GENETIC POLYMORPHISM IN AMPHIBIAN POPULATIONS OF PROTECTED AREAS IN THE SOUTH OF WESTERN SIBERIA AND THE URALS

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Amphibians are an important but vulnerable component of biodiversity. Climatic changes and anthropogenic transformation of the environment can lead to changes in spawning times and habitat boundaries, causing adverse genetic processes in populations. In the present study, we assess genetic variation and differentiation among 184 individuals of four amphibian species (*Rana arvalis, Rana amurensis, Rana temporaria, Bufo bufo*) from seven localities of the south of Western Siberia and the Urals, Russia. To investigate the genetic diversity of these species, we used six primers for inter simple sequences repeat (ISSR) markers. Nei's gene diversity (*h*) varied from 0.169 to 0.311 in the local populations of amphibians; the diversity was the smallest in the common toad *B. bufo* and the highest in the Siberian wood frog *R. amurensis*. Populations of *B. bufo* and the moor frog *R. arvalis* were highly differentiated (mean multilocus G_{ST} = 0.249 and 0.268, respectively). Nei's original measures of genetic identity (*I*) and genetic distance (*D*) among the toad populations were comparable with these indexes among the studied brown frog populations. These results indicate that *B. bufo* and *R. arvalis* have a well-defined population structure with restricted gene flow between populations. We also identified a high level of genetic diversity among eggs of *R. arvalis* not observed in adults. Our results provide genetic evidence that all the studied species have high adaptive potential and genetic structure typical for amphibian populations. The presented data are intended to fill the gap in studying the genetic structure of the amphibian populations of the south of Western Siberia and the Urals. The data on different levels of genetic variability in amphibian populations from protected areas show their different value for conservation management. The presence of genetically impoverished populations requires monitoring of genetic diversity of amphibians. These data will be useful for conservation concerns, especially for developing appropriate management strategies.

Key words: *Bufo bufo,* differentiation, genetic variability, ISSR markers, *Rana amurensis, Rana arvalis, Rana temporaria*

Introduction

The fauna of amphibians of the south of Siberia is not very rich. Nevertheless, in Siberia, there are four amphibian species that are widespread in Eurasia. The moor frog *Rana arvalis* (Nilsson 1842) is the most numerous and ubiquitous species in the studied area. The Siberian wood frog *R. amurensis* (Boulenger 1886) and the common frog *Rana temporaria* (Linnaeus 1758) are rarer species in the studied region. The Siberian wood frog has its western distribution border in Western Siberia; the common frog has its eastern distribution border in this region. The common toad *Bufo bufo* (Linnaeus 1758) is ubiquitous but not a numerous species.

Although the conservation status of these species is not a concern, they are an important component of biodiversity, and monitoring of their genetic resources is needed to prevent decline and diversity loss. Genetic diversity is expected to decrease in peripheral populations due to genetic drift and inbreeding (Edenhamn et al., 2000). Amphibian species usually do not compete for food and territory in the adult state, but there is a competition for spawning ponds (Ruchin, 2013). Most clutches of *R. arvalis* are not fertilised after joint spawning with another species of brown – *R. temporaria* (Trubetskaya, 2014). Changes in the range and timing of spawning due to climate warming pose a threat to the genetic resources of amphibians.

A number of genetic markers have been developed to study the population structure and conservation genetics of *Rana* and *Bufo* species. Most of the works have been based on the analyses of microsatellites (Rowe et al., 1998; Beebee & Rowe, 2000; Simandle et al., 2006; Bessa-Silva et al., 2015; Faucher et al., 2016; Trujillo et al., 2017), amplified fragment length polymorphism (AFLP) (Rogell et al., 2010; Chen et al., 2013), and mitochondrial genes sequence data (Shaffer et al., 2000; Hase et al., 2012; Ozdemir et al., 2014).

Different types of markers give different estimates of diversity. They often show discordant results (Fontenot et al., 2011). Most of them are species-specific or suitable for closely related forms and do not make it possible to compare polymorphism in different species. In this regard, universal markers may be more useful in some cases (Rogell et al., 2010). ISSR method identifies polymorphisms between microsatellites sequences and has a high sensitivity for differentiation (Zietjiewicz et al., 1994).

The purpose of the research is to study genetic variability in populations of four amphibian species inhabiting the south of Western Siberia and the Urals.

Material and Methods

Amphibians were collected in three localities of Western Siberia during the period from July to August in 2011, 2012, 2015, and in four localities of the Southern and Middle Urals, Russia, from July to August in 2016, 2017 (Fig.). The moor frog *Rana arvalis* was collected in Uvatsky area of Tyumen region (n = 27), near Tyumen (n = 26), Chervishevsky Natural Monument (Chervishevsky area, Tyumen region) (n = 19), and near Irbit, Sverdlovsk region (n = 24). The Siberian wood frog *R. amurensis* was found only in Uvatsky area of Tyumen region (n = 33). The common frog *R. temporaria* was collected near Irbit, Sverdlovsk region (n = 9). The common toad *Bufo bufo* was collected by Lake Tavatui Natural Monument, Sverdlovsk region (n = 11), near Sagra settlement, Sverdlovsk region (n = 20), near Irbit, Sverdlovsk region (n = 1), and in Taganay National Park, Chelyabinsk region (n = 14).

In total, 184 individuals of amphibians were sampled (Table 1). In addition, 15 eggs from one clutch of the moor frog were collected near Irbit in May 2017. The species of brown frogs were identified by a manual (Kuzmin, 2012). The animals were treated according to the regulations of Ministry of Health Order 755 of August 12, 1977.

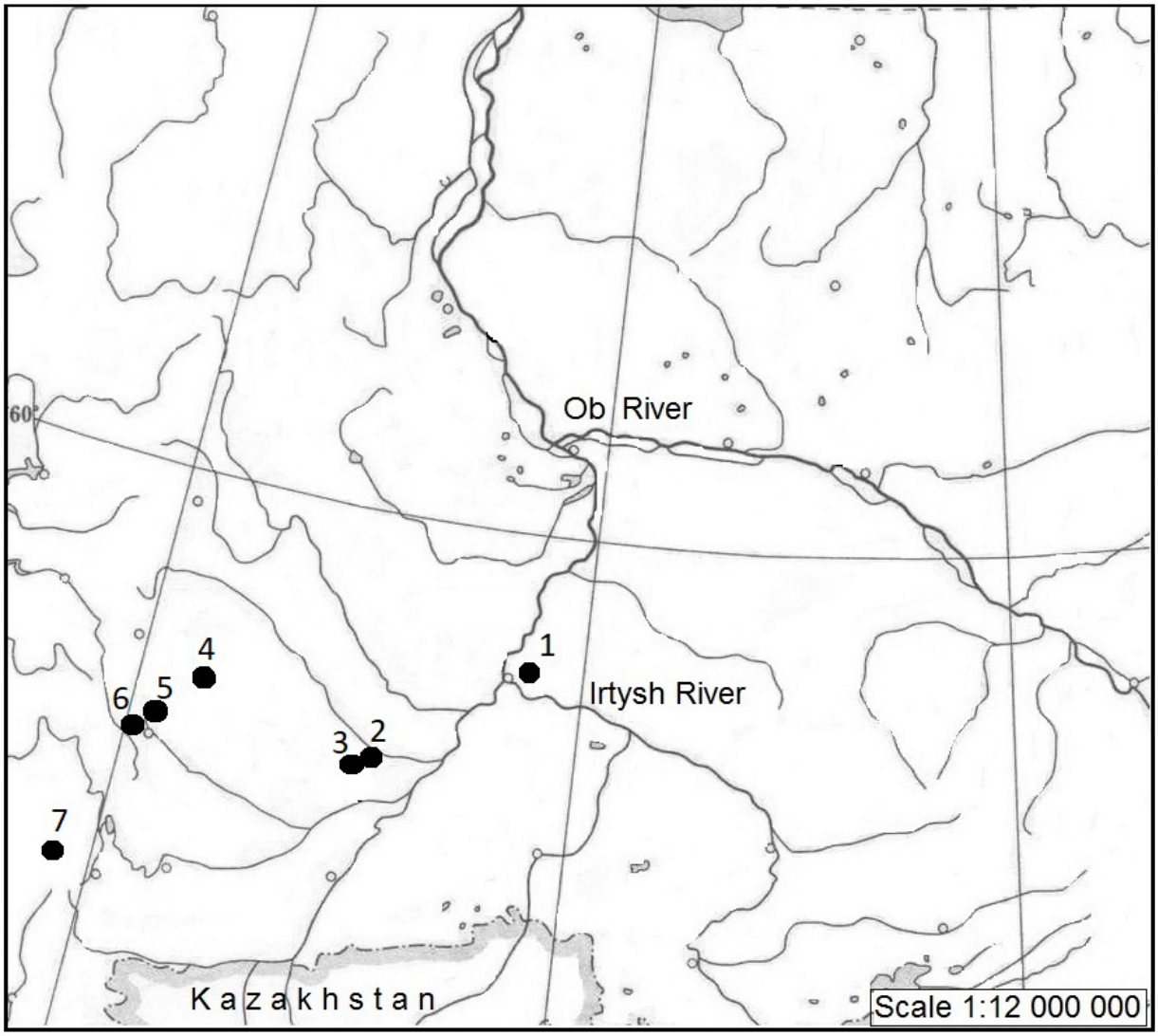

Fig. Places of sample collections: 1 – Uvatsky area, Tyumen region, 2 – Tyumen, 3 – Chervishevsky Natural Monument (Chervishevsky area, Tyumen region), 4 – Irbit, Sverdlovsk region, 5 – Lake Tavatui Natural Monument, Sverdlovsk region, 6 – Sagra settlement, Sverdlovsk region, 7 – Taganay National Park, Chelyabinsk region.

Table 1. Locations and number of animals investigated

Locations	Co-ordinates	Year	Sample size			
			Rana arvalis	*Rana amurensis*	*Rana temporaria*	*Bufo bufo*
Uvatsky area	58°46′N, 68°40′E	2012	27	33		
Tyumen	57°00′N, 65°48′E	2015	26			
Chervishevsky Natural Monument	56°56′N, 65°23′E	2011	19			
Irbit	57°67′N, 63°06′E	2016	24		9	1
Lake Tavatui Natural Monument	57°08′N, 60°10′E	2017				11
Sagra settlement	57°01′N, 60°17′E	2017				20
Taganay National Park	55°13′N, 59°47′E	2017				14
Total			96	33	9	46

Total genomic DNA was extracted from tissues of skeletal muscle fixed in 70% ethanol using the technique of alkaline lysis (Bender et al., 1983). We used the method ISSR-PCR (inter simple sequences repeats polymerase chain reaction) to study genetic polymorphism of the four species and to evaluate differentiation between population. Six primers $(AG)_8C$ (UBC-808), $(AG)_8G$ (UBC-809), $(AG)_8T$ (UBC-807), $(CA)_8G$ (UBC-818), $(AC)_8T$ (UBC-825) and $(TC)_8C$ (UBC-823) were used for ISSR-PCR (Zhigileva, Kirina, 2015). 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»), 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 in 2% agarose gel electrophoresis with Tris-EDTA-Borate buffer. The sizes of the fragments were determined using 100 bp DNA molecular weight markers («Fermentas»).

Population genetic characteristics – the percentage of polymorphic loci ($P_{95\%}$), observed (n_a) and effective number of alleles (n_e), Nei's gene diversity (h), Nei's original measures of genetic identity (I) and genetic distance (D) (Nei, 1972), gene flow (Nm), F-statistics (G_{ST}) – were computed using POPGEN software (Yeh et al., 1999).

Results and Discussion

Using 6 primers, we studied 82 loci in toads and 89 loci in brown frogs. The percentage of polymorphic loci varied from 31% to 93% in different amphibian populations (Table 2). Genetic diversity in the common frog *R. temporaria,* was 0.23. The average genetic diversity in the moor frog *R. arvalis* had a similar value (0.21). A lower level of genetic diversity was detected in the northern population group of the moor frog, which is consistent with the allozyme data (Zhigileva et al., 2014). Low levels of variability were detected in amphibian populations from Northern Europe and can be explained by their Pleistocene history – habitation in a small number of southern refugia and rapid spread to the north and east (Beebee & Rowe, 2000).

Genetic diversity was the highest in the Siberian wood frog *R. amurensis* (h = 0.31). The Siberian wood frog and the other studied amphibian species have fundamentally different distribution areas, Asian and European, respectively. Therefore, they must have had a different Pleistocene history. In particular, their settlement in Siberia occurred, apparently, from different refugia. This issue requires further detailed research.

In different populations of the toad *B. bufo,* genetic diversity varied from 0.169 to 0.275, and was 0.23 on average. The lowest genetic diversity was found in the common toad population from Taganay National Park (Table 2). This may be due to the isolation of this population.

We identified a high level of genetic diversity among eggs of *R. arvalis* not observed in adults (Table 3). This can be an effect of natural selection. The same phenomenon was revealed in the sand lizard *Lacerta agilis* Linnaeus, 1758 (Bolnykh & Zhigileva, 2016). Increased mortality of juvenile individuals was observed in peripheral populations, which explains the loss of their genetic diversity (Edenhamn et al., 2000). Although the level

of variability was estimated using neutral markers, data on low variation can indicate diminution of adaptive properties (Hoglund et al., 2015).

Populations of *B. bufo* and the moor frog *R. arvalis* were highly differentiated (mean multilocus G_{ST} = 0.249 and 0.268, respectively). Nei's original measures of genetic identity (*I*) and genetic distance (*D*) between toad populations were comparable with these indexes between the studied brown frog populations (Table 4).

These results indicate that *B. bufo* and *R. arvalis* have a well-defined population structure with restricted gene flow between populations. A high level of differentiation of populations was found in other *Bufo* species (Rowe et al., 1998; Shaffer et al., 2000; Rogell et al., 2010; Faucher et al., 2016). Restricted gene flow is characteristic of amphibians due to their weak migratory capacity and dependence on spawning water bodies (Palo et al., 2004).

In general, the presented data are intended to fill the gap in studying the genetic structure of the amphibian populations of the south of Western Siberia and the Urals. These data will be useful for conservation concerns, especially for developing appropriate management strategies and defining distinct evolutionary significant units. The data on different levels of genetic variability in amphibian populations from protected areas show their different value for conservation management. Some amphibian populations (such as the common toad population from Taganay National Park) can have reduced genetic diversity. On the one hand, the presence of genetically impoverished populations requires monitoring of genetic diversity of these amphibian populations. The identification of possible reasons for this diversity reduction is also necessary to understand whether it concerns anthropogenic factors or that we have to deal here with a natural process. On the other hand, data on the genetic diversity of amphibians should be taken into account when developing a network of protected areas.

Table 2. Indices of genetic variability in populations of amphibians according to ISSR data

Species	Locations	Sample size	$P_{95\%}$	n_a	n_e	*h*
Rana arvalis	Uvatsky area	27	93.3	1.9	1.3	0.176
	Tyumen	26	30.7	1.6	1.4	0.210
	Chervishevsky Natural Monument	19	63.3	1.6	1.3	0.202
	Irbit	24	73.4	1.7	1.5	0.250
Rana amurensis	Uvatsky area	33	90.0	1.9	1.5	0.311
Rana temporaria	Irbit	9	69.7	1.7	1.4	0.230
Bufo bufo	Sagra settlement	20	79.3	1.8	1.5	0.275
	Lake Tavatui Natural Monument	11	68.3	1.7	1.5	0.253
	Taganay National Park	14	45.1	1.5	1.3	0.169

Note: $P_{95\%}$ – the percentage of polymorphic loci, n_a – the observed number of alleles, n_e – the effective number of alleles, *h* – Nei's gene diversity.

Table 3. Indices of genetic variability in various groups of the moor frog

Stage and age group	$P_{95\%}$	n_a	n_e	*h*
Eggs	71.43	1.71	1.43	0.25
Juvenile	69.72	1.69	1.45	0.25
Adult	53.21	1.53	1.34	0.19

Note: $P_{95\%}$ – the percentage of polymorphic loci, n_a – the observed number of alleles, n_e – the effective number of alleles, *h* – Nei's gene diversity.

Table 4. Indices of genetic differentiation of amphibian populations

Compared groups	*I*	*D*	G_{ST}	*N*m
Populations of *B. bufo*	0.790–0.898	0.107–0.236	0.249	1.50
Populations of *R. arvalis*	0.829	0.187	0.268	1.36

Note: *I* – Nei's original measure of genetic identity, *D* – genetic distance (Nei, 1972), G_{ST} – interpopulation component of genetic variability, *N*m – gene flow.

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References

Beebee T.J.C., Rowe G. 2000. Microsatellite analysis of natterjack toad *Bufo calamita* Laurenti populations: consequences of dispersal from a Pleistocene refugium. *Biological Journal of the Linnean Society* 69(3): 367–381. DOI: 10.1006/bijl.1999.0368

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.

Bessa-Silva A.R., da Cunha D.B., Sodre D., da Rocha T.J.O., Schneider H., Sampaio I., Sequeira F., Vallinoto M. 2015. Development and characterization of microsatellite loci for *Rhinella marina* (Amphibia, Bufonidae) and their transferability to two closely related species. *Conservation Genetics Resources* 7(1): 247–250. DOI: 10.1007/s12686-014-0349-7

Bolnykh I.I., Zhigileva O.N. 2016. Color polymorphism and genetic variability of the sand lizard (*Lacerta agilis*) (Lacertidae, Sauria) in the surroundings of the Tyumen and Sterlitamak cities. *Current Studies in Herpetology* 16(3–4): 107–112. DOI 10.18500/1814-6090-2016-16-3-4-107-112 [In Russian]

Chen C.C., Li K.W., Yu T.L., Chen L.H., Sheu P.Y., Tong Y.W., Huang K.J., Weng C.F. 2013. Genetic structure of *Bufo bankorensis* distinguished by amplified restriction fragment length polymorphism of cytochrome b. *Zoological Studies* 52: 48. DOI: 10.1186/1810-522X-52-48

Edenhamn P., Hoggren M., Carlson A. 2000. Genetic diversity and fitness in peripheral and central populations of the European tree frog *Hyla arborea*. *Hereditas* 133(2): 115–122. DOI: 10.1111/j.1601-5223.2000.00115.x

Faucher L., Gode C., Arnaud J.F. 2016. Development of nuclear microsatellite loci and mitochondrial single nucleotide polymorphisms for the Natterjack Toad, *Bufo (Epidalea) calamita* (Bufonidae), using next generation sequencing and competitive allele specific PCR (KASPar). *Journal of Heredity* 107(7): 660–665. DOI: 10.1093/jhered/esw068

Fontenot B.E., Makowsky R., Chippindale P.T. 2011. Nuclear-mitochondrial discordance and gene flow in a recent radiation of toads. *Molecular Phylogenetics and Evolution* 59(1): 66–80. DOI: 10.1016/j.ympev.2010.12.018

Hase K., Shimada M., Nikoh N. 2012. High degree of mitochondrial haplotype diversity in the Japanease common toad *Bufo japonicus* in Urban Tokyo. *Zoological Science* 29(10): 702–708. DOI: 10.2108/zsj.29.702

Hoglund J., Wengstrom A., Rogell B., Meyer-Lucht Y. 2015. Low MHC variation in isolated island populations of the Natterjack toad (*Bufo calamita*). *Conservation genetics* 16(4): 1007–1010. DOI: 10.1007/s10592-015-0715-3

Kuzmin S.L. 2012. *Amphibians of the former USSR*. Moscow: KMK Scientific Press Ltd. 370 p. [In Russian]

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

Ozdemir N., Gul S., Poyarkov N.A., Kutrup B., Tosunoglu M., Doglio S. 2014. Molecular systematics and phylogeography of *Bufotes variabilis* (syn. *Pseudepidalea variabilis*) (Pallas, 1769) in Turkey. *Turkish Journal of Zoology* 38(4): 412–420. DOI: 10.3906/zoo-1307-27

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.

Rogell B., Thorngren H., Palm S., Laurila A., Hoglund J. 2010. Genetic structure in peripheral populations of the natterjack toad, Bufo calamita, as revealed by AFLP. *Conservation Genetics* 11(1): 173–181. DOI: 10.1007/s10592-009-0021-z

Rowe G., Beebee T.J.C., Burke T. 1998. Phylogeography of the natterjack toad *Bufo calamita* in Britain: genetic differentiation of native and translocated populations. *Molecular Ecology* 7(6): 751–760. DOI: 10.1046/j.1365-294x.1998.00387.x

Ruchin A.B. 2013. The ecological niches of amphibians in the syntopic conditions. *The world of science, culture and education* 38(1): 342–343. [In Russian]

Shaffer G., Fellers G.M., Magee A., Voss R. 2000. The genetics of amphibian declines: population substructure and molecular differentiation in the Yosemite Toad, Bufo canorus (Anura, Bufonidae) based on single-strand conformation polymorphism analysis (SSCP) and mitochondrial DNA sequence data. *Molecular Ecology* 9(3): 245–257.

Simandle E.T., Peacock M.M., Zirelli L., Tracy C.R. 2006. Sixteen microsatellite loci for the *Bufo boreas* group. *Molecular Ecology Notes* 6(1): 116–119. DOI: 10.1111/j.1471-8286.2005.01159.x

Trubetskaya E.A. 2014. Effect of herbal frog (*Rana temporaria* Linnaeus, 1758) on a population of moor frog (*Rana arvalis* Nilsson, 1842) collectively spawning. *Advances in Current Natural Sciences* 2: 46–48.

Trujillo T., Gutierrez-Rodriguez J., Arntzen J.W., Martinez-Solano I. 2017. Morphological and molecular data to describe a hybrid population of the Common toad (*Bufo bufo*) and the Spined toad (*Bufo spinosus*) in western France. *Contributions to Zoology* 86(1): 1–9.

Yeh F.C., Yang R., Boyle T. 1999. *POPGENE, version 1.31*. University of Alberta and Centre for International Forestry Research. Available from https://sites.ualberta.ca/~fyeh/popgene_download.html

Zhigileva O.N., Kirina I.Y. 2015. First data on genetic variability of the Siberian wood frog *Rana amurensis* in Western Siberia and its differentiation from the moor frog *Rana arvalis*. *Ecological genetics* 13(3): 23–27. DOI: 10.17816/ecogen13323-27

Zhigileva O.N., Kirina I.Y., Burakova A.V. 2014. Genetic diversity and differentiation of the moor frog (*Rana arvalis*) in Western Siberia. *Herpetology Notes* 7: 569–574.

Zietjiewicz E., Rafalski A., Labuda D. 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20: 176–183.

ГЕНЕТИЧЕСКИЙ ПОЛИМОРФИЗМ В ПОПУЛЯЦИЯХ АМФИБИЙ ОСОБО ОХРАНЯЕМЫХ ПРИРОДНЫХ ТЕРРИТОРИЙ НА ЮГЕ ЗАПАДНОЙ СИБИРИ И УРАЛА

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Амфибии – важный, но уязвимый компонент биоразнообразия. Изменения климата и антропогенная трансформация среды обитания может привести к изменению сроков размножения и границ распространения и вызвать негативные генетические процессы в популяциях. В настоящей работе мы оценили генетическую изменчивость и дифференциацию 184 особей четырех видов амфибий (*Rana arvalis, Rana amurensis, Rana temporaria, Bufo bufo*) из семи мест юга Западной Сибири и Урала, Россия. Для изучения генетического разнообразия этих видов мы использовали 6 праймеров для последовательностей, ограниченных простыми повторами (ISSR). Генетическое разнообразие Нея (*h*) варьировало от 0.169 до 0.311 в локальных популяциях амфибий; разнообразие было наименьшим у серой жабы *B. bufo* и наибольшим у сибирской лягушки *R. amurensis*. Популяции *B. bufo* и остромордой лягушки *R. arvalis* высоко дифференцированы (среднее мультилокусное G_{ST} = 0.249 и 0.268, соответственно). Индексы генетического сходства Нея (*I*) и генетические дистанции (*D*) между популяциями жабы были сопоставимы со значениями этих показателей изученных популяций бурых лягушек. Эти результаты показывают, что *B. bufo* и *R. arvalis* имеют хорошо выраженную популяционную структуру с ограниченным потоком генов между популяциями. Мы также выявили высокий уровень генетического разнообразия *R. arvalis* на стадии икры, не наблюдаемый среди взрослых особей. Эти результаты свидетельствуют, что все изученные популяции амфибий имеют высокий адаптивный потенциал и генетическую структуру, характерную для амфибий. Представленные данные восполняют пробел в изучении генетической структуры популяций амфибий юга Западной Сибири и Урала. Данные о разных уровнях генетической изменчивости в популяциях амфибий особо охраняемых природных территорий показывают их разное значение для природоохранных мероприятий. Наличие генетически обедненных популяций требует организации мониторинга генетического разнообразия амфибий. Эти данные могут быть полезны для решения природоохранных проблем, особенно для разработки соответствующих стратегий управления.

Ключевые слова: *Bufo bufo,* ISSR маркеры, *Rana amurensis*, *Rana arvalis*, *Rana temporaria*, генетическая изменчивость, дифференциация