Russian Journal of Herpetology Vol. 14, No. 2, 2007, pp. 121 – 132

A CASE OF NATURAL TRIPLOIDY IN EUROPEAN DIPLOID GREEN TOAD (*Bufo viridis*), WITH SOME DISTRIBUTIONAL RECORDS OF DIPLOID AND TETRAPLOID TOADS

Leo J. Borkin,[1] Dmitry A. Shabanov,[2] Oleg V. Brandler,[3] Oleg V. Kukushkin,[4] Spartak N. Litvinchuk,[5] Gleb A. Mazepa,[6] and Jury M. Rosanov[7]

Submitted October 24, 2006.

A triploid female ($3n = 33$) was found in diploid species *Bufo viridis* from the Crimea Peninsula, Ukraine. The case is recognized as an occasional autotriploidy. No morphological differences were found between this triploid female and other diploid specimens of the sample. Three categories of triploids in the *Bufo viridis* group are classified. Based on six green toad samples identified by chromosome number, the distribution of diploid and polyploid toads in Kazakhstan is discussed.

Keywords: Amphibia, Anura, *Bufo viridis* group, polyploidy, karyotype, genome size, the Crimea Peninsula, Ukraine, Russia, Kazakhstan.

INTRODUCTION

Among Eurasian green toads (the *Bufo viridis* group), Asian members of the group provide an intriguing case of so called polyploid speciation. Indeed, tetraploid populations recognized as one or two species are widely distributed from eastern Iran across mountainous areas of Middle Asia to north-western China and western Mongolia. Triploid toads were recorded in various localities in Middle Asia situated in contact zones between diploid and tetraploid species. Moreover, a bisexual triploid species with two subspecies has been discovered in Pakistan (Pisanetz, 1978; Borkin et al., 1986a, 1986b, 2001a; Mezhzherin and Pisanets, 1990; Dujsebayeva et al., 1997, 2004; Castellano et al., 1998; Stöck, 1998; Stöck et al., 1999, 2001a, 2001b, 2005; Litvinchuk et al., 2006).

In this paper we describe a case of natural triploidy in diploid species *Bufo viridis* Laurenti, 1768 from eastern Europe (the Crimea Peninsula) at great distance from the range of polyploid toad species. In addition, we provide some distributional records of diploid and tetraploid toads in Ukraine, Russia and Kazakhstan based on karyology and DNA flow cytometry.

MATERIAL AND METHODS

In 1993 – 1994, in total, 38 specimens from 11 localities of Russia (4 individuals, 2 localities), Ukraine (14 and 3), and Kazakhstan (20 and 6, respectively) were karyotyped (Table 1, Fig. 1).

Somatic chromosome preparations were made from bone marrow, according to methods described by Macgregor and Varley (1986), with our modification. Animals were injected intraperitoneally with 0.2 – 1.0 ml 0.5% colchicine solution for 15 – 20 h before anesthesia. Then, the femur was cut off at their two ends. The

[1] Zoological Institute, Russian Academy of Sciences, Universitetskaya nab. 1, St. Petersburg 199034, Russia; E-mail: lacerta@zin.ru

[2] Department of Zoology and Animal Ecology, Kharkov National University, Svobody sq. 4, Kharkov 61077, Ukraine; E-mail: d_sh@list.ru

[3] Koltzov Institute of Developmental Biology, Russian Academy of Sciences, Vavilova str. 26, Moscow 119334, Russia; E-mail: brandler@nm.ru

[4] Karadagh Nature Reserve, National Academy of Sciences of Ukraine, Nauki str. 24, Kurortnoye, Feodosia 98188, Crimea, Ukraine; E-mail: ecol_monit@pochta.ru

[5] Institute of Cytology, Russian Academy of Sciences, Tikhoretskiy pr. 4, St. Petersburg 194064, Russia; E-mail: slitvinchuk@yahoo.com

[6] Department of Zoology and Animal Ecology, Kharkov National University, Svobody sq. 4, Kharkov 61077, Ukraine; E-mail: mazepaga@mail.ru

[7] Institute of Cytology, Russian Academy of Sciences, Tikhoretskiy pr. 4, St. Petersburg 194064, Russia.

1026-2296/2007/1402-0121

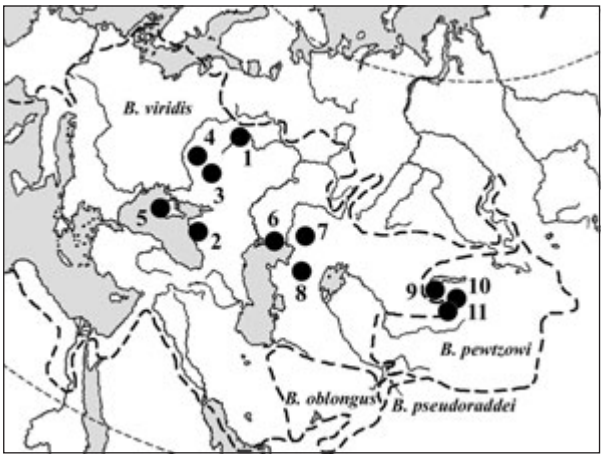

Fig. 1. The distribution of diploid, triploid and tetraploid green toads, the *Bufo viridis* group (range limits are designated as dotted line), with samples based on chromosome study. Diploid *B. viridis* is the localities 1 – 8 and 10, tetraploid *B. pewzowi* is the localities 9 and 11, and the Olenevka locality with diploid and triploid animals is the locality 5 (the Crimea Peninsula, for details see Fig. 2).

marrow cells were washed out by a solution of 0.046 M KCl. Later, we followed standard methods. Slides were air-dried and stained with 2.0% Giemsa in phosphate buffer (pH 6.8) for 15 – 20 min. The number and morphology of chromosome were identified from microphotographs.

In total, 163 specimens (85 females, 69 males, and 9 subadults) were examined for various qualitative and quantitative external characters. They were taken from the same localities, like for chromosome analysis (Table 1). To formalize morphometric and phenetic description of animals, we worked out a standard form. Results were treated by use of computer program Statistica 5 for Windows. The comparison of samples was made based on four groups of characters, namely:

— 20 linear characters, including body length, measurements of head, parotoids, and hind limbs (where L is body length and TD is vertical tympanum diameter);

— 40 qualitative characters, like coloration and dorsal skin gland patterns;

— 8 discrete characters (the number of subarticular tubercles on ventral side of longest toes and the number of spots or bands on fore- and hind limbs); these characters were transformed to 20 quantitative ones, responding to the number of tubercles and of spots;

— 25 indices represented body proportions (linear measurements related to body length or each other).

The majority of characters were used in previous papers of various authors.

Canonical discriminate analysis was used to characterize the degree of morphometric divergence among populations and was performed separately for males and females on natural logarithms of size data. The centroids of each sample were plotted on the first two canonical axes. Variation of qualitative traits was analyzed using a principal coordinate analysis. These analyses were performed by use of the computer program Statistica 6.0.

TABLE 1. List of Localities, Collectors, and Samples Used for Chromosome and Morpho-Phenetic Analyses

No.	Locality, collector, year	Ploidy	Coordinates	Chromosomes	Morphology
	Russia				
1	Chernogolovka, Noginsk District, Moscow Oblast, D. A. Shabanov, 1993	2*n*	56°00′ N 38°22′ E	2 females	9 females, 1 male
2	Vyselki, Krasnodar Kray, D. A. Bukhanovsky, 1993	2*n*	45°34′ N 39°39′ E	2 juveniles	5 subadults
	Ukraine				
3	Kharkov City, subway station "Geroyev Truda," D. A. Shabanov, 1993	2*n*	50°04′ N 36°35′ E	2 females, 2 males	6 females, 3 males
4	Akhtyrka, Sumy Province, N. P. Mushtay, 1993	2*n*	50°30′ N 34°91′ E	2 females	
5	Olenevka, Tarkhankut Peninsula, the Crimea Peninsula, A. Yu. Utevsky, August 1993, and A. M. Tsarevsky, August 1994	2*n*, 3*n* (♀)	45°23′ N 32°31′ E	6 females, 2 males	9 females, 4 males
	Kazakhstan				
6	Isatay, Atyrau Province, I. I. Lyamsin, 1993	2*n*	46°47′ N 50°12′ E	2 females, 1 male	
7	Maqat station, Atyrau Province, I. I. Lyamsin, 1993	2*n*	47°38′ N 53°20′ E	1 female, 2 males	
8	Beyneu, Mangyshlak Province, I. I. Lyamsin, 1993	2*n*	45°19′ N 55°11′ E	2 females, 1 male	3 females, 3 males
9	Kuigan, Ili River, 8 km from Balkhash Lake, Almaty Province, D. A. Shabanov, 1993	4*n*	45°22′ N 74°08′ E	2 females, 1 male, 4 subadults	14 females, 6 male, 4 subadults
10	Qapshaghay (= Kapchagay) Reservoir, Ili River, Almaty Province, D. A. Shabanov, 1993	2*n*	43°55′ N 77°58′ E	1 female, 1 male (large form)	2 females, 2 males (large form)
11	Almaty City, Kazakh University campus, Almaty Province, D. A. Shabanov, 1993	4*n*	43°15′ N 76°55′ E	2 females	6 females, 5 males

The content of nuclear DNA (genome size, *2C*) was determined by means of flow cytometry. In total, 104 specimens from 15 localities across the Crimea Peninsula, Ukraine were studied (Table 2, Fig. 2). After anesthesia, the blood was taken by a micropipette from the heart. Peripheral blood cells of the grass frog (*Rana temporaria*, 10.32 pg), collected in Leningradskaya Oblast' (= St. Petersburg Region), were used as a reference standard. Details of technique have been published previously (Borkin et al., 2001b). The mean coefficient of variation (CV) of technical errors estimated with use of various tissues of the same amphibian specimens was equal to 0.2% (Rosanov and Vinogradov, 1998).

The chromosome analysis was performed in Moscow, morphometric and phenetic analyses were carried out in Kharkov, whereas DNA flow cytometry was applied in St. Petersburg.

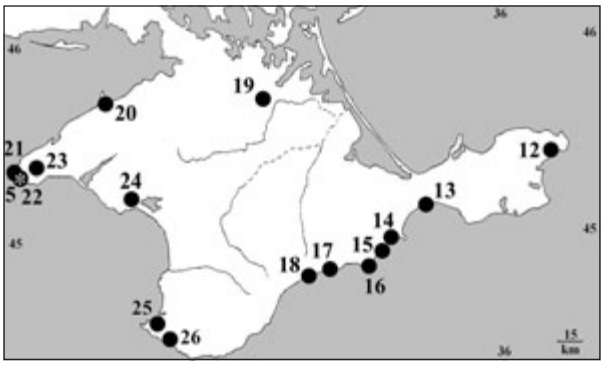

Fig. 2. The records of diploid and triploid green toads (*Bufo viridis*) in the Crimea Peninsula, based on genome size analysis. Solid circles are diploids, an asterisk marks the Olenevka locality with diploid and triploid animals.

RESULTS

Karyotype

In all toads under the karyological study a haploid set was equal to 11 chromosomes (Fig. 3). We detected no sexual heteromorphism in any of the chromosome pairs and, accordingly, used the combined data for both sexes for analysis. Also, we found no constrictions or markers of any kind. Two groups of chromosomes can be recognized. The first group consists of six larger chromosomes, forming rank gradually decreasing on size, all are metacentric, except chromosome 4, which is submetacentric. The second group contains five smaller chromosomes, which are metacentric as well, except submetacentric chromosome No. 8.

According to chromosome account, we found toads with three levels of ploidy (Fig. 3). The majority of samples contained specimens with diploid number, $2n = 22$, NF = 44. However, toads from two Kazakhstan samples

TABLE 2. List of Localities, Collectors, Samples, and Genome Size (pg) Variation in *Bufo viridis* Samples

No.	Locality	Coordinates	Collector	Year	*n*	Mean ± S.D.	Range	CV, %
12	Kerch'	45°22′ N 36°27′ E	L. F. Litvinchuk	2004	1	9.86		
13	Primorskiy	45°06′ N 35°28′ E	D. A. Shabanov	2006	10	9.96 ± 0.03	9.92 – 9.99	0.3
14	Koktebel' (= Planerskoye)	44°56′ N 35°15′ E	A. G. Desnitsky	1985	2	9.86	9.86 – 9.86	
15	Kurortnoye (= Karadagh)	44°55′ N 35°12′ E	O. V. Kukushkin	2003 – 2004	5	9.83 ± 0.05	9.76 – 9.87	0.5
16	Meganom Cape	44°47′ N 35°03′ E	O. V. Kukushkin	2002	2	9.88	9.87 – 9.89	
17	Morskoye	44°50′ N 34°48′ E	D. A. Shabanov	2003	3	9.64 ± 0.15	9.47 – 9.74	1.5
18	Rybach'ye	44°47′ N 34°37′ E	S. N. Litvinchuk	2004	4	9.85 ± 0.02	9.83 – 9.87	0.2
19	Dzhankoy	45°43′ N 34°24′ E	S. N. Litvinchuk	2004	2	9.87	9.86 – 9.88	
20	Bakal'skoye Lake	45°44′ N 33°13′ E	L. F. Litvinchuk	2004	1	9.87		
21	Tarkhankut Cape	45°21′ N 33°30′ E	D. A. Shabanov	1997	1	9.81		
22	Okunevka	45°22′ N 32°44′ E	D. A. Shabanov	2003	19	9.81 ± 0.02	9.77 – 9.84	0.2
23	Krasnosel'skoye	45°25′ N 32°41′ E	D. A. Shabanov	2003	10	9.81 ± 0.02	9.78 – 9.83	0.2
24	Saki	45°08′ N 33°32′ E	S. N. Litvinchuk	1997	7	9.96 ± 0.03	9.92 – 9.99	0.3
25	Sevastopol'	44°37′ N 33°29′ E	O. V. Kukushkin, S. N. Litvinchuk, G. A. Mazepa	1997, 2002, 2003	10	9.87 ± 0.08	9.73 – 9.99	0.8
26	Balaklava	44°29′ N 33°37′ E	O. V. Kukushkin, S. N. Litvinchuk, G. A. Mazepa	1999, 2002, 2003	26	9.82 ± 0.06	8.74 – 9.93	0.6
26	Balaklava*	44°29′ N 33°37′ E	O. V. Kukushkin	1999	1	9.19		
	Total				104	9.83 ± 0.10	9.19 – 9.99	1.5

* The specimen has anomalously low genome size.

Fig. 3. Karyotypes in green toads: **A**, diploid set of chromosomes in a female *Bufo viridis* from Olenevka sample; **B**, triploid set in a female *B. viridis* from Olenevka sample, the Crimea Peninsula; **C**, tetraploid set in a female *B. pewzowi* from Kuigan.

(Kuigan settlement at Balkhash Lake and Almaty City) had tetraploid sets of 44 chromosomes, NF = 88. A sample from Olenevka settlement (Tarkhankut Peninsula, the Crimea Peninsula, Ukraine) was characterized by diploid set ($2n = 22$) in seven animals, although one female displayed a triploid set of chromosomes ($3n = 33$, NF = 66). In all specimens homological chromosomes were similar. We failed to find any differences between chromosomes of di-, tri-, and tetraploid animals.

Genome Size in the Crimean Toads

The content of nuclear DNA in green toads (*Bufo viridis*) taken across the Crimean Peninsula is given in Table 2. All specimens demonstrated genome size values ranging from 8.93 to 9.71 pg. Such values are characteristic for diploid level. Therefore, based on DNA flow cytometry, we found no triploid animals, including a sample ($n = 30$) from the localities at Tarkhankut Peninsula (localities 5, 20, 21).

Morphometric and Phenetic Comparison

Morphometrically, a triploid female provided no significant differences from diploid members of Olenevka sample taken at the Crimea Peninsula, Ukraine (Fig. 4).

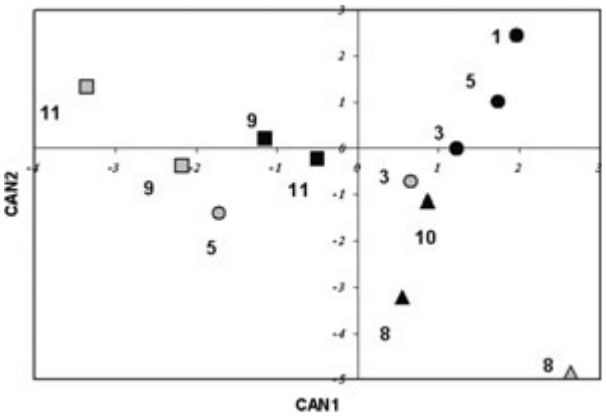

Fig. 4. Distribution of sample centrioles for European diploid and triploid (circles), Asiatic diploid (triangles), and tetraploid (squares) green toads in the space of first and second canonical axes (morphometric characters). Males are designated by gray symbols; females by black ones.

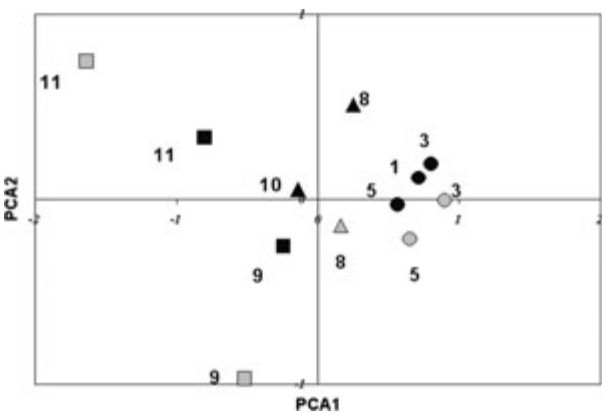

Fig. 5. Distribution of sample centrioles for European diploid and triploid (circles), Asiatic diploid (triangles), and tetraploid (squares) green toads in the space of first and second principal coordinate axes. Males are designated by gray symbols; females by black ones.

The application of canonical analysis demonstrated significant sexual dimorphism in linear parameters in the majority of samples (Fig. 4). Interestingly, the differences between populations were expressed stronger in females rather than in males.

This analysis distinguished three sample groups: a) European diploids, b) Asiatic diploids, and c) Asiatic tetraploids. However, this separation was weaker in a case with males, because Olenevka diploid males (the gray circle *5*) proved to be more similar to tetraploid males rather than to diploid ones (Fig. 4).

The discriminate analysis (Table 3) revealed differences between diploid males and females as well as a triploid female from Olenevka, on the one hand, and

TABLE 3. Results of Discriminate Analysis with Use of Morphometric Characters

Group	Sex	*n*	Percent	Females				Males		
				2*n* + 3*n* (E)	2*n* (M)	2*n* (Q)	4*n*	2*n* (E)	2*n* (M)	4*n*
2*n* + 3*n* (E)	♀	24	92	22	0	0	2	0	0	0
2*n* (M)	♀	3	67	0	2	1	0	0	0	0
2*n* (Q)	♀	4	100	0	0	4	0	0	0	0
4*n*	♀	20	100	0	0	0	20	0	0	0
2*n* (E)	♂	8	100	0	0	0	0	8	0	0
2*n* (M)	♂	3	100	0	0	0	0	0	3	0
4*n*	♂	11	82	0	0	0	1	1	0	9
Total		73	93	22	2	5	23	9	3	9

E, European samples; M, Mangyshlak Province; Q, Qapshaghay, Almaty Province (both in Kazakhstan); 2*n*, 3*n*, and 4*n* are diploid, triploid, and tetraploid toads.

TABLE 4. Values (mean ± S.D. and range) of Body Length (mm) and Index TD/L in Eight Samples of Green Toads

Locality	Ploidy	*n*	Body length (L)	TD/L
			Females	
1. Chernogolovka	2*n*	9	53.9 ± 7.7 (44.9 – 70.8)	5.8 ± 0.7 (4.5 – 6.6)
3. Kharkov	2*n*	6	63.0 ± 6.3 (53.6 – 71.7)	5.8 ± 0.5 (5.0 – 6.3)
5. Olenevka	2*n*	8	65.0 ± 5.8 (58.3 – 73.9)	5.7 ± 0.4 (5.1 – 6.4)
5. Olenevka	3*n*	1	63.6	4.6
8. Beyneu	2*n*	3	78.2 ± 12.6 (63.8 – 68.8)	4.9 ± 0.7 (4.2 – 5.3)
10. Qapshaghay	2*n*	4	66.4 ± 19.2 (49.0 – 83.2)	4.8 ± 0.9 (3.6 – 5.5)
9. Kuigan	4*n*	14	60.6 ± 6.8 (48.6 – 71.2)	5.1 ± 0.6 (4.5 – 6.4)
11. Almaty	4*n*	6	68.0 ± 9.4 (56.2 – 80.1)	5.1 ± 0.4 (4.7 – 5.8)
			Males	
1. Chernogolovka	2*n*	1	61.5	6.2
3. Kharkov	2*n*	3	63.9 ± 2.6 (60.9 – 65.6)	6.3 ± 1.2 (4.9 – 7.2)
5. Olenevka	2*n*	4	64.6 ± 4.3 (58.6 – 68.0)	5.7 ± 0.4 (5.1 – 6.1)
8. Beyneu	2*n*	3	80.1 ± 0.5 (79.7 – 80.6)	5.3 ± 0.6 (4.8 – 6.0)
10. Qapshaghay	2*n*	2	90.3 (80.7 – 99.8)	5.1 (4.8 – 5.4)
9. Kuigan	4*n*	6	60.4 ± 3.2 (55.1 – 63.7)	5.2 ± 0.6 (4.4 – 5.8)
11. Almaty	4*n*	5	64.2 ± 3.5 (58.9 – 67.5)	5.3 ± 0.7 (4.2 – 5.9)

tetraploid toads, on the other hand. Moreover, this technique allocated European and Asiatic diploid samples to different groups. However, we failed to identify any single morphometric character which would allow us to separate clearly diploid and tetraploid toads. The TD/L (Table 4) proved to be most useful for the discrimination between European and Asiatic toads.

The principal coordinate analysis supported the separation between European diploid and Asiatic tetraploid samples, too (Fig. 5). Asiatic diploid samples had an intermediate position.

Also, we observed that the area of dark-green dorsal spots in Asiatic toads was markedly less than in European animals (Figs. 6 – 8). Kuigan toads were characterized by a dorsal coloration pattern consisted of small spots connected each other; often dorsal warts were separated each other and arranged in rows. In contrast, Almaty toads had large and isolated dorsal spots, whereas skin glands were connected and distributed chaotically.

DISCUSSION

Karyotype

In general, gross chromosome morphology based on routine Giemsa staining in diploid toads under the study was similar to that described in the literature, despite some slight contradictions in the identification of individual chromosomes (metacentric or submetacentric), probably due to different rates of spiralization or various

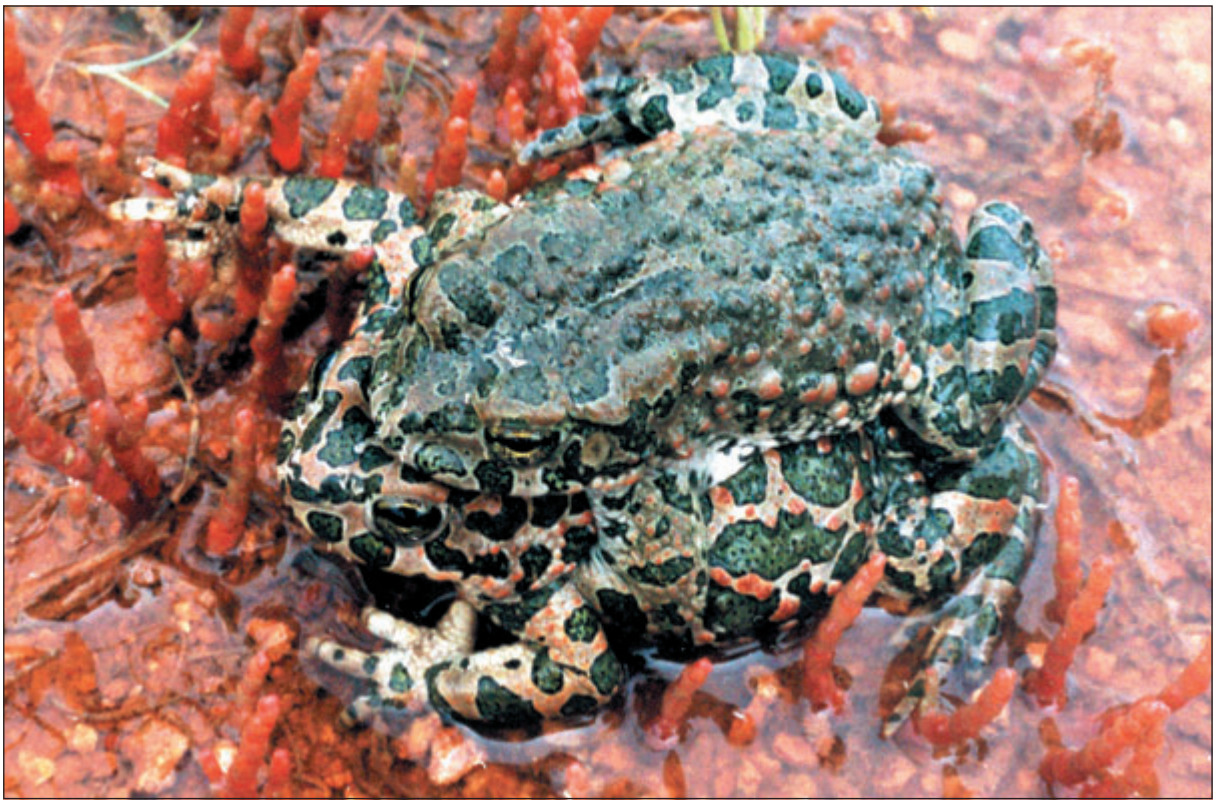

Fig. 6. A pair of diploid green toads (*Bufo viridis*) from Sevastopol' City, the Crimea, Ukraine. Photo by S. N. Litvinchuk, August 2002.

interpretation of the position of adjoining chromosome pairs (e.g., Wickbom, 1945; Morescalchi, 1963; Ullerich, 1966; Mészáros, 1973; Masik et al., 1976; Popov and Banova, 1976; Schmid, 1978; Kryukov et al., 1985; Borkin et al., 1986a; Roth and Ráb, 1986; Pisanets, 1991; Stöck et al., 2005). The pattern with six larger and five smaller metacentric to submetacentric chromosomes is very conservative across the majority of species in the genus *Bufo* as well (e.g., Bogart, 1972).

In diploid karyotypes we found no secondary constrictions or markers of any kind, like many previous authors which also worked with conventionally stained chromosome preparations (Masik et al., 1976; Popov and Banova, 1976; Kryukov et al., 1985; Borkin et al., 1986a; Roth and Ráb, 1986). In contrast, Wickbom (1945, p. 252) showed a secondary constriction on the long arm of chromosome 7 (from smaller chromosome group). According to Bogart (1972, pp. 176 – 177, 375), *B. viridis* from Israel (p. 354) had secondary constrictions (*A*, *D*, and *F*) on the long arms of three large chromosomes 1, 2, and 3. However, other papers did not confirm these data.

Another group of papers provided similar data about long arms of chromosome pair 6. So, Ullerich (1966) detected secondary constrictions at terminal position in *B. viridis* from Braunschweig, Germany. Used silver ($AgNO_3$) staining, Schmid (1973) showed, that in a European *B. viridis* obtained from dealers nucleolar organizing regions (NORs) occupied a telomeric position. Birstein (1981) also identified by means of Ag – As staining the secondary constrictions of both homologues the 6th chromosome pair in *B. viridis* from the Crimea Peninsula ("Planerskoye"), Ukraine, and Alma-Ata (now Almaty), Kazakhstan. However, he mentioned some differences in localization of these constrictions at telomeric zone in comparison with data presented by Schmid (1973). Dr. V. Birstein suggested that slight differences may exist between populations. Roth and Ráb (1987) also found NORs on the long arms of the chromosome pair 6 in diploid *B. viridis* from Hvar ("Yugoslavia," Croatia). Unlike six diploid toads originated from Armenia (Zolokary), Turkmenistan (Ashkhabad and Sarykamysh Lake), and Tajikistan (the settlement Chirik, Shaartuz District), two diploid specimens (male and female) from Tajikistan (the settlement Shaartuz)

Fig. 7. A subadult of diploid green toad (*Bufo viridis*) from Ili River, Qapshaghay District, Almaty Province, Kazakhstan. Photo by S. N. Litvinchuk, August 2006.

Fig. 8. A female of tetraploid green toad (*Bufo pewzowi*) from Taldy-Korgan District, Almaty Province, Kazakhstan. Photo by S. N. Litvinchuk, August 2006.

showed "terminal satellites" in the long arm of large chromosome pair 6. Curiously, diploid karyotype of a male from Chirik of the same administrative district lacked any "satellites" (Pisanets, 1991). Later, diploid toads from the Beshkent sands, south-western Tajikistan, including Chirik and Shaartuz samples, were described as a new species, *B. shaartusiensis* Pisanets, Mezhzherin et Szczerbak, 1996. Therefore, the presence/absence of terminal satellites in these toad samples should be recognized as a geographic variation within a species or even a subspecies because the taxon is currently ascribed to *B. viridis* as *B. v. shaartusiensis* (Stöck et al., 2001b).

In Giemsa-stained karyotype of diploid *B. viridis kermanensis* from Iran, the chromosome pair 6 terminally exhibited a secondary constriction in its long arms (Stöck et al., 2001a). Recently, based on silver ($AgNO_3$)-staining of NORs, Stöck et al. (2005) reported that all diploid karyotypes contained one pair of active homologous NORs, situated terminally in the long arms of chromosome pair 6. Therefore, these data corresponded with some previous data (Roth and Ráb, 1987; Pisanets, 1991). However, in contrast to the latter paper, Stöck et al. (2005) observed no geographic variation in position or size of the NORs in *B. viridis viridis* from Germany (Halle), *B. v. turanensis* from north-eastern Iran (Gorgan and frontier zone near Turkmenistan), Turkmenistan (Ashkhabad) and Kyrgyzstan (Bishkek), and *B. v. kermanensis* from Iran (Kerman).

Many authors mentioned the similarity in chromosome morphology between diploid and tetraploid toads, as well as the lack of secondary constrictions in 4*n* karyotypes (Masik et al., 1976; Kryukov et al., 1985; Borkin et al., 1986b, c; Roth and Ráb, 1986; Borkin and Kuzmin, 1988). In our study, chromosomes in tetraploid karyotypes corresponded to those in diploids as well. We found no obvious heterogeneity between homologous chromosomes within quartets. Based on morphometric comparison of individual chromosomes in the karyotype of a female tetraploid from Faisabad, southern Tajikistan, Pisanets (1991) revealed significant differences within quartet 9 (from smaller chromosome group) only, where the arm ratio value in the largest chromosome proved to be higher than in the smallest homologous one. According to Stöck et al. (2005), there were visible differences in the chromosome size and morphology within one quartet in some tetraploid taxa. They were most obviously expressed in the quartet 6 (from larger chromosome group), in which two chromosomes were distinctly larger than the remaining ones. Other quartets showed minor size differences, which allowed to the authors to divide a quartet in two chromosome pairs.

Interestingly, in the quartet 6 of the same Faisabad tetraploid toad, Pisanets (1991) observed terminal satellites in long arm of two chromosomes only, whereas two other chromosomes of the quartet lacked any satellites. Later, based on own studies, Stöck et al. (2005) suggested that size differences between pairs in the quartet 6 could be caused by the presence or absence of NORs. Indeed, all tetraploid karyotypes contained only one pair homologous NORs. Moreover, 18S + 28S rDNA *in situ* hybridization revealed that potentially duplicated rDNA genes in the 4*n* karyotypes were not only inactive but were physically absent from the two other (homologous)

copies of chromosomes 6. The intensity and/or length of the NOR structures was often found to differ within and between individuals of both diploid and tetraploid toads, even from the same locality (Stöck et al., 2005).

Triploidy

Formerly, the phenomenon of triploidy in green toads (*B. viridis* group) has been known in Asian populations only (Masik et al., 1976; Pisanets, 1978; Mezhzherin and Pisanets, 1990; Borkin et al., 1997, 2001a; Castellano et al., 1998; Stöck et al., 2001a; Odierna et al., 2004; Litvinchuk et al., 2006; our unpublished data). Two categories of triploidy in these animals were recognized (Borkin et al., 2000).

Some triploids occurred in quite narrow zones sandwiched between the parapatric ranges of diploid and tetraploid species. Such contact zones with triploids were registered in south-western Turkmenistan, northern Kyrgyzstan, and south-eastern Kazakhstan. Sometimes, these triploids coexisted with either diploid or tetraploid toads, sometimes, with both species at the same site (e.g., Pisanets, 1978; Borkin et al., 1997, 2001a; Castellano et al., 1998; Stöck et al., 2001a; Odierna et al., 2004; Litvinchuk et al., 2006).

Recently, another kind of triploidy has been discovered in northern Pakistan. A bisexually reproducing species, *B. pseudoraddei*, proved to consist of triploid animals only. Moreover, all-triploid populations were allocated to two various subspecies of the species (Stöck et al., 1999, 2001a, 2001b, 2002).

Clearly, our finding in 1993 of a triploid female in a Crimean population of diploid *B. viridis* cannot be assigned to the categories above mentioned. The study of this case has a long history. In August 1993, A. Yu. Utevsky provided the first four toads from Olenevka settlement at Tarkhankut Peninsula. Unfortunately, two specimens died, two ones were karyotyped, and a female proved to be triploid. After our request, in August 1994, A. M. Tsarevsky captured 9 specimens at the same site. Chromosomes were counted in six toads, and all specimens were diploid. In August 1995, D. A. Shabanov visited Olenevka; however, he failed to collect any toads. This steppe locality is quite arid, and a sole artificial pool, which was used by green toads for breedings, dried up. E. M. Pisanets (oral communication, 1995) observed a diploid set of chromosomes only in a toad sample from Olenevka, karyotyped by him. Formerly, the picture of karyotype of diploid green toads "from Kherson Province and the Crimea" (without exact localities) has been published (Pisanets, 1978).

It is important to underline that Olenevka population exists in severe conditions associated with lower humidity and high salinity of available water (the shore of Black Sea). Largely, local toads were dependent on fresh water supplied by humans for needs of the settlement Olenevka.

Since 1997, using DNA flow cytometry, we started our extensive screening the Crimean green toads in order to identify new triploids. However, we find no triploids among 94 toads taken from 14 localities across the Crimea Peninsula, including Tarkhankut Peninsula (Fig. 2, Table 2). Previously, we recorded diploid toads in two more Crimean localities as well, namely in "Planerskoye" and "Karadagh" (Borkin et al., 1986a). The diploid set of chromosomes in toad(s?) from "Planerskoye" was evidenced by Birstein (1981) as well.

In total, we examined 38 specimens from Tarkhankut Peninsula by means of chromosome and genome size analyses. A sole triploid female provides 2.6% of total sample at the locality and about 1% of all Crimean green toads. Thus, we should recognized our case of triploidy in diploid Crimean *B. viridis* as an occasional occurrence. Importantly, it was the first record of natural triploidy in European populations, quite far from Central Asia with contact zones between diploid and tetraploid species.

Later, two more cases with occasional triploidy were registered in Kibbutz-Mezon, Syria in 1997 and in Vesuvio, Italy in 2000. Thus, these data confirmed the phenomenon of occasional triploidy in diploid *Bufo viridis* and showed quite wide distribution of such a triploidy beyond Central Asia. In the both cases, a diploid sample of 8 males contained one triploid (Odierna et al., 2004) or 12.5% per sample. At the regional level, the incidence of triploidy in Near East ($n = 36$) was equal to 3% and in Europe ($n = 91$) about 1%. These values are similar to the Crimean estimate. For comparison, the incidence of spontaneous autotriploidy in wild populations of five species of urodeles varied between 0.20 and 1.05% per species (Borkin et al., 1996; Litvinchuk et al., 2001).

Therefore, the phenomenon of occasional triploidy in diploid green toads should be classified as the third category, in addition to other two categories above mentioned (Borkin et al., 2000). Taxonomically, the phenomenon was observed in two subspecies of *Bufo viridis*, namely: *B. v. viridis* (the Crimea and Apennines) and *B. v.* "*arabicus*" (Syria).

Origin of Triploidy

Based on sterile specimens from Danata, Turkmenistan, E. M. Pisanets (1978) suggested that the origin of triploids appeared in the contact zone of diploid and tetraploid species was resulted from the hybridization. Theoretically, such an explanation may be accepted as a

reasonable and simple. Indeed, triploids were collected together with diploid and tetraploid toads at the same site in several localities of Turkmenistan, Kyrgyzstan, and Kazakhstan (Pisanets, 1978; Castellano et al., 1998; Borkin et al., 2001a; Odierna et al., 2004; our unpublished data). However, in the parapatric area of 2*n* and 4*n* species, we also found an "asymmetrical" occurrence of triploids with one of presumed parental species only. For instance, in Zhyngyldy locality, south-eastern Kazakhstan, triploids were captured with diploid toads only, and no tetraploids were recorded (Borkin et al., 2001a). Such cases could be explained by inadequate sampling or would need another mechanism of triploidy origin rather than simple hybridization between two parental species.

Indeed, a multi-sided examination of Kok-Jar mixed population of 2*n*, 3*n*, and 4*n* toads from a river canyon near Bishkek City, Kyrgyzstan, discovered by use of DNA flow cytometry and supported by chromosome analysis, gave important results. Morphometric and bioacoustic approaches demonstrated that triploid and tetraploid males form a phenotypically homogeneous group clearly distinguished from diploid males (Castellano et al., 1998). The random amplified polymorphic DNA (RAPD) analysis obviously separated diploid specimens, on the one hand, and polyploid (tri- and tetraploid) specimens, from the other hand, and, thus, provided arguments against hybrid origin of Kok-Jar triploids (Delpero et al., 2000).

The variation in microsatellite locus *BM224* in di-, tri- and tetraploid green toads was examined by Litvinchuk et al. (2006). Triploids from south-eastern Kazakhstan shared allele *b* with local tetraploids, whereas diploids had another allele *c*. Theoretically, hybrids should bear both alleles, because of co-dominant mechanism of satellite inheritance. Thus, we can conclude that these triploids proved to be of non-hybrid origin. The allele *b* was recorded in the Pamirs triploids from Lyangar village, Badakhshan (Tajikistan) as well. However, the same allele *a* was found in di-, tri-, and tetraploid toads in southern Turkmenistan.

Recently all-triploid species of green toads (*B. preudoraddei*) with bisexual reproduction was discovered in northern Pakistan (Stöck et al., 1999, 2002). In Batura toads, a new all-triploid subspecies (*B. preudoraddei baturae* Stöck, Schmid, Steinlein et Grosse, 1999), chromosome 6 proved to be heteromorphic for the presence of the NOR because only two of three chromosomes of the triplet contained NORs. A new mechanism of bisexual reproduction in these toads termed "triploid hybridogenesis" has been proposed. The occurrence of meiotic stages and recombination in both sexes, at least in two-thirds of the whole genome, allows the perpetuation of all-triploidy. It unites characteristics of sexual (genetic recombination) with those of asexual reproduction. Briefly, a triploid toad male provides a haploid NOR-bearing sperm, whereas a triploid female gives diploid eggs with one chromosome set with NOR and another set without NOR (Stöck et al., 2002; Stöck and Lamatsch, 2002).

Obviously, both hybridization and triploid hybridogenesis hypotheses could not be applied to three European and Near Eastern triploids occasionally appeared in various populations of diploid *Bufo viridis*. We suggest that, at least, the Crimean triploid female can be classified as an autotriploid because no other toad species inhabits this peninsula. The autotriploidy would be the most plausible explanation for two triploid males from Italy and Syria as well.

Two mechanisms of origin of autotriploidy could be proposed. First, a parent might produce unreduced gametes. For instance, a diploid male with haploid sperm may mate with diploid female provided unreduced diploid eggs. Conversely, a diploid male supplied diploid sperm may mate with a diploid female with normal haploid eggs. In our opinion, the first scheme should be most probable.

Experimentally, triploids were encountered in many crosses between various species of the genus *Bufo*, including *B. viridis* (Bogart, 1972: Appendix G). Importantly, they were found even in control crosses. Invariably, where it could be determined, the female was responsible for the duplicate set of chromosomes in triploids. Thus, hybrid (allo-) and non-hybrid (auto) triploids were obtained in the laboratory crosses. A small percentage of eggs produced by female *Bufo* species were diploid which was probably the result of suppressed maturation of divisions in some oocytes (Bogart, 1972). According to Bogart (1972, p. 182), *B. viridis* (no locality) also produced a small percentage of eggs which were diploid. Fertilization of a diploid egg would result in the formation of a triploid.

Second, fresh fertilized eggs might be influenced by damaged environmental factors. As it is known, triploidy could be obtained easily in the laboratory when such eggs would be treated by various techniques, e.g., by cold or heat shocks, hydrostatic pressure, etc. (review by Kawamura, 1984).

Thus, we conclude that, first, various categories of triploidy can occur in the *Bufo viridis* group, and, second, triploidy can origin via different cytogenetic mechanisms.

About Morphological Identification of Diploid and Polyploid Green Toads

In Russian literature some external morphometric characters were proposed to discriminate between diploid and tetraploid toads. These were (Pisanets, 1978): parotoid gland length in relation to body length (L.par./L), internarial distance in relation to head width (Sp.n./Lt.c.), and horizontal orbital diameter in relation to the distance between orbit and nostril (L.oc./D.n.o.). Examined lowland and mountain samples of Kyrgyz toads, Fikhtman (1989) recommended three characters: the relative length of parotoid gland (L.par./L), the gleno-acetabular body length measured between fore- and hindlimbs in relation to the distance from glenoid point to mouth angle (Ga/Ao), and forearm length in relation to body length (L/For).

Our comparison of quantitative and qualitative characters in diploid and tetraploid toads, separated by chromosome analysis, distinguished diploid and tetraploid samples (Figs. 4 and 5). Nevertheless, external morphological features, including above mentioned ones, can not be considered diagnostic and may provide incorrect identification of toads with different ploidy. We agree with the conclusion that some morphometric traits exhibit clear differences of the means between diploid and tetraploid toads. However, value ranges showed overlap, and morphometric parameters only are not suitable for exact ploidy determination for each specimen (Stöck, 1997; Stöck et al., 2001a). Moreover, based on examination of about two thousands specimens across vast range of green toads, Roth (1986) pessimistically stated that morphometric characters are, with some exceptions, quite useless for distinguishing various taxa in green toads, without karyological analysis.

Important results were obtained by Castellano et al. (1998), using various morphometric techniques. Diploid and tetraploid toads were reliably separated, in particular if their ploidy was identified karyologically *a priori*. In contrast, triploid and tetraploid specimens could be erroneously classified. For instance, 4*n* males taken at Kok-Jar locality, Kyrgyzstan, were identified as triploids.

Therefore, a prognosis for the purely morphometric classification of diploid and polyploid toads is possible with multivariate methods, and it can be improved in combination with erythrocyte size data (Stöck et al., 2001a). Nevertheless, the application of cytogenetic methods (chromosomes and/or genome size) is the most reliable approach to identify ploidy level in each individual.

Some Comments on Distributional Records

The records provided by our chromosome analysis, confirmed the literature and our unpublished data about the distribution of tetraploid toads in Central Asia only (see a review by Stöck et al., 2001a). Indeed, in eastern Europe (Russia and Ukraine), we found diploid animals only, except an occasional triploid in the Crimea Peninsula.

Three diploid samples from north-western Kazakhstan (Atyrau and Mangyshlak provinces, the localities 6 – 8) showed that the vast territory between Lower Volga River and Turan Lowland harbors diploid toads only. Our chromosome records are in agreement with known data (Borkin et al., 2000; Dujsebayeva et al., 2004; Odierna et al., 2004; Litvinchuk et al., 2006; our unpublished data).

Two samples (Kuigan and Almaty City) with tetraploid toads, evidenced by chromosome count, were found in southern Kazakhstan. According to current taxonomic treatment (Stöck et al., 2001b) supported by cytogenetic (Stöck et al., 2005) and molecular (Litvinchuk et al., 2006) studies, these samples should be assigned to so called "Central Asian tetraploids," *Bufo pewzowi* Bedriaga, 1898.

The Kuigan sample (the locality 9, Table 1, Fig. 1), taken at Ili River in 8 km from its mouth, is situated at intermediate geographic position between the villages Burubaital and Karaoj. The locality confirmed the distribution of tetraploid toads in the Ili River delta and along the southern shore of Balkhash Lake (Dujsebayeva et al., 1997; Borkin et al., 2001a).

Another tetraploid sample, collected in the Kazakh University campus in Almaty City, also supported the finding that the city is inhabited by tetraploid toads (Borkin et al., 1995, 2001a; Dujsebayeva et al., 1997). Previously, both Birstein (1981) and Mezhzherin and Pisanets (1995) reported the occurrence of diploid toads in "Alma-Ata" or in "the vicinity" of the city, respectively. However, the latter diploid specimen was taken, in fact, from the city Zoo area with large park, where green toads of unclear origin gifted by visitors, sometimes, were released by the Zoo staff (see Borkin et al., 2001a: p. 51).

Previously (Borkin et al., 1995), we suggested that Ili River seems to be a lowland contact zone where diploid toads (*B. viridis*) are replaced by tetraploid toads (*B. pewzowi*). Indeed, in the Qapshaghay Reservoir area, situated at Ili River to the north from Almaty City, both diploids and tetraploids were registered. In 1994, triploid specimens were collected by us at the same site with diploids (Borkin et al., 1995). In 1993, D. A. Shabanov found two forms of green toads of a larger and

smaller size in the Qapshaghay area (the locality 10, Fig. 1); they differed by body proportions and coloration. Karyologically, a larger form proved to have a diploid set of chromosomes (Table 1). Unfortunately, two females of a smaller form were not karyotyped; they may belong to a tetraploid species *B. pewzowi*. Among 8 males, diploid (3), triploid (4) and tetraploid (1) specimens were revealed in 1997 at "Kap Chagay" (Odierna et al., 2004: Table 1, the locality 33).

Acknowledgments. We are grateful to D. A. Bukhanovsky (Kharkov, Ukraine), A. G. Desnitsky (St. Petersburg, Russia), L. F. Litvinchuk (St. Petersburg, Russia), I. I. Lyamsin (Kharkov, Ukraine), K. D. Milto (St. Petersburg, Russia), N. P. Mushtay (Akhtyrka, Ukraine), A. Yu. Utevsky (Kharkov, Ukraine), and A. M. Tsarevsky (Kharkov, Ukraine) for the field and technical assistance. This work was partially supported by the Russian Foundation for Basic Research (grants Nos. 05-04-48403 and 05-04-49118), the contract with "Rosnauka" No. 02.452.11.7031 (2006-RI-26.0/001/070), the Joint Research Center "Material Science and Characterization in High Technology," and by the Basic Research Program of the Russian Academy of Science "Biodiversity and Dynamics of Gene Pools."

REFERENCES

Birstein V. I. (1981), "Specific differential staining patterns of chromosomes in the Anura, some species of the genera *Rana* and *Bufo* taken as an example," *Zool. Zh.*, **60**(2), 246 – 256 [in Russian, with English summary].

Bogart J. P. (1972), "Karyotypes," in: W. F. Blair (ed.), *Evolution in the Genus* Bufo, Univ. of Texas Press, Austin – London, pp. 171 – 195.

Borkin L. J. and Kuzmin S. L. (1988), "Amphibians of Mongolia: species account," in: E. I. Vorobyeva and I. S. Darevsky (eds.), *Amphibians and Reptiles of the Mongolian People's Republic. General Problems. Amphibians*, Nauka, Moscow, pp. 30 – 197 [in Russian].

Borkin L. J., Caune I. A., Pisanetz E. M., and Rozanov Y. M. (1986a), "Karyotype and genome size in the *Bufo viridis* group," in: Z. Roček (ed.), *Studies in Herpetology*, Charles University, Prague, pp. 137 – 141.

Borkin L. J., Eremchenko V. K., and Rosanov J. M. (1997), "Distribution of and contact zones in the diploid-tetraploid *Bufo viridis* complex in Central Asia," in: Z. Roček and S. Hart (eds.), *Herpetology'97. Abstrs. of the Third World Congr. of Herpetol.*, *2 – 10 August 1997*, Prague, Czech Republic, p. 26.

Borkin L. J., Eremchenko V. K., Helfenberger N., Panfilov A. M., and Rosanov Yu. M. (2001a), "On distribution of diploid, triploid and tetraploid green toads (*Bufo viridis* complex) in south-eastern Kazakhstan," *Russ. J. Herpetol.*, **8**(1), 45 – 53 [see also Erratum in **8**(3), 246].

Borkin L. J., Litvinchuk S. N., and Rosanov J. M. (1996), "Spontaneous triploidy in the crested newt, *Triturus cristatus* (Salamandridae)," *Russ. J. Herpetol.*, **3**(2), 152 – 156.

Borkin L. J., Litvinchuk S. N., Rosanov J. M., and Milto K. D. (2001b), "Cryptic speciation in *Pelobates fuscus* (Anura, Pelobatidae): evidence from DNA flow cytometry," *Amphibia–Reptilia*, **22**(4), 387 – 396.

Borkin L. J., Rosanov J. M., and Litvinchuk S. N. ("2000," 2001), "Nuclear DNA content in some green toads (*Bufo viridis* complex) of Turkey and Iran," *Russ. J. Herpetol.*, **7**(3), 171 – 180.

Borkin L. J., Rosanov J. M., Terbish Kh., and Caune I. A. (1986c), "Distribution, karyology, taxonomic position and variability of toads of the *Bufo viridis* group in Mongolia," in: E. I. Vorobyeva (ed.), *Gerpetologicheskiye Issledovaniya v Mongol'skoy Narodnoy Respublike* [*Herpetological Research in Mongolian People's Republic*], Institute of Evolutionary Morphology and Ecology of Animals, USSR Academy of Sciences, Moscow, pp. 120 – 143 [in Russian, with German summary].

Borkin L. J., Terbish Kh., and Caune I. A. (1986b), "Tetraploid and diploid toads from the *Bufo viridis* group in Mongolia," *Dokl. AN SSSR*, **287**(3), 760 – 764 [in Russian; also translated in USA in *Dokl. Biol. Nauk*, *Proc. Acad. Sci. USSR*, **287**(1 – 6), 243 – 246 (1986)].

Borkin L. J., Vinogradov A. E., Brushko Z. K., and Kuzmin S. L. (1995), "New records of tetraploid toads (*Bufo viridis* group) from Alma-Ata and Taldy-Kurgan Region, Kazakhstan," *Russ. J. Herpetol.*, **2**(2), 174 – 175.

Castellano S., Giacoma C., Dujsebayeva T., Odierna G., and Balletto E. (1998), "Morphometrical and acoustical comparison between diploid and tetraploid green toads," *Biol. J. Linn. Soc.*, **63**(2), 257 – 281.

Delpero M., Cervella P., Borkin L. J., Dujsebayeva T., Eremchenko V. K., and Balletto E. (2000), "Random amplified polymorphic DNA (RAPD) analysis of Asiatic population of the *Bufo viridis* complex," in: C. Giacoma (ed.), *Atti I Congresso Nazionale della Societas Herpetologica Italica* (*Torino*, *2 – 6 Ottobre 1996*), Museo Regionale di Scienze Naturali, Torino, pp. 299 – 304.

Dujsebayeva T., Castellano S., Giacoma C., Balletto E., and Odierna G. (1997), "On the distribution of diploid and tetraploid green toads of the *Bufo viridis* complex (Anura; Bufonidae) in southern Kazakhstan," *Asiatic Herpetol. Res.*, **7**, 27 – 31.

Dujsebayeva T., Castellano S., Magni P., and Odierna G. (2004), "Distribution of amphibians and reptiles in the Aral Sea Basin and surrounding areas of Kazakhstan. Part I. The green toads of *Bufo viridis* complex (Amphibia: Anura)," *Selevinia*, **2003**, 60 – 65.

Fikhtman B. S. (1989), "Contribution to diagnostics of green toads of the *Bufo viridis* complex in Kirghizia," in: N. N. Szczerbak (ed.), *Voprosy Gerpetologii* [*The Problems of Herpetology*]. *Abstrs. of the 7th USSR Herpetol. Conf.*, Kiev, pp. 263 – 264 [in Russian].

Kawamura T. (1984), "Polyploidy in amphibians," *Zool. Sci.* (*Tokyo*), **1**(1), 1 – 15.

Kryukov V. I., Rzepakovsky V. T., and Astapov V. P. (1985), "Karyotypic characteristics of seven toad samples from Tajikistan," *Dokl. AN TadSSR*, **28**(10), 593 – 595 [in Russian].

Litvinchuk S. N., Rosanov J. M., and Borkin L. J. (2001), "Natural autotriploidy in the Danube newt, *Triturus dobrogicus* (Salamandridae)," *Russ. J. Herpetol.*, **8**(1), 74 – 76.

Litvinchuk S. N., Rosanov J. M., Usmanova N. M., Borkin L. J., Mazanaeva L. F., and Kazakov V. I. (2006), "Variation of microsatellites *BM224* and *Bcal7* in populations of green toads (*Bufo viridis* complex) with various nuclear DNA content and ploidy," *Tsitologiya*, **48**(4), 332 – 345 [in Russian], translated to English: *Cell Tissue Biol.*, 2007, **1**(1), 65 – 79.

Macgregor H. C. and Varley J. M. (1983), *Working with Animal Chromosomes*, Wiley, Chichester.

Masik E. Ju., Kadirova B. K., and Toktosunov A. T. (1976), "Karyotype pattern in the green toad *Bufo viridis* in Kirghizia," *Zool. Zh.*, **55**(11), 1740 – 1743 [in Russian, with English summary].

Mayr E. (1969), *Principles of Systematic Zoology*, McGraw-Hill Book Company, New York, etc.

Mezhzherin S. V. and Pisanets E. M. (1990), "Genetic structure and origin of Asiatic polyploid toads *Bufo danatensis* Pisanetz, 1978," *Dokl. AN USSR Ser. B Geol. Geogr. Khim. Biol.*, **8**, 68 – 71 [in Ukrainian].

Morescalchi A. (1963), "Il corredo cromosom pg e il problema dei cromosomi sessuali in *Bufo viridis* Laur," *Rend. Accad. Sci. Fis. Mat. Ser. 4*, **30**(anno 102), 236 – 240.

Mészáros B. (1973), "Critical studies on karyotypes of eight anuran species from Hungary and some problems concerning the evolution of the order," *Acta Biol. Debrecina*, **10 – 11**(1972 – 1973), 151 – 161.

Odierna G., Aprea G., Capriglione T., Castellano S., and Balletto E. (2004), "Evidence for chromosome and *Pst I* satellite DNA family evolutionary stasis in the *Bufo viridis* group (Amphibia, Anura)," *Chromosome Res.*, **12**, 671 – 681.

Pisanets E. M. (1978), "On a new polyploid species of toads *Bufo danatensis* Pisanetz sp. n. from Turkmenia," *Dokl. AN USSR Ser. B Geol. Geogr. Khim. Biol.*, **3**, 280 – 284 [in Russian] or 277 – 282 [in Ukrainian].

Pisanets E. M. (1991), "The new data on karyology of the *Bufo viridis* complex (Amphibia, Bufonidae) and the questions of origin of Asiatic tetraploid toads," in: V. A. Cherlin and A. Yu. Tsellarius (eds.), *Gerpetologicheskiye Issledovaniya* [*Herpetological Research*], LISS, Leningrad, pp. 41 – 50 [in Russian, with English summary].

Pisanets E. M., Mezhzherin S. V., and Szczerbak N. N. (1996), "Studies on the hybridization and external morphology of Asiatic toads (Amphibia, Bufonidae), and the description of a new species, *Bufo shaartusiensis* sp. nov.," *Dopov. Nazion. AN Ukrainy Mat. Prirod. Tekhn. Nauki*, **6**, 147 – 151 [in Ukrainian].

Popov P. and Banova M. (1976), "Investigation on the karyotype of Amphibia, Anura in Bulgaria. III. Karyotype of *Bufo viridis* Laur.," *Trav. Sci. Univ. Plovdiv* "*Paissi Hilendarski*" Biol., **14**(4), 61 – 68 [in Bulgarian, with English summary].

Rosanov J. M. and Vinogradov A. E. (1998), "Precise DNA cytometry: investigation of individual variability in animal genome size," *Tsitologiya*, **40**(8/9), 792 – 800 [in Russian].

Roth P. (1986), "An overview of the systematics of the *Bufo viridis* group in Middle and Central Asia," in: Z. Roček (ed.), *Studies in Herpetology*, Charles University, Prague, pp. 127 – 130.

Roth P. and Ráb P. (1986), "Karyotype analysis of the *Bufo viridis* group: systematic implications," in: Z. Roček (ed.), Studies in Herpetology, Charles University, Prague, pp. 131 – 135.

Roth P. and Ráb P. (1987), "Sequential chromosome banding studies in the natterjack toad, *Bufo calamita*," in: J. J. van Gelder, H. Strijbosch, and P. J. M. Bergers (eds.), *Proc. of the 4th Ord. Gen. Meeting of the Soc. Eur. Herpetol.*, Nijmegen, pp. 335 – 338.

Schmid M. (1978), "Chromosome banding in Amphibia. I. Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla*," *Chromosoma*, 66, 361 – 388.

Stöck M. (1997), "Untersuchungen zur Morphologie und Morphometrie di- und tetraploider Grünkröten (*Bufo viridis* – Komplex) in Mittelasien (Amphibia: Anura: Bufonidae)," *Zool. Abh. Staatl. Mus. Tierkunde Dresden*, **49**(12), 193 – 222.

Stöck M. (1998), "Tetraploid toads (*Bufo viridis* complex) from north-western China and preliminary taxonomic conclusions for *Bufo nouettei* Mocquard, 1910," *Z. Feldherpetol.*, **5**, 139 – 166.

Stöck M. and Lamatsch D. K. (2002), "Triploide Wirbeltiere. Wege aus der Unfruchtbarkeit oder Eingeschlechtigkeit," *Naturwissenschaftl. Rundschau*, **55**(7), 349 – 358.

Stöck M., Frynta D., Grosse W. -R., Steinlein C., and Schmid M. (2001a), "A review of the distribution of diploid, triploid and tetraploid green toads (*Bufo viridis* complex) in Asia, including new data from Iran and Pakistan," *Asiatic Herpetol. Res.*, **9**, 77 – 100.

Stöck M., Günther R., and Böhme W. (2001b), "Progress towards a taxonomic revision of the Asian *Bufo viridis* group: current status of nominal taxa and unsolved problems (Amphibia: Anura: Bufonidae)," *Zool. Abh. Staatl. Mus. Tierkunde Dresden*, **51**, 253 – 319.

Stöck M., Lamatsch D. K., Steinlein C., Epplen J. T., Grosse W. -R., Hock R., Klapperstück T., Lampert K. P., Scheer U., Schmid M., and Schartl M. (2002), "A bisexually reproducing all-triploid vertebrate," *Nat. Gen.*, **30**(3), 325 – 328.

Stöck M., Schmid M., Steinlein C., and Grosse W.-R. (1999), "Mosaicism in somatic triploid specimens of the *Bufo viridis* complex in the Karakoram with examination of calls, morphology and taxonomic conclusions," *Ital. J. Zool.*, **66**(3), 215 – 232.

Stöck M., Steinlein C., Lamatsch D. K., Schartl M., and Schmid M. (2005), "Multiple origins of tetraploid taxa in the Eurasian *Bufo viridis* subgroup," *Genetica*, **124**, 255 – 272.

Ullerich F. H. (1966), "Karyotyp und DNS-Gehalt von *Bufo bufo*, *B. viridis*, *B. bufo* × *B. viridis* und *B. calamita* (Amphibia, Anura)," *Chromosoma*, **18**(2), 316 – 342.

Wickbom T. (1945), "Cytological studies on Dipnoi, Urodela, Anura, and *Emys*," *Hereditas*, **31**(3 – 4), 241 – 346.