

NEW DATA ON THE DISTRIBUTION OF TWO CRYPTIC FORMS OF THE COMMON SPADEFOOT TOAD (*Pelobates fuscus*) IN EASTERN EUROPE

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The genome size variation in almost four hundred specimens of *Pelobates fuscus fuscus*, collected from 61 localities in Russia, Latvia, Belarus, Ukraine, Moldavia, and Kazakhstan, was studied by DNA flow cytometry. The existence of two geographic groups differed by genome size was confirmed by allozyme analysis. The distribution of the western type (with smaller genome size) and the eastern type (with larger genome size) *P. f. fuscus* is described.

Keywords: Amphibia, Anura, Pelobatidae, *Pelobates fuscus*, distribution, cryptic speciation, genome size, allozymes.

INTRODUCTION

The common spadefoot toad, *Pelobates fuscus* (Laurenti, 1768), is a wide-ranging European anuran species, which is distributed from central France in the west to western Siberia and northwestern Kazakhstan in the east. Like other spadefoot toads, *P. fuscus* is a nocturnal animal, and it practices hidden digging mode of life. Taxonomically, the species consists of two subspecies, namely *P. f. insubricus* endemic to the Po valley in northern Italy, and *P. f. fuscus*, which occupies the remaining huge part of the species' range. The variation in *P. fuscus* is poorly known (Nöllert, 1990, 1997; Borkin, 1998).

In 1997, occasionally, we have found that specimens of *P. f. fuscus* from Pskov and Ryazan provinces (European Russia), treated at the same day, showed pronounced differences in the amount of nu-

clear DNA. Later, based on many samples from various localities, we identified two distinct groups of the common spadefoot toad with different genome size. Geographically, these groups with smaller or larger genome size were found to be distributed in the west and in the east of eastern Europe, respectively (Barabanov et al., 1998; Borkin et al., 2001a, 2001b, 2003; Khalturin et al., 2003).

This study concentrates on the distribution of the western type and the eastern type of *P. f. fuscus* based on numerous genome size data, which have been accumulated since 1995.

MATERIAL AND METHODS

Almost four hundred specimens from 61 localities in Russia (45), Ukraine (9), Belarus (2), Kazakh-

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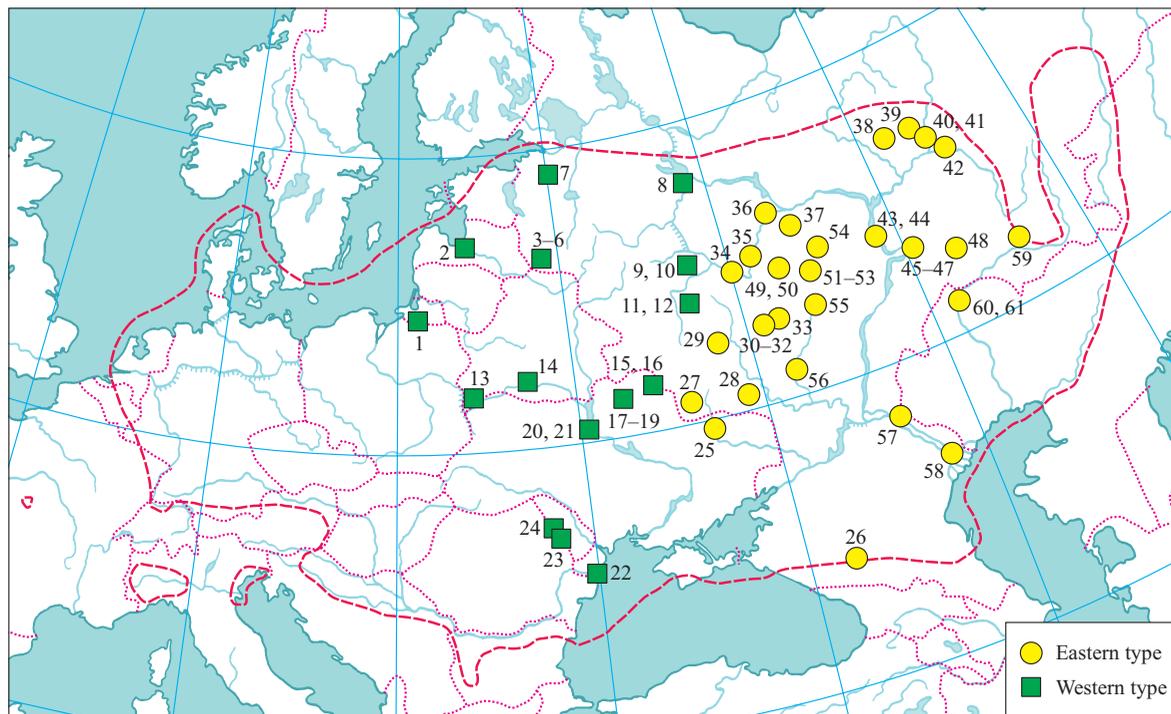


Fig. 1. The distribution of two cryptic forms of *Pelobates fuscus fuscus* (according to genome size data).

stan (2), Moldavia (2), and Latvia (1) were used in the study of genome size variation (Table 1; Fig. 1). The amount of DNA per nucleus was measured by flow cytometry. Red blood cells of the grass frog (*Rana temporaria*), collected in St. Petersburg and Pskov provinces, were used as a reference standard. A detailed description of the technique has been published previously (Borkin et al., 2001b). The numbering of samples under the study is the same in Tables 1–3, and in Figs. 1–4.

RESULTS

According to genome size, there are two distinct groups within *P. f. fuscus* in eastern Europe (Table 1; Fig. 2). Geographically, animals with smaller genome size are distributed in the west (24 samples, $N = 93$), whereas specimens with larger genome size inhabit the eastern part (37 samples, $N = 305$) of eastern Europe (Fig. 1). The average genome size in the western samples was equal to 8.83 pg vs. 9.35 pg in the eastern ones. The differences between means were equal to 5.7%. The variation in genome size ranged between 8.65 and 9.06 pg, as well as between

9.10 and 9.50 pg, in the western group and in the eastern group, respectively. In the two groups, the values of the coefficient of variation (CV) in samples varied from 0.2 to 0.8% (Table 1).

The western type of *P. f. fuscus* was recorded in Latvia, Belarus, Ukraine (Sumy, Chernigov, Kiev, and Odessa provinces), Moldavia, and in the western part of European Russia. These were Kaliningrad, Pskov, Leningradskaya, Yaroslavl, Moscow, and Tula provinces. The easternmost records of this type could be connected by the line: Borok (Yaroslavl Province) – Tugoleskii Bor (Moscow Province) – Tula City (Tula Province) in Russia – Starogutsky Reserve (Sumy Province) – Faivka, Naumovka, Nizhyn (Chernigov Province) – Kiev (Kiev Province) – Vilkovo (Odessa Province) in Ukraine (Table 1; Fig. 1).

The distribution of the eastern type of *P. f. fuscus* covered Kharkov Province of eastern Ukraine, and the southern, central, and eastern provinces of European Russia as well as northwestern Kazakhstan (Table 1; Fig. 1). The territory inhabited by this form is located to the east of the line connected Reshetikha (Nizhny Novgorod Province) – Ryazan City (Ryazan

TABLE 1. Genome Size Variation in Two Types of *Pelobates fuscus fuscus* from Eastern Europe

No.	Country, area	Locality	Latitude	Longitude	Date	N	Range	Mean	SD	CV, %
1	Russia, Kaliningrad	Bagrationovsk	54°23'	20°39'	2002	1		8.65		
2	Latvia	Riga City	56°57'	24°07'	1986	1		8.74		
3	Russia, Pskov	Osino	56°08'	28°36'	1997–1999	12	8.70–8.82	8.75	0.04	0.5
4	—"	Peschanka	56°09'	28°33'	1998	8	8.75–8.83	8.80	0.02	0.3
5	—"	Idritsa	56°20'	28°54'	1998–1999	7	8.74–8.86	8.79	0.04	0.4
6	—"	Rybolovka	56°19'	28°33'	1999	3	8.78–8.84	8.80	0.03	0.4
7	Russia, St. Petersburg	Luga	58°44'	29°51'	2000, 2002	2	8.77–8.81	8.79		
8	Russia, Yaroslavl	Borok	58°00'	38°15'	1999	1		8.82		
9	Russia, Moscow	Gzhel	55°35'	38°30'	2002	1		9.06		
10	—"	Tugolesskii Bor	55°33'	39°48'	2001	1		8.97		
11	Russia, Tula	Tula City (Barsuki)	54°13'	37°37'	1999	4	8.85–9.01	8.92	0.07	0.8
12	—"	Tula City (Skuratovo)	54°11'	37°43'	2002	11	8.80–8.90	8.86	0.03	0.3
13	Belarus, Brest	Medna	51°52'	23°45'	2001	3	8.70–8.74	8.72	0.02	0.3
14	Belarus, Gomel	Borovaya	52°17'	27°40'	2001	10	8.83–8.93	8.90	0.03	0.4
15	Ukraine, Sumy	Antonovka	51°52'	33°29'	2001	6	8.88–8.95	8.91	0.03	0.3
16	—"	Starogutsky National Park	52°18'	33°46'	2000	1		8.87		
17	Ukraine, Chernigov	Naumovka	50°53'	31°32'	2000	2	8.80–8.84	8.82		
18	—"	Nizhyn	51°00'	31°52'	1999	7	8.84–8.96	8.89	0.04	0.5
19	—"	Faivka	58°56'	32°59'	2001	1		8.76		
20	Ukraine, Kiev	Kiev City (Chortoryi)	50°28'	30°32'	2000	1		8.85		
21	—"	Koncha Zaspa	50°15'	30°32'	2001	5	8.78–8.85	8.81	0.03	0.3
22	Ukraine, Odessa	Vilkovo	45°24'	29°35'	1986, 2000	3	8.76–8.80	8.77	0.02	0.2
23	Moldavia	Kantemir	46°17'	28°12'	1995	1		8.90		
24	—"	Ungeny	47°12'	27°48'	1995	1		8.94		
Total:						93	8.65–9.06	8.83	0.08	0.9
25	Ukraine, Kharkov	Kharkov City	49°59'	36°12'	1986	1		9.42		
26	Russia, Stavropol	Stavropol City	45°03'	41°57'	2000	1		9.48		
27	Russia, Belgorod	Borisovka	50°36'	36°01'	1998–1999	5	9.16–9.29	9.21	0.06	0.6
28	Russia, Voronezh	Rossosh	50°12'	39°35'	1999	35	9.24–9.34	9.28	0.03	0.3
29	Russia, Lipetsk	Bukhovee	53°14'	40°04'	2000	3	9.34–9.44	9.38	0.05	0.6
30	Russia, Tambov	Zarech'e	52°52'	41°31'	2000	13	9.30–9.48	9.41	0.06	0.6
31	—"	Chisty Prudy	52°43'	41°30'	1999	2	9.38–9.44	9.41		
32	—"	Bolshaya Lipovitsa	52°33'	41°20'	1999	10	9.37–9.50	9.44	0.04	0.4
33	—"	Orzhevka	52°36'	43°02'	2000	1		9.39		
34	Russia, Ryazan	Ryazan City	54°36'	39°42'	1998	2	9.16–9.19	9.17		
35	—"	Gus' Zheleznyi	55°03'	41°09'	1997	3	9.26–9.39	9.31	0.07	0.8
36	Russia, Nizhny Novgorod	Reshetikha	56°12'	43°19'	1999	4	9.24–9.32	9.29	0.04	0.4
37	—"	Ichalki	55°37'	44°44'	1999	5	9.23–9.32	9.26	0.04	0.5
38	Russia, Udmurtia	Kilmez	57°02'	51°21'	1998	15	9.23–9.36	9.30	0.04	0.4
39	—"	Boyarka	56°04'	54°04'	1998	13	9.31–9.43	9.37	0.04	0.4
40	—"	Krymskaya Sludka	56°04'	51°21'	1999	1		9.30		
41	—"	Izhevsk City	56°50'	53°12'	1999	1		9.38		
42	Russia, Bashkortostan	Amzya	56°15'	54°10'	2000	5	9.36–9.48	9.42	0.04	0.5
43	Russia, Ulyanovsk	Ulyanovsk City	54°18'	48°52'	2001	10	9.38–9.49	9.43	0.03	0.3
44	—"	Dimitrovgrad	54°13'	49°35'	2002	2	9.44–9.46	9.45		
45	Russia, Samara	Nizhnee Sengeleevo	53°29'	49°30'	2001	37	9.36–9.50	9.42	0.03	0.3
46	—"	Timashovo	53°21'	51°12'	2001	7	9.38–9.45	9.41	0.02	0.2
47	—"	Shelekhmet	53°14'	49°51'	2001	4	9.31–9.40	9.34	0.04	0.5
48	—"	Borskoe	53°02'	51°41'	2002	7	9.39–9.43	9.41	0.02	0.2
49	Russia, Mordovia	Andreevka	54°03'	44°02'	2002	25	9.18–9.43	9.32	0.05	0.6

TABLE 1 (continued)

No.	Country, area	Locality	Latitude	Longitude	Date	<i>N</i>	Range	Mean	SD	CV, %
50	—	Staraya Avgura	54°17'	44°13'	2002	1		9.29		
51	—	Rybnyi	54°08'	45°00'	2002	1		9.37		
52	—	Yalga	54°08'	45°06'	2001	2	9.39–9.41	9.40		
53	—	Kamchatka	54°44'	45°30'	2002	14	9.35–9.42	9.38	0.02	0.2
54	—	Simkino	54°10'	46°10'	2002	9	9.24–9.34	9.30	0.03	0.4
55	Russia, Penza	Khoper River	52°49'	44°27'	1999	1		9.41		
56	Russia, Saratov	Rasskazan	51°34'	42°39'	2002	23	9.31–9.48	9.40	0.04	0.4
57	Russia, Astrakhan	Dzhelga	48°18'	46°10'	2001	10	9.17–9.39	9.28	0.08	0.8
58	—	Astrakhan City	46°22'	48°06'	2001	1		9.29		
59	Russia, Orenburg	Maiorskoe	51°59'	55°16'	2002	2	9.10–9.22	9.16		
60	Kazakhstan, Uralsk	Uralsk City	51°13'	51°25'	2002	28	9.27–9.40	9.32	0.03	0.3
61	—	Peremetnoe	51°11'	50°50'	2002	1		9.33		
Total:						305	9.10–9.50	9.35	0.07	0.8

TABLE 2. The Genome Size Variation in Two Cryptic Types of *Pelobates fuscus* Revealed in our Studies (Ranges and Means, pg)

Reference	Date	<i>N</i> _{pop}	<i>N</i> _{ind}	Genome size variation	
				Western	Eastern
Borkin et al., 2001a	1986–1999	25	143	8.74–8.93 (8.84)	9.17–9.43 (9.32)
Borkin et al., 2001b	1986–2000	34	173	8.69–9.00 (8.80)	9.16–9.50 (9.33)
Khalturin et al., 2003	1986–2001	46	260	8.69–9.00 (8.80)	9.16–9.50 (9.33)
This paper	1986–2002	61	398	8.65–9.06 (8.83)	9.10–9.50 (9.35)

Province) – Bukhovoe (Lipetsk Province) – Borisovka (Belgorod Province) in Russia – Kharkov City (Kharkov Province) in Ukraine (Table 1; Fig. 1).

Therefore, the eastern type of *P. f. fuscus* is characteristic for the Volga River Basin area (Borkin et al., 2003), Central Chernozem [“black soil” — *russ.*] Territory of Russia, and the southern lowland area of Russia (Stavropol Territory) adjacent to the northern Caucasus. The animals with larger genome size were also recorded in the Ural River area in Orenburg Province (Russia) and northwestern Kazakhstan (Table 1; Fig. 1).

DISCUSSION

Genome Size Variation

This study provides far more numerous data in comparison with our previous papers (Table 2). Nev-

ertheless, our present data supported the published results. For instance, the existence of two geographically distinct groups with different genome sizes was confirmed. Interestingly, despite markedly increased sampling, the ranges of the genome size variation in the two groups remained basically at the same level and did not overlap (Fig. 2). However, the “gap” between both types of *P. f. fuscus* became somewhat narrower as compared with previous data [0.04 pg vs. 0.16 pg, see (Borkin et al., 2001b)]. Curiously, the closest values of genome size between the western type and the eastern type of the common spadefoot toad were revealed in the easternmost samples of both forms: 9.06 pg in Gzhel, Moscow Province (Russia) vs. 9.10 pg in Maiorskoe, Orenburg Province (Russia), respectively. These localities are separated by distance of approximately 1200 km (Table 1; Fig. 1).

Geographic Distribution

Our new data, especially collected in years 2001 and 2002, allowed us to re-shape the areas inhabited by two cryptic forms of *P. f. fuscus* (Fig. 1; Table 1). Indeed, currently, the genome size records cover a huge territory of the former Soviet Union, which is situated between the longitudes 20°39' E (Kaliningrad Province) and 55°16' E (Orenburg Province), and between the latitudes 58°44' N (Leningradskaya Province) and 45°03' N (Stavropol Territory), i.e. the area with size about 2300 × 1700 km.

This paper provides the first records of the western type of *P. f. fuscus* for Kaliningrad Province, the westernmost part of Russia, and for Belarus (Brest

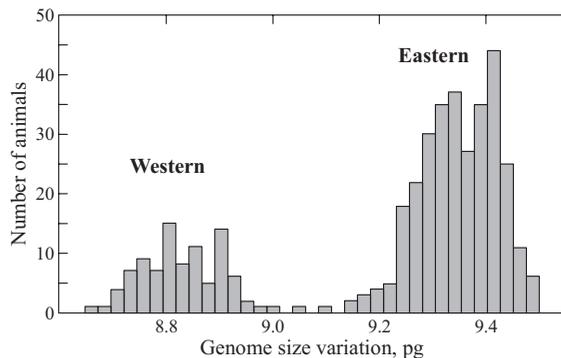


Fig. 2. The distribution of genome size values in *Pelobates fuscus fuscus* of eastern Europe ($N = 398$).

and Gomel provinces). Besides, we confirmed the occurrence of this type in Moscow Province; the former information has been based on a presumed record published by Mazin (1980) and interpreted by Borkin et al. (2001b). Currently, the westernmost record of the western type of *P. f. fuscus* is Bagrationovsk, a small town in the southwest of Kaliningrad Province (approximately $20^{\circ}39'$ E). This point is situated in distance of about 850 km to the southwest from Riga, Latvia (see map in Borkin et al., 2001b).

The first records of the eastern type of *P. f. fuscus* were made for several provinces located in the eastern part of European Russia. These are Ulyanovsk, Samara, Saratov, Astrakhan, and Orenburg provinces, as well as the Republic of Mordovia. Moreover, we identified the eastern type in northwestern Kazakhstan (Peremetnoe and Uralsk City). This is the first record for the republic. Thus, our data demonstrated that the eastern type of *P. f. fuscus* is distributed both in the right side and in the left side of Volga River as well as in the Ural River area. The village Maiorskoe in Orenburg Province (Russia) is the easternmost record of the eastern type of the common spadefoot toad. This locality is situated in distance of approximately 480 km to the southeast from Amzya, northern Bashkortostan (Borkin et al., 2001b, 2003; Khalturin et al., 2003).

Allozyme Data

Apart from genome size approach, some samples of *P. f. fuscus* were also examined by means of allozyme analysis. This technique is especially useful in the detection of cryptic speciation (Borkin et al., 2001a; Khalturin et al., 2003). In total, we examined 149 specimens from 30 samples, collected in Russia,

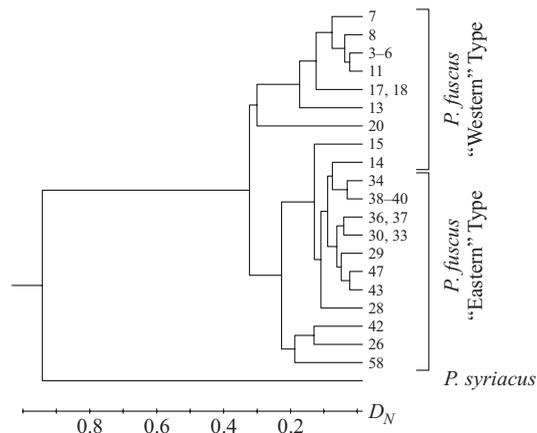


Fig. 3. Dendrogram of 28 primary samples of *Pelobates fuscus fuscus* from eastern Europe ($N = 149$) constructed by the UPGMA method using Nei's (1972) genetic distances.

Belarus and Ukraine. Unfortunately, some samples were too small, and 30 primary samples were arranged in 20 larger samples, united specimens from geographically close populations. A sample of *P. syriacus* from Azerbaijan was used for comparison (an out-group). Twenty-three presumptive loci encoding for 14 enzymes were assayed. Three loci (sMDH-1, PGDH, and PGM) were monomorphic, and the remaining 20 loci were polymorphic in *P. f. fuscus*.

Protein data clearly indicated the presence of two main groups among populations of *P. f. fuscus* of eastern Europe (Fig. 3). Geographically, as a rule, one group proved to consist of the western samples, while another group included the eastern samples (Fig. 4). Either group was characterized by six predominant alleles (sAAT-1, AAT-L, CK, EST-1, GLUD-1, and G6PD-1). In contrast, *P. syriacus* differed by eight diagnostic loci from the western type, and by seven diagnostic loci from the eastern type of *P. f. fuscus* (Khalturin et al., 2003).

The composition of the two groups, identified both by genome size data and by allozyme approach, was compared (Table 3). Among 30 primary samples, the majority of samples (27) were assigned by both techniques to the same — either the western or the eastern — group. Therefore, allozyme data verified the reality of two cryptic groups formerly identified by DNA flow cytometry.

However, in three cases, the results of application of these techniques were discordant (Table 3). Three samples of the western type (according to genome size) were arranged by allozyme data to the eastern

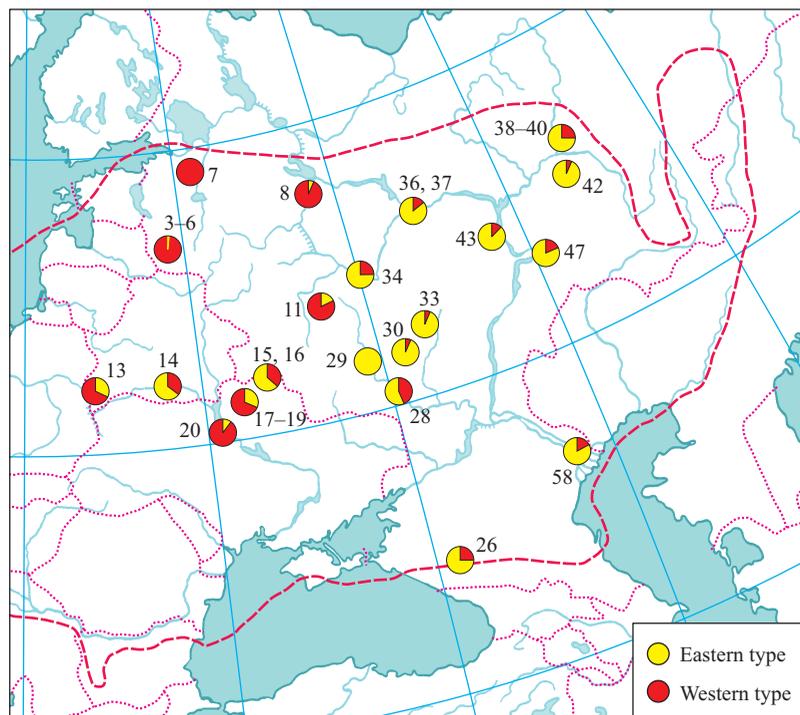


Fig. 4. The distribution of the “western” and “eastern” alleles in two cryptic forms of *Pelobates fuscus fuscus* in eastern Europe.

type of *P. f. fuscus*. Among them, two samples (Antonovka, Sumy Province, and Faivka, Chernigov Province) were located at the eastern border of the distribution of the western type of *P. f. fuscus* in Ukraine (Fig. 1). However, the third sample from Belarus (Borovaya, Gomel Province) was situated quite far from this presumed border. Interestingly, these discrepancies were associated with genomic western type of *P. f. fuscus*, while both techniques gave the same results of identification for the samples of the eastern type (Table 3).

The dendrogram based on allozyme data (Fig. 3) showed that eastern populations were clustered in two main groups. One group united three geographically distant populations, namely one sample collected in the south (No. 26, Stavropol Territory), and two samples taken at the eastern part of the distribution of the eastern type of *P. f. fuscus*. (No. 42, Bashkortostan, and No. 58, Astrakhan). Another allozyme group contained all remaining samples of the eastern type of *P. f. fuscus*. In one part, this group can be divided into two subgroups. In contrast, the western type of the common spadefoot toad proved to be more homogeneous.

The mapping of allozyme data (Fig. 4) demonstrated pronounced geographic heterogeneity of samples under the study. Generally, from the west to the east, the alleles, which were characteristic (predominant) for the western type tended to be replaced by that of the eastern type. However, the frequency of the “eastern” alleles proved to be quite high in some western samples identified by DNA flow cytometry, and, vice versa, the “western” alleles were recorded in populations of the eastern type (Fig. 4).

An introgression effect might be suggested to explain this phenomenon. Indeed, some samples were taken at the border between both types of *P. f. fuscus*. It should be mentioned, that minimum distance between the western group and the eastern group was equal to 105 km (Fig. 1; the sample Tugolesskii Bor vs. Ryazan). However, the increased frequency of “alien” alleles was revealed in some southern and northern samples taken at the eastern part of the distribution of the western type of *P. f. fuscus*. Moreover, the frequency of “alien” alleles in the eastern type was on the decrease in some populations geographically close to the western type (Fig. 4).

TABLE 3. The Identification of Samples of *Pelobates fuscus fuscus* Determined by Use of Genome Size and Allozyme Data

No.	Country, Province	Locality	Types	
			Genome size, sample size	Allozymes, sample size
3	Russia, Pskov	Osino	western (12)	western (4)
4	—”—	Peschanka	western (8)	western (5)
5	—”—	Idritsa	western (7)	western (6)
6	—”—	Rybolovka	western (3)	western (3)
7	Russia, St. Petersburg	Luga	western (2)	western (1)
8	Russia, Yaroslavl	Borok	western (1)	western (1)
11	Russia, Tula	Tula City (Barsuki)	western (4)	western (4)
13	Belarus, Brest	Medna	western (3)	western (3)
14	Belarus, Gomel	Borovaya	western (10)	eastern (10)
15	Ukraine, Sumy	Antonovka	western (6)	eastern (6)
16	—”—	Starogutsky Reserve	western (1)	western (1)
17	Ukraine, Chernigov	Naumovka	western (2)	western (1)
18	—”—	Nezhin	western (7)	western (7)
19	—”—	Faivka	western (1)	eastern (1)
20	Ukraine, Kiev	Kiev City (Chertoryi)	western (1)	western (1)
26	Russia, Stavropol	Stavropol City	eastern (1)	eastern (1)
28	Russia, Voronezh	Rossoosh	eastern (35)	eastern (32)
29	Russia, Lipetsk	Bukhovoe	eastern (3)	eastern (3)
30	Russia, Tambov	Zarech'e	eastern (13)	eastern (13)
33	—”—	Orzhevka	eastern (1)	eastern (1)
34	Russia, Ryazan	Ryazan City	eastern (2)	eastern (2)
36	Russia, Nizhniy Novgorod	Reshetikha	eastern (4)	eastern (4)
37	—”—	Ichalki	eastern (5)	eastern (5)
38	Russia, Udmurtia	Kilmez	eastern (15)	eastern (7)
39	—”—	Boyarka	eastern (13)	eastern (7)
40	—”—	Krymskaya Sludka	eastern (1)	eastern (1)
42	Russia, Bashkortostan	Amzya	eastern (5)	eastern (5)
43	Russia, Ulyanovsk	Ulyanovsk City	eastern (10)	eastern (9)
47	Russia, Samara	Shelekhmet	eastern (4)	eastern (4)
58	Russia, Astrakhan	Astrakhan City	eastern (1)	eastern (1)

Such a complicated picture might be impacted by various factors, like possible gene exchange between populations of the western and eastern types, parallel genetic polymorphisms, historical dispersion of both types *P. f. fuscus* from presumed refugia, etc. It is clear that further comprehensive study of the cryptic speciation in *P. f. fuscus* would be much needed, especially in the transition area between two population groups. Moreover, it would be also very important to learn about populations of *P. f. fuscus* from the central and western parts of Europe in terms of genome size and allozyme variation.

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