Phylogeography of a widespread terrestrial vertebrate in a barely-studied Palearctic region: green toads (*Bufo viridis* subgroup) indicate glacial refugia in Eastern Central Asia

Yi-Jun Zhang · Matthias Stöck · Peng Zhang · Xiu-Ling Wang · Hui Zhou · Liang-Hu Qu

Abstract The phylogeography of western Palearctic species is relatively well studied, but data on Eastern Central Asia are scarce. We present one of the first data sets from a widespread terrestrial vertebrate (*Bufo pewzowi*) inhabiting Eastern Central Asian mountains and deserts to gain knowledge on its phylogeography in this region. We applied combined phylogenetic and demographic analyses to understand the evolutionary history using mitochondrial DNA D-loop variation of toads from 37 locations. Genetic structure of *Bufo pewzowi* is strongly affected by landscape: we found three haplotype groups in eastern Kazakhstan, Dzungaria and Tarim Basin, divided by the Tian Shan and Dzungarian Alatau ranges. A vicariant hypothesis may explain divergence among groups. The divergence time of the three major clades was estimated about 0.9 million years ago (confidence interval 0.5–1.4), and is discussed with respect to Quaternary uplifting and glaciation in the Tian Shan. Demographic analyses provided evidence for both historical bottlenecks and population expansions and suggested Pleistocene signatures. Glacial refugia were inferred in the Tarim Basin (around the Turpan depression), in southern Dzungaria (Urumqui region), at the northern foot of the Tian Shan (Gongnaisi) and perhaps at the Altai range (Terekti). Regional Post-Last Glacial Maximum dispersal patterns are proposed. A taxonomic hypothesis is presented. This study provides a detailed history of how a widespread terrestrial vertebrate responded to geological change and Quaternary glacial events in Eastern Central Asia and may have significance for future phylogeographic research in this understudied region.

Keywords D-loop · Eastern Central Asia · Glacial refugia · Green toads · Historical biogeography · phylogeography · Post glacial expansion

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Introduction

While the phylogeography of terrestrial species in the northwestern Palearctic is relatively well studied (reviews: Hewitt 2000, 2004; Petit et al. 2003; Taberlet et al. 1998), data from Central Asia are extremely scarce: "The extent of our knowledge of the Holarctic is considerable for Europe and North America, but Asia is little studied, and so too the deserts..." (Hewitt 2007). It can be assumed, however, that orogenic history and Pleistocene climatic oscillations have also affected the geographic distributions, demography, and thus evolution of species in Central Asia. Its complex

landscape history includes the dramatic high mountain formations initiated by the Indian collision (starting 70 Mya), the formation of the Paleozoic Dzungarian basin, and the construction of the Cenozoic Tibetan plateau (50–40 Mya) with the Himalaya in the south, and the Kunlun Shan and the Qilian Shan in the north (Yin and Harrison 2000). In the Cenozoic, also the Tarim Basin developed as a complex foreland basin in association with mountain building in Tibet and Tian Shan (Yang and Liu 2002). At least four main Quaternary glaciations were detected in the Tian Shan (Shi 2002). The early Pleistocene glaciation history of some central regions is not well understood (Ehlers 2004; Lehmkuhl and Strauch 2004). During Middle and Late Pleistocene, the permafrost of the northern and central plains of Kazakhstan merged with the permafrost of the mountains in the south (Aubekerov and Gorbunov 1999), but island patterns of permafrost may have left refugia. During the last glacial maximum, permafrost came down to ~900 m a.s.l. (Aubekerov and Gorbunov 1999) in Kazakhstan but only reached ~1800 m a.s.l. in the Tarim Basin (Yang et al. 2002) suggesting larger refugia in the latter. Climate changes correlated with glacial cycles influenced distribution of regional faunas (e.g., Hewitt 2000). However, the phylogeography of terrestrial species in Eastern Central Asia including the Tarim Basin (with the Taklamakan Desert) and Dzungaria has been scarcely studied. The few examples on poikilothermic vertebrates come from phylogenetics in *Teratoscincus* (Macey et al. 1999, 2005) and *Phrynocephalus* lizards (Pang et al. 2003; Wang and Fu 2004; Guo and Wang 2007), lacking an explicit phylogeographic focus but indicated the barrier effect of the Tian Shan, and showed Dzungaria to be colonized from the West (Wang and Fu 2004). Among mammals, phylogeography of red deer (*Cervus elaphus*; Mahmut et al. 2002; Ludt et al. 2004) has revealed a major genetic difference between Tarim Basin and Dzungarian populations, with the western Tarim clade being more closely related to European populations and the lineages from Eastern Tarim, Tian Shan and Dzungaria showing relationship to East Asian and American ones. Recently, a study in *Rhombomys* gerbils (Ning et al. 2007) detected a separation (93 ka ago) of Dzungarian from Mongolian populations and assumed a population expansion about 12 ka. None of these studies attempted to test for the occurrence of glacial refugia in Eastern Central Asia. To our knowledge, no other phylogeographic studies have been conducted on other widespread terrestrial vertebrates. Therefore, phylogeographic insights from terrestrial species, as bufonid frogs, from this region can be of interest for the knowledge on the climatic history and biogeography of its range as well as for that species' biology.

Green toads (*Bufo viridis* subgroup) are widespread in the Palearctic including northwestern China and western Mongolia (Borkin 1999; Stöck et al. 2006). The *B. viridis* subgroup is the only known amphibian species complex that comprises diploid, triploid and tetraploid bisexually reproducing taxa (Stöck et al. 2005). *Bufo pewzowi* (Bedriaga 1898) is a tetraploid member distributed in Central Asia, including Xinjiang (China), western Mongolia, and the entire Tian Shan range of Kazakhstan and Kyrgyzstan (Stöck et al. 2001). Only few previous studies have looked at genetic variation in the *B. viridis* subgroup (Liu et al. 2000; Nevo et al. 1975; Stöck et al. 2005). To study mitochondrial relationships of diploids to infer their phylogeography and the maternal ancestry of polyploids, Stöck et al. (2006) put a cytogenetic dataset from western and northeastern Central Asia in a molecular framework (mtDNA) and applied phylogenetic and demographic methods. However, only few data from Eastern Central Asia were available for that study.

Here we aimed to study how geological history and Pleistocene glaciation of temperate Eastern Central Asia influenced the genetic structure of *B. pewzowi* by testing two alternative hypotheses: (1) the species invaded the Tarim Basin and Dzungaria very recently after the last glacial maximum (LGM) and would therefore represent a scarcely structured group or (2) it had glacial refugia in one or both basins and spread from these into its large range during more favorable climatic conditions and therefore shows detectable phylogenetic structure.

For this purpose, we sequenced a fragment of the mitochondrial DNA (mtDNA) D-loop in toads sampled from throughout the species range in China and Eastern Kazakhstan, and analyzed their genealogy and demography.

Materials and methods

Sample collection

Samples from 37 localities across the entire species distribution range were obtained in the field or from existing collections (Fig. 1, Table 1). Samples (toe clips preserved in 95% ethanol) from Xinjiang were collected in 2004. Samples from Kazakhstan and in part from Xinjiang (blood and muscle, preserved in ethanol) were collected in 1996 or were available from the frozen tissue collection of the Museum of Vertebrate Zoology (MVZ, University of California, Berkeley).

DNA extraction and sequencing of the mitochondrial control region

Genomic DNA was extracted following a standard phenol/chloroform technique (Sambrook et al. 1989) or with the
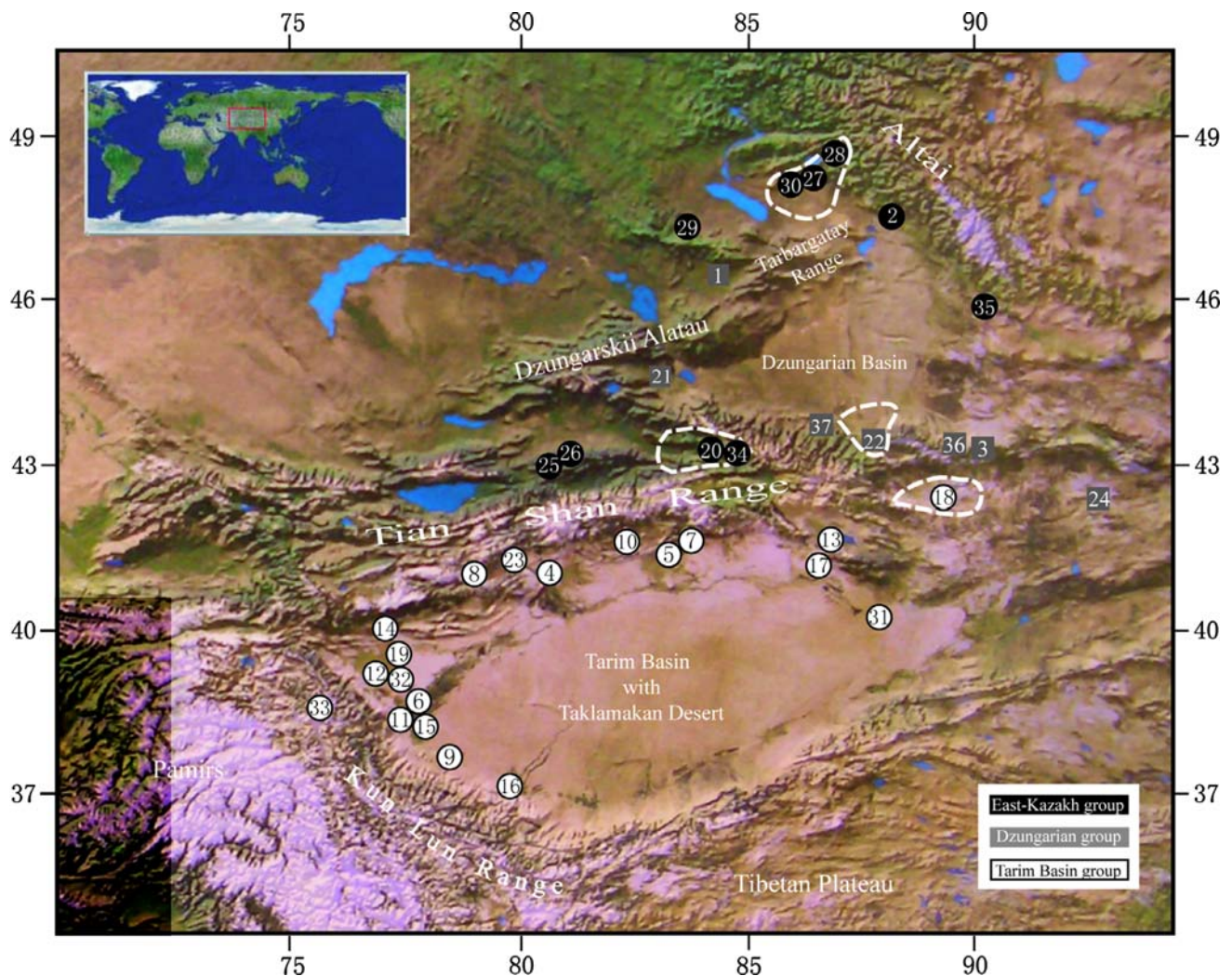
Fig. 1 Map of East Central Asia containing geographical features and localities of samples for this study. The numbers correspond to location numbers listed in Table 1. Haplotype group assignments for populations based on phylogenetic analysis and likely glacial refugia (*dashed circles*) are shown. Relative position of this area is shown on the top left corner

Qiagen DNeasy™ kit. Partial mitochondrial D-loop was amplified in two thirds of the samples with the self-designed primer FCM (5′–GATTAAGCTATR TRAG-TCCATAGATTC-3′) and the published primer CytbA-L (5′-GAATYGGRGGWCAACCAGTAGAAGACCC-3′; Goebel et al. 1999); with ControlB-H and CytbA-L (Goebel et al. 1999) in the remaining. The PCRs (20 μl) were carried out using standard conditions: 2.0 mM MgCl$_2$, 0.5 mM dNTPs, 1 unit ExTaq DNA polymerase (TAKARA), 0.75 μM of each primer, and approximately 50 ng DNA. After 3 min of initial denaturation, each of 30 cycles consisted of the following steps: 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, followed by a 10 min final extension at 72°C. PCR products were purified using the QIAGEN purification kit and sequenced in one direction with the primer FCM on an ABI 377 Genetic Analyzer. Products from CytbA-L and ControlB-H PCRs

were obtained and sequenced as described (Stöck et al. 2006). All sequences were checked with SeqMan (implemented in DNAStar software package) and aligned using ClustalX (Thompson et al. 1997).

Phylogenetic analyses

Phylogenetic trees of unique haplotypes were constructed using Bayesian analysis with the program MrBayes ver. 3.1 (Ronquist and Huelsenbeck 2003). MrModeltest ver. 2.2 (Nylander 2004; modified from Modeltest 3.6 (Posada and Crandall 1998)) was employed to choose the appropriate model of sequence evolution. Two independent analyses were conducted to ensure the result, every analysis consisted of two runs each containing four Markov chains that ran for 10^7 generations, sampled every 1000 generations,

Table 1 *B. pewzowi* samples information in this study

Locality (population) number and sample letter	Locality	Elevation (Feet)	Museum collection voucher number / Sample ID	Haplotype (This paper)	GenBank Accession number
1	China, Xinjiang, Tacheng; Kazak Auto. Prefecture, Tacheng Dist., along Liu Su stream at Liu Su Gou, 29 km E of Miao'ergou Autonomous Region, R. Macey, T. Papenfuss leg.	1778	CAS 171493 (4n199)	Hap-2	DQ629786
2	China, Xinjiang, Aletai	2812	Bufo 02	Hap-13	EU490372
3	China, Xinjiang, Mulei	4168	Bufo 04	Hap-4	EU490374
4	China, Xinjiang, Akesu	3601	Bufo 09	Hap-21	EU490375
5	China, Xinjiang, Xinhe	3330	Bufo 10	Hap-22	EU490376
6	China, Xinjiang, Shache	4038	Bufo 12	Hap-23	EU490377
7	China, Xinjiang, Kuche	3473	Bufo 13	Hap-24	EU490378
8	China, Xinjiang, Aheqi	5996	Bufo 14	Hap-25	EU490379
9	China, Xinjiang, Pishan	4503	Bufo 15	Hap-26	EU490380
10	China, Xinjiang, Baicheng	4047	Bufo 16	Hap-27	EU490381
11	China, Xinjiang, Zepu	4183	Bufo 17	Hap-17	EU490382
12	China, Xinjiang, Yingjisha	4241	Bufo 18	Hap-20	EU490383
13	China, Xinjiang, Bohu	3467	Bufo 19	Hap-28	EU490384
14a	China, Xinjiang, Atushi	4263	Bufo 20	Hap-29	EU490385
14b			Bufo 21	Hap-30	EU490386
15	China, Xinjiang, Yecheng	4438	Bufo 22	Hap-21	EU490387
16	China, Xinjiang, Hetian	4546	Bufo 23	Hap-31	EU490388
17	China, Xinjiang, Kuerle	3067	Bufo 24	Hap-32	EU490389
18	China, Xinjiang, Turpan	0	Bufo 25	Hap-33	EU490390
19	China, Xinjiang, Yuepuhu	3963	Bufo 26	Hap-34	EU490391
20a	China, Xinjiang, Gongnaisi forestry centre	4262	Bufo 27	Hap-14	EU490392
20b			Bufo 29	Hap-14	EU490393
20c			Bufo 30	Hap-15	EU490394
21	China, Xinjiang, Wenquan	4434	Bufo 36	Hap-3	EU490395
22a	China, Xinjiang, Changji	1883	Bufo 38	Hap-5	EU490396
22b	China, Xinjiang, Changji Hui Auto. Prefecture, canyon above Dayou, 8.1 km S of Dayou, Tian Shan		CAS 171676 (4n200)	Hap-3	DQ629787
23	China, Xinjiang, Wushi	4644	Bufo 39	Hap-35	EU490397
24a	China, Xinjiang, Hami	2509	Bufo 40	Hap-3	EU490398
24b			Bufo 41	Hap-3	EU490399
24c	China, Xinjiang, Hami-Barkol, Kazakh Autonomous County (town), J.R. Macey, T. Papenfuss leg.		CAS 171053 (4n197)	Hap-1	DQ629762
25	Kazakhstan, SE, near Kokpak,	6143	328	Hap-10	DQ629796
26a	Kazakhstan, SE, Shalkudysu river, M. Chirikova leg.	8180	327	Hap-9	DQ629795
26b			329	Hap-11	DQ629797
27	Kazakhstan, Altai range, environs of Terekti (formerly Alexeevka) village, T. Dujsebajeva leg.	–	302	Hap-8	DQ629794
28	Kazakhstan, Altai, Pakhmanovskiye Klyuchi N boundaries of S Altay range, T. Dujsebayeva leg.	–	307	Hap-8	DQ629793
29	Kazakhstan, E, Tarbagataj, Tebiske river, M. Chirikova leg.	3236	333	Hap-12	DQ629799
30	Kazakhstan, S foothills of Altai range, Prirechnoye village, T. Dujsebajeva leg.	–	301	Hap-7	DQ629790
31	China, Xinjiang Uygur Autonomous Region, Bayingolin Mongol Autonomous Prefecture, Center of Tikanlik, J.R. Macey, T. Papenfuss leg. 1988	–	CAS 167832 (4n198)	Hap-20	DQ629761

Table 1 continued

Locality (population) number and sample letter	Locality	Elevation (Feet)	Museum collection voucher number / Sample ID	Haplotype (This paper)	GenBank Accession number
32a	China, Kashgar, Xinjiang, China, M. Stöck leg.	4500	ZSM 107/1998 (4n22)	Hap-16	DQ629782
32b		–	ZSM 108/1998 (4n23)	Hap-17	DQ629781
33a	China, NW-China, Taxkurgan, E-Pamir, M. Stöck leg., T. Papenfuss, J.R. Macey leg.	11167	ZSM 110/1998 (4n25)	Hap-18	DQ629756
33b		–	CAS 197007 (193)	Hap-18	DQ629757
33c		–	CAS 197008 (194)	Hap-19	DQ629758
33d		–	CAS 197010 (196)	Hap-18	DQ629760
34	China, E-Tien-Shan, NW-China, Xinjiang, M. Stöck leg.	7150	ZSM 109/1998 (4n31)	Hap-6	DQ629785
35	China, Xinjiang, Altai, Qinghe	–	Bufo 42	Hap-7	EU490400
36	China, Xinjiang, Qitai, Biliu River	–	Bufo 43	Hap-3	EU490401
37	China, Xinjiang, Kuitun	–	Bufo 44	Hap-2	EU490402

Locality numbers as in Fig. 1. Letters indicate multiple samples from single locality. Haplotype of each sample and elevation of each locality are given. *Abbreviations*: ZSM = Zoologische Staatsammlung Munich, Germany; CAS = California Academy of Sciences, San Francisco, USA

with a random starting tree, default priors and equal branch lengths. The trees corresponding to the first two million generations were discarded. Three homologous sequences of green toads from the *Bufo viridis* subgroup were chosen as outgroups: *Bufo boulengeri* (from North Africa), *Bufo zugmayeri* (from Pakistan: Pishin) and *Bufo pseudoraddei baturae* (from Pakistan: Pasu) (see Stöck et al. (2006) for nomenclature and phylogenetic trees qualifying these taxa as appropriate outgroup species).

Nested clade analyses

To detect an association between geography and haplotype frequency and to infer evolutionary processes causal for genetic structure, we conducted a nested-clade analysis (NCA; Templeton et al. 1995). First, we used the program TCS ver. 1.21 (Clement et al. 2000) to create a network of haplotypes (gaps were treated as missing data) using the 95% statistical parsimony method of Templeton et al. (1992). The network was converted into a nested design following Templeton et al. (1987). For all clades within the network the significance levels for associations between haplotype and geography were calculated using the program GeoDis ver. 2.4 (Posada et al. 2000). Random permutation tests of the data against a null hypothesis created from a random geographic distribution of all clades within each nesting level (generated using 1,000 permutations) were used to infer association between phylogeny and geography. Significance was determined at the 5% and 10% level. For clades, for which the null hypothesis was rejected, we followed GeoDis ver 2.4 (2004) to infer biological causes for the observed associations.

Demographic analyses

Three haplotype groups recovered by the phylogenetic analyses occupied non-overlapping geographical regions (Fig. 1) and were treated as separate units to analyze regional genetic diversity and demographic history. To understand the level of gene flow among three haplotype groups, F_{st} value was estimated using DNAsp ver.4.0 (Rozas et al. 2003). For comparisons of within-region genetic diversity, haplotype diversity (h) and nucleotide diversity (π) were calculated using Arlequin ver. 2.0 (Schneider et al. 2000) and DNAsp ver.4.0 (Rozas et al. 2003). We also calculated Tajima's D (Tajima 1989) and performed Fu's Fs test (Fu 1997) using Arlequin to confirm demographic expansions. Histograms of the frequency of observed pairwise differences among sequences were plotted using Arlequin, and the shape of the observed mismatch distribution was tested against the null hypothesis of population expansion (Slatkin and Hudson 1991). Mismatch distributions were plotted for all samples, including identical haplotypes, for each haplotype group. Populations which have gone through a period of expansion or growth are expected to have a star-like phylogeny and a unimodal shape to the mismatch distribution reflecting similarity in the amount of divergence among all pairs of haplotypes (Rogers and Harpending 1992; Slatkin and Hudson 1991). Populations which have been stable over time are predicted to have a more balanced phylogenetic shape and a bi- or multimodal mismatch distribution (Slatkin and Hudson 1991). The fit between the observed and expected distributions was tested using the sum of squared deviations (Schneider and Excoffier 1999).

Divergence time estimates

Divergence time of *B. pewzowi* was estimated using BEAST (Drummond and Rambaut 2003; Drummond et al. 2002, 2006). The molecular clock was relaxed by allowing the rate to vary throughout the tree in an autocorrelated manner, with the rate in each branch being drawn from an exponential distribution whose mean was equal to the rate in its parent branch. Unlike previous relaxed-clock methods for rate estimation, BEAST does not require a user-specified tree topology. The model of sequence evolution used for the divergence time estimates was identical to that in the Bayesian phylogenetic analysis (GTR + G). A relaxed molecular clock model (Uncorrelated Exponential) and coalescent prior were used for the tree, and constant population size models were tested. Following a burn-in of 1,000,000 cycles, time of the most recent common ancestor (TMRCA) were sampled once every 1000 cycles from 10,000,000 MCMC steps. We used the conservative paleogeographic calibration from Stöck et al. (2006), who estimated the evolutionary rate of the mitochondrial D-loop in green toads as 1.278% divergence per My. Convergence of chains was checked with the program Tracer ver. 1.3 (Rambaut and Drummond 2004). The effective sample sizes for each parameter sampled from the MCMC analyses were almost always found to exceed 1000.

Results

Partial control regions of 48 individuals from 37 populations of *B. pewzowi* yielded an alignment of 833 bp. We identified 42 polymorphic sites, which comprize 35 unique haplotypes. Haplotypes shared across populations were found at geographically proximate localities (Table 1, Fig. 1).

Phylogenetic analyses

A GTR + G model of evolution was selected for the Bayesian analysis by MrModeltest using the Akaike information criterion (AIC). The average standard deviations of split frequencies of two independent analyses were 0.0028 and 0.0036, respectively. An identical branch topology was suggested by two independent analyses and is shown in Fig. 2. The Bayesian consensus tree revealed three well-supported genetically distinct haplotype groups: (i) the East-Kazakh (EK) group, (ii) the Tarim Basin (TB) group and (iii) the Dzungarian (DZ) group (Fig. 2). The posterior support values of all major haplotype groups exceeded 95. The TB group comprises all individuals from the Tarim Basin; the
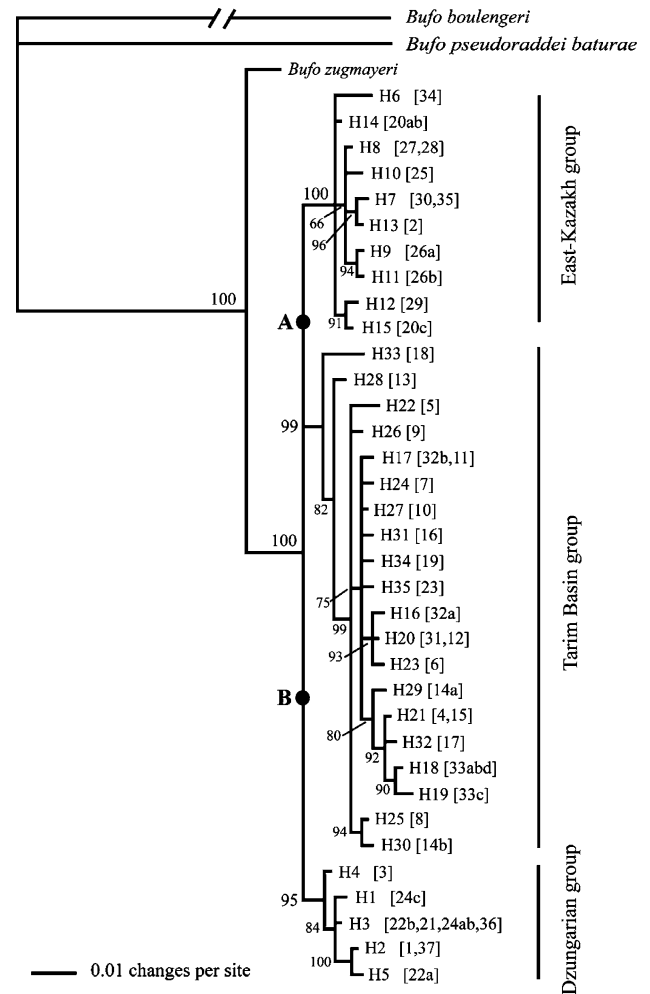
Fig. 2 Consensus tree derived from the Bayesian analysis of haplotype sequences. Posterior probabilities (greater than 50%) for branches are shown. Locality numbers and sample IDs are given behind each haplotype. Vertical bars indicate the haplotype groups corresponding geographical distribution in Fig. 1. Point A and B illustrate positions of outgroups found when the tree was rooted with different outgroup species
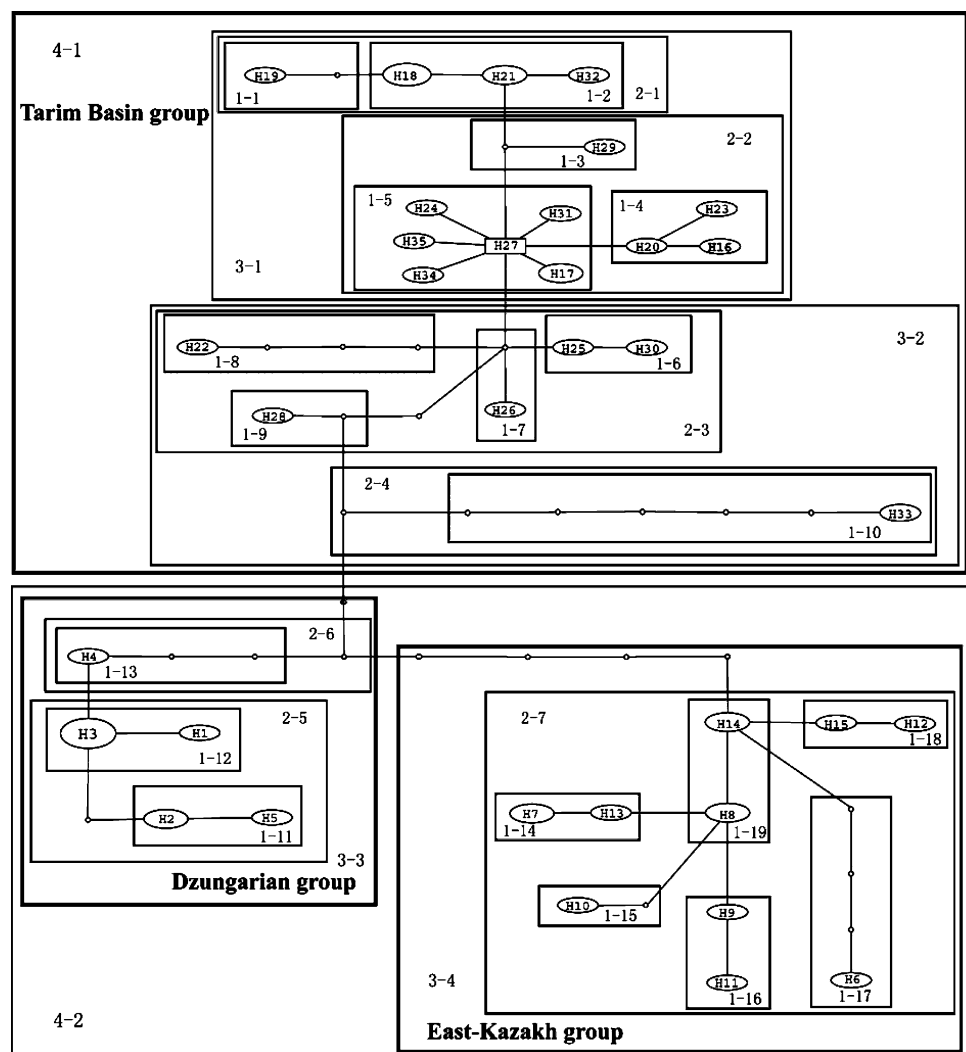
DZ group mainly spans the northern slope of the Eastern Tian Shan, while the EK group is distributed in the plains and basins to the north of the Tian Shan, west and north of the Dzungarian Alatau and Tarbagatay range and south of the Altai range (Fig. 1, Table 1). In this tree, all three haplotype groups were reciprocally monophyletic. The problem of tree reconstruction and rooting is known to be challenging, especially when dealing with rapid radiations, where there is a combination of short and long branches. To further confirm our phylogenetic results, we reanalyzed our data with different outgroups. These outgroup candidates were also from the *Bufo viridis* subgroup, but showed different levels of divergence to the ingroup (see Stöck et al. 2006). After excluding poorly rooted trees (i.e., trees rooted with too close or too distant outgroups), we found the outgroup position to

be unstable relative to the ingroup. When the outgroup was pinned at position A (Fig. 2), the TB group and DZ group formed a monophyletic clade with EK as a sister clade. When the outgroup point was constrained to position B, the TB group and EK groups formed a monophyletic clade with DZ as a sister clade. However, a monophyletic clade consisting of DZ and EK group was not found in our study. Although a monophyletic clade consisting of two haplotype groups was produced in some cases, the Bayesian posterior support value of this clade was always low (<65). The difficulty to find a close relationship among the three haplotype groups suggests reciprocal monophyly may most probably reflect their true phylogenetic relationship. However, more data from other rapid-evolution loci may be helpful to confirm this conclusion.

Nested clade analyses

Figure 3 shows the intraspecific network and nesting design obtained using the algorithm of Templeton et al.

(1987). One reticulation caused by an inferred haplotype connecting haplotypes H26 (loc. 9) and H14 (loc. 20) was removed (criteria of Crandall and Templeton 1993) for determining the most plausible of alternative networks. The third nesting level (clade 3–3, 3–4) illustrated the break between DZ and EK groups; clade 3–1, 3–2 described two clades within the Tarim Basin group. At the fourth nesting level one clade united haplotypes from the TB group and another included both the DZ and the EK groups. The association of the DZ and EK groups in clade 4–2 might result from relatively few mutational steps within each group as compared to the TB group. Five mutational steps are needed to connect the TB and DZ groups, while seven mutational steps are required for the connection between TB and EK. Outgroup weight calculations indicated that clade 1–5 contained the inferred ancestral haplotype (H27, loc. 10). Haplotype H33 (loc. 18, 1–10) showed deep divergence from the rest (up to six mutational steps from its closest interior) of the TB group (clade 4–1). The null hypothesis of no association between geography and

Fig. 3 Statistical parsimony (95%) network for 35 mitochondrial DNA haplotypes identified for *Bufo pewzowi*. The network is grouped into nesting clades. Each line in the network represents a single nucleotide substitution. Small circles indicate undetected intermediate haplotype states. The relative size of the boxes and ovals (at haplotype level) represent the frequency of these haplotypes (boxes around haplotypes denote those haplotypes with the highest outgroup weight). The boxes indicate nesting level (e.g., 1–4, nesting level one, grouping number four). Alphanumeric haplotype names correspond to those in Fig. 1, Table 1. Three haplotype groups corresponding to Fig. 2 are highlighted by thick-line black boxes

Table 2 Inference for all clades showing significant association from nested clade analysis results, using the key implemented in GeoDis ver. 2.4 (July 2004)

Clade	Chain of inference	Inference
Zero-step clades nested in Clade 1–2	1-19-20-2-3-4 NO	Restricted gene flow with isolation by distance
One-step clades nested in Clade 2–7	1-2-11-17-4 NO	Restricted gene flow with isolation by distance
Three-step clades nested in Clade 4–2	1-19 NO	Allopatric fragmentation
Four-step clades nested in entire cladogram	1-19 NO	Allopatric fragmentation
One-step clades nested in Clade 2–5*	1-2-3-4 NO	Restricted gene flow with isolation by distance
Two-step clades nested in Clade 3–1*	1-2-11-12 NO	Contiguous range expansion
Three-step clades nested in Clade 4–1*	1-2-3-4 NO	Restricted gene flow with isolation by distance

* The significance level of the permutational contingency test is set as $P < 0.10$

haplotype position in clades was rejected for four clades (5% significance level) and three more clades (10%; Table 2). Nevertheless, the null hypothesis was rarely rejected in low level clades (e.g., 1-step clade) showing that sampling was partly inadequate to achieve significance (Templeton et al. 1995; Table 2), especially regarding the number of samples per location. The biological processes inferred to have caused the significant associations are listed in Table 2. Most often, restricted gene flow with isolation by distance was invoked. However, we also inferred allopatric fragmentation for splits between EK and DZ group (clade 4–2), and the TB group from both (Fig. 3).

Demographic analyses

The pairwise *Fst* values for the three haplotype groups were 0.6707, 0.6971 and 0.7379 respectively (Table 3). Levels of haplotype and nucleotide diversity were found unequal among geographic regions. Haplotype diversity (*h*) and nucleotide diversity (*π*) are significantly lower in the Dzungarian group than in the other two (Table 4). Nucleotide differences within populations are the highest in the Tarim Basin (0.53%), followed by the E-Kazakh group (0.40%). The Dzungarian group showed the lowest nucleotide differences (0.22%). Between populations, nucleotide differences did not show obvious bias among the three

groups, with an average at ∼1% (Table 3). Significance of neutrality tests varied depending on statistics and region. Fu's *Fs* test was highly significant for all geographic regions, whereas, Tajima's *D* only approached significance in the Tarim Basin group ($P = 0.044$). Nevertheless, nominal values of Tajima's *D* within the Dzungarian and E-Kazakh groups were negative, suggesting demographic expansions. Mismatch distribution analyses yielded similar results with neutral tests, all three groups showed unimodal mismatch distributions for the control region (Fig. 4). However, none of the distributions was significantly different from the null expectation of population growth (Table 4).

Divergence time estimates

We estimated that the three haplotype groups split from their most recent common ancestor 0.9 million years before present (MYBP), with a 95% confidence interval of 0.5–1.4 MYBP. The effective sample size (ESS) for this estimation was 1695.15 from the analysis using the program BEAST.

Discussion

Our dataset is one of the few phylogeographic studies, which uses multiple sample data from widespread, small terrestrial vertebrates from Eastern Central Asia. Here, we discuss our data in context with geological and landscape history data and the few available publications from this region.

Evolutionary history of green toads in Eastern Central Asia

We found geographically isolated Tarim Basin, Dzungarian Basin and East-Kazakh haplotype groups (Fig. 2) that

Table 3 Pairwise differences and *Fst* values between haplotype groups for the D-loop region

	DZ group	EK group	TB group
DZ group	0.22	0.7379	0.6707
EK group	1.02	0.40	0.6971
TB group	0.97	1.14	0.53

Below diagonal: corrected average pairwise differences in percent (PiXY − (PiX + PiY)/2). *P* values for all corrected PiXY is 0. Diagonal elements: pairwise differences within population (PiX). Upper diagonal: *Fst* values between haplotype groups

Table 4 Results of demographic parameters including h, π, Fu's Fs, Tajima's D, and SSD (95% CI and P-values given where applicable), grouped by geographic region

Haplogroup	n	n_H	Haplotype diversity, h (±95% CI)	Nucleotide diversity, π (±95% CI)	Fu's Fs (P-value)	Tajima's D (P-value)	SSD	P (Sim. Ssd ≥ Obs.Ssd)
DZ group	10	5	0.756(0.017)	0.0019(0.00044)	−12.216(**0.000**)	−0.531(0.330)	0.0038	0.8590
EK group	13	10	0.962(0.002)	0.0035(0.00054)	−13.500(**0.000**)	−0.546(0.319)	0.0026	0.8790
TB group	25	20	0.980(0.000)	0.0053(0.00079)	−25.768(**0.000**)	−1.626(**0.044**)	0.0055	0.3340

n, number of individuals

n_H, number of haplotypes

SSD, sum of squared deviations of mismatch distribution from prediction under population expansion mode

Significance P-value in bold face

Fig. 4 Mismatch histograms for the control region. Observed pairwise substitutional differences (*grey boxes*) are compared with values simulated under a population expansion model (*black line with dots*). Details in text
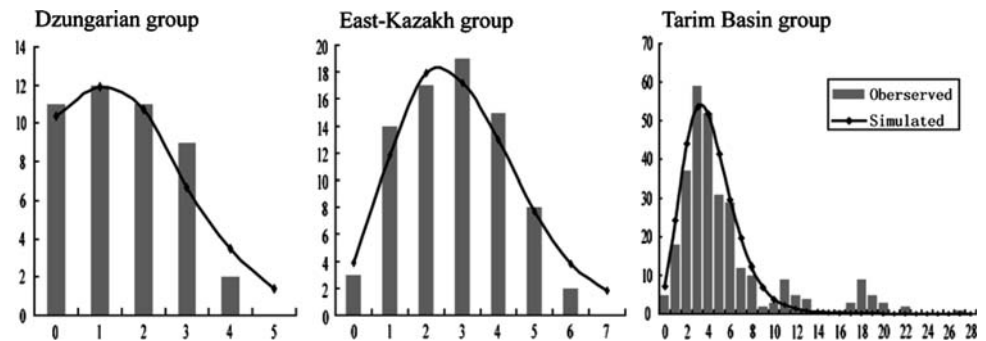
appear divided by the Tian Shan, Dzungarian Alatau and Tarbagatay ranges (Fig. 1). As expected for amphibians (e.g., Hoffman and Blouin 2004; Shaffer et al. 2004), the genetic structure of *B. pewzowi* shows a strong phylogeographic signature. The phylogeny exhibits a polytomy containing three very well supported monophyletic haplotype groups and suggests a vicariant event (or serial events within a short period of time) split *B. pewzowi* into these lineages. This finding rejects a very recent invasion of the Tarim Basin (hypothesis 1). Instead, the phylogenetic structure suggests that green toads survived in glacial refugia and spent there sufficient periods of time to form three distinct groups.

The Tian Shan lies in the actively deforming part of the India-Asia collision zone, which is regarded as one of the most active geological areas. Through crustal shortening and thickening caused by the India-Asia collision, the Tian Shan underwent uplifting in four main ages: about 65.6, 45.22 and 32.60 Ma (initial uplift), and between 24.74 and 15.98 Ma, 6.7–0.73 Ma and less than 0.73 Ma (Liu et al. 2004; see also Yang and Liu 2002). Stöck et al. (2006) estimated that the single mitochondrial lineage that lead to all Central Asian taxa of the *B. viridis* subgroup (eastern Iran to western Mongolia, including *B. pewzowi*), diverged in the Pliocene (between 4.4 and 3.1 Mya) from proximate toads in Anatolia. In our study, inferences from NCA for all groups (the entire network) and clade 4–2 (EK and DZ

group) suggested allopatric fragmentation caused by topography. In accordance to the occurrence of three reciprocally monophyletic groups (Fig. 2), we favor a vicariant rather than a dispersal event to have shaped the phylogeographic structure of *B. pewzowi*. Divergence time estimation suggested that different lineages of *B. pewzowi* diverged from their most recent common ancestor at ca. 0.9 Mya, which corresponds to the end of the third uplifting time intervals (6.7–0.73 Mya) of the Tian Shan (Liu et al. 2004). We inferred (NCA) that H27 (loc. 10) was the ancestral haplotype in the Tarim Basin, suggesting toads may have first reached the basin from the north of the Tian Shan or were forced into a glacial refugium in this region. Therefore, we propose a scenario on the biogeographic history of *B. pewzowi* comprising three stages: (i) the lineage to *B. pewzowi* reached eastern Central Asia before the Quaternary mountain building and Pleistocene cooling and aridization, perhaps in the late Pliocene (~3 Ma). (ii) Toads may have entered the Tarim Basin across western mountain passes from the currently non-sampled Fergana valley, or reached the Tarim along the Tian Shan foothills from the north. Few founders may have surmounted the passes near the Turpan basin to the north and gave rise to the Dzungarian group; to the northeast they likely spread along the western foothills of the Dzungarian Alatau and further occupied the upper margin of the Dzungarian Basin. At that time, there was perhaps still

gene flow among populations. (iii) The advance of Pleistocene cooling and aridization accompanied by Plio-Pleistocene mountain building in the Tian Shan until ~2.58 Ma (Sun et al. 2004) and adjacent mountain ranges restricted genetic contact increasingly until populations were isolated into different regions, perhaps in lower or middle Pleistocene (1.8–1 Ma).

Strong restriction of gene flow between haplotype groups was suggested by both *Fst* values and NCA. The *Fst* values between regions were high (0.67–0.74), suggesting a barrier effect of the Tian Shan, most probably along with cooling and aridization during glacial periods of the Pleistocene that disrupted the gene flow. This is also supported by a lack of shared haplotypes between ranges of the three clades (one exception). Interestingly, a Dzungarian haplotype from TaCheng (loc. 1) in the range of the E-Kazakh group (Fig. 1) suggests a westward dispersal of Dzungarian haplotypes. Both ranges appear connected via the Dzungarian Gate (ca. 45.25 N; 82.50 E). 'Restricted gene flow with isolation by distance' was inferred for the highest level clade of each haplotype groups by NCA (Table 2), suggesting the difficulty of genetic connection through the range of each group. Furthermore, restricted gene flow within a one-step clade (1–2) nested in TB group was also detected. The basal branch of the TB clade, haplotype H33 from Turpan depression (loc. 18) and H28 (loc. 13), showed notable divergence from the remaining ones, suggesting the genetic contact to other regions of the Tarim Basin has been disrupted for a large number of generations. A gecko of the genus *Teratoscincus* occurring in the Turpan depression (Macey et al. 2005) seems to show similar phylogeographic isolation but may have persisted in Turpan much longer periods of time.

The widely distributed TB group reflects the relative ecological connectivity of the Tarim river drainage and its tributaries. The climate in the Tarim Basin may have been mostly arid since the Tertiary; however, an episodically connected ring of natural oases along the foothills of the surrounding high mountains was probably developed throughout Tertiary and Quaternary (Zhang et al. 2003). In addition, more recent humid periods and changing river flow from west to east (and vice versa, Zhang et al. 2003) and a river (Keriya), crossing the basin's Taklamakan desert centrally from the south to the northern Tarim river as recently as during the Chinese Xihan dynasty (~200–25 B.C.; Yang et al. 2002), probably contributed to dispersal of some green toads while others persisted in temporarily isolated oases.

Demographic history and Pleistocene signatures

Demographic analyses show evidence of both population expansions and historical bottlenecks that are likely caused by Pleistocene glacial oscillations. A recent growth of the Tarim Basin group is indicated by significant negative value of Fu's *Fs* and Tajima's *D* (Table 4), as well as unimodal mismatch distributions (Fig. 4, Table 2). We also deduced this from the star-like haplotype network (clade 1–5 in clade 3–1, Fig. 3). In the Dzungarian group, recent range expansion is suggested only by Fu's *Fs* test and the unimodal mismatch distribution curve; this ambiguity may result from a relatively short expansion time. Demography of the E-Kazakh is similar to the DZ group (Table 4). However, a star-like haplotype network in clade 2–7 (Fig. 3) shows a stronger signature of range expansion in the E-Kazakh one, consistent with a stronger glacial retreat and a recent postglacial expansion in this region.

Loss of alleles and homozygosity are consequences of founder events from postglacial expansions (Hewitt 2000). Our analyses suggested that the northern Dzungarian region was most affected followed by the E-Kazakh region, and as expected the southern Tarim Basin region was least affected. The pairwise differences within DZ, EK and TB groups were 0.22%, 0.40% and 0.53% respectively (Table 3). A similar pattern was found for haplotype diversity (h) and nucleotide diversity (π) (Table 4).

Genetic differentiation triggered by the Tian Shan has been reported from several animal species, such as red deer (*Cervus elaphus*), gekkonid (*Teratoscincus*) and agamid lizards (*Phrynocephalus*) (Mahmut et al. 2002; Ludt et al. 2004; Macey et al. 1999, 2005; Pang et al. 2003), with the major vicariant events estimated to be of Late Miocene or Pliocene age (Macey et al. 1999, 2005; Pang et al. 2003). By implication, vicariant groups had to survive the Pleistocene glacial cycles in the basins north and south of the Tian Shan that must have played key roles as glacial refugia for relatively cold-tolerant species. Accordingly, Guo and Wang (2007) considered the Tarim and Dzungarin Basins "secondary divesification centers" for *Phrynocephalus* lizards. However, glacial refugia and post-glacial expansion patterns of Eastern Central Asia have been rarely investigated. In our study, the haplotype network suggests glacial refugia for each of the three major lineages. During glacial advance, distribution ranges of species were restricted to refugia. Since refugia served as sources of post-glacial expansions, haplotypes harbored here are supposed to be older than derived ones emerging during colonization. In the haplotype network, inferred ancestral haplotypes have a strong tendency to be immediately interior to derived ones (Templeton 1998). Hence, these interior haplotypes have a high probability to be localized in refugia, especially those in the center of a star-like radiation. Therefore, we conclude that last-glacial maximum (LGM) refugia were perhaps situated in the Changji region (loc. 22) of the DZ group (see Fig. 1). Two potential separate refugia were inferred at the foot of the

Tian Shan (Gongnaisi, loc. 20) and at the Altai range (Terekti, loc. 27, 28) of the EK group and in the Turpan depression (loc. 18) for the TB group. In the Changji region, identical haplotypes (H3) were found in four populations (loc. 22, 21, 24, 36), partly as distantly as 1,100 km. This suggests rapid expansions into available habitats immediately after the glacial maximum. However, Rhode et al. (1996) found a late Pleistocene humid episode (radiocarbon-dated at 37,000–32,000 year B.P.) in Dzungaria. Although this first wet episode was followed by a period of extreme aridity (LGM), the Early-Mid Holocene record again indicates conditions wetter than today. Such wetter periods provided climatic conditions that may have facilitated green toad dispersal, and may explain our data of a rapid expansion of the toads. An inferred refugium in the Altai range (Terekti, loc. 27, 28) may have been crucial for the maintenance of the EK lineage and its rapid colonization of higher latitudes after the last glaciation. In this case, toads that survived here may have undergone a fast post-LGM recolonization to the Tarbagatay range and southern Altai range. The postglacial expansion from the Turpan refugium was apparently restricted to the basin, as proximate populations showed significant genetic distance (e.g., loc.13, 36).

Our data on green toads refer to glacial refugia and may be especially significant to identify regions with seasonally available liquid water (for reproduction of toads). The observed distribution patterns within each green toad lineage may result from fragmentation, contraction and subsequent expansion of populations associated with the glacial and interglacial cycles. From these refugia, lineages re-expanded and spread in the basins as the Tarim Basin, Dzungaria and the region west and north of the Tarbagatay range and south of the Altai. To verify if these data for the region are of general significance for other species will be a challenging future research topic.

Taxonomic implications

Stöck et al. (2001) provided information on name-bearing types, type localities, nomenclatural and systematic histories, morphology, ploidy level, bioacoustics, distribution, proposed current taxonomic status, and a tentative identification key. Seven nominal taxa (for details Stöck et al. 2001) have been described in the geographic scope of our study. If one likes to apply subspecific names, we note on the haplotype groups found that: (1) The Tarim Basin group represents the nominotypical subspecies *Bufo pewzowi pewzowi* (Bedriaga 1898). (2) The restricted type locality of *B. pewzowi strauchi* (Bedriaga, 1898) to the "lower reaches of Tarim" by Stöck et al. (2001) is covered by this range, making this name (*strauchi*) a junior

subjective synonym of the *B. p. pewzowi*. The same synonymy fits to the nominal subspecies *Bufo pewzowi taxkorensis* (Fei et al. 1999), a tetraploid form as shown by Stöck et al. (2001) that is deeply nested with the TB clade (*Bufo pewzowi pewzowi*). (3) The E-Kazakh group could be termed *Bufo pewzowi unicolor* (Kashchenko 1909), however, as Stöck et al. (2006) have shown, the mtDNA phylogeny of this group is intertwined with that of diploid *Bufo turanensis* (Hemmer et al. 1978) and does not fit into classical nomenclatural concepts (cf. Litvinchuk et al. 2006). Further research is ongoing to better understand the interaction between diploid and tetraploid green toads in this region. (4) Taxonomic proposals of Stöck et al. (2001) for *Bufo nouettei* (Mocquard 1910) and *Bufo tianschanicus* (Toktosunov 1984) were supported by our analyses, making both junior subjective synonyms of *Bufo pewzowi*.

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