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Multiple origins of tetraploid taxa in the Eurasian *Bufo viridis* subgroup

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Abstract

We used Q-banding and analyzed nucleolar organizing regions (NORs) to study the cytogenetic evolution of tetraploids within the Palearctic *Bufo viridis* subgroup, the only known amphibian complex comprising di-, tri- and tetraploid bisexually reproducing taxa. We examined three diploid (2*n*) nominal taxa (*Bufo viridis viridis*, *B. v. turanensis*, *B. v. kermanensis*) from five Eurasian localities and six tetraploid (4*n*) nominal taxa (*B. oblongus*, *B. o. danatensis*, *B. pewzowi pewzowi*, *B. p. taxkorensis*, *B. p. unicolor*, *B. p. strauchi*) from eight Central Asian localities. Homeologous chromosomes of 2*n* and 4*n* toads exhibit a similar morphology. Silver-staining and *in situ* hybridization revealed terminal NORs in the long arms of chromosomes 6 in all 2*n* but in only two out of four chromosomes 6 in all 4*n* taxa. Q-banding and a rapidly evolving mitochondrial marker suggest at least two origination events for Asian 4*n* toads: "Western Central Asian tetraploids" (*B. oblongus* Nikolsky, 1896) exhibit distinct differences within some chromosome quartets, which are divisible into pairs of chromosomes and may be allopolyploid. In contrast, "Central Asian tetraploids" (*B. pewzowi* Bedriaga, 1898) showed homogenous Q-banding patterns within each quartet, suggesting autopolyploidy. In Northeastern Iran, we discovered a zone of either common ancestry or hybridization of 2*n* and Western Central Asian 4*n* toads. This raises intriguing questions about how diploid and tetraploid taxa may evolve by exchanging genetic material.

Abbreviations: 2*n* – diploid; 2*n*s – diploids; 3*n* – triploid; 4*n* – tetraploid; 4*n*s – tetraploids; CA-4*n*s – Central Asian tetraploids (= *Bufo pewzowi* and related taxa); DAPI – 4′-6-diamidino-2-phenylindole; MTD – Museum Tierkunde Dresden, Germany; MVZ – Museum of Vertebrate Zoology, University of California, Berkeley, USA; WCA-4*n* – Western Central Asian tetraploids (= *Bufo oblongus* and related taxa); ZSM – Zoologische Staatsammlung Münich, Germany.

Introduction

Currently, the *B. viridis* subgroup is the only known amphibian species complex that comprises diploid (2*n*), triploid (3*n*) and tetraploid (4*n*) bisexually reproducing taxa. However, neither sufficient cytogenetic nor molecular data were available to investigate the evolution and phylogeny of the *B. viridis* subgroup or to address speciation within its different groups. Since Laurenti described *Bufo viridis* (1768), the morphological variability of green toads in their Palearctic range

(Borkin, 1999) resulted in the description of numerous nominal taxa. In Asia, however, cryptic 2*n*, 4*n* (Mazik, Kadyrova & Tokotosunov, 1976) and even 3*n* bisexual taxa (Stöck et al., 1999, 2002) occur, and complicated mixed breeding systems (e.g., 2*n*/3*n* or 3*n*/4*n* etc.) may exist in northern Central Asia (e.g., Borkin et al., 2001b; Stöck, Günther & Böhme, 2001b; Cavallo et al., 2002). Several authors reviewed the taxonomy of *B. viridis* and related taxa (Eiselt & Schmidtler, 1973; Hemmer et al., 1978), and provided karyological (Roth, 1986; Borkin & Kuzmin, 1988; Borkin et al., 2001a) and/or chorological data (Borkin, 1999; Stöck et al., 2001a). Here, we use the nomenclature as revised for Asian green toads by Stöck et al. (2001b) who provided information on name-bearing types, type localities, nomenclatural and systematic histories, ploidy level, bioacoustics, distribution, proposed current taxonomic status, and a tentative identification key. Among 2*n* toads, they tentatively distinguished the taxa: (1) *B. viridis* with nominal subspecies *B. v. kermanensis*, *B. v. shaartusiensis*, *B. v. turanensis* and *B. v.* ssp. [formerly '*arabicus*']), and (2) *B. latastii latastii*. They distinguished two 4*n* species: (I) *B. oblongus* (with *B. o. oblongus* and *B. o. danatensis*) provisionally called 'Western Central Asian tetraploids' (WCA-4*n*), and (II) *B. pewzowi* (with nominal subspecies *B. p. pewzowi*, *B. p. unicolor*, *B. p. strauchi* and *B. p. taxkorensis*) – termed 'Central Asian tetraploids' (CA-4*n*). Bisexual triploid taxa are apparently represented by *B. pseudoraddei pseudoraddei* and *B. p. baturae*. Because of contradictory data or unknown ploidy, the status of *B. asiomontanus* and *B. zugmayeri* remained unclear (for further details: Stöck et al., 2001b).

In this paper we show, using cytogenetic and molecular techniques on Eurasian 2*n* and 4*n* taxa, that Asian 4*n* green toads have evolved independently at least twice. We also report on the discovery of a zone of common ancestry or hybridization of 2*n* and 4*n* taxa of these toads.

Materials and methods

Animals

We sampled either topotypic individuals from various Asian type localities or from other Central Asian regions. Because of logistical difficulties, only a limited number of specimens could be transported alive to the laboratory (Table 1; GenBank accession numbers and linked voucher information). If the taxonomic position appears not completely clarified (designated in Table 1 as: '?') taxa were grouped provisionally.

Chromosome preparation

Chromosomes were prepared from bone marrow, intestine and testes tissue as described by Schmid (1978). Giemsa and Silver ($AgNO_3$)-staining of nucleolar organizing regions (NORs) followed Schmid (1978, after Goodpasture & Bloom 1975). We also performed either distamycin A/mithramycin counterstaining or non-radioactive 18S + 28S rDNA *in situ* hybridization. The latter was applied to somatic and testicular chromosomes with the plasmid pXIr101, a derivative of pMB9 (4800 bp), into which one complete ribosomal RNA gene of *Xenopus laevis* was inserted (Morgan, Macgregor & Colman, 1980). Distamycin A/DAPI (4′-6-diamidino-2-phenylindole) staining, distamycin A/mithraycin counterstaining, Quinacrine mustard (Q)-banding, and centromere (C)-banding, followed Schmid et al. (1983). Fluorescence was selectively obtained by excitation with UV light in the 450–490 nm range (Schmid et al., 1993). *In situ* hybridization on telomeres $(TTAGGG)_n$ followed Meyne, Ratliff and Moyzis (1989). We used a Zeiss Axiophot and a Zeiss III photomicroscope and Agfaortho 25 (15 DIN) film.

DNA amplification and sequencing

Genomic DNA was extracted from frozen or ethanol-preserved liver or blood of at least one of the karyotyped specimens from each locality using a phenol–chloroform extraction or the DNeasy™ tissue kit (Qiagen). A fragment of about 880 bp was amplified comprising nearly the entire mitochondrial control region (primers ControlB-H and CytbA-L; PCR: 96°C, 2 min, denaturation; 52°C, 45 s, annealing; 72°C, 2 min, extension; 38 cycles of [94°C, 30 s, denaturation, 52° C, 45 s annealing, 72°C, 1.5 min, extension]; 72°C, final extension, 5 min; Goebel, Donelly & Atz, 1999). Products were sequenced in both directions on an ABI 3730 sequencer.

Table 1. Localities of toads examined (see Figure 8)

Locality	Geographic data (+ number in Appendix and Figure 1 of Stöck et al., 2001a)	Ploidy	Nominal taxon	Voucher number (if available)	*N* (karyo-typed)	GenBank acc. no.
Halle	Germany, Saxony-Anhalt	2*n*	*B. viridis viridis* Laurenti, 1768	MVZ 241555	1	AY830079
Kerman	Iran, Kerman Province, Kerman, 30°18′ N, 57°05′ E, 1860 m a.s.l. (13c)	2*n*	Topotypes of *B. viridis kermanensis*, Eiselt & Schmidtler, 1971	MTD 40730, 40731	7	AY830082 AY830083
Baghestan	Iran, 10 km NE Baghestan, Khorasan, N of Kuh-e-Kalat, 34°09′ N, 58°25′ E, 1900 m a.s.l. (13)	4*n*	*B. oblongus oblongus* Nikolsky, 1896 (Western Central Asian tetraploids)	–	1	–
Birjand	Iran, Khorasan, Birjand, 32°33′ N, 59°10′ N, about 1500 m a.s.l. (13a)	4*n*	Topotypes of *B. oblongus oblongus* Nikolsky, 1896	MTD 41347	5	AY830080 AY830081
Bolshoi Balkhan	Turkmenistan, Nebit-Dagskii Rayon, N-slope of Bolshoi Balkhan, approx. 15 km S of Oglanly village, 39°43′ N, 54°29′ E, 500 m a.s.l. (4)	4*n*	? (Western Central Asian tetraploids)	–	2	AY830075 AY830076
Gorgan	Iran, N-slope of Elburz mountains, valley 15 km S Gorgan, approx. 1100 m a.s.l. (2)	2*n*	*B. viridis* cf. *viridis* Laurenti, 1768	–	2	AY830071
NE-Iran	Iran, NE, frontier zone near Turkmenistan, approx. 50 km NE Gonbad-e-Kavus, 37°38′ N, 55°29′ E, 250 m a.s.l. (3)	2*n*	transition zone *B. viridis viridis*/ *B. viridis turanensis*	–	3	AY830073
Danata	Turkmenistan, Ashgabadskaya oblast, stream 2–4 km SE of Danata village and warm spring approx. 4 km SE of Danata, 39°06′ N, 55°06′ E, 200 m a.s.l. (5)	4*n*	Topotypes of *B. oblongus danatensis* Pisanets, 1978 (Western Central Asian tetraploids)	MTD 39400	2	AY830072 AY830074
Ashgabad	Turkmenistan, Ashgabad (11)	2*n*	*B. viridis turanensis* Hemmer et al., 1978	MTD 44397	1	AY830062
Issyk-Kul	Kyrgyzstan, Chu-valley, approx. 20 km W Rybache, near Kokmoynok, approx. 1600 m a.s.l., Kyrgyzstan, Issyk-Kul, S-bank near Tamga, 1670 m a.s.l. (53, 55)	4*n*	Including topotypes of *B. pewzowi unicolor* Kashchenko, 1909 (Central Asian tetraploids)	MTD 40012	2	AY830067 AY830068

Table 1. (Continued)

Bishkek	Kyrgyzstan, Bishkek (39)	2*n*	*B. viridis* cf. *turanensis*	–	1	AY830077
Nuratau	Uzbekistan, Dzhisakskaya oblast, Rayon Farish, Nuratau-Reserve, N-slope of Nuratau Range, 40°35′ N, 66°30′ E, 900–1600 m a.s.l. (17)	4*n*	*Bufo pewzowi* ssp. (Central Asian tetraploids)	MTD 39406, 40010	3	AY830065 AY830069 AY830070
Kashgar	China, Kashgar, 39°29′ N, 76°02′ E, 1350 m a.s.l. (43)	4*n*	*Bufo pewzowi pewzowi* Bedriaga, 1898 (Central Asian tetraploids)	ZSM 107/1998, 108/1998	2	AY830063 AY830064
E-Tien Shan	China, E-Tian Shan (E-Narat Shan), Künas 43°14′ N, 84°40′ E, 2145 m a.s.l. (63)	4*n*	*Bufo pewzowi strauchi Bedriaga*, 1898 (Central Asian tetraploids)	ZSM 109/1998	1	AY830078
Taxkurgan	China, E-Pamir, Taxkurgan, 37°47′ N, 75°14′ E, 3350 m a.s.l. (44)	4*n*	Topotypes of *Bufo pewzowi taxkorensis* Fei et al. 1999 (Central Asian tetraploids)	ZSM 110/1998	1	AY830066

Phylogenetic analysis

Sequences were aligned using Sequencher, vers. 4.1.2 (Gene Codes). We analyzed DNA comprising 815 characters of the mitochondrial control region of 21 karyo-typed specimens from the *B. viridis* subgroup. *Bufo calamita* from Spain (3.1 km S Benalup de Sidona, MVZ 186039, GenBank AY830061) was used as an outgroup. Phylogenetic analyses were carried out using parsimony, and likelihood methods as implemented in PAUP, vers. 4.0b10 (Swofford, 2002) and MrBayes (Huelsenbeck & Ronquist, 2001). We applied Modeltest 3.06 (Posada & Crandell, 1998) to infer the best fitting model of sequence evolution under the PAUP likelihood settings and MrModeltest (Nylander, 2004). Support was estimated by bootstrapping with 1000 pseudo-replicates; branches were collapsed if support was lower than 60%. Likelihood settings and the best-fit model of sequence evolution (HKY; Hasegawa, Kishino & Yano, 1985) incorporating a gamma shape distribution for variable sites (HKY + I + G) were selected by hLRT in Modeltest [nucleotide frequencies A = 0.3143, C = 0.0891, G = 0.1939, T = 0.4027, gamma = 0.3419]; and HKY + I + G in MrModeltest 2.0. [A = 0.3231, C = 0.0841, G = 0.1985, T = 0.3943, gamma = 0.4197] for MrBayes.

Results

Cytogenetic inference

Conventionally stained diploid and tetraploid karyotypes

To test for geographic variability in chromosome morphology and to compare with previously published data, we applied Giemsa staining. Diploid karyotypes (Figures 1–3, 5) consist of six pairs of larger metacentric to submetacentric chromosomes and five pairs of smaller metacentric to submetacentric chromosomes. This shows chromosome morphology to be conservative within the range of 2*n* green toads. The morphology in 4*n*s (Figures 1–3, 6 and 7) is similar to 2*n*s, that is, each chromosome pair of the 2*n* karyotype corresponds to a group of four chromosomes (= quartet) of the 4*n*. However, there are visible differences in the chromosome size and morphology within one quartet in some of the 4*n* taxa. The most obvious one is in quartet 6 (e.g., Figure 1c), in which two chromosomes are distinctly larger

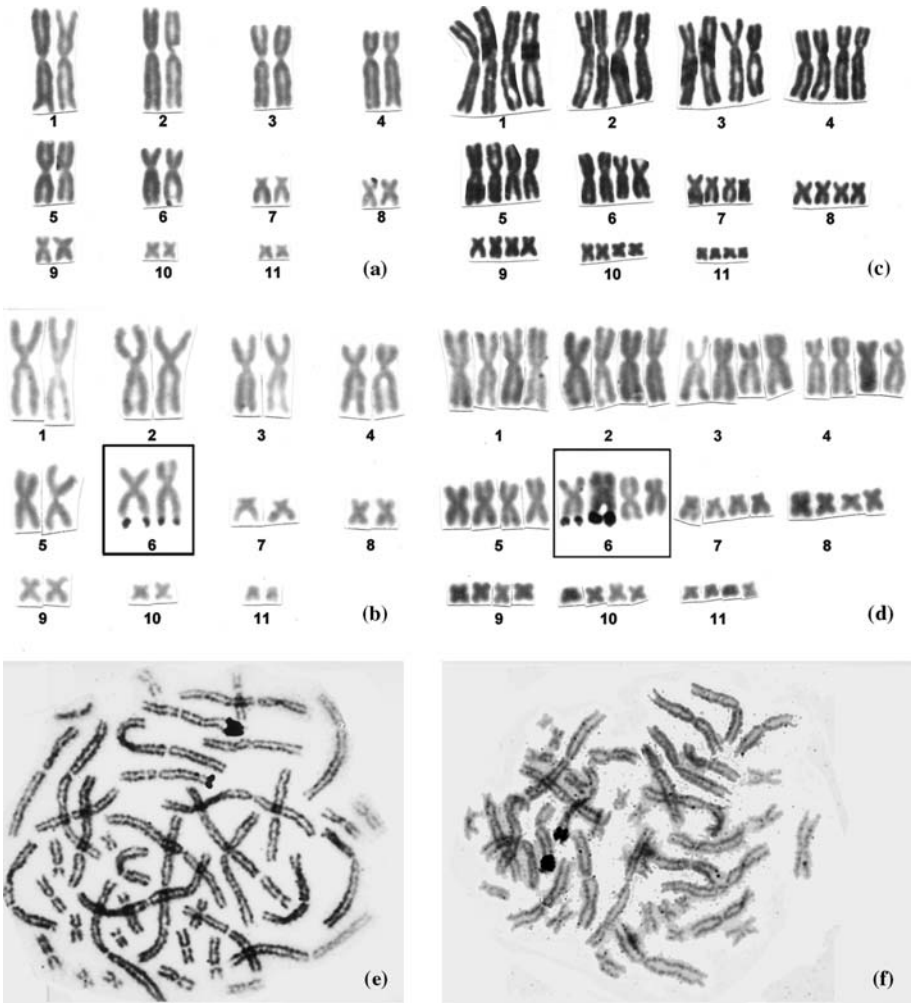

Figure 1. 2*n* and 4n karyotypes. (a, c): Giemsa staining, (b, d, e, f): silver staining. a: *Bufo viridis kermanensis* (Iran, Kerman), (b): *B. viridis viridis/turanensis* (Iran, near Gonbad-e-Kavus), (c, e): *B. oblongus oblongus* (Iran, Birjand), (d): *B. oblongus danatensis* (Turkmenistan, Danata), (f): *B. pewzowi pewzowi* (China, Kashgar).

than the others. This size difference seems mainly caused by the presence or absence of NORs (see below). Other quartets show minor size differences, which allow the division of a quartet in two chromosome pairs.

Variation of size and intensity of nucleolar organizing regions

All 2*n* karyotypes contained only one pair of active homologous NORs, situated terminally in the long arm of chromosomes 6 (Figures 1b, 3a–c and 5). We observed no geographic variation in position or size of the NORs among all 2*n* taxa we tested (*B. viridis viridis* (Germany), *B. v. turanensis* (NE-Iran, Ashkhabad, Bishkek), and *B. v. kermanensis* (Kerman)). All of the NOR-carrying copies of chromosome 6 in the 2*n* karyotypes showed Q-bands in their short arms (see below).

Interestingly, all 4*n* karyotypes contained only one pair of homologous NORs (Figures 1d–f, 3b, d, 4c and 6a, b). 18S + 28S rDNA *in situ* hybridization revealed that potentially duplicated rDNA genes in the 4*n* karyotypes are not only inactive but are indeed physically absent from the two other (homeologous) copies of chromosomes 6. Furthermore, NORs were only found on the long arms

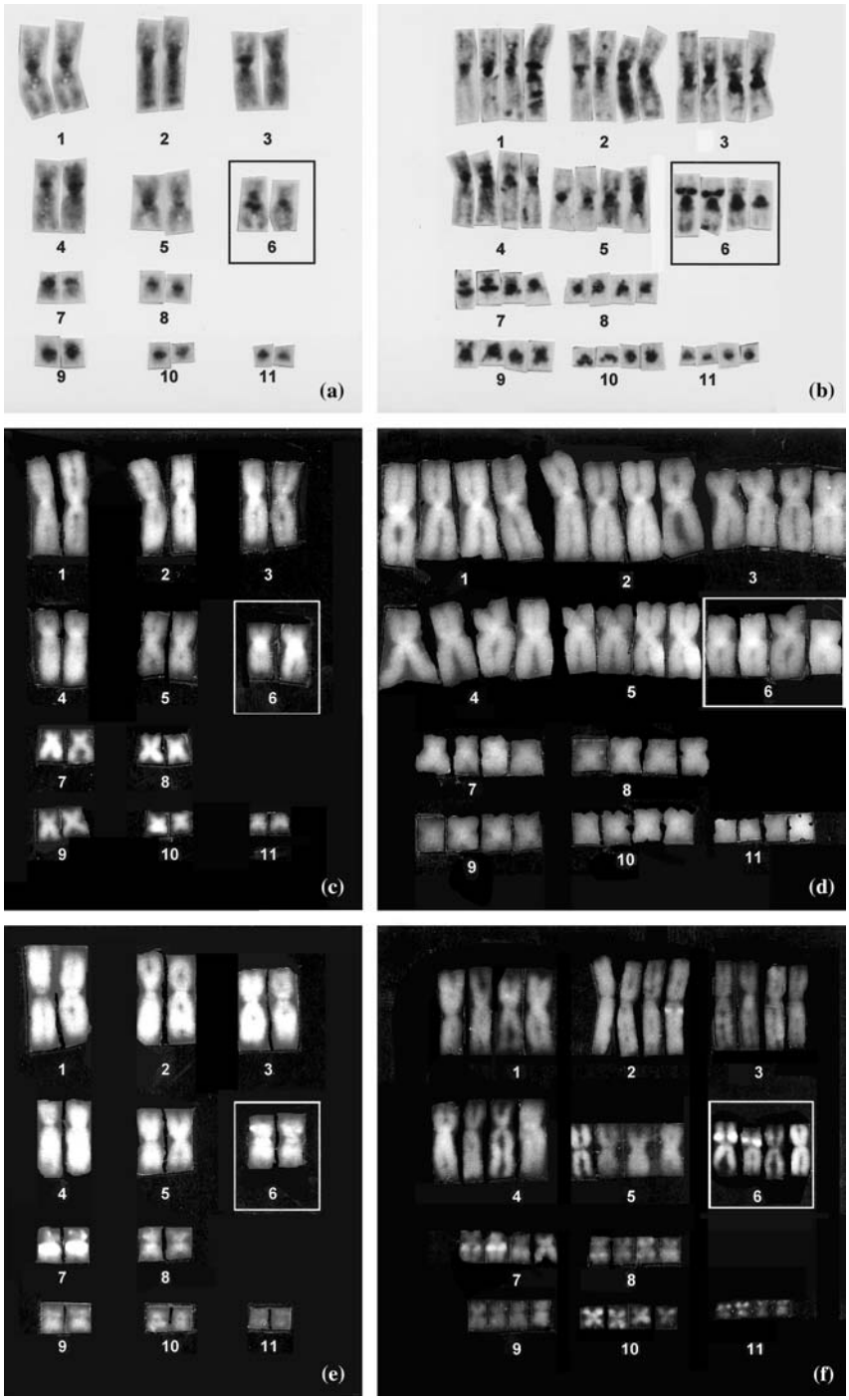

Figure 2. 2*n* and 4n karyotypes. (a, b): C-banding, (c, d): Distamycin A/DAPI (4′-6-diamidino-2-phenylindole) staining; (e, f): Q-banding. (a, c): *Bufo viridis viridis*/*turanensis* (Iran, near Gonbad-e-Kavus); (b, f): *B. oblongus danatensis* (Turkmenistan, Danata); (e): *B. viridis kermanensis* (Iran, Kerman), (d): Central Asian tetraploid (Uzbekistan, Nuratau-Range).

of those 6th chromosomes that showed brightly fluorescing Q-bands in their short arms (see below). The intensity and/or length of the NOR structures was often found to differ within and between individuals of both 2*n* and 4*n* toads, even from the same locality (e.g., Figures 5b and 6a).

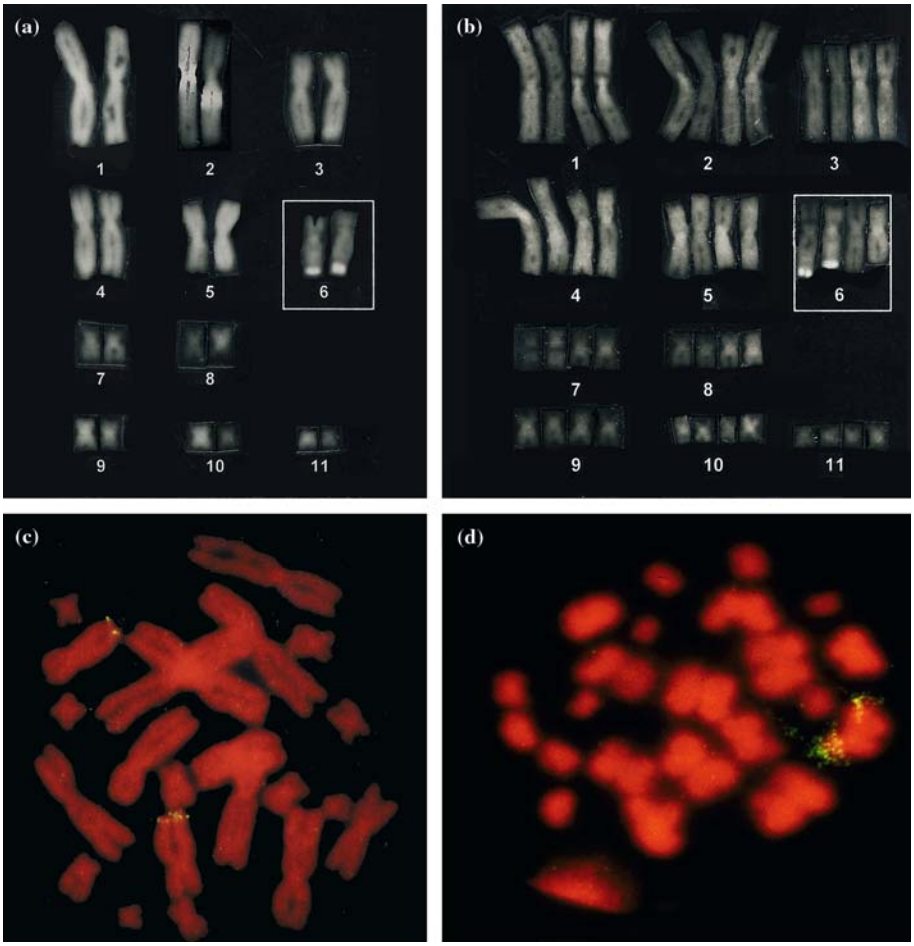

Figure 3. 2*n* and 4*n* karyotypes. (a): Distamycin A/mithramycin counterstaining (note slight manual correction in pair 2); (b): Distamycin A/mithramycin counterstaining (chromosomes 1 + 3 in quartets 1 + 3 were photographically duplicated since they showed substantial overlap), (c, d): 18 + 28 S rDNA *in situ* hybridization. (a): *Bufo viridis viridis*/*turanensis* (Iran, near Gonbad-e-Kavus); (b): *B. oblongus danatensis* (Turkmenistan, Danata); (c): *B. viridis viridis* (Germany, Halle); (d): Central Asian tetraploid (Uzbekistan, Nuratau-Range).

C-bands

The comparison of 2*n* and 4*n* karyotypes (Figures 2a, b) revealed darkly stained regions of constitutive heterochromatin, which slightly varied in size, on both sides of the centromere in all chromosomes. However, within quartet 6 of 4*n*s, large, heavily labeled C-bands (Figure 2b) were observed in the short arms in only two out of four copies of chromosome 6 but never in those of the 2*n* toads. A similar situation was found in two chromosomes of quartet 7 in 4*n*s, having C-bands in their long arms, which were never observed in chromosomes of 2*n*s. In WCA-4*n*, similar to Q-bands (Figures 2f and 6b), the karyotype (Figure 2b) showed distinct differences between two pairs of homeologous pairs in some quartets, especially 6, 7 and 10. Comparison between C- and Q-bands (see below) showed that brightly fluorescing heterochromatic regions are mostly C-band-positive. Therefore, we used the more contrasting Q-banding method for a more detailed karyological comparison of various taxa.

Q-bands

In 2*n* karyotypes, interstitial Q-bands occurred in chromosomes 6–11 (Figure 5), but distinct bright fluorescence was only found in short arms of chromosomes 6 and short and long arms of chromosomes 7. We observed some geographic

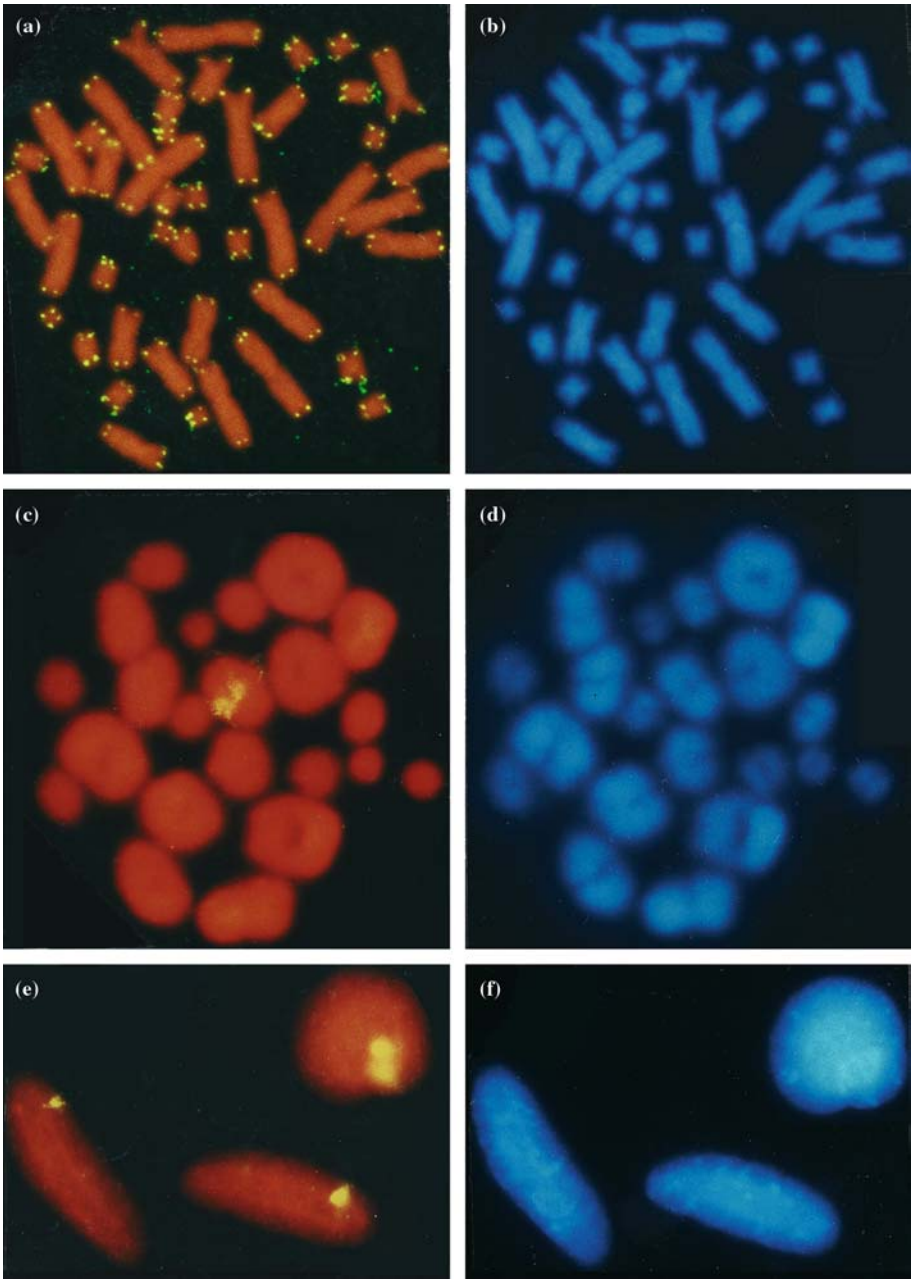

Figure 4. 4*n* chromosomes and nuclei. (a): *In situ* hybridization on telomeres $(TTAGGG)_n$, (b): DAPI-fluorescence of metaphase from (a, c): 18 + 28S rDNA *in situ* hybridization meiotic metaphase I, (d): DAPI-fluorescence of metaphase I shown in (c, e): 18 + 28S rDNA *in situ* hybridization of a spermatogonium second order with two NOR-signals (right above) and sperm nuclei (below) with one NOR-signal each; (f): DAPI-fluorescence of e. (a, b): Western Central Asian tetraploids (Turkmenistan, Bolshoi Balkhan); (c–f): Central Asian tetraploids (Uzbekistan, Nuratau-Range).

variation between different 2*n* taxa, with the intensity of Q-bands becoming weaker towards the East: The representative of *Bufo viridis viridis* (Germany) showed the strongest fluorescence, Q-bands in *B. v. kermanensis* chromosomes (Central Iran) appeared not as bright, and *B. v. turanensis*

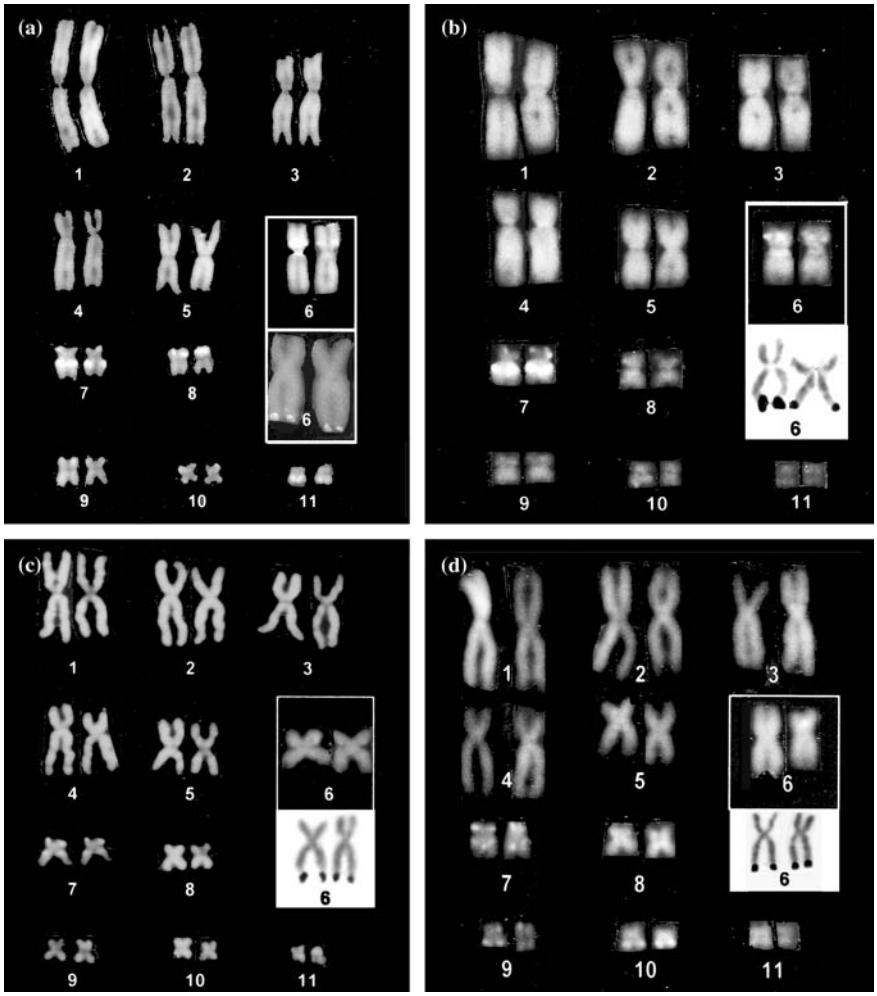

Figure 5. 2*n* karyotypes. (a): Q-banding, 18 + 28S rDNA *in situ* hybridization on NORs of chromosomes 6; (b, c, d): Q-banding, silver staining of NORs. (a): *Bufo viridis viridis*, (b): *B. viridis kermanensis* (Iran, Kerman), (c): *B. viridis viridis*/*turanensis* (Iran, near Gonbad-e-Kavus), (d): *B. viridis turanensis* (Kyrgyzstan, Bishkek).

(NE-Iran and Kyrgyzstan) exhibited the most inconspicuous Q-bands (Figure 5).

The Q-banding patterns of 4*n*s were consistent with those of homeologous chromosomes in 2*n*s. However, we also found brightly fluorescing bands in some chromosomes that did not show any brilliant bands in 2*n*s, for example, one chromosome of quartet 2 in tetraploids from Bolshoi Balkhan (Figure 6a). A single chromosome of quartet 1 in 4*n*s (*B. oblongus*) from Birjand (Figure 7a), and a pair of chromosomes of quartet 2 in tetraploids (*B. oblongus danatensis*) from Danata (Figure 6b) contained these unusual additional Q-bands. Most interestingly, we observed significant variation in the karyotypes of 4*n*s. In those from Bolshoi Balkhan (Figure 6a), Danata (*B. oblongus danatensis*, Figure 6b), and Birjand (*B. o. oblongus*, Figure 7a), all WCA-4*n*, distinct differences in Q-bands within some quartets occurred. This allowed us to divide them into two chromosomes pairs. In contrast, CA-4*n*s from Nuratau (Figure 6c), Issyk-Kul (*B. v. pewzowi unicolor*, Figure 6d), Kashgar (*B. pewzowi pewzowi*, Figure 7b) and Taxkurgan (*B. pewzowi taxkorensis*, Figure 7c) showed homogeneity of Q-bands within quartets. We found the most striking differences in quartet 6. In WCA-4*n* (B. Balkhan, Birjand), it consists of two

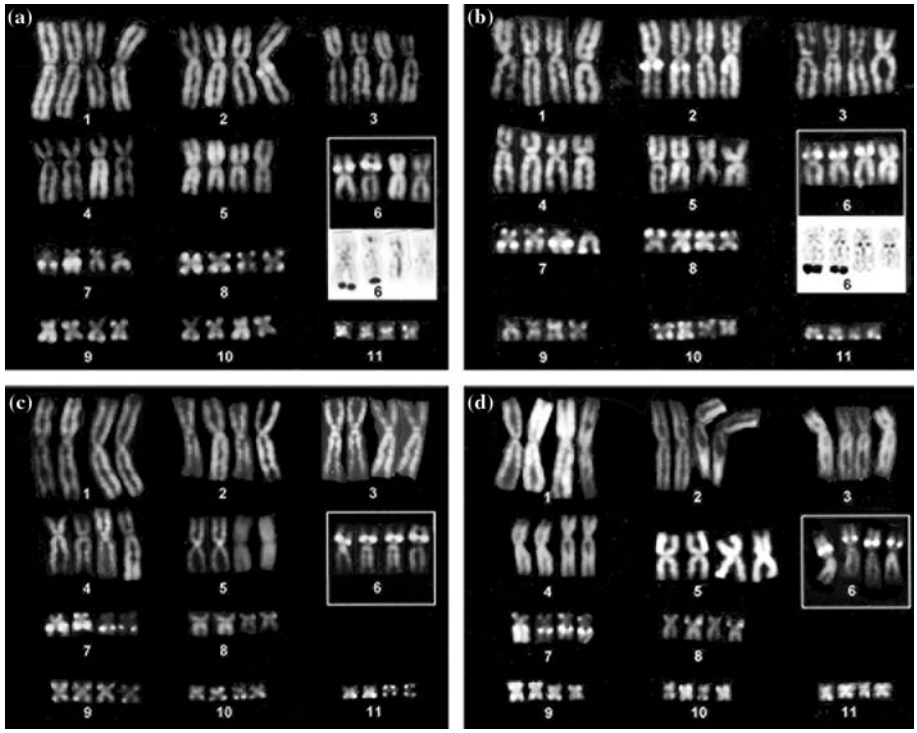

Figure 6. 4*n* karyotypes. (a–d): Q-banding, (a, b): silver staining of NORs of the same chromosomes 6 from Q-banded metaphases. (a): Western Central Asian tetraploids (Turkmenistan, Bolshoi Balkhan), (b): *Bufo oblongus danatensis* (Turkmenistan, Danata), (c): Central Asian tetraploids (Uzbekistan, Nuratau-Range), (d): *B. pewzowi unicolor* (Kyrgyzstan, Issyk-Kul).

chromosomes with Q-bands in their short arms, and two remaining chromosomes lacking bright fluorescence. While some of the tetraploids from Danata exhibited this pattern (Figure 2f), others had a third Q-positive chromosome 6 in this quartet (Figure 6b). At least one chromosome showed no Q-bands in all 4*n*s. Silver staining of originally Q-banded metaphases (Figures 6a, b) of WCA-4*n* revealed NORs to occur exclusively in Q-positive chromosomes 6 while the reverse was not true since more than two NORs were never detected. Individual variation (long arms of only one or two chromosomes showed bright Q-bands) was observed in quartet 2 of WCA-4*n* as seen in specimens from Danata (*B. oblongus danatensis*, Figures 2f, 6b) and B. Balkhan (Figure 6a) and in quartet 1 as observed in a toad from Birjand (*B. oblongus*, Figure 7a). In contrast, karyotypes of the CA-4*n* from Nuratau (*B. pewzowi* ssp., Figure 6c), Issyk-Kul (*B. pewzowi unicolor*, Figure 6d), Kashgar (*B. pewzowi pewzowi*, Figure 7b) and Taxkurgan (*B. pewzowi taxkorensis*, Figure 7c) showed exclusively Q-positive chromosomes 6, but only two out of four carried NORs.

Telomeres and meiosis in tetraploids

In the 4*n* mitotic metaphase, hybridization to the telomere motif $(TTAGGG)_n$ in all chromosomes was observed regardless of chromosome size (Figure 4a). Furthermore, we did not find any intrachromosomal sites of hybridization with the telomere sequence.

To check for multivalent formation or unusual (e.g. hybridogenetic) patterns of gametogenesis, we also examined male meiosis in both groups of tetraploids. Both, WCA-4*n* and CA-4*n* showed exclusively bivalents in male meiosis I (Figures 4c, d). This included the pairing of the two NOR-carrying chromosomes 6, their subsequent segregation into two different spermatids and, finally, in two different sperm nuclei. Therefore, the 2*n* sperm of the tetraploids (Stöck, 2001a, for DNA content – Figure 2 in Stöck et al., 2002) contained only one

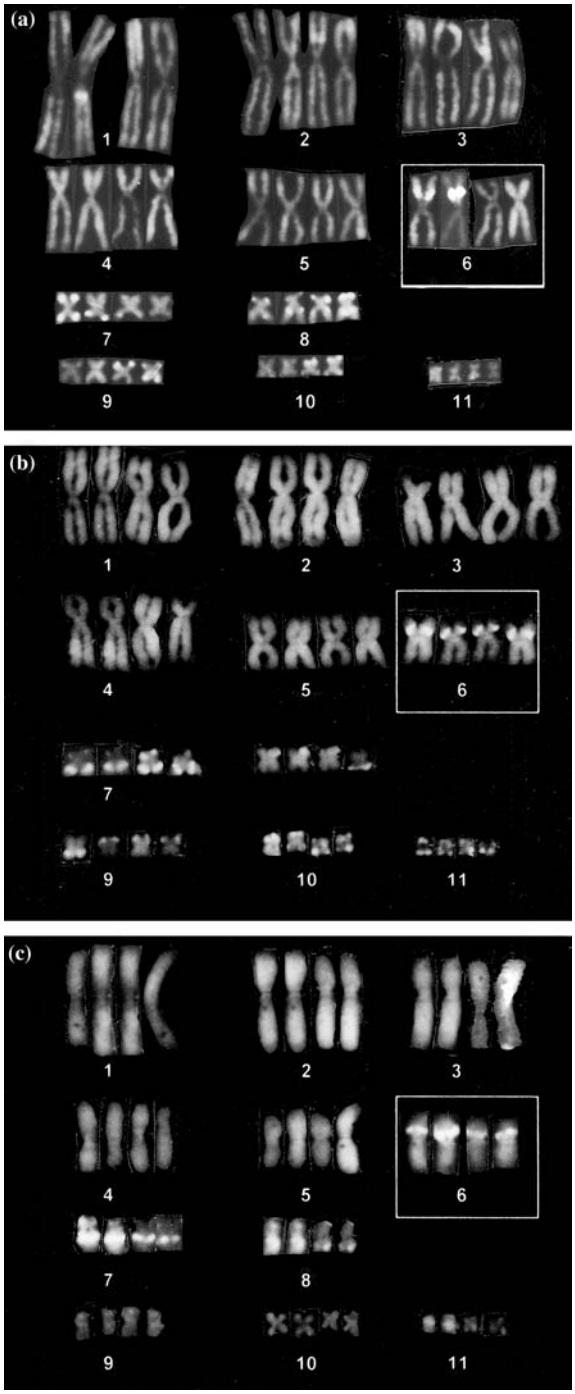

Figure 7. 4*n* karyotypes. (a–c): Q-banding. (a): *Bufo oblongus oblongus* (Iran, Birjand), (b): *B. pewzowi pewzowi* (China, Kashgar), (c): *B. pewzowi taxkorensis* (China, Taxkurgan).

NOR-signal (Figure 4e), whereas somatic nuclei and spermatogonia showed two NOR-signals after 18S + 28S rDNA *in situ* hybridization.

Phylogenetic inference

Of the 815 characters, 504 were invariant, 80 of the variable characters were parsimony-informative, and 231 uninformative. Because likelihood settings (as those in MrBayes) do not allow using gaps in the sequences as taxonomic characters and had to be treated as 'missing data', the maximum likelihood (ML) and Bayesian tree (MB; Figure 9) trees shows slightly less resolution than the MP tree. However, general topology of ML and MB trees are very similar to the MP tree. A heuristic search resulted in 5 most parsimonious trees of which we show the strict consensus tree (MP; Figure 9).

The CA-4*n* from Kashgar (*B. pewzowi pewzowi)* and Taxkurgan (*B. p. taxkorensis*) form a weakly supported clade. Specimens from Issyk Kul (*B. p. unicolor*), where 4*n*s occur exclusively (Borkin, 1989), form a well-supported clade. Tetraploids from Nuratau (*B. pewzowi* ssp.) show some variation and appear not very distinct from those from Kashgar and Taxkurgan, and the single sample from E-Tian Shan (*B. pewzowi strauchi*). The single 2*n* toad from Bishkek is geographically close to all these 4*n*-taxa; however, its mitochondrial genotype is slightly distinctive from them. The WCA-4*n*s cluster in three well supported subclades. Haplotypes of *Bufo o. oblongus* from Birjand, a region where 4*n*s may occur exclusively, are different from those of all remaining 4*n*s, including 4*n*s from Danata (*B. o. danatensis*). However, the two 4*n* individuals from the latter locality each exhibit a common haplotype with individuals from close (< 300 km) populations of 2*n*s (Gorgan, Gonbad, Ashkhabad). Sequences of both 4*n* individuals from Bolshoi Balkhan are very distinctive. Haplotypes of 2*n*s (*B. viridis kermanensis*) in the West of the Central Iranian deserts are strongly differentiated from both 2*n* and the 4*n*s in Eastern Iran.

Discussion

Cytogenetic inference

Chromosome morphology

General chromosome morphology corresponds with results from 2*n B. viridis* reported earlier (Beccari, 1926; Stohler, 1927; Ullerich, 1966;

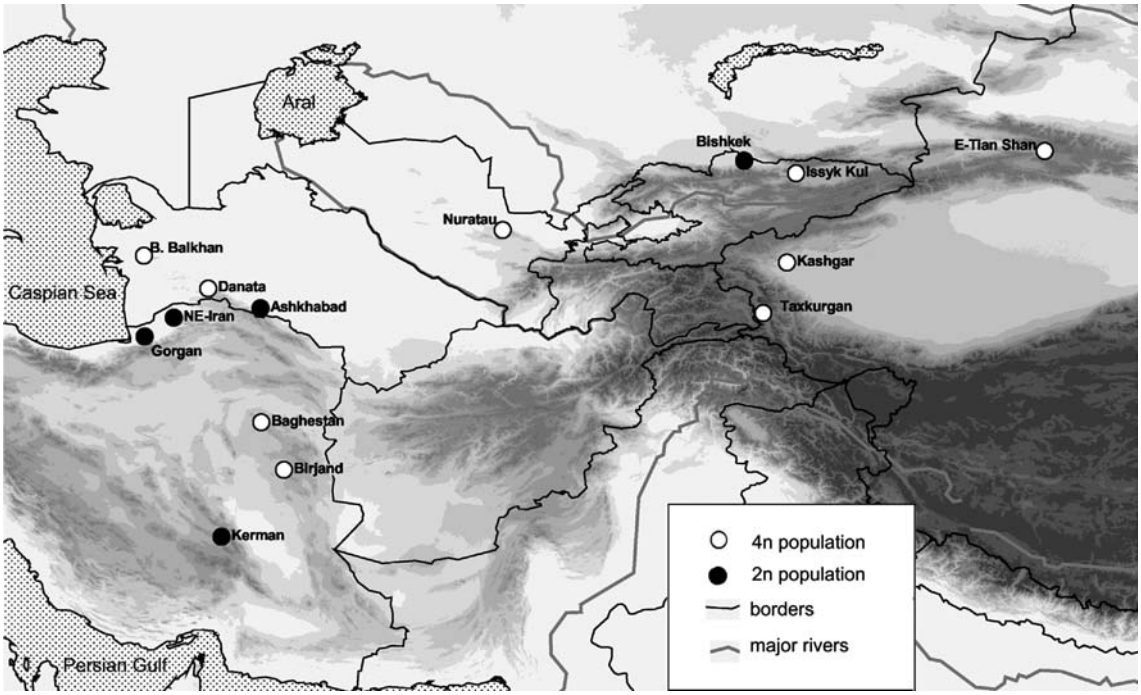

Figure 8. Map of Central Asia and parts of the Middle East with localities described in Table 1 and text.

Birstein, 1981; Bogart, 1972; Roth & Ráb, 1987; Schmid, 1978). The conventionally-stained 4*n* karyotypes reported by several authors (Mazik, Kadyrova & Tokotosunov, 1976; Pisanets, 1978; Borkin et al., 1986a, b; Toktosunov, 1984; Orlova & Uteshev, 1986; Roth & Ráb, 1986, 1987; Wu & Zhao, 1987; Borkin & Kuzmin, 1988; 1991; Stöck, 1998; 2001; Stöck et al., 2001a) show consistent size differences within quartets. However, these stains are not sensitive enough to reveal any taxonomic differences among them and do not allow further comparison with our data.

Nucleolar organizing regions

Since silver staining only marks NORs that were active in the preceding interphase, we also applied in situ hybridization (18S + 28S rDNA) and distamycin A/mithraycin, both sensitive to inactive NORs (e.g., Schmid & Guttenbach, 1988). Our findings were consistent across all three methods. NOR length polymorphism reflects intraspecific (and even individual) variation as frequently demonstrated in higher Urodela, Anura (ref. in Schmid, Vitelli & Bastoni, 1987) and 3*n Bufo pseudoraddei baturae* (Stöck, 2001; Stöck et al., 2002). In mammals, the relative amount of rDNA, as revealed by *in situ* hybridization, determines its level of expression, as detected by silver staining, and also its probability of becoming active (Zurita et al., 1998, 1999); however, in green toads inactive NORs have never been found. While variation in position and numbers of NORs is known in 4*n Hyla versicolor* (Wiley et al., 1989, 2000), and among different 4*n Neobatrachus* (Mahoney & Robinson, 1980; Mahoney & Roberts, 1986), terminal NOR positioning on chromosome 6 appears constant in all three ploidy levels in the *B. viridis* subgroup. Also, in 2*n Bufo latastii*, the NOR-carrying chromosome 5 appears morphologically homologous to chromosome 6 in all other green toads (Stöck et al., 2001b). If this is true, no switch of the NOR to a different *B. latastii* chromosome would have happened, and NOR position may be invariable in the whole *B. viridis* subgroup.

Unusual additional Q-bands in single chromosomes of Western Central Asian tetraploids

Whether this observation (i) reflects an ancestral polymorphism or (ii) is evidence for genome evolution in progress, or (iii) may be a sign of hybridization as common mtDNA sequences of 2*n*s and 4*n*s suggest, remains to be examined (see below).

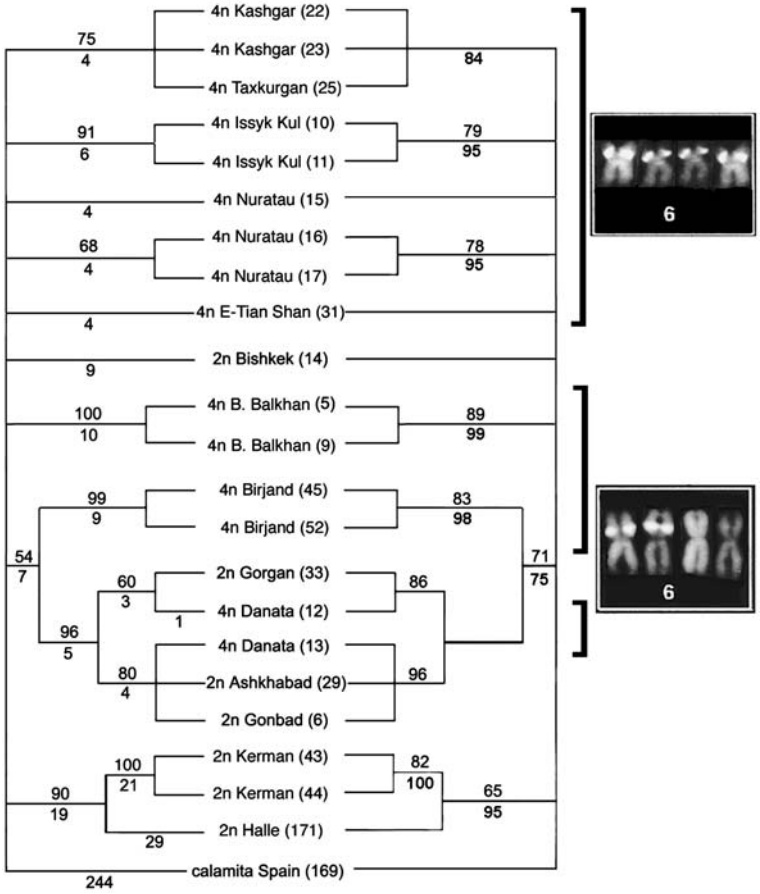

Figure 9. Phylogenetic trees, 815 characters of mtDNA control region, 21 2*n* and 4*n* toads of the Eurasian *Bufo viridis* subgroup (*B. calamita* = outgroup). Left: Consensus of the five MP trees with bootstrap values for 10,000 pseudoreplicates, above, and numbers of changes below branches; right: ML tree with bootstrap values for 100 pseudoreplicates above and posterior probabilities of Bayesian analysis; further right: Q-banded chromosome 6 of 4*n* karyotypes above *B. pewzowi* and related CA-4*n*, below *B. oblongus* and related WCA-4*n*. For locality names see Figure 8 and Table 1; after locality name (in parentheses) individual sample number.

Telomeres

The telomere sequence consists of repetitive $(TTAGGG)_n$ in all vertebrates (Meyne, Ratliff & Moyzis, 1989). Application of telomere probes to 4*n* toads suggests the absence of (i) chromosome fusion of ancestral species or (ii) amplification of retained stretches (latent telomeres) of the tandem $(TTAGGG)_n$ sequence originating from ancestral karyotypes. However, our fluorescence *in situ* hybridization (FISH) technique may not be sensitive to inserts shorter than 1 kb (Schmid et al., 2002). Interstitial telomeric sequences were described, for example, in the 4*n Hyla versicolor* (Meyne et al., 1990; Wiley et al., 2000). In amphibians, telomeric DNA fragments may vary in size between tissues and individuals as reported in *Xenopus* (Bassham, Beam & Shampay, 1998), but in green toads we only examined preparations from single bone marrow cells.

Indications for diploidization in tetraploids and comparisons with other polyploid anurans

The presence of only two NORs in all 4*n* toads demonstrates loss of rRNA genes (= of rDNA) as a sign of diploidization, regardless of their allo- or autopolyploid origin (see below). (Re-)Diploidization has been considered important for stabilizing new gene loci created by duplication events (Ohno, 1970; Schmid, Haaf & Schempp, 1985). Polyploidy may make possible 'myriads of genetic

interactions' as Wendel (2000) recently reviewed for plants and Mable (2004) and Gregory and Mable (2005) showed for animals.

In anurans, diploidization has various consequences and evolutionary stages. While haemoglobin and RNA content, as well as lactate dehydrogenase activity showed equal levels in 2*n* and 4*n* *Odontophrynus* (Becak & Pueyo, 1970; Becak & Goissis, 1971), 4*n* nuclei exhibited four active NORs, which were not (yet) reduced as they would be after complete diploidization (Schmidtke, Becak & Engel, 1976). Although nuclei of 4*n* *Hyla versicolor* have twice as much DNA as 2*n* *H. chrysoscelis*, Bachmann and Bogart (1975) found that 4*n* cells contained less than twice the amount of RNA, histones, and proteins found in the 2*n*s, and NOR number in 4*n* nuclei varies from three to four (Cash & Bogart, 1978) or one to four (Wiley et al., 1989). Mahony and Robinson (1980) observed a similar reduction of NORs in the 4*n* *Neobatrachus kunapalari* (Mahony & Roberts, 1986). Schmid, Haaf, and Schempp (1985) showed diploidization by loss of up to four NORs per octet in 8n *Ceratophrys ornata*. The presence of only two NORs was interpreted as an indication of strong diploidization in ancient 4*n* *Xenopus laevis* (Schmid & Steinlein, 1991).

Allopolyploidy or autopolyploidy of the tetraploid karyotypes

Our data on WCA-4*n* suggest allopolyploidy, and possibly autoploidy in CA-4*n*. However, this is no final solution for the auto-/allopolyploid controversy. While Mezhzherin and Pisanets (1990, 1995a, b) argued for a allopolyploidy of 4*n* green toads using allozymes, conventional karyology (Roth, 1986; Roth & Ráb, 1986), isozymes (Borkin et al., 1986a; Lattes A., 1997, Abstr. 3rd World Congr. Herpetol., Prague: p. 123) and cytometry (Kudryavcev et al., 1988) suggested autoploidy of certain 4*n*s. Some supporters of autotetraploidy exclusively examined toads from the range of CA-4*n* (uniform Q-bands): Borkin et al. (1986a) studied toads from Mongolia, Lattes (1997) from Kyrgyzstan, and Kudryavcev et al. (1988) from Tashkent (Uzbekistan). The latter found protein and RNA content per cells in 4*n*s is twice that of 2*n*s, and concluded auto- rather than alloploidy. However, Mezhzherin and Pisanets (1990, 1995a, b), who studied allozymes presumably in WCA-4*n* and CA-4*n* toads only detected alloploids. Theory predicts (e.g., Roth & Ráb, 1986) that autotetraploids relative to allotetraploids, experience stronger selective pressure leading to fast diploidization, including a molecularly divergent development of similar or identical chromosomes. Bivalents then may quickly replace initial tetravalents during meiosis I to prevent deleterious effects in segregation. In fact, only bivalents and one pair of homologous NORs were observed in both groups of 4*n* toads, so neither allo- nor autoploidy is clearly supportable.

Evolutionary and taxonomic implications

The Q-banding differences showed the evolutionarily and taxonomically most meaningful finding: two groups of 4*n*s, each with fundamental karyological characters: (i) CA-4*n* taxa including toads from Nuratau (*B. pewzowi* ssp.), Issyk-Kul (*B. pewzowi unicolor*), Kashgar (*B. pewzowi pewzowi*) and Taxkurgan (*B. pewzowi taxkorensis*) have uniformly Q-banded quartets, and (ii) WCA-4*n*, including 4*n*s from Birjand (*B.o. oblongus*), Danata (*B. oblongus danatensis*), and the unnamed form from Bolshoi Balkhan show Q-banded karyotypes easily divisible into chromosome pairs. Evidence from flow cytometry (Borkin et al., 1986b, 2001a), isozymes (Mezhzherin & Pisanets, 1995a, b) and morphology (Stöck, 1997, some present karyotypes stem from these toads) also suggested the occurrence of at least two groups of 4*n* toads in Central Asia (details: Stöck et al., 2001b: 269). Since cytogenetic techniques are only 'appropriate under limited circumstances' (Hillis, Mable & Moritz, 1996) to resolve intraspecific geographic variation, differences indicate interspecific differentiation or at least reproductive isolation (e.g. in allopatry). Consequently, Q-banding differences and aforementioned data may reflect more than intraspecific variation but species-level distinctiveness between WCA-4*n* [*Bufo oblongus* Nikolsky, 1896 (Nikolsky, 1896, 1897)] and CA-4*n* (*Bufo pewzowi* Bedriaga, 1898), each probably showing further divergence according to our mtDNA data.

Phylogenetic inference

Common mtDNA haplotypes in diploids and tetraploids in Turkmenistan and Iran

The pattern of common mtDNA haplotypes in 2*n*s and 4*n*s may be explained by either (i) a close common maternal ancestor of 2*n*s and 4*n*s in NE-Iran and Western Turkmenistan or (ii) by

introgression of mtDNA between the gene pools of 2*n*s and 4*n*s. Under scenario (i), both the mitochondrial haplotype and the Q-banding positive karyotype of the 2*n*s would represent the maternal ancestor of WCA-4*n*. These are considered allopolyploids which received their second, Q-banding negative, genome from an unknown paternal ancestor. In the case of (ii), hybridization between 2*n* and 4*n* toads leading to fertile offspring is required. Triploids are reported from one of our localities (Danata; Pisanets, 1978). Stöck et al. (2002) found other fertile triploids in the *B. viridis* subgroup. In addition, unreduced gametes in 2*n* toads may sometimes occur (e.g. Bogart, 1972), fusion of these 2*n* gametes with 2*n* gametes of 4*n*s (Stöck et al., 2002) may sometimes lead to 4*n* offspring. Scenario (ii) is also more likely, because 4*n*s from Danata only have a different mtDNA haplotype but share the karyotype characteristics and morphology with 4*n*s from Birjand. However, more samples must be examined before we can definitively distinguish between these scenarios.

Multiple origins of tetraploid green toads

Our phylogenetic trees are an initial hypothesis. Inferring phylogeny from mtDNA, which is not always exclusively maternally inherited and may include ancient lineage sorting (see the recent review by Ballard & Whitlock, 2004), is especially problematic in allopolyploid taxa, because several evolutionary scenarios (hybridization events) can result in the same tree (e.g. Kobel et al., 1998; Evans et al., 2004; cf. Alves, Coelho & Collares-Pereira, 2001). However, the mitochondrial information combined with the karyotype data supports the hypothesis that at least two tetraploidization events may have taken place with further differentiation caused by geographic isolation.

At least two origins of 4*n*s were also inferred in *Hyla versicolor* (Ptacek, Gerhardt & Sage, 1994) and in *Neobatrachus* (Mable & Roberts, 1997) and more complicated evolutionary scenarios with multiple origins followed by further differentiation appear possible in green toads. This is especially apparent in light of the current knowledge about genome evolution in polyploid plants (e.g. Soltis & Soltis, 1999; Wendel, 2000; Mable, 2003). For a comprehensive understanding of the evolution of the *B. viridis* subgroup, molecular data from the entire Palearctic range appear crucial and such a study is in preparation.

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