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Influence of environmental conditions on the distribution of Central Asian green toads with three ploidy levels

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Abstract

We studied the distribution of Palearctic green toads (*Bufo viridis* subgroup), an anuran species group with three ploidy levels, inhabiting the Central Asian Amudarya River drainage. Various approaches (one-way, multivariate, components variance analyses and maximum entropy modelling) were used to estimate the effect of altitude, precipitation, temperature and land vegetation covers on the distribution of toads. It is usually assumed that polyploid species occur in regions with harsher climatic conditions (higher latitudes, elevations, etc.), but for the green toads complex, we revealed a more intricate situation. The diploid species (*Bufo shaartusiensis* and *Bufo turanensis*) inhabit the arid lowlands (from 44 to 789 m a.s.l.), while tetraploid *Bufo pewzowi* were recorded in mountainous regions (340–3492 m a.s.l.) with usually lower temperatures and higher precipitation rates than in the region inhabited by diploid species. The triploid species *Bufo baturae* was found in the Pamirs (Tajikistan) at the highest altitudes (2503–3859 m a.s.l.) under the harshest climatic conditions.

Key words: Polyploidy – genome size – environmental conditions – *Bufo viridis* subgroup

Introduction

Polyploidy has played a significant role in the evolution and speciation of fungi (yeast), plants and animals. Although in animals polyploidy is rarer than in plants, numerous polyploidization events have been documented in vertebrates (Otto and Whitton 2000; Gregory and Mable 2005). Among anurans, 36 naturally occurring bisexual polyploid species have been reported, belonging to 16 genera of eight families (King 1990; Otto and Whitton 2000).

Speciation by polyploidization involves hybridization between two species (allopolyploidy) or, alternatively, the duplication of a single species' genome (autopolyploidy). To be evolutionarily established, a polyploid lineage must successfully compete with its parental species or be able to colonize new environments. For all these reasons, the occurrence of many polyploid species in arid, arctic and alpine habitats suggests that polyploids have an increased tolerance for harsh environments (Beaton and Hebert 1988). Among amphibians, the polyploid complexes for which environmental conditions have been comparatively examined, there are the Nearctic tree frogs of the *Hyla versicolor* complex, where tetraploid *H. versicolor* usually occupies areas with harsher conditions (higher

elevation, colder temperatures and lower precipitation) than diploid *Hyla chrysoscelis* (Otto et al. 2007).

Polyploidy is the most important process involved in the evolution of the Western Palearctic green toads of the *Bufo viridis* subgroup. The taxonomy of this subgroup is still under debate, and some authors placed them in the new genus *Pseudepidalea* or subgenus *Bufotes* (see Frost et al. 2006; Dubois and Bour 2010). This species complex comprises diploid, triploid and tetraploid bisexually reproducing taxa (Stöck et al. 2001, 2006, 2010; Litvinchuk et al. 2006). Diploid and tetraploid toads reproduce meiotically (Stöck et al. 2005), while triploid *Bufo baturae* use a reproductive mechanism related to hybridogenesis, with females producing diploid eggs and males generating haploid sperm (Stöck et al. 2002).

It is usually considered that the Amudarya River drainage is mainly inhabited by diploid *Bufo turanensis* Hemmer, Schmidtler and Böhme, 1978 and tetraploid *Bufo pewzowi* Bedriaga, 1898 (Stöck et al. 2006). Based on allozyme and crossing experiments, diploid toads from the Beshkent sands (south-western Tajikistan) were described as a separate species (e.g.: *Bufo shaartusiensis* Pisanets, Mezhzherin & Shcherbak, 1996). The southernmost part of Tajik Pamirs is inhabited by triploids (Litvinchuk et al. 2006). Finally, the bisexual triploid species *B. baturae* Stöck, Schmid, Steinlein & Grosse, 1999 has been recorded in the neighbouring territory of Pakistan (Stöck et al. 2001, 2006, 2010).

Based on allozyme data, Mezhzherin and Pisanets (1995) suggested that tetraploids (referred to as *Bufo danatensis* Pisanets 1978) as well as triploids (considered by these authors as tetraploids) from the Tajik Pamirs are the results of hybridization involving diploid *B. turanensis* (referred to as

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B. viridis turanensis) and an unknown species (*Bufo* sp.), later described as *B. shaartusiensis*. Stöck et al. (2010) revealed that tetraploid *B. pewzowi* from northern Kyrgyzstan represent allopolyploids, whose ancestral maternal parent is the large-sized widespread Central Asian diploid taxon (*B. turanensis*).

Diploid *B. turanensis* and tetraploid *B. pewzowi* have similar mitochondrial DNA, while triploid *B. baturae* belong to a different lineage, and the divergence time between these lineages was estimated to be between 5.7 and 10.8 Mya (Stöck et al. 2006). A complex of closely related taxa including a triploid, a tetraploid and two diploid species inhabits the Amudarya River drainage. Borkin and Darevsky (1980) speculated whether these might represent stages of possible reticulate speciation, while other authors considered triploid green toads to be the results of ongoing interactions and hybridization (e.g. Pisanets 1978; Borkin et al. 2001b), as recently shown in Kyrgyzstan (Stöck et al. 2010). Given the occurrence of toads of three ploidy levels in the large range of the Amudarya drainage characterized by wide altitudinal and various climatic conditions, we choose this region to study the influence of environmental conditions on the distribution of the diploid and polyploid amphibian species.

Material and Methods

DNA flow cytometric analysis

Two hundred and fifty-three specimens of green toads from 46 localities from the Amudarya River drainage were used for DNA flow cytometry. After anaesthesia, blood was taken from the femoral vein. Blood of grass frogs, *Rana temporaria* Linnaeus, 1758 were used as a reference standard. The details of our technique have been previously published (Borkin et al. 2001a; Litvinchuk et al. 2004). Voucher specimen locations are given in Appendix S1.

Chromosome counts

The karyotypes of green toads from six localities, which were previously studied by DNA flow cytometry, were analysed. We counted chromosomes in a male of *B. turanensis* from Uchquduq settlement (Uzbekistan; 8 metaphases), a male of *B. shaartusiensis* from Aivodzh village (Tajikistan; 4 metaphases), a female of *B. pewzowi* from Pakhtakor village (Tajikistan; 15 metaphases) and *B. baturae* from Yashilkul' Lake (Tajikistan; a female, 10 metaphases), Langar village (Tajikistan; six males, 56 metaphases) and Ishkashim village (Tajikistan; a male, 14 metaphases). Chromosomes were prepared from intestine and testes tissues, as described by Schmid (1978). Giemsa staining followed Schmid (1978). Previously published karyological data from 28 localities (Kryukov et al. 1985; Borkin et al. 1986; Roth and Ráb 1986; Pisanets 1991, 1992; Odierna et al. 2007) were added to our data set (see Appendix S1).

Mitochondrial DNA analysis

To evaluate relationships between diploid and polyploid green toad taxa, we analysed a fragment of the mitochondrial 16S rRNA gene (ca. 480 bp). The total genomic DNA of five specimens of *B. shaartusiensis*, one specimen of *B. turanensis* (Ashgabad: 37°56'N, 58°22'E), four specimens of *B. baturae* from Tajikistan and Pakistan (Karimabad: 36°18'N, 74°41'E), three specimens of *B. pewzowi* from Tajikistan (Khujand: 40°17'N, 69°38'E) were extracted using a proteinase K digestion (10 mg ml⁻¹) followed by a standard salt-extraction protocol (Bruford et al. 1992). PCR was used to amplify a fragment of 16S rRNA gene following the protocol used in Vences et al. (2000). Sequencing was carried out using BigDye version 3.1 chemistry (Applied Biosystems, Foster City, CA, USA) on an ABI 3130XL sequencer. Sequences were edited and aligned using MEGA 4.1 (Kumar et al. 2008). GenBank accession numbers are presented in Appendix S1.

The best-fit model of nucleotide evolution was determined under Akaike information criterion in jModeltest (Posada 2008). Maximum likelihood (ML) trees were generated using PHYML version 2.4.5 (Guindon and Gascuel 2003) under a GTR model. We choose a BioNJ as a starting tree and the options to optimize the topology, branch length and rate parameters. All other parameters were used as in the default setting of the program (<http://www.atgc-montpellier.fr/phyml/> for details). We generated bootstrap values based on 1000 resampled data sets. Phylogeny was also inferred using the program MRBAYES (v. 3.1.2; Ronquist and Huelsenbeck 2003), running four chains for 10 million generations, with tree sampling every 1000 generations. The 'burnin'-value was selected by visualizing the log likelihoods associated with the posterior distribution of trees in the program Tracer. All trees generated before the log likelihood curve flattened out were discarded. We constructed 16S rRNA gene trees using thirteen newly provided sequences and previously published samples (Liu et al. 2000; Van Bocxlaer et al. 2009): *B. pewzowi* from China (Hutubi, Xinjiang; AF160796) and Uzbekistan (FJ882811); *Bufo raddei* from China (Gulang, Gansu; AF160795); and *Bufo surdus* from Iran (Sistan-Baluchistan; FJ882810). The two last species were used as outgroups.

Environmental data sets

We extracted the altitude and climatic data (1950–2000; ~1 km resolution; WORLDCLIM 1.4, <http://www.worldclim.org/current>; Hijmans et al. 2005), as well as percentage of tree, herbaceous (Herb) and bare (without vegetation) land covers (2000–2001; ~0.5 km resolution; <http://www.landcover.org>; Hansen et al. 2002) for each locality from a set of GIS layers using the program ENVI 4.3[®]. Eight climatic characters with high physiological importance for the studied species were used in statistical analyses: total annual precipitation, precipitation of the 3 months (April–June) corresponding to the breeding season, precipitation during 3 months of winter, mean annual temperature, mean breeding season (April–June) temperature, mean winter temperature, minimum temperature of the coldest month and maximum temperature of the warmest month.

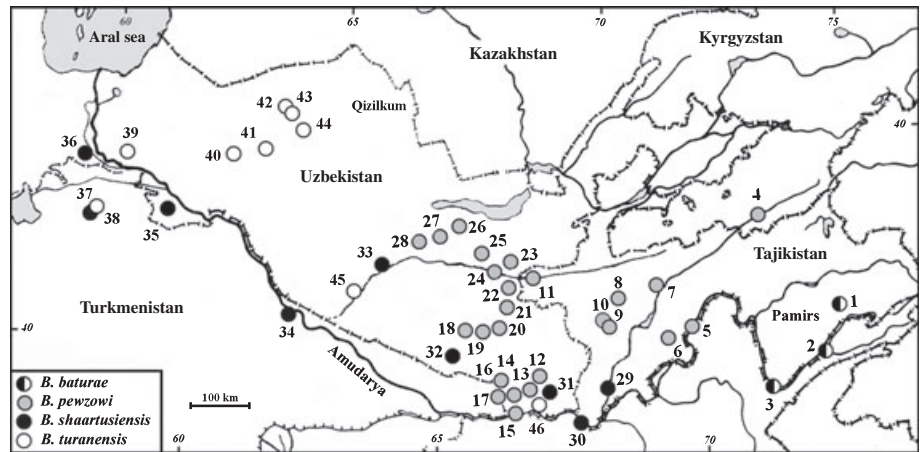
Statistical analyses and environmental modelling

Reliability of differences between sample means was estimated using the Kolmogorov–Smirnov test. To test the segregation of distributional patterns according to ploidy along the climatic variables, vegetation land covers and elevation, a principal component (PCA) and a partial least squares regression components (PLSR) analyses were performed. For the principal component analysis, land covers percentage data were recalculated to scores of a principal component axis. Statistical analyses were performed with STATISTICA 6.0. Maximum entropy modelling (Phillips et al. 2006) of geographical distribution was computed with Maxent 3.3.3a software (<http://www.cs.princeton.edu/~schapire/maxent>) using the altitudes and 19 bioclimatic variables (Bio1–19). In Maxent analysis, eleven additional localities of *B. baturae* from the adjacent territory of Pakistan (Stöck et al. 2006; Ficetola et al. 2010) were used. Models were developed using 75% of the data randomly selected as training data and the remaining 25% for testing the model.

Results

In the Amudarya River drainage (Fig. 1), we found four groups of green toads with different genome size. The group with the largest amount of nuclear DNA (19.57–21.26 pg; Table S1) contained tetraploid *B. pewzowi* ($4n = 44$). Triploid *B. baturae* ($3n = 33$) were characterized by intermediate genome size (14.86–16.05 pg). Two groups with lower nuclear DNA contents consisted of diploids ($2n = 22$). Diploid green toads with the largest genome size (11.23–11.85 pg) were found in the Beshkent sands (south-western Tajikistan, locality 30 in Table S1), from where *B. shaartusiensis* was described. Diploids with the smallest genome size (9.69–10.21 pg) were

Fig. 1. Localities of *Bufo shaartusiensis*, *Bufo turanensis*, *Bufo baturae* and *Bufo pewzowi* in the Amudarya River drainage. Numbers of localities as in Table S1



attributed by us to *B. turanensis*. The nuclear DNA content in *B. shaartusiensis* samples does not overlap with those in *B. turanensis* (Fig. 2). The gap between the largest genome size in *B. turanensis* and the smallest in *B. shaartusiensis* was equal to 1.02 pg. The range of nuclear DNA content in triploid *B. baturae* does not overlap with any other genome sizes of the studied species. The gap between the highest value in *B. shaartusiensis* and the lowest one in *B. baturae* comprised 3.01 and 3.52 pg between the highest value in *B. baturae* and the lowest in *B. pewzowi*. The differences between means of all these species were significant ($p < 0.001$).

Maximum likelihood (ML)-based and Bayesian (MB) phylogenetic analyses distinguished two well-supported clades (ML: bootstrap values 99%, MB: posterior probability support 96–99%). Diploid and polyploid toads were found in both clades. Diploid *B. turanensis* and tetraploid *B. pewzowi* belonged to one clade, whereas diploid *B. shaartusiensis* and triploid *B. baturae* to another one (Fig. 3). Triploids from Tajikistan belonged to the same clade of the *B. baturae* from Pakistan.

In the Amudarya River drainage, the distribution of green toads with different ploidy levels clusters with the altitude (Tables S2 and S3; Fig. 4), with a clear dominance of polyploids at higher elevations (diploids: 44–789 m a.s.l.; polyploids: 340–4000 m a.s.l.; see Appendix S1). Diploid populations are widespread in deserts in the western part of the region. Among diploids, both species inhabit lowlands along Amudarya River and the lower part of Zerafshan River (Fig. 1). However, *B. shaartusiensis* was not recorded in the Qizilkum (=Kyzylkum) desert, and *B. turanensis* was not found in the Tajik Depression. In its range, *B. turanensis* is common near desert springs with very scarce vegetation (Table S2). Sympatric populations of both diploid species were only found in the vicinities of Shasenem village (Turkmenistan; localities 37 and 38 in Table S1). Polyploids occupy the eastern mountains of the Amudarya drainage. Altitudinal limits of distribution for tetraploid *B. pewzowi* are 340–3492 m a.s.l. The uppermost high-altitude localities were inhabited only by triploid *B. baturae* (2503–3859 m a.s.l.). In the Amudarya River drainage, this species was only observed in mountains in

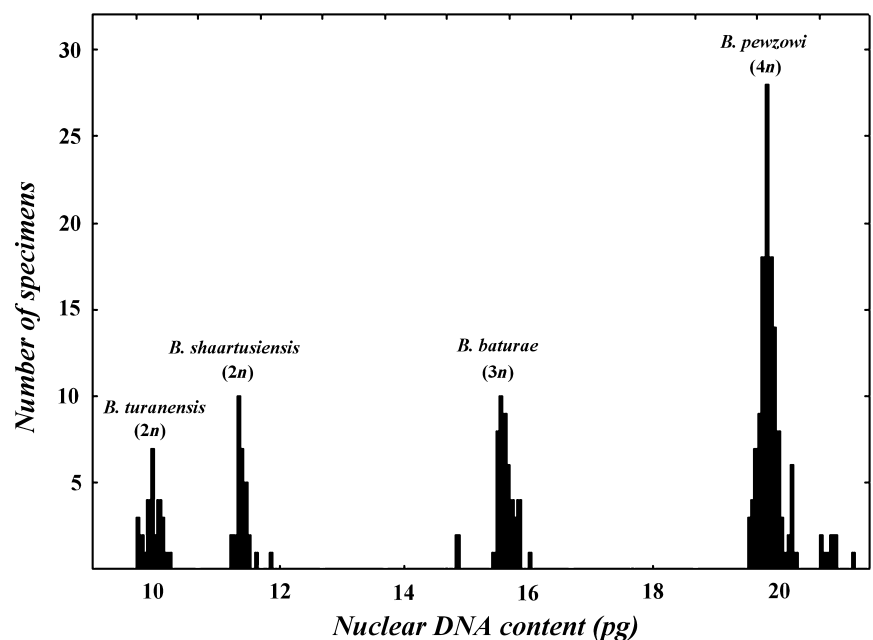


Fig. 2. Distribution of nuclear DNA content values in green toads from the Amudarya River drainage

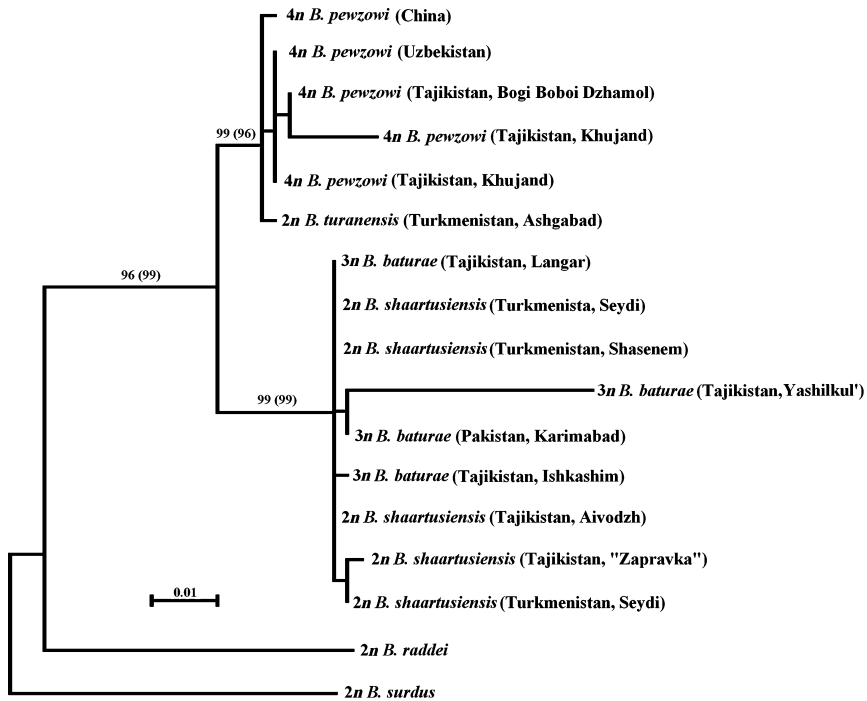


Fig. 3. Maximum likelihood-based phylogram (16S rRNA gene) for various species of diploid, triploid and tetraploid green toads. Numbers at nodes are bootstrap values in per cent from maximum likelihood bootstrap analyses; numbers in parentheses are posterior support values as obtained in the program MRBAYES 3.1.2; only bootstrap values >90% are reported

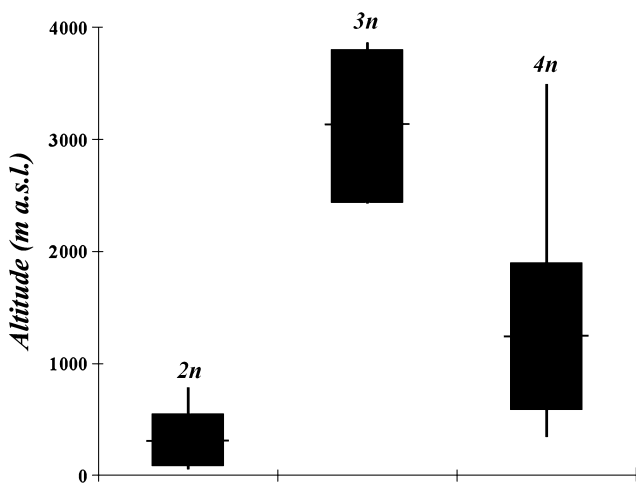


Fig. 4. Variation (mean \pm SD and range) of elevations for diploid, triploid and tetraploid green toads from the Amudarya River drainage

the south-western part of the Tajik Pamirs (south of the Rushan and North-Alichur ridges).

The mean temperatures at localities inhabited by diploids were the highest (Table S2). In average, localities of tetraploid toads were characterized by 0.5–3.7°C lower temperatures. The lowest temperatures were registered for localities inhabited by triploids (on average 9.6–12.9°C lower than for diploids and 8.1–9.1°C lower than for tetraploids). The mean precipitations at localities inhabited by diploid and triploid green toads were 1.4–2.4 times lower than at localities inhabited by tetraploids (Table S2).

Kruskal–Wallis ANOVA tests revealed that all measured environmental variables significantly affect the distribution of green toads with different ploidy levels (Table S3). The first PCA axis, explaining 61% of the total variance, differentiated altitude and temperature, and the second axis (29%) winter

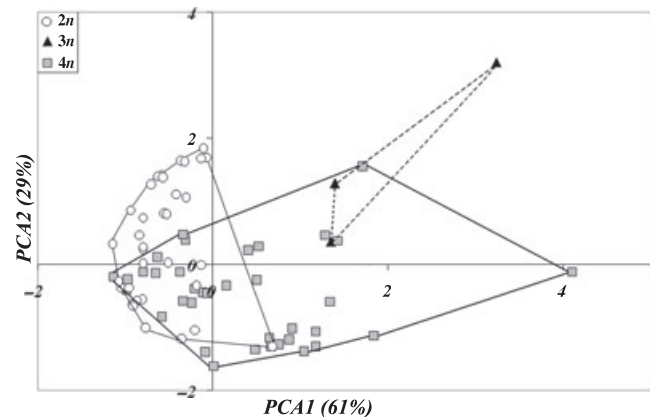


Fig. 5. Principal component analysis plot performed on elevational, vegetation land covers and climatic parameters for different localities of green toads from the Amudarya River drainage

precipitation and vegetation land covers. In the PCA plot (Fig. 5), the environmental space occupied by diploids does not overlap with that of triploids. At the same time, the space of tetraploids overlaps with both diploids and triploids. PLSR analysis provided two significant components. The first component accounted for a major proportion of the explained variance (92.6%) and is mainly associated with precipitation (especially in winter). The information content of the second component (7.4%) was mainly associated with altitude (Table S3).

A heuristic estimate of relative contributions of the climatic and elevation parameters to the Maxent models revealed that distributions of green toads with different ploidy levels are mostly associated with the summer (explained 59.6% of the distribution of diploids, 13.4% of tetraploids and 22.2% of triploids) and winter (42.0% of tetraploids and 13.3% of triploids) precipitations. The impact of other parameters was lower than

12.9%. Exceptions were the diurnal range (16.0%) and annual (15.9%) mean temperatures for triploids. The accuracy of the Maxent models (potential ranges are given in Fig. 6) was very high (training data AUC: 98% for diploids, 100% for triploids and 98% for tetraploids).

Discussion

It is usually assumed that polyploids are capable of tolerating and invading harsher environments than their diploid counterparts (Soltis and Soltis 2000; Comai 2005). Patterns of habitat segregation between diploids and polyploids consistently show that polyploids occur in relatively harsher habitats (Schlosser et al. 1998; Johnson et al. 2003), and the establishment of polyploid populations in 'higher quality' habitat is probably prevented by direct competition with the progenitor(s), while range expansion of the progenitor into areas occupied by the polyploids might be limited by the physical environment (Otto and Whitton 2000; Adamowicz et al. 2002).

In accordance with this observation, in the Amudarya River drainage, we found that the polyploid species occur in mountainous areas with presumably harsher environments. However, by comparing detailed climatic conditions, we found a more intricate picture. The majority of localities with relatively higher precipitation and lower temperatures are inhabited by tetraploids (Table S2). In contrast, diploids are

usually widespread in deserts with distinctly more arid conditions (scarce vegetation, low precipitation and high temperatures). Low rainfall is typical of territories inhabited by diploids, which usually breed in temporary swamps of flooding regions or in ephemeral water bodies of oases (our field observations). On the other hand, triploid *B. baturoae* are distributed at high-altitude deserts with the most challenging environments (low precipitation and temperatures, scarce vegetation; Table S2). In winter, localities inhabited by this species can be characterized by thin snow layer, strong winds, and temperatures can decrease down to -32°C (observations of the Bulunkul' meteorological station, 2000–2005). Our anecdotal evidence might confirm this result: during a fieldtrip in the vicinities of Yashilkul' Lake (June 2, 2009, Tajikistan), one of us (G.M.) observed several *B. baturoae* freezing overnight, which survived this natural experiment without visible problems.

Green toads are considered typical 'steppe species' (e.g. Stöck et al. 2008). Low temperatures coupled with higher precipitation in high mountains might be 'extreme' for diploid green toads, which usually inhabit drier desert and steppe regions. Triploidy in the Amudarya River drainage might be an ecological transitional stage from diploidy to tetraploidy (Borkin and Darevsky 1980; Otto and Whitton 2000).

The nuclear genome compositions of triploids and tetraploids are largely unknown. Once the genetic material of two species is present in the same nucleus, a variety of intergenomic exchange events ('intergenomic recombination') can be expected to occur (Mable 2007), as it has been shown in hybridogenetic frogs (Ogielska et al. 2004) and gynogenetic/kleptogenetic salamanders (Bi and Bogart 2006). Therefore, such interactions may also contribute to the observed distributional patterns.

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Zusammenfassung

Einfluss von Umweltbedingungen auf die Verbreitung zentralasiatischer Wechselkröten mit drei Ploidiestufen

Wir untersuchen die Verbreitung Paläarktischer Wechselkröten (*Bufo viridis* subgroup), einer Gruppe eng verwandter Anurenarten mit drei Ploidiestufen, in Zentralasien die Einzugsgebiet des Amu-Darja bewohnen. Unterschiedliche Methoden (uni- und multivariate und Komponentenvarianzanalyse, Maximum-Entropie-Model) wurden genutzt, um den Einfluss von Höhenlage, Niederschlag, Temperatur und Landbedeckung auf die Verbreitung der Kröten abzuschätzen. Es wird allgemein angenommen, dass polyploide Pflanzen- und Tierarten in Regionen mit härteren Klimabedingungen vorkommen (höhere Breiten, grössere Höhenlagen, etc.). Die Situation ist bei Grünkröten jedoch komplizierter. Die diploiden Arten (*Bufo shaartusiensis* und *B. turanensis*) bewohnen die ariden Tieflandsgebiete (Höhen zwischen 44 und 789 m über dem Meeresspiegel), während die tetraploiden



Fig. 6. Predicted potential geographical distribution for diploid (*Bufo shaartusiensis* and *B. turanensis*), triploid (*Bufo baturoae*) and tetraploid (*Bufo pewzowi*) species of green toads inhabiting the Amudarya River drainage and the adjacent territories made using all known records (squares) and 20 environmental variables. All grey areas likely represent suitable environmental conditions (dark areas are high suitability, and light ones are less suitable)

B. pewzowi in Gebirgsregionen gefunden wurden, die gewöhnlich niedrigere Temperaturen und höheren Niederschlag aufwiesen als die Verbreitungsgebiete der diploiden Formen. Triploide *B. baturae* wurden im Pamir (Tadschikistan) in den grössten Höhenlagen (2503–3859 m a.s.l.) und unter den extremsten Klimabedingungen angetroffen.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Nuclear DNA content (pg) in various populations of diploid (*Bufo shaartusiensis* and *B. turanensis*), triploid (*Bufo baturae*) and tetraploid (*Bufo pewzowi*) species of green toads inhabiting the Amudarya River drainage.

Table S2. Environmental characters variation and reliability of differences between sample means (the Kolmogorov–Smirnov test; $p > 0.05$ is ‘–’; $p < 0.05$ is ‘+’; $p < 0.01$ is ‘++’; $p < 0.001$ is ‘+++’) for localities of toads (*Bufo viridis* subgroup) with various ploidy levels in the Amudarya River drainage.

Table S3. Results of the Kruskal–Wallis one-way analysis of variance (KWA) and weights of each variable in the first and second partial least squares regression (PLSR) components analysis.

Appendix S1. List of localities, ploidy levels (PL), geographical co-ordinates, altitudes, methods of ploidy determination (FC is flow DNA cytometry, and CH is chromosomes count), GenBank numbers, museum collection numbers and references.

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1 Supporting Information

2 Table S1. Nuclear DNA content (pg) in various populations of diploid (*Bufo shaartusiensis* and *B. turanensis*), triploid (*B.*
3 *baturae*), and tetraploid (*B. pewzowi*) species of green toads inhabiting the Amudarya River Drainage.

N	State, Province	Locality	Lat	Long	Alt	n	Mean ± SD	Range	CV%
<i>Bufo baturae</i> (3n)									
1	Tajikistan, Badakhshon	Yashilkul' Lake	37°44'	72°53'	3859	20	15.56 ± 0.08	15.47 – 15.88	0.54
2	--	Langar	37°01'	72°39'	2982	25	15.68 ± 0.28	14.86 – 16.05	1.77
3	--	Ishkashim	36°43'	71°37'	2503	19	15.68 ± 0.14	15.43 – 16.00	0.89
Total for the species						64	15.62 ± 0.21	14.86 – 16.05	
<i>Bufo pewzowi</i> (4n)									
4	Kyrgyzstan, Issyk-Kul'skaya	Daroot-Korgon	39°33'	72°11'	2462	2	19.65	19.62 – 19.67	–
5	Tajikistan, Badakhshon	Bogi Boboi Dzhamol	38°02'	70°22'	1642	9	19.68 ± 0.11	19.57 – 19.84	0.54
6	Tajikistan, Khatlon	Pakhtakor	38°00'	69°43'	790	2	19.76	19.69 – 19.83	–
7	Tajikistan, RRS	Obi-Garm	38°43'	69°41'	1493	6	19.84 ± 0.06	19.77 – 19.94	0.33
8	--	Ziddi	38°46'	69°22'	3492	1	21.26	–	–
9	--	Dushambe	38°32'	68°49'	749	11	20.00 ± 0.13	19.81 – 20.23	0.67
10	--	Luchob	38°42'	68°41'	1130	8	20.83 ± 0.10	20.70 – 20.95	0.46

11	Tajikistan, Sugd	Panjikent	39°29'	67°35'	1017	8	19.86 ± 0.06	19.78 – 19.99	0.32
12	Uzbekistan, Surxondaryo	Istara	37°42'	67°17'	397	8	19.88 ± 0.08	19.80 – 20.01	0.39
13	--	Sherobod	37°42'	67°04'	406	3	19.72 ± 0.04	19.67 – 19.74	0.20
14	--	Xalqobod	37°28'	66°55'	340	4	19.89 ± 0.05	19.83 – 19.93	0.24
15	--	Pashkhurd Valley	37°43'	66°43'	923	4	20.09 ± 0.07	20.03 – 20.20	0.35
16	--	Kugitang Mts.	37°54'	66°39'	2049	1	20.29	–	–
17	--	Kelif-Sherabad Mts.	37°45'	66°33'	1979	6	20.23 ± 0.04	20.16 – 20.27	0.18
18	Uzbekistan, Qashqadaryo	Khanabad	38°52'	66°00'	402	12	19.94 ± 0.05	19.84 – 20.00	0.25
19	--	G'uzor	38°37'	66°15'	540	3	19.73 ± 0.05	19.69 – 19.79	0.28
20	--	Kattatal	38°46'	66°37'	731	3	19.70 ± 0.06	19.63 – 19.75	0.31
21	--	Yukary-Akbai	39°11'	66°59'	740	5	19.80 ± 0.09	19.68 – 19.91	0.46
22	Uzbekistan, Samarqand	biostantsiya SGU	39°22'	67°20'	1262	1	19.88	–	–
23	--	Jumabozor	39°31'	67°13'	790	2	19.96	19.92 – 20.00	–
24	--	Samarqand	39°40'	66°57'	694	4	19.86 ± 0.09	19.76 – 19.98	0.46
25	--	Karateri	39°49'	66°36'	574	8	19.86 ± 0.08	19.77 – 19.99	0.40
26	--	Nuratau Ridge	40°35'	66°30'	1188	6	19.84 ± 0.08	19.74 – 19.93	0.42
27	Uzbekistan, Navoiy	Aktepa	40°21'	65°26'	582	10	19.82 ± 0.09	19.60 – 19.84	0.44

28	--	Kanimekh	40°16'	65°09'	322	4	19.84 ± 0.09	19.76 – 19.93	0.45
Total for the species						131	19.94 ± 0.30	19.57 – 21.26	

Bufo shaartusiensis (2n)

29	Tajikistan, Khotlon	“Zapravka”	37°30'	68°27'	357	4	11.37 ± 0.05	11.33 – 11.44	0.40
30	--	Aivodzh	36°58'	68°01'	315	8	11.41 ± 0.09	11.35 – 11.64	0.83
31	Uzbekistan, Surxondaryo	Jarqo'rg'on	37°31'	67°27'	324	2	11.49	11.46 – 11.52	–
32	Uzbekistan, Qashqadaryo	Talimardzhan	38°23'	65°38'	385	1	11.31	–	–
33	Uzbekistan, Buxaro	Jondor	39°45'	64°12'	207	1	11.35	–	–
34	Turkmenistan, Lebap	Seydi	39°25'	62°54'	175	3	11.33 ± 0.03	11.31 – 11.37	0.28
35	Uzbekistan, Xorazm	Khonqa	41°29'	60°46'	90	8	11.39 ± 0.11	11.23 – 11.51	–
36	Uzbekistan, Qoraqalpog`iston	Qunghirot	42°49'	59°00'	53	1	11.85	–	–
37	Turkmenistan, Dasoguz	Shasenem	41°35'	58°43'	62	2	11.40	11.40 – 11.40	–
Total for the species						30	11.41 ± 0.12	11.23 – 11.85	

Bufo turanensis (2n)

38	Turkmenistan, Dasoguz	Shasenem	41°35'	58°43'	62	5	10.08 ± 0.09	9.98 – 10.21	0.93
39	Uzbekistan, Qoraqalpog`iston	Nukus	42°22'	59°34'	71	2	9.83	9.77 – 9.89	–
40	Uzbekistan, Navoiy	Dzharakuduk	42°06'	62°36'	80	1	9.85	–	–

41	--	Mingbulak	42°13'	62°51'	44	5	9.81 ± 0.11	9.87 – 9.96	1.15
42	--	Bakhaly	42°40'	63°23'	448	6	9.92 ± 0.03	9.87 – 9.96	0.31
43	--	Jamanuru	42°39'	63°19'	387	1	10.00	–	–
44	--	Uchquduq	42°07'	63°31'	193	2	9.82	9.69 – 9.96	–
45	Uzbekistan, Buxaro	Kyzyl-Ravat	39°35'	63°59'	201	4	10.09 ± 0.04	10.05 – 10.15	0.42
46	Uzbekistan, Surxondaryo	Katta-Kum Sands	37°23'	67°18'	327	2	10.18	10.14 – 10.21	–
Total for the species							28	9.96 ± 0.15	9.69 – 10.21

4 Lat: latitude North; Long: longitude East; Alt: altitude (in meters above sea level).

5 Table S2. Environmental characters variation and reliability of differences between sample means (the Kolmogorov-Smirnov
6 test; $p > 0.05$ is “-”; $p < 0.05$ is “+”; $p < 0.01$ is “++”; $p < 0.001$ is “+++”) for localities of toads (*Bufo viridis* subgroup) with
7 various ploidy levels in the Amudarya River Drainage.

Ploidy	n	Alt	AP	BSP	WP	AT	BST	WT	MTC	MTW	Bare	Herb	Tree
2n	34	297 ± 220 (44–789)	221 ± 143 (109–689)	61 ± 43 (30–222)	79 ± 50 (29–234)	14.5 ± 2.1 (10.6–17.7)	21.3 ± 1.5 (16.8–23.3)	1.3 ± 3.5 (-5.0–5.5)	-5.0 ± 3.5 (-10.7–-0.9)	36.2 ± 1.6 (32.0–38.8)	58 ± 35 (7–100)	41 ± 34 (0–88)	1 ± 2 (0–6)
2nS	9	219 ± 131 (53–385)	167 ± 59 (109–272)	43 ± 13 (30–69)	64 ± 30 (29–110)	15.1 ± 1.9 (11.7–17.2)	22.0 ± 1.1 (19.8–23.3)	2.4 ± 3.0 (-2.7–5.5)	-4.1 ± 3.1 (-9.2–-1.0)	37.0 ± 1.3 (34.7–38.8)	47 ± 31 (23–100)	52 ± 30 (0–75)	1 ± 1 (0–2)
2nT	8	219 ± 154 (44–448)	129 ± 23 (109–176)	37 ± 4 (32–43)	45 ± 15 (30–75)	13.4 ± 2.0 (10.9–17.0)	21.1 ± 1.4 (19.2–23.3)	-1.0 ± 3.4 (-4.4–5.4)	-7.4 ± 3.3 (-10.7–-1.3)	36.2 ± 1.3 (34.6–38.5)	88 ± 22 (50–100)	12 ± 22 (0–49)	0 ± 0 (0–1)
3n	3	3114 ± 688 (2503–3859)	289 ± 96 (219–398)	107 ± 33 (86–145)	87 ± 33 (65–125)	4.1 ± 6.0 (-2.7–8.2)	8.8 ± 5.9 (2.0–12.7)	-8.4 ± 6.3 (-15.6–-3.8)	-14.6 ± 6.4 (-21.9–-10.0)	23.3 ± 5.9 (16.5–27.1)	54 ± 40 (27–100)	45 ± 39 (0–71)	1 ± 1 (0–2)
4n	36	1147 ± 671 (322–3492)	461 ± 215 (175–1030)	145 ± 80 (39–364)	159 ± 64 (73–316)	12.2 ± 3.8 (-1.6–17.2)	17.7 ± 4.1 (2.8–23.5)	0.7 ± 3.8 (-12.7–5.5)	-5.5 ± 3.9 (-19.1–-0.9)	32.5 ± 4.1 (17.4–38.4)	32 ± 15 (7–84)	66 ± 14 (16–91)	2 ± 2 (0–8)
2n/3n		++	-	+	-	++	++	+	+	++	-	-	-
2n/4n		+++	+++	+++	+++	+	+++	-	-	+++	+	+	+
3n/4n		+	-	-	-	+	+	+	+	+	-	-	-
2nS/2nT		-	-	-	-	-	-	-	-	-	+	+	-

8 Mean ± standard deviation and range (in round squares). n is number of localities studied. Alt is altitude (m a.s.l.); AP is total
9 annual precipitation (mm); BSP is precipitation of the three months (April–June) corresponding to the breeding season (mm);
10 WP is precipitation during three months of winter (mm); AT is mean annual temperature (°C); BST is mean breeding season
11 (April–June) temperature (°C); WT is mean winter temperature (°C); MTC is minimum temperature of the coldest month (°C);
12 and MTW is maximum temperature of the warmest month (°C). 2nS is *B. shaartusiensis*, and 2nT is *B. turanensis*.

13 Table S3. Results of the Kruskal-Wallis one-way analysis of variance (KWA) and weights of each variable in the first and
 14 second partial least squares regression (PLSR) components analysis.

Analysis		Alt	AP	BSP	WP	AT	BST	WT	MTC	MTW	Bare	Herb	Tree
KWA	<i>H</i>	44.2800	26.4214	28.5635	26.3747	15.1985	25.5611	7.4466	7.2457	29.2391	8.3809	7.8631	9.8308
	<i>p</i>	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0005	< 0.0001	0.0242	0.0267	< 0.0001	0.0151	0.0196	0.0073
PLSR	Comp1	0.133	0.298	0.283	0.336	0.034	0.084	0.001	0.002	0.086	0.181	0.179	0.094
	Comp2	0.613	0.304	0.301	0.338	0.323	0.427	0.207	0.201	0.447	0.183	0.180	0.094

15 Elevational and climatic data abbreviations as in Table 2.

16

Appendix. List of localities, ploidy levels (PL), geographic co-ordinates, altitudes, methods of ploidy determination (FC is flow DNA cytometry, and CH is chromosomes count), GenBank numbers, museum collection numbers and references.

N	Locality	PL	Latitude	Longitude	Altitude	Method	GB	Source
1	Yashilkul' Lake	3n	37.7253	72.8873	3859	FC	HQ843038	KhNU
2	Lyangar	3n	37.0215	72.6545	2982	--/	HQ843039	--/
3	Ishkashim	3n	36.7194	71.6195	2503	--/	HQ843037	--/
4	Daroot-Korgon	4n	39.5500	72.1833	2462	--/	–	ZISP
5	Bogi Boboi Dzhamol	4n	38.0378	70.3693	1642	--/	HQ843042	KhNU
6	Pakhtakor	4n	38.0000	69.7167	790	--/	–	ZISP
7	Obi-Garm	4n	38.7174	69.6862	1493	--/	–	KhNU
8	Ziddi	4n	39.0500	69.0167	3492	--/	–	ZISP.6344
9	Dushanbe	4n	38.5260	68.8209	1310	--/	–	KhNU
10	Luchob	4n	38.7000	68.6833	1130	--/	–	ZISP.5142
11	Panjikent	4n	39.4908	67.5960	1017	--/	–	KhNU
12	Istara	4n	37.7032	67.2865	397	--/	–	--/
13	Sherobod	4n	37.6667	67.0167	406	--/	–	--/
14	Xalqobod	4n	37.4667	66.9167	340	--/	–	ZISP
15	Pashkhurt River	4n	37.7200	66.7200	923	--/	–	ZISP.5743
16	Kugitang Ridge	4n	37.9000	66.6500	2049	--/	–	ZISP
17	Kelif-Sherabad Ridge	4n	37.7500	66.5500	1979	--/	–	--/
18	Khanabad	4n	38.8564	66.0020	402	--/	–	KhNU
19	G'uzor	4n	38.6167	66.2800	540	--/	–	--/
20	Kattatal	4n	38.7667	66.6167	731	--/	–	--/
21	Yukary-Akbai	4n	39.1833	66.9833	740	--/	–	--/
22	Biostation	4n	39.3667	67.3333	1262	--/	–	--/
23	Jumabozor	4n	39.5167	67.2167	790	--/	–	--/
24	Samarqand	4n	39.6667	66.9500	694	--/	–	--/
25	Karateri	4n	39.8167	66.6000	574	--/	–	--/
26	Nuratau Nature Reserve	4n	40.5833	66.6833	900	--/	–	ZISP.5523
27	Aktepa	4n	40.3512	65.4367	582	--/	–	KhNU
28	Kanimekh	4n	40.2697	65.1507	322	--/	–	--/
29	Zappravka	2n	37.5082	68.4600	357	--/	HQ843036	--/

30	Aivodzh	2n	36.9708	68.0070	315	--/	HQ843040	--/
31	Jorqo'rg'on	2n	37.5203	67.4419	324	--/	-	--/
32	Talimardzan	2n	38.3833	65.6333	385	--/	-	--/
33	Jondor	2n	39.7388	64.1889	207	--/	-	--/
34	Seydi	2n	39.4167	62.9000	175	--/	HQ843044, HQ843045	ZISP
35	Khonqa	2n	41.4743	60.7590	90	--/	-	KhNU
36	Qunghirot	2n	42.8081	58.9917	53	--/	-	--/
37	Shasenem	2n	41.5833	58.7167	62	--/	HQ843047	ZISP.6461
38	Shasenem	2n	41.5833	58.7167	62	--/	-	ZISP.5163
39	Nukus	2n	42.3676	59.5713	71	--/	-	KhNU
40	Dzharakuduk	2n	42.1000	62.6000	80	--/	-	ZISP.7399
41	Mingbulak	2n	42.2167	62.8500	44	--/	-	KhNU
42	Bakhaly	2n	42.6690	63.3866	448	--/	-	--/
43	Jamanuru	2n	42.6515	63.3090	387	--/	-	--/
44	Uchquduq	2n	42.1167	63.5167	387	--/	-	--/
45	Kyzyl-Ravat	2n	39.5833	63.9833	201	--/	-	ZISP
46	Katta-Kum Sands	2n	37.3833	67.3000	327	--/	-	ZISP.5743
47	Khovaling	4n	38.3411	69.9794	1434	CH	-	I. A. Caune, pers. com.
48	Fayzobod	4n	38.5500	69.3167	1211	--/	-	Pisanets 1991
49	Lyangar	4n	38.7667	69.3667	1327	--/	-	I. A. Caune, pers. com.
50	Romit Canyon	4n	38.7333	69.3167	1198	--/	-	Borkin et al. 1986
51	Postigav	4n	39.4167	69.2333	1931	--/	-	Kryukov et al. 1985
52	Komarov River	4n	39.1333	70.2000	1333	--/	-	Borkin et al. 1986
53	Javroz	4n	38.7490	69.3580	1249	--/	-	Kryukov et al. 1985
54	Esanboy	4n	38.0833	68.6833	537	--/	-	Pisanets 1992
55	Aktau Ridge	4n	37.9167	68.4000	1809	--/	-	Kryukov et al. 1985
56	Chashmaobzan	4n	38.3000	66.7333	1344	--/	-	I. A. Caune, pers. com.
57	Tupalang River	4n	38.4500	67.7667	944	--/	-	Borkin et al. 1986
58	Dushanbe airport	2n	38.6333	68.8500	789	--/	-	Roth & Rab 1986
59	50 km W Dushanbe	2n	38.4833	68.2167	664	--/	-	I. A. Caune, pers. com.
60	24-90 km SW Dushanbe	2n	38.4333	68.5500	667	--/	-	Borkin et al. 1986
61	Kurgan-Tyube	2n	37.8333	68.7667	423	--/	-	Kryukov et al. 1985
62	Dusti	2n	37.3500	68.6667	351	--/	-	--/

63	Vakhsh River	2n	37.9167	68.7833	421	--/	–	--/
64	Chirik	2n	37.2167	68.2000	376	--/	–	Borkin et al. 1986
65	Shaartuz	2n	37.2667	68.1333	366	--/	–	Pisanets 1991
66	Samarqand	2n	39.6500	66.9333	720	--/	–	Borkin et al. 1986
67	Chilyanzor	2n	37.9333	67.0333	738	--/	–	I. A. Caune, pers. com.
68	Kuyu Mazar Canal	2n	39.9000	64.7000	256	--/	–	Odierna et al. 2007
69	Buxaro	2n	39.7667	64.4333	228	--/	–	Roth & Rab 1986
70	Nukus	2n	42.5167	59.6000	76	--/	–	--/
71	Kanal	2n	43.0667	62.4000	86	--/	–	Odierna et al. 2007
72	Karatereng	2n	43.5000	60.4500	51	--/	–	--/
73	Sudochye Lake	2n	43.1667	58.4000	54	--/	–	--/
74	Sarykamysh Lake	2n	41.9000	57.5000	112	--/	–	Borkin et al. 1986

Latitude is given in degrees north, longitude is given in degrees east, and altitude is in meters above sea level. Specimens studied are deposited at the Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia (ZISP) or at the Museum of Nature, Kharkiv National University, Kharkiv, Ukraine (KhNU).