Cryptic speciation in *Pelobates fuscus* (Anura, Pelobatidae): evidence from DNA flow cytometry

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Abstract. The amount of nuclear DNA in 173 specimens of *Pelobates fuscus* from 34 localities in Russia, Ukraine, Moldavia and Latvia was determined by DNA flow cytometry. Two distinct groups with different genome sizes were identified. The ranges of the genome size variation in the two groups did not overlap. Geographically, these groups with smaller or larger genome size are distributed in the west and in the east of eastern Europe, respectively.

Introduction

The common spadefoot toad, *Pelobates fuscus,* has been described by Laurenti in 1768 as *Bufo fuscus.* Currently, two subspecies of the species are recognized. The range of the nominotypical subspecies, *P. f. fuscus* (Laurenti. 1768), covers a huge territory from France to western Siberia and northwestern Kazakhstan, whereas *P. f. insubricus* occupies a small and isolated area in the Po valley, northern Italy (Nöllert, 1990, 1997; Borkin, 1998, 1999a).

In the territory of the former Soviet Union, more than 370 localities of *P. f. fuscus* are known (Barabanov et al., 1998). The range mainly encompasses zones of the broad-leaved and mixed forests as well as of the forest-steppe, reaching the southern taiga zone northward and the steppe zone southward and eastward. Several very isolated larval records from the former Soviet Central Asia (Kazakhstan. Uzbekistan, and Kyrgyzstan) formerly assigned by various authors to *P. fuscus* were re-identified as giant tadpoles of *Rana ridibunda* (Borkin. 1979, 1984: Borkin et al., 1984).

Traditionally, the taxonomy of *P. fuscus* was (and is) considered to be quite clear despite some discussion associated with the status of the Italian subspecies (see details in Nöllert, 1990). The common spadefoot toad practices a nocturnal, hidden digging mode of life. The

variation in *P. f. fuscus* is poorly known. Any data on geographic variation might be useful for the species protection policy. A few years ago, we have revealed significant differences in genome size between some populations of *P. f. fuscus* from the European part of the former USSR (Barabanov et al., 1998; Borkin, 1999b).

The purpose of this paper is to describe the genome size variation in *P. f. fuscus* of eastern Europe and to provide preliminary analysis of distribution of two kinds of the common spadefoot toad with different genome size.

Materials and Method

One hundred seventy-three specimens from 34 localities in Russia (25), Ukraine (6), Moldavia (2), and Latvia (1) were used in the study of genome size (table 1; fig. 1). They are deposited at the collection of the Zoological Institute, Russian Academy of Sciences, St. Petersburg (ZISP).

The amount of DNA per nucleus (genome size) was measured by DNA flow cytometry. Red blood cells were taken by a micropipette from the heart. Tested cells were mixed with reference cells and assayed simultaneously. Therefore, in such a mixture, both kinds of cell samples were stained and measured in the same conditions. Peripheral blood cells of the grass frog, *Rana temporaria,* collected in St. Petersburg and Pskov provinces were used as a reference standard.

The cell samples were suspended in phosphate buffer saline, supplemented with 0.7 mM EDTA (pH 7.5), with a total cell concentration of approximately 10^6 cells/ml. The cells were lysed by addition of Triton X-100 (Ferak, Berlin) at a final concentration of 0.1%, and stained with a mixture of olivomycin (OM, Moscow Medicine Plant) and ethidium bromide (EB, Calbiochem) at the following final concentration: 20 μg/ml EB, 40 μg/ml OM, 15 mM $MgCl_2$. Stained cell samples were measured after 24 h (at 4°C).

Flow cytometry was performed by use of microscope-based flow fluorimeter with mercury arc lamp as a light source, constructed at the Institute of Cytology, Russian Academy of Sciences, St. Petersburg. The conditions of fluorescence excitation and registration were optimized for each method of staining. DNA-histograms were acquired with a multichannel analyzer connected with a microcomputer. The analysis rate was 100-200 cells per second; four runs were done on each sample, with the total number of cells measured per sample being above 10000. The peaks of DNA histograms were fitted to the Gaussian curves by means of the least-square technique. Coefficients of variation of these peaks were in the range 1.5-2.0%. In arbitrary units, genome size of an individual under the study was determined as the ratio of its cell peak mean to that of *R. temporaria* (Rt-index).

To convert genome size from the relative units (Rt-index) to picograms, it is necessary to have data about genome size of reference cells. Such data should be obtained without using stains by means of biochemical analysis, ultraviolet cytophotometry, etc. Unfortunately, the data available today, mentioned by various authors, do not correspond to each other. Moreover, difficulties in recalculation of relative units to absolute ones are connected with the base-pair-specificity of some stains widely used in flow cytometry. To exclude the influence of AT/GC-structure, it is necessary to use corrections (Vinogradov, 1998). On the other hand, such corrections themselves may be a source of additional errors. Only using of ethidium bromide (EB) or propidium iodide (PI) does not apply the corrections (Vinogradov and Borkin, 1993). Our estimations of genome size of some mammals *(Homo sapiens, Mus musculus, Rattus norvegicus)* were the closest to that mentioned by Bianchi et al. (1983). In this work we used the genome size *of Mus musculus* as a basic reference standard and considered that this value is 6.8 pg. Therefore, to obtain the data in absolute units, the mixtures of red blood cells of *P. fuscus* and *Rana temporaria* as well as the mixtures of red blood cells of *Rana temporaria* and spleenocytes of *Mus musculus* (C57B1) were examined after staining with ethidium bromide. Cell mixtures were stained by addition of Triton X-100, EB and $MgCl_2$ at the final concentration accordingly 0.1%, 20 μg/ml and 15 mM, respectively. The stained samples were measured after 4-6 h (at 4°C). The mean genome size of *R. temporaria* was determined as 10.32 pg (6.8 pg to 0.6592 Rt). Some other details of the technique have been published previously (Vinogradov et al., 1990; Rozanov and Vinogradov, 1998).

Results

The average genome size in *P. f. fuscus* in eastern Europe was equal to 9.17 pg. The samples were allocated to two main distinct groups with different genome size (fig. 1; table 1). The data ranges of the groups did not overlap (fig. 2), and the gap between both groups was 0.16 pg. The geographic distribution of these two groups of samples was, obviously, not chaotic, and the "western" and "eastern" groups were recognized. Therefore, both groups were identified both by genome size and geographically. The "western" samples were characterized by smaller genome size in comparison with the eastern ones: 8.69-9.00 pg vs. 9.16-9.50 pg; the means are 8.80 pg vs. 9.33 pg. The differences were equal to 5.8%.

The coefficient of variation in samples studied varied from 0.2% to 0.8% (table 1). Among the "western" samples, it ranged between 0.2% (Odessa Province) and 0.8% (Tula Province) versus between 0.3% (Voronezh Province) and 0.8% (Ryazan Province) in the "eastern" ones. Thus, the within-population variation in both geographic groups was similar. Levels of geographic ("between-population") variation in genome size in the "western" and the "eastern" kinds *of P. f. fuscus* were also similar (CV = 0.8%).

Genome size correlated slightly negatively with latitude changes *(r* = —0.13), and positively with longitude changes in the "western" type (r = +0.60), and with longitudes only in the overall samples *of P. f. fuscus (r* = +0.80). The "eastern" populations did not demonstrate any significant correlations with neither latitudes nor longitudes.

Discussion

Genome size in Pelobates fuscus

Several authors published values of the nuclear DNA content (table 2). According to Olmo (1973), genome size in *P. fuscus* was equal to 73.10 ± 5.03% of that of *Rana "esculenta"* [*R. lessonae?*] taken as a standard species. He converted it to 8.19 ± 0.56 pg [8.2 pg in Morescalchi et al., 1977; Olmo, 1983], genome size of man being considered as 7 pg. Olmo (1973) also reported unpublished data supplied by Sexsmith (1968). Using "biochemical and histophotometric methods", the latter has calculated the content of nuclear DNA in *P. fuscus* as 0.88 arbitrary units in relation to genome size of *Rana pipiens* as a standard. The value expressed in absolute units was equal to 8.9 pg because *Rana pipiens* was considered to have about 10.1 pg. According to Mazin (1980), *P. fuscus* has a genome size of 7.8 ±0.9 pg. This estimation was based on *Xenopus laevis* as a standard species (6.3 pg). Vinogradov (1998) determined genome size in *P. fuscus* as 8.23 pg. The primary value was obtained in the comparison with *Rana temporaria* as an internal standard and, then, was converted to picograms based on 7.0 pg genome size in *Homo sapiens.*

Thus, the literature values of genome size in *P. fuscus* expressed in absolute units varied between 7.8 and 8.9 pg. The authors mentioned above also provided data for *Xenopus laevis* and *Rana temporaria.* Judging from the authors' data, we calculated

Table 1. Genome size variation in two types of *Pelobates fuscus fuscus* from eastern Europe.

Locality	Latitude	Longitude	Date	*n*	Min	Max	Mean	*s*	CV, %
The "Western" Type									
Latvia:									
1. Riga	56°57'	24°07'	1986	1			8.73		
Pskov Province, Russia:									
2. Osyno	56°08'	28°36'	1997-1999	12	8.69	8.83	8.74	0.04	0.5
3. Peschanka	56°09'	28°33'	1998	8	8.74	8.82	8.78	0.02	0.3
4. Idritsa	56° 20'	28°54'	1998-1999	7	8.73	8.84	8.77	0.04	0.4
5. Rybolovka	56° 19'	28°33'	1999	3	8.76	8.82	8.78	0.03	0.4
St. Petersburg Province, Russia:									
6. Luga	58°44'	29°51'	2000	1			8.79		
Yaroslavl Province, Russia:									
7. Borok	58°00'	38°15'	1999	1			8.80		
Tula Province, Russia:									
8. Tula City	54° 13'	37°37'	1999	4	8.84	9.00	8.91	0.07	0.8
Sumy Province, Ukraine:									
9. Starogutsky Reserve	52° 18'	33°48'	2000	1			8.85		
Chernigov Province, Ukraine:									
10. Naumovka	50°56'	31°35'	2000	2	8.79	8.83	8.81		
11. Nezhin	51°02'	31°53'	1999	7	8.83	8.94	8.88	0.04	0.5
Kiev Province, Ukraine:									
12. Kiev City	50°27'	30°32'	2000	1			8.84		
Odessa Province, Ukraine:									
13. Vilkovo	45°24'	29°35'	1986, 2000	2	8.75	8.78	8.76	0.02	0.2
Moldavia:									
14. Kantemir	46° 17'	28°12'	1995	1			8.88		
15. Ungeny	47° 12'	27°48'	1995	1			8.92		
Total:				*53*	*8.69*	*9.00*	*8.80*	*0.07*	*0.8*
The "Eastern" Type									
Kharkov Province, Ukraine:									
16. Kharkov City	49°59'	36°12'	1986	1			9.40		
Stavropol Province, Russia:									
17. Stavropol City	45°03'	41°57'	2000	1			9.48		
Belgorod Province, Russia:									
18. Borisovka	50°36'	36°01'	1998-1999	4	9.16	9.26	9.20	0.05	0.5
Voronezh Province, Russia:									
19. Rossosh	50° 12'	39°35'	1999	35	9.24	9.34	9.28	0.03	0.3
Lipetsk Province, Russia:									
20. Bukhovoe	53° 14'	40°04'	2000	3	9.34	9.44	9.38	0.05	0.6
Tambov Province, Russia:									
21. Zarechye	52° 52'	41°31'	2000	13	9.30	9.48	9.41	0.06	0.6
22. Chistye Prudy	52° 43'	41°30'	1999	2	9.38	9.44	9.41		
23. Bolshaya Lipovitsa	52°33'	41°20'	1999	10	9.37	9.50	9.44	0.04	0.4
24. Orzhevka	52° 36'	43°02'	2000	1			9.39		
Penza Province, Russia:									
25. Khopyor River	52°49'	44°27'	1999	1			9.41		
Ryazan Province, Russia:									
26. Ryazan City	54°36'	39°42'	1998	2	9.16	9.19	9.17		
27. Gus Zheleznyi	55°03'	41°09'	1997	3	9.26	9.39	9.31	0.07	0.8

Table 1. (Continued).

Locality	Latitude	Longitude	Date	*n*	Min	Max	Mean	*s*	CV, %
Nizhniy Novgorod Province, Russia:									
28. Ichaiki	55°37'	44°44'	1999	5	9.23	9.32	9.26	0.04	0.5
29. Reshetikha	56°12'	43°19'	1999	4	9.24	9.32	9.29	0.04	0.4
Udmurtia, Russia:									
30. Kilmez	57°02'	51°21'	1998	15	9.23	9.36	9.30	0.04	0.4
31. Boyarka	56°04'	54°04'	1998	13	9.31	9.43	9.37	0.04	0.4
32. Krymskaya Sludka	56°04'	51°21'	1999	1			9.30		
33. Izhevsk	56°50'	53°12'	1999	1			9.38		
Bashkiria, Russia:									
34. Amzya	56°15'	54°10'	2000	5	9.36	9.48	9.42	0.04	0.5
Total:				*120*	*9.16*	*9.50*	*9.33*	*0.08*	*0.8*

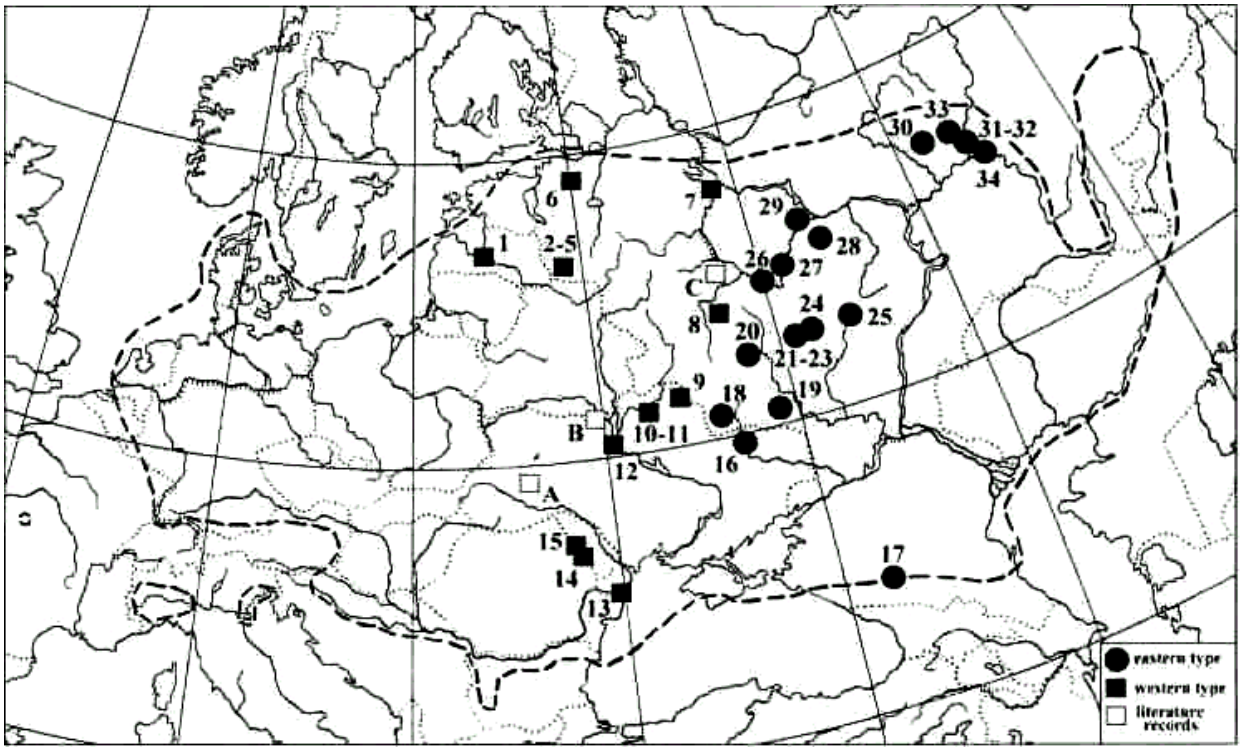

Figure 1. Geographic distribution of *Pelobates fuscus fuscus* (the locality numbers are the same as in table 1). The species' range is shown as a dashed line. The literature records (open squares) are: A — Ternopol, Ukraine; B — Strakholesie, Kiev Province, Ukraine and C — Moscow, Russia.

the ratio of genome size of *P. fuscus* to that of these species (table 2). Obviously, the published values are controversial, and the ranking rows in picograms and in the relative units are not parallel. The contradictions may be explained by an application of different techniques (Feulgen vs. flow cytometry) and dyes, as well as by laboratory conditions (cell preparation, devices for measurements, etc.). It has been shown that genome size measured with fluorochromes of different specificity may differ markedly (e.g., Johnston et al., 1987; Vinogradov and Borkin, 1993). For instance, the determination of genome

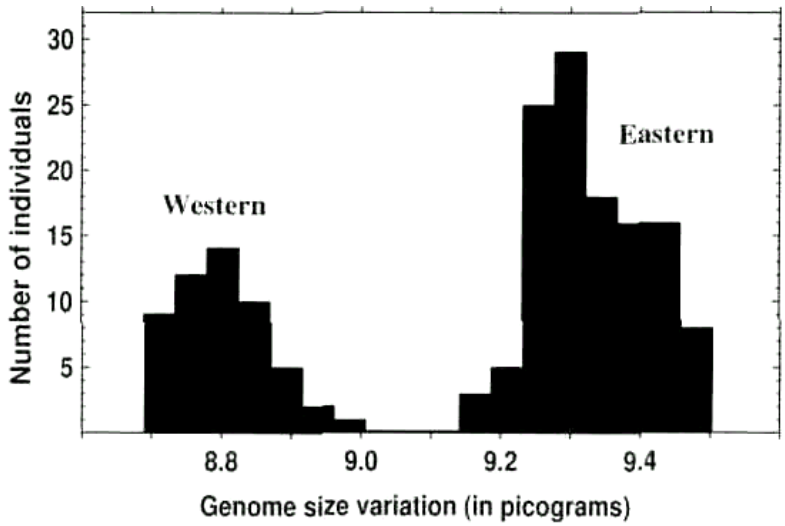

Figure 2. Distribution of genome size values in *Pelobates fuscus fuscus* in eastern Europe (n = 173).

Table 2. Genome size in *Pelobates fuscus fuscus* expressed in picograms (2c, pg) and in the relation to that of *Xenopus laevis* (Xl-index) or of *Rana temporaria* (Rt-index).

Author	Method	Reference species	2c, pg	Xl[a]	Rt[a]
Sexsmith (1968)	Feulgen	*R. pipiens*	8.9	1.62	-
Olmo (1973, 1983)	Feulgen	*R. "esculenta"*?	8.19	1.32	0.98
Mazin (1980)	Feulgen	*X. laevis*	7.8	1.24	0.80
Vinogradov, Borkin (1993)	Flow cytometry	*R. temporaria*	-	1.33	0.781[b]
Vinogradov (1998)	Flow cytometry	*R. temporaria*	8.23	1.15	0.769[b]
Present study	Flow cytometry	*R. temporaria*	8.69-9.50	-	0.770-0.842[b]

[a] the values of both indices were calculated by us;
[b] the values were obtained with the olivomycin technique by authors.

size by means of flow cytometry for cell samples of *P. fuscus* stained with olivomycin, a GC-specific fluorochrome, and with Hoechst, an AT-specific fluorochrome, provided 0.769 and 0.906 arbitrary units, respectively; *Rana temporaria* was taken as an internal reference (Vinogradov, 1998).

Moreover, previous authors used various species as a reference standard (table 2). However, these species could differ by DNA composition (DNA base frequencies). Vinogradov and Borkin (1993) listed the AT- and GC-pair specific DNA contents (C_{AT} and C_{GC}) for many species of amphibians. C_{AT}/C_{GC} was equal to 1.13 in *P. fuscus,* 1.42 in *Xenopus laevis,* 0.99 in *Rana pipiens,* 1.03 in *Rana temporaria* and 1.00 in *Rana lessonae* [= *R. "esculenta"* of Olmo?].

Unfortunately, previous authors did not supply any information about sample size and localities. We may suggest that Olmo (1973, 1983) and Sexsmith (1968) would study *P. fuscus* collected in western Europe rather than in the territory of the former USSR, whereas,

certainly, Mazin (1980) and Vinogradov (1998) have used animals from the Soviet Union. Therefore, some differences in genome size might be influenced by geographic variation. Our values of the nuclear DNA content in *P. fuscus* are higher (table 1 and 2) than in previous authors, although the minimal value overlaps with Sexsmith's data.

Geographic distribution

Thus, the samples of *P. f. fuscus* in eastern Europe shape two geographic groups (table 1; figs. 1 and 2). The "western" type was recorded in the Baltic area (Latvia and St. Petersburg Province), the north-western (Pskov and Yaroslavl provinces) and the central parts (Tula Province) of European Russia, in Ukraine (Sumy, Chernigov, Kiev and Odessa provinces), as well as in Moldavia. Thus, this type of *P. f. fuscus* ranges from the Baltic Sea in the north to the Danube River Delta in the south.

The distribution of the "eastern" type of *P. f. fuscus* covers central European Russia (Ryazan and Penza provinces), the Volga River area (Nizhniy Novgorod Province, Udmurtia and Bashkiria Republics), the Central Chernozem ["black soil"] Territory of Russia (Tambov, Lipetsk, Voronezh and Belgorod provinces), the North Caucasus area (Stavropol Province) and north-eastern Ukraine (Kharkov Province).

However, the geographic distances between the closest localities of the "western" and the "eastern" kinds of *P. f. fuscus* vary markedly. Estimated by means of the geographic map, the distances are as follows (in straight line distances):

a) in the north — approximately 475 km between Borok, Yaroslavl Province vs. Reshetikha, Nizhniy Novgorod Province;
b) in the center — approximately 142 km between Tula vs. Ryazan towns; and
c) in the south — approximately 225 km between Starogutsky Reserve, Sumy Province, Ukraine vs. Borisovka, Belgorod Province, Russia.

Unfortunately, these "gaps" lack any sample studied by flow cytometry. However, zoogeographically, at the first glance, there exist no significant barriers which could prevent the dispersal of the "western" kind of *P. f. fuscus* to the south-east and the dispersal of the "eastern" kind to the north-west. Indeed, records of the common spadefoot toad are known from the intermediate area. Therefore, both kinds of *P. f. fuscus* may contact in the central part of the East European Plain. Additional sampling would be much needed to identify populations geographically intermediate between the two groups.

Mazin (1980), Vinogradov and Borkin (1993), and Vinogradov (1998) published data, which seem to be associated with the "western" type of *P. f. fuscus.* Judging from Mazin's value in picograms, we calculated genome size of *P. fuscus* (7.8 pg) to that of *Rana temporaria* (9.8 pg) because we used the latter species as a standard. Genome size expressed in arbitrary units is equal to 0.80 (table 2). Indeed, this value coincides with the maximum in the "western" type (0.797). In 1976 in the correspondence with one of us (L.J. Borkin), A.L. Mazin has mentioned that two samples of *P. fuscus,* with the same mean amount of nuclear DNA, were collected in Moscow, Russia and in Ternopol, a town

in western Ukraine. The Moscow sample was taken at the eastern limit of the distribution of the "western" type of *P. f. fuscus*. Therefore, taking into consideration the contents of nuclear DNA (in arbitrary units) and the geographic position of both localities, Mazin's samples could be assigned to the "western" type of *P. f. fuscus*.

Based on the same specimen, Vinogradov and Borkin (1993), and Vinogradov (1998) have reported the values of 0.781 and 0.769 arbitrary units, respectively (table 2). These data are within the range of genome size variation in the "western" type of *P. f. fuscus* (0.770-0.797). Both values were based on the use of olivomycin-stained cells and *Rana temporaria* as an internal reference. Certainly, the authors mentioned above have examined a female specimen, which has been taken by L.J. Borkin in autumn 1989, at the Strakholesie village (Kiev Province, Ukraine), nearby the Chernobyl disaster area.

Therefore, with some caution, all three localities could be added to our list as the "western" type of *P. f. fuscus* (fig. 1).

Taxonomic implications

The intraspecific variation in cellular DNA content among amphibians is suggested to be typically low. Therefore, despite morphological similarity or ambiguity, individuals with substantially different DNA amount are probably not conspecific. Thus, the flow cytometry approach can facilitate to detect cryptic species, which were suspected, for instance, among some anurans (Borkin et al., 1986; MacCulloch et al., 1996; Murphy et al., 1997). However, a comprehensive estimation of evolutionary (genetic) distinctness of such genomically different samples should precede any taxonomic conclusions.

The case of *P. f. fuscus* in eastern Europe is of special interest. Indeed, we revealed two geographic groups with different genome size. The levels of within-population variation (CV) are similar in the "western" kind and in the "eastern" kind *of P. f. fuscus* (table 1). The overall within-group variation is also similar (CV is 0.8% in both groups). The ranges of the genome size variation in the two groups did not overlap. Therefore, we could consider these groups as two different taxonomic entities, at least, at the subspecific rank because of their geographic separation.

Formerly, two names were coined for the common spadefoot toad of eastern Europe. *"Rana vespertina"* Pallas, 1771 has been described from the Middle Volga River area, nearby Samara City. Later, Sewertzow (1856) has proposed the name *"Pelobates campestris"* for the individuals from Voronezh Province, the black-soil ("chernozem") area of European Russia. Both names could be applied to the "eastern" type of *P. f. fuscus* (fig. 1); however, *"Rana vespertina"* is the senior available name.

The further study of the geographic "gap" between the two groups should be crucial to make a final taxonomic decision. If both kinds of *P. f. fuscus* were genetically independent in a presumed contact zone, i.e. with no gene flow among these types, the two entities should be recognized as two species. Intervening populations not yet discovered might show intermediate states by genome size and other characters. If both kinds would

hybridize, the question would arise whether this area is a transition zone between two subspecies (i.e. geographic races), or a hybrid zone between two species.

In the nearest future, we intend to study *P. f. fuscus* by means of allozyme analysis. Now, it is clear that we face a case of the so-called cryptic speciation because of morphological uniformity of *P. f. fuscus.*

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