia-Reptilia **3**: 267–275.

Litvinchuk, S.N., Borkin, L.J., Rosanov, J.M. (2008): Genome size variation in *Rana arvalis* and some related brown frog species, including taxonomic comments on the validity of the *R. arvalis* subspecies. Zeitschrift für Feldherpetologie **3**: 95–112.

Mantel, N. (1967): The detection of disease clustering and a generalized regression approach. Cancer Research **27**: 209–220.

Maurer, H.R. (1971): Disc electrophoresis and related techniques of polyacrylamide gel electrophoresis, 2nd Edition. Berlin, New York, Walter de Gruyter&Co.

Merilä, J., Söderman, F., O'Hara, R., Räsänen, K., Laurila, A. (2004): Local adaptation and genetics of acid-stress tolerance in the moor frog, *Rana arvalis.* Conservation Genetics **4**: 513–527.

Mezhzherin, S.V. (1992): Comparative analysis of allozyme variability of vertebrates. Zhurnal Obshchei Biologii **4**: 549–555.

Nei, M. (1972): The genetic distance between populations. The American Naturalist **106**: 283–291.

Palo, J.U., Oõhara, R.B., Laugen, A.T., Laurila, A., Primmer, C.R., Merilä, J. (2003): Latitudinal divergence of common frog (*Rana temporaria*) life history traits by natural selection: evidence from a comparison of molecular and quantitative genetic data. Molecular Ecology **12**: 1963–1978.

Palo, J.U., Schmeller, D.S., Laurila, A., Primmer, C.R., Kuzmin, S.L., Merilä, J. (2004): High degree of population subdivision in a widespread amphibian. Molecular Ecology **13**: 2631–2644.

Peacock, A.C., Dingman, C.W. (1967): Resolution of multiple ribonucleic acid species by polyacrylamide gel electrophoresis. Biochemistry **6**: 1818–1827.

Rafiski, J., Babik, W. (2000): Genetic differentiation among northern and southern populations of the moor frog *Rana arvalis* Nilsson in central Europe. Heredity **84**: 610–618.

Richardson, B.J., Baverstock, P.R., Adams, M. (1986): Allozyme electrophoresis: A handbook for animal systematics and population studies. London, Academic Press.

Roček, Z., Šandera, M. (2008): Distribution of *Rana arvalis* in Europe: a historical perspective. Zeitschrift für Feldherpetologie **13**: 135–150.

Sas, I., Covaciu-Marcov, S.D., Kovács, É.H., Radu, N.R., Tóth, A., Popa, A. (2006): The populations of *Rana arvalis* Nills. 1842 from the Ier Valley (The Western Plain, Romania): present and future. North-Western Journal of Zoology **1**: 1–16.

Shapovalov, S.I., Zhigileva, O.N. (2002): Spatial variability of morphological and biochemical characters of moor frog (*Rana arvalis*) from three research locations. Contemporary Problems of Ecology **6**: 729–734.

Sinsch, U., Eblenkamp, B. (1994): Allozyme variation among *Rana balcanica, R. levantina*, and *R. ridibunda* (Amphibia: Anura): Genetic differentiation corroborates the bioacoustically detected species status. Journal of Zoological Systematics and Evolutionary Research **1**: 35–43.

Sjogren-Gulve, P., Berg, L.M. (1999): Allozyme variation as a demographic predictor at high latitudes: the moor frog and the pool frog at 60 degrees N. Hereditas **3**: 317–323.

Song, J.Y., Matsui, M., Chung, K.H., Oh, H.S., Zhao, W. (2006): Distinct specific status of the Korean brown frog, *Rana amurensis coreana* (Amphibia: Ranidae). Zoological Science **2**: 219–224.

Toda, M., Nishida, M., Matsui, M., Wu, G.F., Ota, H. (1997): Allozyme variation among East Asian populations of the Indian rice frog *Rana limnocharis* (Amphibia: Anura). Biochemical Systematics and Ecology **2**: 143–159.

Trakimas, G., Matsui, M., Nishikawa, K., Kasugai, K. (2003): Allozyme variation among populations of *Rana pirica* (Amphibia: Anura). Journal of Zoological Systematics and Evolutionary Research **2**: 73–79.

Veith, M., Kosuch, J., Vences, M. (2003): Climatic oscillations triggered post-Messinian speciation of Western Palearctic brown frogs (Amphibia, Ranidae). Molecular Phylogenetics and Evolution **26**: 310–327.

Yeh, F.C., Yang, R., Boyle, T. (1999): POPGENE, version 1.31. University of Alberta and Centre for International Forestry Research.

Zhang, M., Jia, X., Ma, Y., Ma, J. (2010): Genetic diversity and differentiation of the Dybowski's frog (*Rana dybowskii*) in Northeast China. Journal of Forest Research **2**: 239–245.

Zhigileva, O.N. (2010): Characteristics of the genetic structure of parasite and host populations by the example of helminthes from moor frog *Rana arvalis* Nilsson. Parazitologiya **5**: 377–388.

Zhigileva, O.N., Burakova, A.V. (2005): Indicators of developmental stability, parasitic infestation and genetic variability of populations of moor frog *Rana arvalis* in urban and natural areas. Tyumen State University Herald **5**: 178–184.

Zhigileva, O.N., Baranova, O.G., Pozhidaev, V.V., Brol, I.S., Moiseenko, T.I. (2013): Comparative analysis of using isozyme and ISSR-PCR-markers for population differentiation of cyprinid fish. Turkish Journal of Fisheries and Aquatic Sciences **1**: 159–168.

Accepted by Diogo Provete