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Molecular Phylogenetics and Evolution 41 (2006) 663–689
MOLECULAR PHYLOGENETICS AND EVOLUTION
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Evolution of mitochondrial relationships and biogeography of Palearctic green toads (*Bufo viridis* subgroup) with insights in their genomic plasticity

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Received 2 February 2006; revised 19 May 2006; accepted 22 May 2006
Available online 2 June 2006

Abstract

Taxa involving three bisexually reproducing ploidy levels make green toads a unique amphibian system. We put a cytogenetic dataset from Central Asia in a molecular framework and apply phylogenetic and demographic methods to data from the entire Palearctic range. We study the mitochondrial relationships of diploids to infer their phylogeography and the maternal ancestry of polyploids. Control regions (and *tRNA*s between *ND1* and *ND2* in representatives) characterize a deeply branched assemblage of twelve haplotype groups, diverged since the Lower Miocene. Polyploidy has evolved several times: Central Asian tetraploids (*B. oblongus*, *B. pewzowi*) have at least two maternal origins. Intriguingly, the mitochondrial ancestor of morphologically distinctive, sexually reproducing triploid taxa (*B. pseudoraddei*) from Karakoram and Hindukush represents a different lineage. We report another potential case of bisexual triploid toads (*B. zugmayeri*). Identical d-loops in diploids and tetraploids from Iran and Turkmenistan, which differ in morphology, karyotypes and calls, suggest multiple origins and retained polymorphism and/or hybridization. A similar system involves diploids, triploids and tetraploids from Kyrgyzstan and Kazakhstan where green toads exemplify vertebrate genomic plasticity. A new form from Sicily and its African sister species (*B. boulengeri*) allow internal calibration and divergence time estimates for major clades. The subgroup may have originated in Eurasia rather than Africa since the earliest diverged lineages (*B. latastii*, *B. surdus*) and earliest fossils occur in Asia. We delineate ranges, contact and hybrid zones. Phylogeography, including one of the first non-avian datasets from Central Asian high mountains, reflects Quaternary climate and glaciation.

Keywords: Polyploidy; d-loop; Phylogeny; Population genetics; Palearctic; Phylogeography

1. Introduction

Gene and genome duplications are a major source of evolutionary innovation and diversity. In vertebrates, two aspects can be distinguished: (i) whole genome doubling during early evolution (Ohno, 1970) with accumulating evidence (e.g. Meyer and Schartl, 1999; Taylor and Raes, 2005; McLysaght et al., 2002; Furlong and Holland, 2002; Jaillon et al., 2004), including number and history of duplications (Dehal and Boore, 2005). (ii) More recently evolved polyploids, which provide an opportunity to understand the evolutionary consequences of large genomic changes. In animals, recent polyploids (Gregory and

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doi:10.1016/j.ympev.2006.05.026

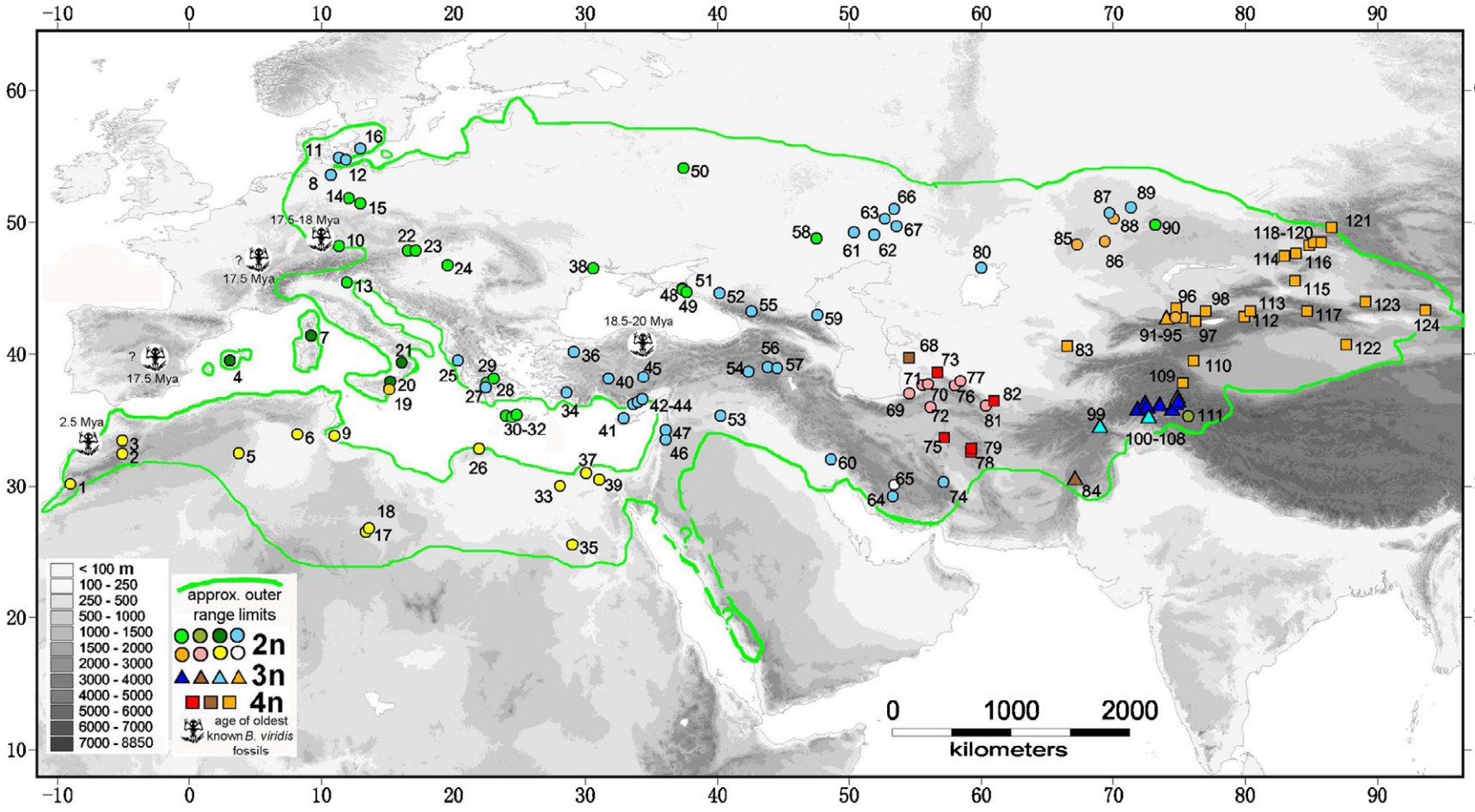


Fig. 1. Geographic range of the *Bufo viridis* subgroup with sampling localities and sites of oldest known fossils of the *B. viridis* subgroup. See legend and Appendix A for locality IDs. Approximate range limits after Balletto et al. (1985), Bons and Geniez (1996), Gasc et al. (1997), Borkin (1999), Kuzmin (1999), Schleich et al. (1996), and Stöck et al. (2001a,b).

Mable, 2005) are known among turbellarians, annelids, mollusks, insects and crustaceans. Among vertebrates, cytogenetic studies revealed numerous clades of polyploids in teleosts (Schultz, 1980; Le Comber and Smith, 2004), amphibians and reptiles (Bogart, 1980), but not in mammals (Contreras et al., 1990; Svartman et al., 2005). Natural polyploids are especially frequent in amphibians in which they evolved in Urodela and Anura (Bogart, 1980; Schmid, 1980; Kawamura, 1984; Vences and Wake, in press).

A challenge for the formation of polyploid animals is the duplication of sex determining loci (for a recent discussion: Mable, 2004; Coyne and Orr, 2004), often resulting in deviations from sexual and/or meiotic reproduction. In vertebrates, reproductive modes without (so far identified forms of) recombination are parthenogenesis (Suomalainen et al., 1987; Dawley and Bogart, 1989; Simon et al., 2003) and gynogenesis (Beukeboom and Vrijenhoek, 1998). True parthenogenesis among vertebrates is apparently restricted to reptiles with all-female clones producing identical daughters that lack any paternal contribution. In gynogenesis, found in fishes and amphibians, embryogenesis is triggered by sperm from allo-specific males, but usually without their genetic input. "Hybridogenetic reproduction in a broad sense" (Stöck and Lamatsch, 2002) can be termed different hemiclonal (Schultz, 1969) and meroclonal (Vinogradov et al., 1990) mechanisms, characterized by elimination of complete chromosome sets and clonal or meiotic inheritance of the remaining sets (e.g. Alves et al., 2001; Günther et al., 1979; Günther, 1990; Stöck et al., 2002). Remarkably, several vertebrate complexes (mainly fishes, amphibians and few reptiles) include animals of various ploidy levels and show common occurrence of these main reproductive modes, in which clonal, hemiclonal and/or meroclonal as well as sexual mechanisms contribute to genetic diversity, interaction of ploidy levels and evolution (e.g. Darvesky et al., 1989; Alves et al., 2001; Günther, 1990; Zhou et al., 2000; Bogart and Klemens, 1997; Goddard et al., 1998; Bogart, 2003; Rab et al., 2000; Vasilev et al., 2003; Ogielska et al., 2004; Plötner, 2005). This genomic diversity and plasticity and in some systems the interaction of individuals with sexual and asexual reproduction makes them ideal systems to address an enormous variety of evolutionary questions. This includes consequences of gene and genome doubling, evolutionary genetics of hybridization with the interplay of two or more foreign genomes in one nucleus and one organism, hybrid fertility and fitness, sex determination, occurrence or absence of meiosis, crossing over and consequences of the partial loss, or maintenance, of recombination, and finally hybrid ("recombinational", Coyne and Orr, 2004, or "collective" Morjan and Rieseberg, 2004) speciation and evolution.

In this comparative context green toads of the Palearctic *Bufo viridis* subgroup (Borkin, 1999; Stöck et al., 2001a) stand out. They are the only known complex of amphibians that comprises diploid (2n),[1] tetraploid (4n; Mazik et al., 1976) and even triploid (3n; Stöck et al., 1999, 2002) bisexually reproducing taxa. Whereas 2n and 4n toads reproduce meiotically, one of the three chromosome sets of South Asian 3n *Bufo pseudoraddei* seems neither to participate in crossing over nor in random segregation (recombination) during meiosis but rather is clonally inherited (females) or eliminated (males; Stöck et al., 2002), a mechanism related to hybridogenesis. In High Asia, i.e. in the eastern Pamirs, Karakoram, Hindukush and western Himalayas, green toad taxa of all three ploidy levels occur allopatrically in similar high mountain environments (Stöck et al., 2001b). Cytogenetic data (Stöck et al., 2005) suggest that Central Asian 4ns have evolved independently at least twice, and there is morphological evidence for two separate 3n taxa (Stöck et al., 2001a).

For the Asian green toads, we use the nomenclature as revised by Stöck et al. (2001a) 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 diploid 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 recognized two tetraploid species: (I) *B. oblongus* Nikolsky, 1896, with *B. o. oblongus* and *B. o. danatensis*—provisionally called "Western Central Asian Tetraploids", and (II) *B. pewzowi* Bedriaga, 1898, with nominal subspecies *B. p. pewzowi*, *B. p. unicolor*, *B. p. strauchi* and *B. p. taxkorensis*,—termed "Eastern Central Asian Tetraploids". In the literature of the 1980s and 1990s, all Central Asian tetraploids (now *B. oblongus*, *B. pewzowi*) were mostly called "*B. danatensis*", a younger subjective synonym of *Bufo oblongus*. Bisexual triploid taxa are represented by *B. pseudoraddei* (Mertens, 1972), with *B.p. pseudoraddei* and *B. p. baturae*. Because of contradictory data or unknown ploidy, the status of *B. asiomontanus* and *B. zugmayeri* remained unclear (further details: Stöck et al., 2001a).

The polytypic *B. viridis* subgroup (sensu Stöck et al., 2001a, see Section 4) inhabits an enormous Palearctic range, and previous morphological and general biogeographic knowledge suggest the occurrence of multiple

[1] *Abbreviations used:* 2n, diploid; 4n, tetraploid; 3n, triploid; 2ns, diploids; 3ns, triploids, 4ns, tetraploids; LGM, last glacial maximum of the Pleistocene; MRCA, most recent common ancestor; My, Million years, Mya, million years ago; ML, Maximum likelihood analysis, MP, Maximum Parsimony analysis; MB, Bayesian analysis using MrBayes; mtDNA, mitochondrial DNA; BMNH, British Museum of Natural History London, United Kingdom; MTD, Museum Tierkunde Dresden, Germany; MVZ, Museum of Vertebrate Zoology, University of California, Berkeley, USA; ZSM, Zoologische Staatsammlung Munich, Germany, HNHM, Hungarian National History Museum, Budapest, Hungary; ZFMK, Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn, Germany; CUP, Charles-University, Praha, Czech Republic; NME, Naturkundemuseum Erfurt, Germany; CAS, California Academy of Sciences, San Francisco, USA; CS, Collection Schmidtler, private collection (will be transferred to ZSM) of Josef Friedrich Schmidtler, Munich, Germany.

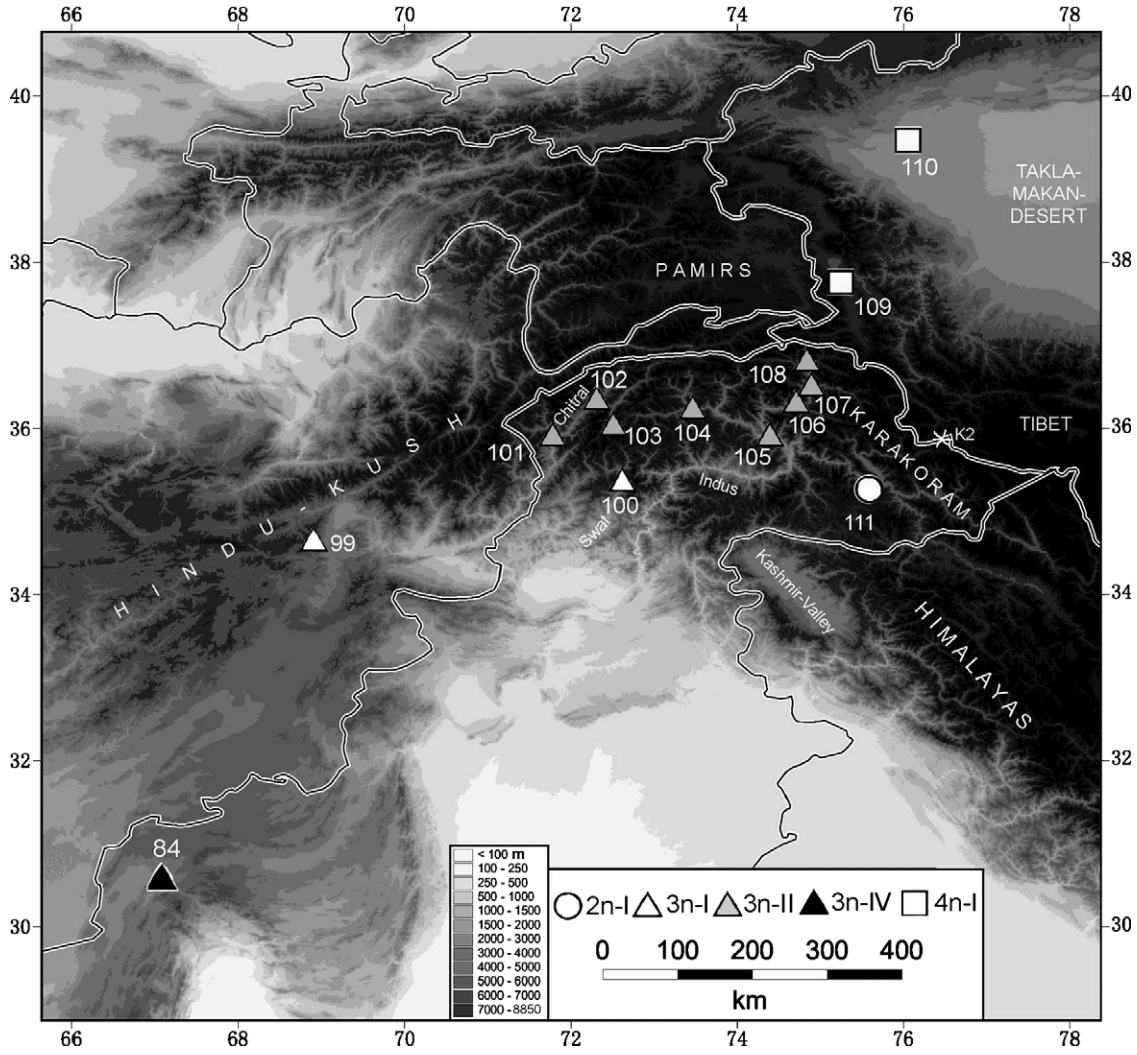


Fig. 2. Geographic range of the *Bufo viridis* subgroup in High Asia with sampling localities. See legend and Appendix A for locality IDs.

lineages, as there has been no range-wide analysis of molecular variation. Consequently, a first step towards the understanding of the evolution of the subgroup is to generate a comprehensive phylogenetic and phylogeographic hypothesis of the 2n lineages, and to analyze the mitochondrial relationships of the polyploids.

Two hypothesis can be tested: (i) The bisexually reproducing 3n south of the Karakoram–Hindukush watershed and the 3n forms in north central Asia, geographically close to 2n and 4n toads, are derived from the same recent mitochondrial ancestor or (ii) all these forms represent descendents of several polyploidizations involving different lineages.

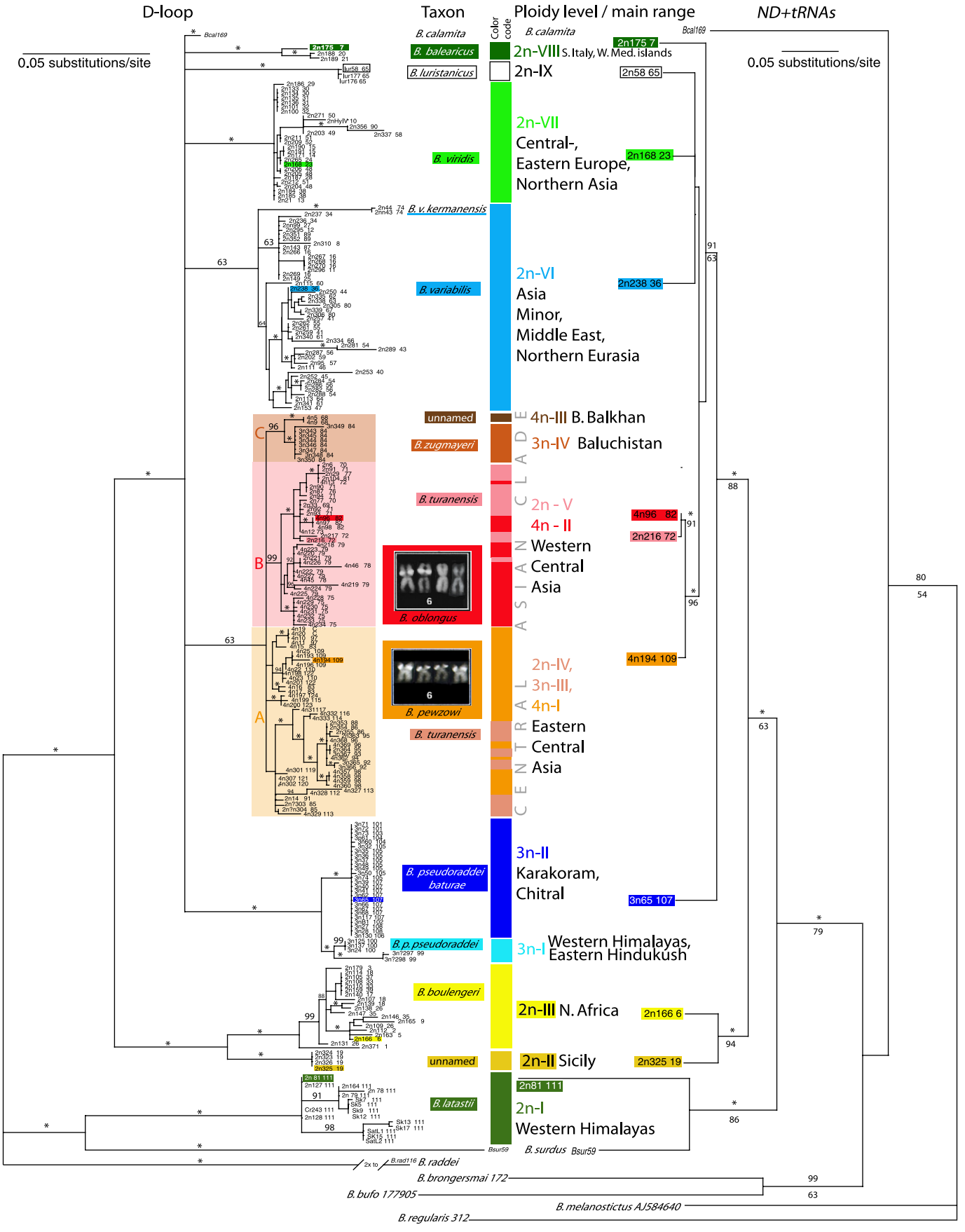
In this paper, we present mtDNA evidence for separate matrilineal ancestry of 3n *B. pseudoraddei* and 4n Central Asian polyploid taxa (*B. pewzowi, B. oblongus*) and report the discovery of a new likely sexually reproducing 3n taxon (*B. zugmayeri*), which also belongs to the Central Asian lineage. Our phylogeographic analysis of this subgroup also provides a comparative historic biogeographic perspective derived from one of the most widespread amphibian complexes of the Palearctic realm.

2. Materials and methods

2.1. Sampling, DNA extraction, PCR conditions

A total of 325 specimens [135 2ns (+2 presumably 2ns), 114 3ns (+3 presumably 3ns), 71 4ns] of the *B. viridis* sub-

Fig. 3. Phylograms resulting from a Bayesian analysis of d-loop sequences (left) and a ML-analysis of *ND* + *tRNA*s (right). D-loop tree (left): ploidy levels (e.g. 4n), sample ID (max. three digits, see Appendix A), and locality ID as in (Figs. 1 and 2). F_1 individuals resulting from crosses are designated by a "C" or "Hy" instead of a locality ID. Note potentially applicable taxon names for many groups. Color-labeled individuals in the d-loop tree (left) are identical to those that yielded the *ND* + *tRNA* sequences for the tree on the right. Groups A–C all belong to the "Central Asian clade". For both trees, Bayesian posterior support values are shown above major nodes (∗ = 100), below Maximum likelihood bootstrap values (*ND* + *tRNA*s). For the Central Asian Clade, Q-banded chromosomes 6 of two tetraploid forms (*B. oblongus*, *B. pewzowi*) are depicted.


D-loop
0.05 substitutions/site
Taxon
Color code
Ploidy level / main range
ND+tRNAs
0.05 substitutions/site
B. calamita
B. balearicus
B. luristanicus
2n-VIII S. Italy, W. Med. islands
2n-IX
2n-VII
Central-,
Eastern Europe,
Northern Asia
B. viridis
B. v. kermanensis
2n-VI
Asia
Minor,
Middle East,
Northern Eurasia
B. variabilis
unnamed
4n-III B. Balkhan
B. zugmayeri
3n-IV Baluchistan
B. turanensis
2n - V
4n - II
Western
Central
Asia
B. oblongus
B. pewzowi
2n-IV,
3n-III,
4n-I
B. turanensis
Eastern
Central
Asia
3n-II
Karakoram,
Chitral
B. pseudoraddei baturae
B. p. pseudoraddei
3n-I Western Himalayas, Eastern Hindukush
B. boulengeri
2n-III N. Africa
unnamed
2n-II Sicily
B. latastii
2n-I
Western Himalayas
B. surdus
B. raddei
B. brongersmai 172
B. bufo 177905
B. regularis 312
B. melanostictus AJ584640
CENTRAL ASIAN CLADE
A
B
C

group, originating from 124 (85 for 2n, 11 for 3n, 28 for 4n) localities (Figs. 1 and 2) throughout the Palearctic range were included (see Appendix A). For most toads from the range of the polyploids we have karyotypic as well as microdensitometric or flow cytometric data on ploidy of sequenced specimens (Stöck, 1997, 1998a,b, Stöck et al., 1999–2005). Several additional specimens were karyotyped (data not shown). Green toads from the *B. viridis* subgroup, which occur west of a line between western Iran and northwestern Kazakhstan, were considered 2n because no previous study has revealed polyploids (Borkin, 1999; Stöck et al., 2001b), except as rare accidental cases (Odierna et al., 2004) presumably resulting from unreduced ova (Bogart, 1972). In addition, diploidy of several green toads from North Africa was confirmed by flow cytometry. If no ploidy data were available, all samples east of 80° E were considered 4n, because neither 2n nor 3n have been detected in that region (Borkin et al., 2001; Stöck et al., 2001b). In a small number of cases, tissue samples from remote localities without ploidy information were included. Toads were collected by the authors or provided by natural history museums and many colleagues (see Acknowledgements). We also included crosses (F_1) resulting from two chromosomally different 4n taxa (*B. oblongus* father × *B. pewzowi* mother), from 3n (*B. pseudoraddei baturae* father) with 2n (*B. latastii* mother) toads obtained in the laboratory, and from a natural cross (*B. calamita* father × *B. v. viridis* mother), in order to test for maternal inheritance of the mitochondrial marker. All tested F_1 from these three interspecies crosses (Appendix A for details) shared their marker with their mothers (Fig. 3: "C", "Hy") and confirmed that d-loop sequences represent authentic mtDNA. Many specimens from the 3n *B. pseudoraddei baturae* and 2n *B. latastii* were released after blood sampling but vouchers are available from these and many other populations (Appendix A). From some localities, toe clips or tail tip samples of tadpoles were used. In order to test for common ancestry and maternal contribution to the formation of polyploids of the *B. viridis* subgroup, we also sequenced d-loops and *ND + tRNA*s of several taxa previously considered to be related or even closely related members of the subgroup (*B. arabicus, B. brongersmai, B. calamita, B. luristanicus, B. mauritanicus, B. raddei, B. surdus* and *B. stomaticus*). While *B. raddei* served as the "outgroup" taxon for the phylogenetic analyses of the d-loops, *B. regularis* was used for rooting the "*ND + tRNA*s" tree (see below).

Genomic DNA was extracted from frozen or ethanol preserved blood, liver, muscle tissue, pooled organs (tadpoles) and muscle of vouchers from scientific collections using a phenol–chloroform extraction (~20% of samples) or the Quiagen DNeasy™ kit. About 880 bp were amplified, comprising most of the mitochondrial control region (= "d-loop"; primers ControlB-H, CytbA-L; PCR: 96 °C, 2 min, denaturation; 52 °C, 45 s, annealing; 72 °C, 2 min, extension; cycle [94 °C, 30 s, denaturation, 52 °C, 45 s annealing, 72 °C, 1.5 min, extension]38times; 72 °C, final extension, 5 min; Goebel et al., 1999). In representatives from most clades (and ploidy levels), as revealed from analyses of d-loop sequences (see below), as well of *B. calamita, B. brongersmai, B. bufo* and *B. regularis*, we sequenced an additional 1100 bases of mtDNA extending from *ND1* through the $tRNA^{Ile}$, $tRNA^{Gln}$, and $tRNA^{Met}$ genes to *ND2* (termed "*ND + tRNA*s" here), as described by Macey et al. (1998a,b). All PCR-products were sequenced in both directions on an ABI 3730 sequencer. Sequences were aligned using Sequencher, v. 4.1.2 and adjusted by eye using MacClade 4.06.

2.2. Phylogenetic and phylogeographic analysis

The complete alignment of d-loop sequences comprised 898 characters. Because of questionable alignment, characters 125–166 were excluded from the analyses. We applied MrModeltest (vers. 2.0 modified from Modeltest, Posada and Crandall, 1998, by J.A. Nylander, Uppsala Univ., Sweden) for determining the best fitting model of sequence evolution (HKY+I+G, AICE). We inferred phylogeny with Bayesian statistics (MB, MrBayes v. 3.0b4; Huelsenbeck and Ronquist, 2001), running four chains for 10 million generations, with tree sampling every 1000 generations. We also estimated relationships using neighbor joining (NJ, 1000 bootstrap pseudoreplicates) and parsimony methods (MP, 100 bootstrap steps) as implemented in PAUP, vers. 4.0b10 (Swofford, 2002). For analysis of *ND + tRNA*s, we used ML-settings from Modeltest in PAUP (HKY+I+G, Nst = 2, TRatio = 6.7648, Rates = gamma Shape = 0.7208, Pinvar = 0.5616, 100 bootstrap steps). For some d-loop clades, we used the parsimony-based network analyses program TCS (vers. 1.18, Clement et al., 2000).

2.2.1. Genealogical analysis, estimations of evolutionary rates and minimum divergence times

We estimated the age of population expansion for green toad groups as found in a certain geographical region using *Fluctuate* (Kuhner et al., 1998) by obtaining maximum likelihood estimates for θ ($2N\mu$; μ is DNA substitution rate per site per generation, N is the current female effective population size) and g (the historical exponential growth parameter). Repeated analyses to ensure stability of estimates were run with random seeds, 10 short Monte Carlo chains of 4000 steps, and five long chains of 20,000 steps. Growth was inferred using logarithmic likelihood ratio tests with one degree of freedom (Huelsenbeck and Rannala, 1997). If a no-growth model was rejected, g values were used to approximate the time at which effective population size was 10% of the current effective size by applying our DNA substitution estimate. Estimates of exponential growth (g in units of μ^{-1}) were used to approximate population size at time t in the past from $N_t = \theta^{e-(g\mu)t}$ where N_t is the effective size at time t in the past (Kuhner et al., 1998; Wares and Cunningham, 2001). Using this equation, t was estimated by substituting N_t with $N_t/N_{t=0} = 0.1$. We also calculated Tajima's D using the program Arlequin 2.000 (Schneider et al., 2000). For each estimate we define a population by the geographical region in which a clade is found. We assume that the time at which the effective size was 10% is an approximation of a population's

minimum age. We also assume that each clade's current distribution encompasses its place of origin, and that each clade is defined by an ancestral haplotype and all regional descendants. As another indication of population growth, we report Tajima's D, which is expected to be significantly negative under demographic expansion or a recent selective sweep at a linked locus (Tajima, 1989).

Estimated rates of anuran mtDNA-substitution (Macey et al., 1998a,b), comparisons of coding and d-loop-mtDNA (Liu et al., 2000; Sumida et al., 2000) and major geological events such as the last connection of Africa and Europe and the fossil record served for rough calibrations. We estimated the d-loop (and *ND* + *tRNA*) rate(s) by assuming that the last landbridge between North Africa and Sicily broke off about 5.3 Mya, i.e. the Strait of Sicily was formed at the end of the Messinian salinity crisis at the Miocene/Pliocene boundary (e.g. Jaeger et al., 1987; Krjigsman et al., 1999; Butler et al., 1999; Dobson and Wright, 2000; F. Rögl pers. comm.). By comparing populations from north and south of the Strait of Sicily, d-loop and *ND* + *tRNA* rates were estimated from the average pairwise genetic divergences between the respective samples using the best fitting substitution model (Modeltest). Because the mtDNA MRCA is likely to precede the population divergence (Arbogast et al., 2002), we corrected d-loop sequence divergence for ancestral polymorphism by assuming that the ancestral divergence is equivalent to the divergence found in current African samples. Using this rate estimate, other divergence times among pairwise regional groups were estimated with $\mu = (\pi_b - \pi_w)/2\tau$, where τ is the divergence time, μ is the DNA substitution rate per locus per generation, π_b is the average number of pairwise differences between sampled populations, and π_w is the average number of pairwise differences within populations (Nei and Li, 1979). In all cases, pairwise genetic distances were based on the best-fit model of DNA evolution. This method assumes migration among regions to be negligible, a reasonable assumption given regional monophyly. Divergence times were estimated among all green toad "regional taxa", defined by the most inclusive haplotype clades associated with major geographic regions. We also conducted log-likelihood ratio tests (Huelsenbeck and Rannala, 1997) using PAUP to test for a molecular clock in D-loop and in *ND* + *tRNA*s alignments using different *Bufo*-species as outgroups and tested these in Bayesian and ML-trees, respectively.

3. Results

Bayesian, NJ, and MP analyses distinguished twelve major mitochondrial clades (d-loop), most of which are well supported and differ substantially from each other (Fig. 3). In clades of diploids and clades containing polyploids both mitochondrial markers reflect deep divergences in an old group with substantial structure and up to ~30% divergence, and >2% between the youngest sister clades (Table 1). Most clades are distributed allo- or parapatrically. Only two 2n clades (2n-VI, 2n-VII) exhibit apparent wide range overlap (Fig. 1). Polyploids belong to two major clades, the 'Central Asian clade' (Fig. 3) and the clade comprising two nominal subspecies of 3n *B. pseudoraddei* (3n-I, 3n-II). The geographic distribution of clades (Fig. 1) reflects the expected higher genetic diversity in the south than in the north, with all clades present between 30° and 40° but only three between 45° and 55° N.

Likelihood ratio tests for clocklike evolution were sensitive to different outgroup species, suggesting difficulties in estimating the correct model of sequence evolution and/or correct alignment. When the *ND* + *tRNA*s ML-tree for all green toads was rooted with *B. calamita*, clocklike evolution was found. We estimated divergence for the Pi_{NET}rate = 0.06777 per d-loop per 5.3 My (ca. 1.278% divergence per My), Pi_{BTW}rate = 0.08712 per d-loop per 5.3 My (ca. 1.644. % divergence per My) and the Pi_{BTW}rate = 0.0571 per *ND* + *tRNA*s (ca. 1.077% divergence per My).

Below, we characterize the various clades based on ploidy, geographic ranges and/or taxonomic identity using the Bayesian d-loop phylogram (Fig. 3).

3.1. Groups of diploid green toads

Mitochondrial DNA of the 2n taxa belongs to nine major clades which cover the entire Palearctic range with the exception of the Asian high mountain and eastern continental desert areas that are dominated by polyploids (3n, 4n).

3.1.1. 2n-I Western Himalayas

MtDNA of the geographically isolated high mountain 2n *Bufo latastii* from the western Himalayas (Ladakh, loc. 111) is most closely related to that of a single specimen of the poorly known *B. surdus*, and both form an early diverged sister group to all remaining green toad mitochondrial lineages. *Bufo latastii* exhibits slight chromosomal differences but close bioacoustic and morphological affinities to other *B. viridis* subgroup taxa (Dubois and Martens, 1977; Stöck et al., 2001a). Substantial haplotype structure in clade 2n-I may be explained by an origin of toads from different tributaries of the Indus, which meet at Skardu/Ladakh (loc. 111). A zero growth model could not be rejected by *Fluctuate* and Tajima's D (0.096). This lineage is of considerable age and might have diverged from the mtDNA-lineage to all remaining green toads more than 20 Mya (Table 1).

3.1.2. 2n-II Southeastern Sicily

Four identical d-loop sequences of unnamed green toads from the San Leonardo river in southeastern Sicily (loc. 19), isolated by the Mt. Etna massif from northern Sicily (loc. 20 of 2n-VIII), represent a sister clade to the mtDNA of all African green toads (2n-III). This old relationship is also confirmed by the *ND* + *tRNA*s (Fig. 3) and was used to calibrate the divergence time estimates, assuming the most recent common ancestor (MRCA) with clade 2n-III may have existed at least 5.3 Mya.

Table 1
Average divergence between different groups of toads and minimum divergence time estimates based on mitochondrial sequences

Nominal taxa			*B. variabilis* (2n)			*B. viridis* (2n)			*B. boulengeri* (2n)			unnamed (2n)			*B. balearicus* (2n)			*B. pewzowi* (4n) + *B. turanensis* (2n) + 3n (F1-hybrids?)			*B. oblongus* (4n) + *B. turanensis* (2n)			unnamed (4n), *B. zugmayeri* (3n)			*B. pseudoraddei baturae* (3n)			*B. pseudoraddei pseudoraddei* (3n)			*B. latastii* (2n)		
D-loop clades, **ploidy groups included,** *Individual sample number for ND+tRNA marker*			Asia Minor			Europe			Africa			Sicily			S-Italy, W-Mediterranean Islands			E-Central Asia			W-Central Asia			B. Balkhan, N-Baluchistan			Karakoram, Chitral			W-Himalaya, E-Hindukush			W-Himalayas		
	2n-VI	*2n238*				4.06	3.16	*4.48*	9.88	7.69	*10.67*	10.83	8.42	*10.23*	4.59	3.57	*6.43*	3.94	3.07	*4.1*	4.1	3.19	*4.10; 4.27*	4.44	3.46	7.97	6.2	*6.47*	7.63	5.93	19.58	15.23	*25.09*
Europe	**2n-VII**	*2n168*	5.19	6.92	*4.82*				10.11	7.87	*10.18*	10.45	8.13	*11.26*	4.87	3.79	*5.79*	4.65	3.62	*3.59*	4.56	3.54	*3.51; 3.36*	4.22	3.28	7.93	6.17	*6.52*	7.25	5.64	20.72	16.12	*27.81*
Africa	**2n-III**	*2n166*	12.64	14.92	*11.49*	12.93	14.31	*10.97*				NA*	NA*	*NA**	10.08	7.84	*12.91*	9.08	7.06	*9.38*	8.87	6.9	*9.72; 9.88*	9.24	7.19	9.99	7.77	*11.22*	9.99	7.77	23.51	18.29	*29.71*
Sicily	**2n-II**	*2n325*	13.84	15.16	*11.02*	13.36	13.77	*12.13*	7.74	8.71	*5.71*				12.51	9.73	*12.78*	9.53	7.41	*10.37*	9.64	7.5	*10.94; 10.75*	10.5	8.17	11.64	9.05	*9.74*	11.27	8.76	23.46	18.25	*29.15*
S Italy, W. Med. Isl.	**2n-VIII**	*2n175*	5.86	8.00	*6.93*	6.53	7.46	*6.24*	12.89	14.67	*13.71*	16.00	16.62					5.24	4.07	*5.76*	5.11	3.97	*6.02; 5.58*	5.48	4.26	7.27	5.66	*8.58*	6.89	5.36	21.34	16.6	*30.87*
E. Central Asia	**A** (2n-IV, 3n-III, 4n-I)	*4n194*	5.04	7.12	*4.42*	5.95	7.13	*3.97*	11.61	13.35	*10.10*	12.18	12.95	*11.17*	6.70	8.29	*6.20*				2.23	1.73	*1.14; 0.86*	0.76	0.59	7.33	5.7	*6.47*	7.01	5.46	21.04	16.37	*27.73*
W. Central Asia	**B** (2n-V, 4n-II)	*2n216, 4n96*	5.24	7.26	*4.60; 4.42*	5.82	6.94	*3.79; 3.62*	11.34	13.01	*10.64; 10.10*	12.33	13.03	*11.79; 11.58*	6.53	8.06	*6.49; 6.02*	2.85	4.32	*1.23; 0.93*				1.19	0.92	7.87	6.13	*6.33; 6.47*	7.44	5.78	19.68	15.31	*27.77; 28.03*
B. Balkhan, Baluchistan	**C** (3n-IV, 4n-III)	/	5.68	7.3	/	5.40	6.11	/	11.82	13.09	/	13.42	13.73	/	7.01	8.13	/	0.97	2.04	/	1.52	2.53	/			6.96	5.42	/	6.72	5.23	21.19	16.48	/
Karakoram	**3n-II**	*3n65*	10.20	11.53	*6.98*	10.14	10.57	*7.02*	12.78	13.77	*12.09*	14.88	14.90	*10.49*	9.30	10.15	*9.24*	9.37	10.16	*6.97*	10.07	10.80	*6.82; 6.97*	8.90	9.23				1.58	1.23	25.36	19.72	*29.00*
W. Himalaya E. Hindukush	**3n-I**	/	9.75	11.86	/	9.27	10.47	/	12.77	14.53	/	14.41	15.20	/	8.81	10.42	/	8.97	10.53	/	9.51	11.01	/	8.60	9.69	2.02	2.84	/		1.59	25.06	19.49	/
W. Himalaya	**2n-I**	*2n81*	25.03	27.2	*27.04*	26.50	27.76	*29.97*	30.06	31.88	*32.01*	30.00	30.85	*31.41*	27.28	28.96	*33.27*	26.91	28.53	*29.87*	25.17	26.73	*29.93; 30.20*	27.09	28.25	32.42	33.30	*31.24*		33.68			

Upper right triangle: Minimum divergence time estimates (My); light columns: estimates based on divergence rate Pi_{NET}rate = 0.067765 per d-loop per 5.3 My [ca. 1.278% divergence per My]; gray columns: estimates based on divergence rate Pi_{BTW}rate = 0.08711833 per d-loop per 5.3 My [ca. 1.644% divergence per My]; gray columns with numbers in *italics*: Pi_{BTW}rate = 0.05710676 per *ND* + *tRNA*s [ca. 1.077% divergence per My]; dark frame marks sister relationship used for calibration. Lower left triangle: Average divergence between groups (%), light columns: Pi_{NET} distances; gray columns: Pi_{BTW} distances.

* Because the four Sicilian samples lacked any sequence differences and the ancestral polymorphism was likely more similar to the current African sample, we based the π_w value on this only.

Table 2
Demographic analysis of various green toad taxa and groups as revealed from mtDNA d-loop analysis

Mitochondrial d-loop clade (or ploidy group)	Taxon	N	Theta	g	Ln (likelihood) for L_{max}	Ln (likelihood) for zero growth	$2(L_{max} - L_g = 0)$	No growth can be rejected	Tajima's D	p	MinAge estimate for corrected rate (Pi_{NET}) (Mya)	MinAge estimate for non-corrected rate (Pi_{BTW}) (Mya)
Western Himalayas (2n-I)	*B. latastii*	16	0.0143	45.465	0.0051	−0.1354	0.281	No	0.09572	0.40	NA	NA
North Africa (2n-III)	*B. boulengeri*	19	0.0935	11.62	0.0091	−0.3864	0.791	No ($p \sim 0.35$)	−0.93585	0.19	NA	NA
Europe (2n-VII)	*B. viridis*	27	0.0154	66.407	0.0698	0.001	0.1376	No ($p \sim 0.11$)	−1.02858	0.16	NA	NA
Asia Minor (2n-VI)	*B.variabilis*	46	0.1589	87.934	0.011	−4.578	9.178	Yes	−1.31292	0.09	2.44	1.17
(A) Eastern Central Asia (2n-IV + 3n-III + 4n-I)	Group A	44	0.1006	262.377	0.1066	−7.0139	14.241	Yes	−0.27707	0.41	0.681	0.471
Eastern Central Asia (2n-IV)	*B. turanensis*	7	0.1269	−2.8881	0.249	−4.0623	8.6226	NA	−1.1176	0.15	NA	NA
Eastern Central Asia (3n-III)	hybrids (2n × 4n)?	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Eastern Central Asia (4n-I)	*B. pewzowi*	32	0.0935	242.0563	0.0041	−4.7767	9.5616	Yes	−0.1615	0.45	0.678	0.464
(B) Western Central Asia (2n-V + 4n-II)	Group B	37	0.0955	103.87	0.0769	−3.717	7.5878	Yes	−1.28285	0.10	1.682	1.16
Western Central Asia (2n-V)	*B. turanensis*	14	0.0456	410.0382	0.0390	−2.1518	4.3816	Yes	−0.64087	0.27	0.285	0.184
Western Central Asia (4n-II)	*B. oblongus*	23	0.0474	86.2195	0.0199	−1.8963	3.8324	No ($p \sim 0.06$)	−1.17946	0.12	1.39	0.90

3.1.3. 2n-III 2n North Africa

Toads from western Morocco to eastern Egypt differ substantially from the geographically nearest diploids in the Middle East (e.g. loc. 46, 47), and 2n toads (2n-VIII) on western Mediterranean islands (loc. 4, 7, 20) and in southern Italy (loc. 21). However, clade 2n-III is sister taxon to Sicilian 2n-II. Neither *Fluctuate* nor Tajima's D reflected population growth (Table 2). The sister clades 2n-II and 2n-III form a well-supported group, which is the sister taxon to all 2n, 3n and 4n green toads [except *B. latastii* (2n-I) and *B. surdus*] and probably diverged from these between 7 and 12 Mya (Table 1). The application of the name *Bufo boulengeri* Lataste, 1879 [*nomen nudum* according to Frost, 2004] to African green toads is justified because the *B. boulengeri* type is extant in BMNH (Clarke, pers. comm.).

3.1.4. Diploids in the Central Asian clade

For analyses of maternal ancestry of the two 4n taxa, we divided the Central Asian clade into three groups (Fig. 3: groups A–C), mainly based on specific chromosomal characters in each of the 4n taxa in groups A and B (see below). This approach also results in a somewhat artificial subdivision into two diploid groups (2n-IV, 2n-V), whose differences may simply reflect isolation by distance. The name *B. turanensis* is applied (Stöck et al., 2001a) to these large-sized diploid toads (2n-IV, 2n-V), but data from the type locality (Dushanbe, Tajikistan) are lacking.

3.1.4.1. 2n-IV Eastern Central Asia. Diploid toads in this paraphyletic group A were detected in the semi-desert and steppe of northern Kazakhstan (loc. 85, 86, 88) and N to the Tian-Shan of northern Kyrgyzstan (loc. 91, 94). Analysis of seven d-loops of 2ns provided a negative value for growth ($g = -2.88$, *Fluctuate*) but also negative Tajima's D (close to significance). The mtDNA phylogeny is tightly intertwined with groups 3n-III and 4n-I (*B. pewzowi*) of the same region. We treat them as a paraphyletic "group A" (Figs. 3 and 4). The closest relatives of group A are clades B (2n-V + 4n-II) and C (4n-III + 3n-IV), with which they form the moderately supported "Central Asian clade" (Fig. 3).

3.1.4.2. 2n-V Western Central Asia. Mitochondrial sequences of 2n-V in northeastern Iran (loc. 69–72) and the Kopet Dagh range of eastern Iran and western Turkmenistan (loc. 76, 77, 81) show high growth values ($g = 410$) based on *Fluctuate* and a negative Tajima's D (−0.64; but not significant) and suggest expansion of this population, an event that dates between ~0.2 and 0.3 Mya. This is only half the estimated (expansion) age of the partly syntopic 4n-II (see below). The 2n-V individuals cluster together with those of 4n-II (*B. oblongus*), with which they form subclade B.

3.1.5. 2n-VI Asia Minor, Middle East and northern Eurasia

This is the only haplotype group found in Anatolia. It also occurs on Cyprus (loc. 41), in the Middle East and western Iran (loc. 60, 64). Toads of this clade are separated from subclade B (2n-V, 4n-II) by the central Iranian

deserts. The clade 2n-VI occurs in the Caucasus and to the northeast in the steppe zone of northwestern Kazakhstan (loc. 61–67), the northern Aral Sea (loc. 80) and further east (loc. 87, 89). MtDNA in the most northern populations in Scandinavia (loc. 8, 11, 12, 16) belongs to the same clade, either representing a range disjunction or a connection via Eastern Europe (see 4.4), from which data are missing. Toads from western central Iran (loc. 74, *B. v. kermanensis*) differ from all remaining members of this clade. A specimen from eastern Syria (loc. 53) yielded a shorter sequence (not in tree) but clearly belongs to this group. Separation of the 2n-VI mitochondrial lineage from European (2n-VII), western Mediterranean (2n-VIII) and all Central Asian groups (A–C) is estimated to have happened between Lower and Middle Pliocene, while its separation from mitochondrial ancestors of African (2n-III, 2n-II) and South Asian clades (3n-I, 3n-II, 2n-I) probably dates back to Middle to Lower Miocene (Table 1). Population growth (Table 2) led us to estimate a minimum expansion age of 2n-VI between 1.2 and 2.4 Mya.

We tentatively refer to these populations as *Bufo variabilis* (Pallas, 1769), since their range (loc. 8) includes the type locality (Lübeck; Stöck et al., 2001a).

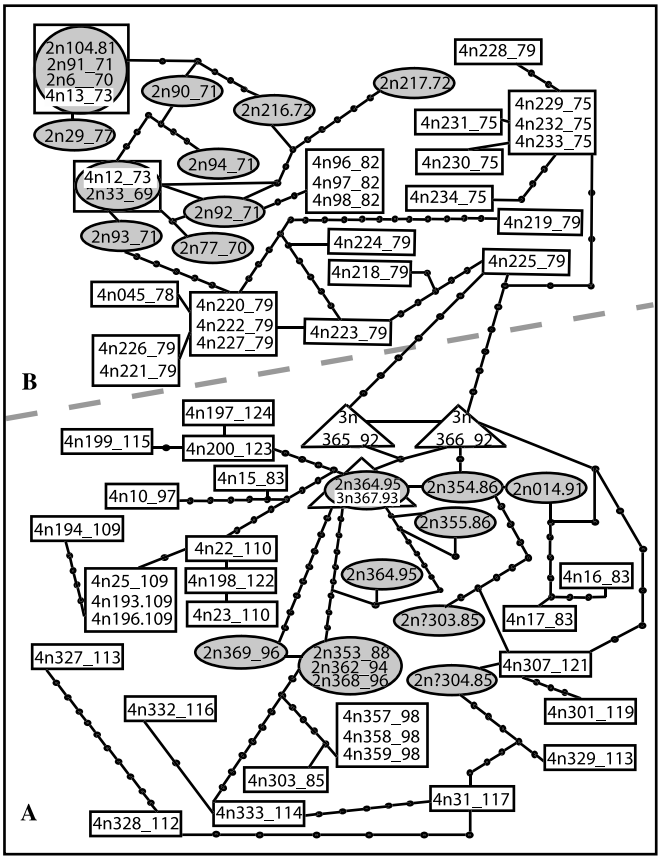


Fig. 4. Parsimony-based haplotype network obtained with the program TCS 1.18 using d-loop sequences of groups A and B of the tree shown in Fig. 3 (left). Gray ellipsoids represent 2n, white rectangles 4n and white triangles indicate 3n toads. Black dots represent hypothetical haplotypes not sampled. Ploidy levels (e.g. 4n), sample ID (max. three digits, see Appendix A), and locality ID as in (Figs. 1 and 2).

3.1.6. 2n-VII Central, southeastern Europe and northern Asia

This clade was detected on the Greek mainland, Crete and in northeastern Italy (loc. 13). It apparently dominates most parts of Central Europe (loc. 10, 14, 15, 22–24), occurs in southeastern Europe (loc. 38) and northwest of the Caucasus (loc. 48, 49, 51). In Russia it reaches the northern edge of the range of the subgroup (loc. 50). Possibly disjunct populations were also found in northeastern (loc. 58) and north central Kazakhstan (loc. 90) where the clade meets 2n-VI and 2n-IV toads. The potentially ancestral haplotype inferred from a network analysis (TCS, not shown) was found in the southern Ukraine (loc. 38) and northeastern Italy (loc. 13) and is only one mutational step apart from Greek and Crete haplotypes. Divergence-time estimates of 2n-VII from all other clades are nearly identical to those of the Asia Minor clade (2n-VI), suggesting that a contemporaneous event was responsible for initial vicariance of 2n-VI and 2n-VII. Although moderate growth was detected, is was not significantly different from a zero growth model (Table 2). Clade 2n-VII represents *B. viridis viridis* (loc. 22, 23 near the type locality (Vienna).

3.1.7. 2n-VIII Southern Italy and West Mediterranean islands

MtDNA of 2n toads from several islands (Corsica, loc. 7; northern Sicily, loc. 20) and the southern Apennine Peninsula (Calabria, loc. 21) clustered together. A museum specimen from Mallorca (loc. 4) provided a shorter readable sequence (not shown in tree) and is assigned to this clade. The small sample size prevented us from application of *Fluctuate*. This lineage probably diverged from ancestors leading to all European (2n-VII), Asia Minor (2n-VI) and Central Asian groups (A–C) between the Lower and Middle Pliocene. It may have split from the last common mitochondrial ancestor with the South Asian clades (3n-I, 3n-II, 2n-I) during Lower to Upper Miocene. The name *Bufo balearicus* Boettger, 1880 is applicable to this taxon (e.g. Garcia-Paris et al., 2004).

3.1.8. 2n-IX Bufo luristanicus

Three sequences of toads from a single locality (loc. 65) form a well supported clade. A phylogenetic position close to *B. viridis* (2n-VI) and *B. variabilis* (2n-VII) is shown by *ND + tRNA*s. As compared to the 2n clades, this taxon differs cytogenetically from them (M. St. unpubl. data).

3.2. Groups of tetraploid green toads

Tetraploid toads exclusively originated from a single, mitochondrial lineage in Central Asia (Fig. 3: "Central Asian clade"). We label these groups based mainly on morphology (Stöck, 1997) and chromosomal characters (Stöck et al., 2005): 4n-I, 4n-II and 4n-III.

3.2.1. 4n-I Eastern Central Asia

4n toads of this group are widely distributed from Uzbekistan (loc. 80) to the west across the Tian Shan (loc. 97–98, 112–113), to the western Altay range (loc. 118–121) and the Dzungarian Gobi of Mongolia in the East (loc. 123, 124) and southwards to the eastern Pamirs of northwestern China (loc. 109, 110). Although mitochondrial data are lacking, based on morphology and 4n karyotypes the haplotype group reaches the southeastern most edge of the subgroup's range in the Kun-Lun (northern Tibet). Towards the south, the >5000 m high Karakoram range isolates 4n-I from 3n-II toads (Fig. 2). MtDNA phylogeny of 4n-I is tightly linked to that of 2n-IV and 3n-III (group A) as shown by a haplotype network (Fig. 4). Growth values (242; Table 2) yielded minimum population-growth estimates between 0.5 and 0.7 Mya. Tetraploids of group A share uniform Q-banding patterns (Stöck et al., 2005), differ morphometrically from 4n-II toads (Stöck, 1997) and represent *B. pewzowi* (Stöck et al., 2001a).

3.2.2. 4n-II Western Central Asia

4ns in northeastern Iran (loc. 75, 78, 79, 82) and western Turkmenistan (loc. 73) show considerable range overlap with 2n-V. In group B, toads of both ploidy levels (2n-V, 4n-II) share a mtDNA subclade and sometimes possess identical haplotypes (Figs. 3 and 4). However, we know only a few localities (81, 82) with syntopic occurrence of 2ns and 4ns. Moderate growth (Table 2) led to minimum expansion age estimates of 4n-II between 0.9 and 1.1 Mya, which is older than estimates for other Central Asian toad groups (4n-I, 2n-IV). 4n-I toads of clade B share distinct Q-banding differences in some chromosome quartets (Stöck et al., 2005; our Fig. 3). They represent *B. oblongus* (details: Stöck et al., 2001a).

3.2.3. 4n-III Bolshoi Balkhan

4n toads from the isolated mountain Bolshoi Balkhan in the western Karakum desert of Turkmenistan (loc. 68) share the karyotype characteristics (Stöck et al., 2005) of *B. oblongus* (4n-II) but are morphologically distinct (Stöck, 1997) and lack a name. They represent the sister taxon of 3n-IV, with which they form the well supported clade C, sister group to all Central Asian 2n, 3n and 4n toads in groups A and B (Fig. 3). For 4n-III this is consistent with a long geographical isolation (see Section 4).

3.3. Groups of triploid green toads

Triploid toads belong to two very different mitochondrial lineages: one well supported clade comprising two sexually reproducing triploid South Asian high mountain taxa (3n-I, 3n-II), and the others in the "Central Asian clade" (Fig. 3). In the latter, we detected 3ns in groups A (3n-III) and C (3n-IV). The 3n-I/3n-II clade, known from high mountain valleys in Karakoram and Hindukush, has no close diploid or tetraploid relatives.

3.3.1. 3n-I Western Himalayas and eastern Hindukush

This well supported clade consists of a population of all-triploid males and females from the isolated upper Swat valley (Fig. 2: loc. 100, western Himalayas) and two morphologically similar toads of unknown ploidy from west of Kabul (loc. 99). *Fluctuate* yielded negative growth values (apparently caused by the inability of the program to deal with genetic uniformity). The divergence time estimates suggest that the mtDNA lineage of 3n-I separated from all other green toad clades (except the South Asian 3n-II and 2n-I) between Middle and Lower Miocene (Table 1), while the lineage leading to 3n-I perhaps diverged during the early Pleistocene from 3n-II but earlier than the Lower Miocene from 2n-I. Based on morphology, Stöck et al. (1999) found 3n-I to be different from 3n-II and the name *B. pseudoraddei pseudoraddei* to be valid (Stöck et al., 2001a).

3.3.2. 3n-II Karakoram and Chitral

108 triploid toads (only 26 in tree) from eight populations (Fig. 2: 101-107) in the Karakoram and Hindukush valley of Chitral, inhabiting three different high mountain drainages (Chitral, Gilgit, Hunza river), show almost no variation of d-loop sequences. This indicates a single origin of all 3n-II toads and suggests their recent range expansion to these localities (101–107), although a formal test (*Fluctuate*) is precluded by the extraordinarily low genetic diversity. The valid name is *B. pseudoraddei baturae* (Stöck et al., 1999, 2001a). It represents a mitochondrial sister clade of *B. p. pseudoraddei* (3n-I), from which it was separated during the Pleistocene (Table 1). In contrast, the lineage leading to *B. p. baturae* (3n-II) separated very early (at least Lower Miocene, Table 1) from the mitochondrial lineage of geographically close *B. latastii* (2n-I, western Himalayas, Figs. 1 and 2: loc. 111). Although the minimal absolute distance is below 100 km, mtDNA genotypes of 3n-II are isolated by the Kunyerab Pass (>4600 m), and therefore are highly differentiated from *B. pewzowi* (4n-I) in China (loc. 109, 110), which reached the eastern Pamirs (loc. 109) from the north and represents a clade which may have shared a most recent common mitochondrial ancestor with the 3n-I + 3n-II lineage in the late Miocene (7.3–5.7 Mya). The Central Asian clade includes triploids from a single site in northern Baluchistan (loc. 84) and triploids in zones of range overlap between diploid (2n-IV) and tetraploid (4n-I) in northern central Asia.

3.3.3. 3n-IV Northern Baluchistan

Eight toads from the type locality of *B. zugmayeri* (Pakistan, Pishin, loc. 84) have a very similar d-loop sequence. Triploidy based on chromosome preparations of three males and one female suggests the discovery of a second bisexually reproducing all-triploid taxon. Toads from Pishin (loc. 84) are the sister group to 4n-III (loc. 68) and this group C is sister group to all other Central Asian green toads (groups A + B) of our study.

3.3.4. 3n-III Eastern Central Asia

3ns of group B of the Central Asian clade appear to be more closely related to diploids (2n-V) than to tetraploids (4n-I), suggesting they may be 3n F_1-hybrids resulting from 2n female × 4n male crosses (Fig. 4).

3.4. Species excluded as maternal ancestors of polyploid green toads

D-loop sequences of *Bufo arabicus*, *B. brongersmai*, *B. luristanicus*, *B. mauritanicus*, *B. raddei*, *B. surdus* and *B. stomaticus* differ substantially from that in all polyploid *B. viridis* subgroup taxa. This excludes *B. stomaticus* from being the recent maternal ancestor of *B. pseudoraddei baturae*, with which they are sympatric in the Hindukush (Chitral city, loc. 101). *Bufo raddei* also did not contribute mitochondrially to allo- or parapatric *B. pewzowi* (4n-I) in northwestern China and Mongolia (Stöck, 1998b). In addition, *B. luristanicus* and *B. surdus*, occurring in sympatry with 2n *B. variabilis* (2n-VI) in western and southern Iran, cannot represent the maternal ancestor of western Central Asian *B. oblongus* (4n-II). Based on their d-loop sequences, *B. luristanicus* and *B. calamita*, appear in a polytomy with several other green toads (Fig. 3, left). Nevertheless, *B. calamita* is not closely related to them based on the *ND* + *tRNA*s (Fig. 3, right), bioacoustic (Stöck et al., 2001c) and chromosomal differences. Similarly, *ND* + *tRNA*s show *B. luristanicus* to be more closely related to European *B. v. viridis* (2n-VII) and *B. variabilis* (2n-VI) than to Central Asian 4ns, but further biological data on this rarely collected species are needed.

4. Discussion

Our study is a geographically comprehensive genetic analysis of the *B. viridis* subgroup. The subgroup is notable for its broad geographic range, its unsettled taxonomic and nomenclatural history and, especially, for the presence of polyploids, including sexually reproducing triploid lineages. Our main aims were to (i) provide insights into the historical biogeography of the diploid taxa, and (ii) to investigate the matrilineal history of the diverse polyploid lineages. As expected for such a widespread species group, we found extensive geographically structured variation among diploid populations, consistent with other studies of Eurasian phylogeography, which generally show much greater diversity in the south than in the north. We demonstrate that the polyploids (3n, 4n) have multiple maternal origins. The Central Asian populations show close mtDNA affinity among diploids (*B. turanensis*) and tetraploids (*B. oblongus*, *B. pewzowi*) as well as rare triploids. The sexually reproducing triploids (*B. pseudoraddei*) have mtDNA that is highly divergent from any sampled diploid population and themselves consist of two divergent clades.

Here, we place the mtDNA results for diploids in the context of the unusually dense fossil record for Eurasian green toads to shed light on geographic origins of the complex and its biogeographic history. We also identify regions with close geographic proximity or overlap of major mtDNA phylogroups that should be the focus of future studies. Regarding the polyploid lineages, we use the mtDNA evidence, together with karyotypic and genetic data to infer origins, biogeographic history and interactions of the polyploid forms. Our results highlight the dynamic history of these lineages, including likely continuing interactions among diploid and polyploid forms.

4.1. Phylogeographic diversity, origins and historical biogeography of diploid taxa

Within the *B. viridis* group (*B. viridis*, *B. calamita*, *B. raddei*, *B. surdus*, *B. latastii*, *B. luristanicus*; Borkin, in Frost, 1985, added *B. brongersmai*), a widely used term coined by Inger (1972), *B. viridis* itself is nested within multiple, closely related lineages. Stöck et al. (2001a) distinguished these lineages as the *B. viridis* subgroup, based on bioacoustic, cytogenetic and morphological evidence and included *B. latastii*, *B. oblongus*, *B. pewzowi*, *B. viridis viridis*, *B. turanensis*, *B. pseudoraddei pseudoraddei*, *B. p. baturae* and we now add *B. balearicus*, *B. boulengeri*, *B. variabilis*, the unnamed taxon from southeastern Sicily, *B. luristanicus*, and *B. surdus*. These taxa form a mitochondrial clade based on *ND* + *tRNA*s.

Within this *B. viridis* subgroup, we report evidence (D-loop, *ND* + *tRNA*s) that the diploids belong to nine mitochondrial clades. The earliest diverged western Himalayan *B. latastii* (2n-I) and its southern Iranian sister species *B. surdus* represent descendents of an Upper Oligocene/Lower Miocene split (Table 1) from the MRCA with African (2n-III) and all other green toad clades. Isolation by mountain uplifting and speciation of *B. latastii* in the Kashmir/Ladakh region might be well linked to the Indian collision with Asia and the rise of the Himalayas.

4.2. Origin of the Bufo viridis group and subgroup in context with the fossil record

Provided our divergence times estimates are correct, green toads (lineages *B. latastii*, *B. surdus*) were likely present in Asia before the Afro-Arabian plate first touched Eurasia (~18 Mya; Rögl, 1998; Tchernov, 1988) since during the entire Oligocene (33.7–23.8 Mya) and early Miocene (23.8 to ~18 Mya), the Mediterranean was a remnant of the Western Tethys, which connected Indo-Pacific and Atlantic and thus separated Afro-Arabia from Eurasia (Rögl, 1998, 1999).

The presence of bufonids north of this sea barrier before Afro-Arabia and Eurasia collided is also supported by the fossil record, because the oldest Old World bufonid fossils come from Paleocene of northern France (Rage, 2003) and middle Oligocene of Kazakhstan (Chikvadze, 1985). Therefore, (1) arrival of bufonids in Eurasia based on fossils and our divergence time estimates seem to reject a "pure Miocene out of Africa hypothesis" for Eurasian toads. One alternative hypothesis (2) of a trans-Beringian invasion of Eurasian toads from the Nearctic (e.g. Blair, 1972; Borkin, 1999: Oligocene) was rejected by Pauly et al. (2004), who

reviewed previous hypotheses on the origin of Nearctic toads but found no close relationship of a single *B. viridis* to recent Nearctic species. However, (3) Pramuk (2006), using molecular and morphological evidence, finds that Eurasian *Bufo* lineages form a basal sister clade to a New World radiation. Thus, Miocene bufonids may have reached Europe [as well as the Nearctic?] from Asia (Sanchiz, 1997; Rage and Rocek, 2003). This latter hypothesis is further supported by the fact that all living *B. viridis* group taxa occur in Eurasia, and two (*B. calamita, B. raddei*) diverged even earlier, before the *B. viridis* subgroup arose (Fig. 3). All *B. viridis* group and subgroup species occur in temperate Palearctic climate (Borkin, 1999). During the estimated early Miocene split of the subgroup, similar to recent climatic conditions probably prevailed (van der Made, 1999), suggesting adaptation of the *B. viridis* group to temperate environments throughout its history. The northwest African *B. brongersmai* can no longer be considered close to the subgroup since karyotype (Herrero et al., 1993), advertisement calls (Bogaerts, 2001, call recording in litt.) and larval morphology (Grillitsch et al., 1989), as well as our *ND + tRNA*-phylogeny (Fig. 3), reject close relationships.

The surprisingly rich fossil record for *B. viridis* or "*Bufo* aff. *viridis*" (for overview: Sanchiz, 1998; Kordikova, 1998; Rocek and Rage, 2000; Rage and Rocek, 2003; Böhme, 2003) also shows the oldest known remains to be found in Eurasia (Fig. 1) rather than in Africa (although the latter fossil record is poorly known, Rocek and Rage, 2000). The oldest fossils (Fig. 1) of *B.* aff. *viridis* (Claessens, 1997; Rocek and Rage, 2000) come from the Lower Miocene of northern Anatolia (18–20.5 Mya, Böhme, M. in litt.), the Lower Miocene of southeastern France (Rage and Rocek, 2003), central Iberia (Rage and Rocek, 2003; doubtful: Sanchiz, 1998), and southern Germany (17.5–18 Mya; Böhme, 2003). *B. calamita* is also known from the Lower Miocene of Spain (Rage and Rocek, 2003). Pre-Pleistocene *B. viridis* fossils are lacking from Central Asia, but *B. raddei* is reported from the Upper Miocene to the Lower Pleistocene of northeastern Kazakhstan (Chikvadze, 1985; Kordikova, 1998; Sanchiz, 1998). In North Africa, the oldest known *B. viridis* fossils are only from the Pliocene of Morocco (Bailon, 2000), much younger then the oldest from Eurasia. Taken together, all early fossils were found in Eurasia and are either older than or contemporaneous to the collision of Afro-Arabia and Eurasia (18–19 Mya).

4.3. Miocene and Pliocene splits

Our data show the mitochondrial lineages of the *B. viridis* subgroup to have diversified since the Oligocene/Early Miocene (Table 1), with five major extant lineages [*B. surdus, B. latastii* (2n-I), *B. boulengeri* (2n-III), unnamed Sicilian taxon (2n-II), *B. pseudoraddei* (3n-I, 3n-II)] likely to have diverged during the Oligocene/Miocene (>23.8–5.3 Mya). All of these occur in the southern part of the range. During the Pliocene (5.3–1.8 Mya), four major haplotype groups [*B. balearicus* (2n-VIII), *B. variabilis* (2n-VI), *B. viridis* (2n-VII), the Central Asian clade] evolved.

4.4. Late Quaternary recolonization of higher latitudes

The *B. viridis* subgroup (clades 2n-VI, 2n-VII, 2n-VIII) reflects biogeographic patterns which were shown for many Palearctic animal and plant species (reviews: Taberlet et al., 1998; Hewitt, 2004; Petit et al., 2003). This includes "extensive extinction and recolonization in higher latitudes and altitudinal shifts and complex refugia nearer the tropics" (Hewitt, 2004). The "southern peninsulas of [...] Italy and the Balkans–Greece, along with the Caspian/Caucasus region [represent] refugia, and taxonomic and genetic diversity [is found] in and among these regions" (Hewitt, 2004). During maximal Pleistocene glaciations, Central European green toads were probably extinct or forced to retract to southern refugia. Therefore, European Miocene fossils (see above) and extant haplotype groups (2n-VI, 2n-VII) cannot be linked. By Late Pleistocene (Early Weichselian), *B. viridis* had returned to Central Europe (fossils: Mlynarski et al., 1978; Böhme, 1991).

Our study shows potential refugia of clade 2n-VII on the Balkan Peninsula (loc. 13, 30), perhaps extended to the southern Ukrainian steppe (loc. 38), and a Post-Pleistocene northwestern range expansion to its current western range limit at the Rhine. This scenario fits the shallow structure of 2n-VII and its star-like radiation (TCS, not shown) from inferred ancestral haplotypes at refugial localities (loc. 38, 13). The most divergent haplotypes (eastern loc. 58, 90 of 2n-VII) may represent haplotypes at a different glacial refugium or isolates from a previous glacial cycle.

The occurrence of a second northern European clade (2n-VI) in Sweden, Denmark and northern Germany (loc. 8, 11, 12, 16) underlines mobility of green toads and shows the dynamics of postglacial colonization. We propose two alternative hypotheses: (i) Clade 2n-VI spread during a previous interglacial cycle and reached the western range in the upper Rhine valley, and colonized Scandinavia to the northeast after the last glacial maximum (LGM). The Rhine/Rhone area is a well-known "major refugium" (Hewitt, 2004). (ii) Alternatively, toads reached southeastern Scandinavia, perhaps via an eastern Carpathian corridor, and/or crossed the Baltic Sea. Under (i) clade 2n-VI is expected in southwestern Germany. If, in contrast, hypothesis (ii) applies, we predict its presence in northern Poland/northeastern Germany.

4.5. Clade boundaries and possible interactions of diploids

We have outlined major dimensions of clade distribution, but identification of exact clade boundaries and contact zones is a challenge for future research. Studying postglacial colonization will likely reveal several Central and/or east European contact zones (e.g. in Germany) between 2n-VI and 2n-VII, which also meet between Abrau Peninsula (loc. 48, 49, 51) and the Caucasus (loc.

52). High allozyme variation (Karakousis and Kyriakopoulou-Sklavounou, 1995) on the Greek mainland (loc. 25, 27-29) may represent both clades or may stem from their hybridization. Serum albumins of toads (called '*B. viridis arabicus*', Flindt and Hemmer, 1968) from southeastern Turkey (Adana, i.e. 2n-VI, close to loc. 42–44) differ from Central-European *B. viridis* (our 2n-VII). In northwestern Kazakhstan (loc. 58, 61–63, 66, 67), where only 2ns are reported (Dujsebayeva et al., 2003), both clades (2n-VI, 2n-VII) are found in close proximity and in north central Kazakhstan (loc. 85–90) these clades meet a third (2n-IV) in a region where *Stipa* grass steppe turns into *Artemisia* semi-desert. While most data suggest geographic sorting (not in Greece 2n-VII, 2n-VI; unclear for northern Kazakhstan), future research should address whether and where these two (or three) mitochondrial clades are broadly sympatric (as e.g. in *B. gargarizans*, Fu et al., 2005) or even admixed and might represent cases of non-polyploid reticulate evolution.

Hybridization of small-sized 2n-VI and large-sized 2n-IV toads in northern Kazakhstan is suggested by a few individuals that exhibit reciprocal haplotypes and phenotypes. The Asia Minor (2n-VI) and the African (2n-III) clades, which may have separated as long as 10 Mya (Table 1), may contact on Sinai, "a major barrier for amphibian dispersal between Africa and Asia" (Borkin, 1999). While this statement is primarily supported by our data, Werner (1982, cit. in Borkin, 1999) listed *B. viridis* as "the only amphibian [occurring at] the northeastern corner of Sinai as in the Negev" desert. Maxson (1981), using immunology, which also revealed differences between *B. viridis* from Tunisia (our 2n-III), Yugoslavia (2n-VI or 2n-VII) and Israel (2n-VI), differentiated *B. viridis* from northeastern Sinai (El Arish, but also Nahal Kzib, northern Israel) from most other Israeli green toads, raising the possibility that these southwestern populations are of African origin. Size differences (Nevo, 1972) could have the same explanation. For many terrestrial African and Eurasian vertebrates Sinai was an intercontinental crossroad (Tchernov, 1988) until the Pleistocene.

4.6. Origin, interaction and dynamics of lineages containing polyploids

4.6.1. Pliocene origin of and Pleistocene expansions in the Central Asian clade

In the *ND* + *tRNA*s tree (Fig. 3, right), the three Central Asian representatives *B. oblongus* (4n-II), *B. turanensis* (2n-V) and *B. pewzowi* (4n-I) form a well supported sister clade of the *B. v. viridis* (2n-VII)/*B. variabilis* (2n-VI)/*B. luristanicus* clade. Divergence time estimates (Table 1) based on d-loop sequences suggest that the Central Asian clade diverged from geographically neighboring Asia Minor (2n-VI) populations in the Pliocene (4.4–3.1 Mya, Table 1), contemporaneous with global cooling and drying, which led to the spread of grasslands, potentially in favor of green toads, a steppe species.

Strong signatures of population growth (4n-I, 4n-II) suggest that mtDNA-lineages of polyploids expanded in the Pleistocene (Table 2), a previously proposed time of 4n formation (Mezhzherin and Pisanets, 1995), when further cooling and increasing aridity may have resulted in sudden selective advantage of the polyploids, which currently dominate the climatically extreme high mountains and continental cold winter eastern deserts of Central Asia (Stöck et al., 2001b), where they show high temperature tolerance (−30 to 45 °C; Kuzmin, 1999) and inhabit elevations <3700 m a.s.l. (Stöck et al., 2001b). Island patterns of Pleistocene lowland permafrost in Central Asia, as low as 900 m a.s.l. (Aubekerov and Gorbunov, 1999), potentially left space for scattered Pleistocene refugia of cold-tolerant toads. By implication, distinct lowland desert gecko species (*Teratoscincus*), evolved during the Tertiary northwest and southeast of the Tian Shan (Macey et al., 1999), also must have had Pleistocene refugia in the region.

4.6.2. Hypothetical matrilineal origin of Bufo pewzowi (4n-I) and Bufo oblongus (4n-II)

Bufo turanensis (2n-V) exhibits Q-bands in both copies of chromosome 6, while *B. oblongus* (4n-II) has a karyotype containing two Q-positive and two Q-negative chromosomes 6, suggesting their allopolyploid (hybrid) origin (Stöck et al., 2005). Some Q-positive 2n toads may have been one of the ancestral forms of these 4n toads. Because the common mitochondrial haplotype in group B of the Central Asian Clade is found in the Q-banding-positive 2ns, they likely represent the maternal ancestors of western Central Asian 4n-II (*B. oblongus*). If this is correct, then the other, Q-negative paternal ancestor is unknown or may no longer exist.

In group A, *B. pewzowi* (4n-I) have four Q-banding positive chromosomes 6, suggestive of autopolyploidy. Some Q-positive 2ns must be their ancestors, and the extant *B. turanensis* (2n-IV) are candidates.

4.6.3. Possible interactions between diploids and tetraploids within the Central Asian clade

In the Central Asian clade, diploid *B. turanensis* (2n-IV, 2n-V), which are differentiated by calls (Castellano et al., 1998; Stöck, 1998a), morphology (Stöck, 1997) and allozymes (Mezhzherin and Pisanets, 1995) from their tetraploid counterparts (4n-I, 4n-II), show close mtDNA affinities to either *B. pewzowi* (4n-I, in group A) or *B. oblongus* (4n-II, in group B). A haplotype network (Fig. 4) reflects these similar situations in groups A and B with most 4ns being at least two mutational steps from 2ns. Pairwise F_{ST} values between 4n-II and 2n-V (0.2) and 4n-I and 2n-IV (0.38; Table) suggest some separation between gene pools of diploids and tetraploids of both groups (A and B). However, two toads of 4n-II (*B. oblongus*) from Danata (loc. 73, Turkmenistan) have identical mtDNA-haplotypes with 2n-V from several northeastern Iranian localities (loc. 69–71, 81), implying multiple origins and retained ancestral polymorphism or some degree of recent

Table 3
Population pairwise F_{ST} values in groups of the Central Asian clade

	2n-V	4n-II	2n-IV	3n-III
2n-V (Western Central Asia)	0			
4n-II (Western Central Asia)	0.20191	0		
2n-IV (Eastern Central Asia)	0.75292	0.57400	0	
3n-III (Eastern Central Asia)	0.77040	0.56410	0.02613 (ns)	0
4n-I (Eastern Central Asia)	0.48036	0.38231	0.34358	0.35531

hybridization. If 2n-V and 4n-II toads rarely hybridize, "mismatings" may be limited by different calls (Castellano et al., 1998; Stöck, 1998a). Triploid females, reported from one locality (73; Pisanets, 1978), may be fertile, as are other 3n toads (Stöck et al., 2002) or waterfrogs (e.g. Günther, 1990). Alternatively, rare unreduced 2n gametes of 2n toads (e.g. Bogart, 1972) and their fusion with normal 2n gametes of 4ns (Stöck et al., 2002) may lead to hybrid meiotic 4n offspring carrying nuclear and mitochondrial genes from the 2n-V into the 4n-II gene pool. Stöck et al. (2005) found some 4n-II karyotypes with one or three instead of two homologous Q-positive chromosomes 6, consistent with introgression of Q-positive chromosomes from the 2n-V into the 4n-II gene pool, possibly restricted to single unreduced eggs of 2n-V.

Similarly, all unequivocally 2n-IV toads (*B. turanensis*) are at least four mutational steps apart from the closest 4n-I individuals (*B. pewzowi*), while rare triploids (3n-III from Northern Kyrgyzstan, loc. 92, 93) share the mtDNA with some 2n-IV (or are only one mutational step apart, Fig. 4) and therefore might be F_1 hybrids (2n-IV female × 4n-I male). Indeed, the pairwise F_{ST} value (Table 3) found to be ~0.35 between 4n-I and 2n-IV or 3n-III, was only 0.026 between 2n-IV and 3n-III, implying a separation of gene pools of 2n-IV and 4n-I but almost no separation of 2n-IV and 3n-III within group A. Rare 3n-III, which comprise ~5% of some regional populations, may consist of males only (Borkin et al., 2001; pers. observation). Even if 3n hybrids are all-male or hypothetical 3n females are sterile, mtDNA introgression might still result (as proposed for clade B) from single unreduced eggs of 2n-IV females, which are fertilized by 4n (or 3n) males and contribute to introgression of 2n-IV mtDNA in the 4n-I gene pool.

These intriguing questions of possible gene exchange and/or continuing reticulation between 2n and 4n toads in a system of "porous" gene pools in diverging taxa, which may occasionally exchange genetic material via rare triploids, require further analyses with rapidly evolving nuclear markers (e.g. microsatellites) from a close contact zone of 2n and 4n toads.

4.6.4. Distinctiveness of tetraploid toads from Bolshoi Balkhan (4n-III)

Distinctive d-loops in green toads from Bolshoi Balkhan (loc. 68) match the separate phylogeographic position of agamids (Macey et al., 1998a,b, 2000) from that island mountain, which also exhibits high plant endemism (Proskuriakova, 1971). This evidence supports a biogeographic separation of Bolshoi Balkhan from Kopet Dagh and eastern Iran (Khorasan). Caspian Sea transgressions and temporary western drainage ("Uzboi") of the Amur-Darya kept the region isolated (Atamuradov, 1994). Nevertheless, the close mitochondrial relationship of 4n-III to 3n-IV toads (*B. zugmayeri*), a potentially second sexually reproducing triploid taxon from northern Baluchistan, is currently unexplained but suggests former range connections across the non-sampled southwest of Afghanistan.

4.6.5. Matrilineal origin of 3n Bufo pseudoraddei in High Asia

Morphologically distinguishable *B. pseudoraddei pseudoraddei* (3n-I) and *B. p. baturae* (3n-II) share a common maternal ancestor, which is only distantly related to green toads (2n, 3n 4n) of the Central Asian clade (Fig. 3) and to geographically proximate *B. latastii* (2n-I). No living recent 2n or 4n maternal ancestor of the 3n *B. p. pseudoraddei* and *B. p. baturae* is known. Although their mtDNA genotype also differs substantially from all other mitochondrial clades, this is not necessarily evidence for their great evolutionary age (see below).

4.6.6. Glaciation in Karakoram, Hindukush and western Himalayas caused speciation and late invasion of green toads

We sampled *B. p. pseudoraddei* (3n-I) at two sites in the lower Kabul and Swat river drainages (Fig. 2: loc. 99, 100), and *B. p. baturae* (3n-II) in Hunza, Gilgit, and Chitral valleys (loc. 101–108). Both high mountain taxa split off in early Pleistocene (Table 1), suggesting that glaciation forced them into different refugia. Since both taxa are 3n, triploidy might have evolved before their separation (1.6–1.3 Mya). Triploidy, however, may be younger than the monophyletic 3n-I + 3n-II mitochondrial lineage, if the mitochondrial lineage diverged and evolved earlier in an unknown 2n ancestor.

Current Himalaya and Transhimalaya (the Karakoram represents its western end) contain the highest non-polar concentration of glaciers. However, changing monsoon influence caused Quaternary glaciations to be dynamic and asynchronous to northern cycles. Pleistocene Hunza valley (Fig. 2: loc. 106–108) was so heavily glaciated (Owen et al., 2000a) that extant *B. p. baturae* (3n-II) could not have spent the ice age at its current sites (2000–3000 m a.s.l.) but must have invaded from southern refugia. During the northern last glacial maximum (LGM), Himalayan glaciations were limited (Ref. in Benn and Owen, 2002). Indeed, Upper Hunza (loc. 106–108) first became ice free postglacially (Gulkin I stage) during the last glacial maximum (Owen et al., 2000a). Owen et al. (2000b) concluded that all of late Pleistocene Chitral (loc. 101, 102) was filled with a huge glacier system above 1300 m (Drosh stage), which would have made continued existence of 3n-II toads in Chitral impossible. Glaciation here (upper Mastuj valley: 102) lasted at least until early Holocene. The Shandur Pass (loc. 103; 3720 m a.s.l.) was ice-covered as recently as middle or late Holocene (Owen et al., 2000b). Therefore, 3n-II toads must have crossed this pass

(loc. 103) later. Far downstream of Chitral, towards the other clade (3n-I: loc. 99, 100), modern climate is too hot for temperate-adapted green toads, and consequently genetically uniform 3n-II must have invaded Chitral (loc. 101, 102) from the East (loc. 105, 104), crossing Shandur (loc. 103). This implies a Pleistocene refugium of 3n-II in the Himalayan Indus valley (S of loc. 105), which was ice-free south of ~35° N in late Pleistocene (Kamp and Haserodt, 2004). *B. p. pseudoraddei* (3n-I) from Paghman (loc. 99) and Swat (loc. 100), forming two subclades of 3n-I, supposedly reached these sites from southern refugia in the Kabul river drainage during Holocene warming and may no longer be in genetic contact. Their occurrence close (loc. 99) to the Salang Pass (3880 m, slightly above the maximum elevation of green toad records) in the Hindukush raises questions whether this lineage could surmount the range to the North and would then be found in northern Afghanistan and western Tajikistan. A range limit of *B. p. baturae* (3n-II) exists between their easternmost record (loc. 105) and the westernmost record (loc. 111) of *B. latastii* (2n-I) in the rock gorge of the Indus river (Stöck et al., 2001b), where both taxa occur allopatrically. However, Baig (1998) reported sympatry of *B. latastii* and *B. pseudoraddei* (3n-I or 3n-II?) in the "Neelam (= Jhelum) valley" of Azad Kashmir (eastern Pakistan).

5. Conclusions: phylogenetic and evolutionary implications of polyploidy in the Bufo viridis subgroup

We have provided evidence for ancient splits of mitochondrial lineages at different time depths. Several clades meet each other geographically and their interactions, especially the extant of hybridization and/or polyploidization, offer appealing research opportunities. Our mtDNA analyses show that polyploidy in the *B. viridis* subgroup evolved several times. Cytogenetic data (Stöck et al., 2005) suggest at least two origins of 4ns (*B. oblongus*, 4n-I; *B. pewzowi*, 4n-II). Unique to the present data is the demonstration that the mitochondrial ancestor contributing to morphologically distinctive (Stöck et al., 1999, 2001a) triploid toads from Karakoram and Hindukush (*B. p. pseudoraddei*, 3n-I; *B. p. baturae*, 3n-II) is different from the lineage leading to different groups of Central Asian tetraploids. This fascinating genomic diversity is further highlighted by the apparent discovery (further tests ongoing) of a new potentially sexually reproducing 3n taxon (*B. zugmayeri*, 3n-IV) within the Central Asian clade and especially by the probable continuing interactions of parapatric diploid and tetraploid toads. In their contact zones, diploid, triploid and tetraploid genotypes interact (and likely co-evolve), and show that vertebrate evolution can not only tolerate but readily incur major changes in genome size and composition, which signals genomic plasticity. Given the relatively rare occurrence of polyploidy in *Bufo* (e.g. Tandy et al., 1985; King, 1990; Vences and Wake, in press), this also suggests that certain propertiesof the *B. viridis* genome may be especially suitable for the appearance of polyploidy, a hypothesis that deserves further investigation (e.g. by artificial polyploidization experiments). *Bufo asmarae* (Tandy et al., 1982), a tetraploid species from the *B. regularis* group with 4n = 40 chromosomes, is an evolutionary parallel of possibly hybrid origin (Tandy et al., 1985) in which comparative research with molecular methods would be of great interest.

Multiple origins of polyploids in green toads may be the rule rather than the exception, as in several other polyploid vertebrate groups (see introduction; Cunha et al., 2004; Ptacek et al., 1994; Evans et al., 2004, 2005; Tsigenopoulos et al., 2002). The role of hybridization and reticulation in animal evolution is still debated and restricted by our currently limited access to genomes of non-model organisms. Easily observable quantitative differences in diploid/polyploid complexes make us aware of the fluidity and plasticity of genomes and this "view of the tip of the ice berg" could mean that introgression, fusion and hybridization are not only tolerable but may be a major component of animal evolution (cf. Morjan and Rieseberg, 2004; Mavárez et al., 2006; Patterson et al., 2006).

Acknowledgments

This work was supported by a research fellowship (Sto 493/1-1) from the Deutsche Forschungsgemeinschaft (DFG) to M. Stöck and the University of California, Berkeley (Craig Moritz and David Wake).

The following people helped with collecting and/or sending specimens or tissue samples: C. Andrén (Alvhem), M. Auer (Dresden), A. Basheeva (Karaganda), W. Böhme (Bonn), S. Bogaerts (Nijmegen), D. Buckley (Berkeley), M. Chirikova (Almaty), L. Choleva (Libechov), T. Dieterich (Astana), R. Dressel (Dresden), U. Fritz (Dresden), K. Fog (Vekso), F. Glaw (Munich), E. Gnidenko (Almaty), R. Günther (Berlin), W.-R. Grosse (Halle), C. Herden (Lauenburg), C. Klütsch (Bonn), L. Kratochvil (Prague), S. Kuzmin (Moscow), N. Lutzmann (Zurich), C. Miaud (Le Bourget du Lac), M. Ogielska (Wrozlaw), Zh. Oralov (Astana), J. Martens (Mainz), J.F. Parham (Berkeley), E. Recuero (Madrid), U. Scheidt (Erfurt), J.F. Schmidtler (Munich), H. Veith (Halle), J. Vörös (Budapest), S. Voitel (Eisleben), T. Woeltjes (The Netherlands). D.K. Lamatsch (Brussels) and C. Steinlein (Würzburg) helped with ploidy determination by flow cytometry. M. Stöck is grateful for references or information from B. Clarke (London), A. Ohler (Paris), J. Pramuk (Provo), C. Muster (Leipzig), L. Owen (Cincinnati), E. Roitberg (Rostock), Y.L. Werner (Jerusalem); for references and discussion on fossil *B. viridis*: M. Böhme (Munich); for correspondence on Mediterranean paleogeography: F. Rögl (Vienna); for veterinary services: F. Mutschmann (Berlin), and for discussion, the choice of the mitochondrial marker and various support: M. Schartl (Würzburg). M. Stöck wishes to thank many colleagues in the MVZ (Berkeley) who helped with some methodological approaches, especially N.M. Belfiore, B. Slikas, R. Bingham, M. Brandley, C. Conroy, T. Devitt, M. Fujita, J. Kapoor, A. Leaché, J.B. MacKenzie, I. Martínez-Solano and J. McGuire. R. Hijmans provided valuable cartographic support.

Appendix A

Map	Voucher (if available)	GenBank Acc. No.	Taxon	Locality	Ploidy	*N*	Sample-ID (see Figs. 3 and 4)	LAT	LONG	ELEVATI ON (if known)
1	—	DQ629730	*B. boulengeri*	Morocco, AitBaha, E. Recuero leg.	2n	1	371	30.130	−9.080	
2	MTD45286	DQ629718	*B. boulengeri*	Morocco, High Atlas, D. Frynta leg.	2n	1	112	32.427	−5.156	
3	CUP\AMPH\MOR\01	DQ629704	*B. boulengeri*	Morocco, High Atlas, D. Frynta leg.	2n	1	179	33.427	−5.150	1438
4	ZFMK 37856	—	*B. balearicus*	Spain,Balearic islands, Mallorca, Caps Andraixs, C.A.Raehmel leg.1982	2n	1	160	39.500	3.000	
5	ZFMK49652	DQ629720	*B. boulengeri*	Algeria, Ghardaia, W. Bischoff, U. Joger leg.	2n	1	163	32.483	3.667	
6	MVZ 235680	DQ629721, DQ629602	*B. boulengeri*	Tunisia, Nefta oasis, Tawzar (= T0zeur) Governorate, T. Papenfuss 6 Feb 2002 leg.	2n	1	166	33.917	3.133	45
7	ZSM 6/2004	DQ629731, DQ629598	*B. balearicus*	France, S-Corsica, near Bonifacio, 19 June 2002, F. Glaw, K. Schmidt. leg.	2n	1	175	41.383	9.150	
8	—	DQ629645	*B. variabilis*	Germany, Schleswig-Holstein, Woltersdorf near Lübeck, C. Herden leg.	2n	1	310	53.583	10.633	
9	ZFMK 14704	DQ629719	*B. boulengeri*	Tunesia, Djerba island, Kiehlmann leg. 1974	2n	1	165	33.800	10.900	
10	ZSM 5/2004	DQ629671	Natural cross *B. calamita* male x *B. viridis* female	Germany, Fürstenfeldbruck, E. Andrä leg.	2n	1	Hyb-IV	48.180	11.250	
11	—	DQ629670	*B. variabilis*	Denmark, NW Lolland, K. Fog leg.	2n	1	296	54.900	11.250	
12	MVZ 247648 (tissue)	DQ629632	*B. variabilis*	Denmark, Falster, a few km S Nykøbing Falster, K. Fog leg.	2n	1	295	54.750	11.800	
13	—	DQ629687	*B. viridis*	Italy, Padua, University of Würzburg 1995 leg.	2n	1	21	45.417	11.883	
14	MVZ 241555	DQ629674	*B. viridis*	Germany, Halle (Saale), Martin-Luther-University Halle-Wittenberg, Botanical Garden, W. Grosse and M. Stöck leg.	2n	1	171	51.833	12.000	
15	NME 974/02	DQ629672, DQ629673	*B. viridis*	Germany, Thuringia, Falkenhain, opencast, mining "Phönix Nord" leg. A. Nöllert	2n	2	190, 191	51.417	12.883	
16	MVZ 244350, 244354, 244355 (tadpoles)	DQ629659, DQ629662, DQ629663, DQ629664, DQ629665, DQ629666	*B. variabilis*	Sweden, Malmö, Limhamn, C. Andren leg.	2n	7	241, 242, 266	55.583	12.900	
17	—	DQ629717	*B. boulengeri*	Libya, Al' Fiayi, Sabah Province, D. Frynta leg.	2n	1	140	26.533	13.317	
18	—	DQ629705, DQ629706, DQ629707	*B. boulengeri*	Libya, Gabroon Lake, D. Frynta leg.	2n	2	139, 107, 114	26.800	13.533	
19	—	DQ629726, DQ629727, DQ629728, DQ629729, DQ629608	Unnamed	Italy, Sicily, E of Lentini, near mouth of San Leonardo River, 500 m from coast inland	2n	4	323, 324, 325, 326	37.333	15.067	
20	NME 912/01	DQ629732	*B. balearicus*	Italy, Sicily, N Francavilla di Sicilia, stream valley, T. Zavianni, A. Nöllert leg.. 16 April 1995	2n	1	188	37.900	15.133	
21	NME 913/01	DQ629733	*B. balearicus*	Italy, W coast, Calabria, Paola, A. Nöllert leg., 17 April 1995	2n	1	189	39.350	16.033	
22	ZFMK 65102	DQ629661	*B. viridis*	Austria, moutainous country above Eisenstadt	2n	1	144	47.850	16.516	

(*continued on next page*)

Appendix A (*continued*)

Map	Voucher (if available)	GenBank Acc. No.	Taxon	Locality	Ploidy	*N*	Sample-ID (see Figs. 3 and 4)	LAT	LONG	ELEVATI ON (if known)
23	MVZ 164718	DQ629686, DQ629606	*B. viridis*	Austria, MVZ frozen tissue collection (FC 13312), 3.2 km E Podersdorf Buraenland, Austria: R. D. Sage leg.	2n	1	168	47.850	16.833	
24	HNHM2004.94.2	DQ629678	*B. viridis*	Hungary, Central Hungary, Orgovany, May 2004, L. Forro leg.	2n	1	265	46.750	19.467	
25	ZFMK 62479	DQ629722	*B. variabilis*	Greece, Epirus, S Igoumenitsa, Patraia, W. Böhme leg. 1996	2n	1	149	39.500	20.266	
26	MTD 45036,45281	DQ629710, DQ629711, DQ629712	*B. boulengeri*	Libya, Shahhat (Ancient Cyrene), Binghazi Province, D. Frynta leg.	2n	3	109, 131, 138	32.817	21.867	
27	—	DQ629630	*B. variabilis*	Greece, Peloponnes, J. Plötner leg.	2n	1	99	37.516	22.367	
28	NME 901/01	DQ629675	*B. viridis*	Greece, Peloponnes, Kióna, E-Bank Stymphalian Lake, leg. A. Nöllert, 10 April 1996	2n	1	187	37.850	22.450	
29	NME 900/01	DQ629654	*B. viridis*		2n	1	186	38.133	23.000	
30	NME A 1037/03 (2nd + 3rd indivi	DQ629655, DQ629656	*B. viridis*	Greece, Crete, Omalos, U. Scheidt leg.	2n		133, 134	35.333	23.900	
31	—	DQ629657, DQ629658	*B. viridis*	Greece, Crete, Aradena village, 19 April 2003, leg. U. Scheidt	2n	2	135, 136	35.200	24.083	
32	MTD 45275,45276	DQ629667, DQ629668	*B. viridis*	Greece, Crete, via J. Plötner	2n	2	100, 101	35.417	24.750	
33	MTD 45280, 45282	DQ629714, DQ629715	*B. boulengeri*	Egypt, Matrouh, via E. J. Bentley	2n	2	108, 110	30.000	28.000	
34	MVZ 230206, 230207	DQ629621, DQ629624	*B. variabilis*	Turkey, Cicekli Köyü, 7 km E (by road) Ula Mugla Prov., T. Papenfuss leg.	2n	2	236, 237	37.066	28.500	
35	ZFMK 77600, 77601	DQ629708, DQ629709	*B. boulengeri*	Egypt, Oasis Dakhla (Dakhilah, Al Wahat ad), N. Lutzmann leg.	2n	2	146, 147	25.553	28.948	
36	MVZ 230208	DQ629623, DQ629600	*B. variabilis*	Turkey, Osman Gazi, Bursa, Bursa Prov., T. Papenfuss leg.			238	40.167	29.083	
37	MTD 45277	DQ629713	*B. boulengeri*	Egypt, 70 km S Alexandria, via J. Bentley	2n	1	105	31.000	30.000	
38	MTD42716,42717	DQ629684, DQ629685	*B. viridis*	Ukraina, Cherson Oblast, Golija Pristan, U. Fritz leg.	2n	2	184, 185	46.516	30.516	
39	ZFMK 50909	DQ629716	*B. boulengeri*	Egypt, Alexandria, El Menoufia (via U. Sinsch), 1989	2n	1	159	30.500	31.000	
40	CS96V:4	DQ629625	*B. variabilis*	Turkey, Central Turkey, S. Doganhisar, Prov. Konya, 1650 m, 31 May 1996, Central Turkev. J.F Schmidtler leg.	2n	1	253	38.150	31.683	
41	CS73V:1	DQ629636	*B. variabilis*	Greece, Cyprus, Lefka, 16 April 1973, J.F. Schmidtler leg.	2n	1	259	35.117	32.850	
42	CS98V:1	DQ629651		Turkey, Tepeköy, NW Mersin, 5 April 1998, J F. Schmidtler leg.	2n	1	257	36.217	33.566	1250
43	MVZ 247506 (tissue)	DQ629648	*B. variabilis*	Turkey, Kizakalesi Korykos, Kizkalesi, Silifke 19 July 2004, L. Choleva leg.	2n	2	289	36.360	33.930	
44	CS96V:1	DQ629626	*B. variabilis*	Turkey, Limonlu, 50 km W Mersin, 300 m; 9 April 1996, J.F. Schmidtler leg.	2n	1	250	36.566	34.250	
45	CS96V 3	DQ629627	*B. variabilis*	Turkey,Güzelyurt, Pr. Akhisar, 1550 m; 29 May 1996, J F. Schmidtler leg.	2n	1	252	38.283	34.383	
46	NME A 1039/03	DQ629724	*B. variabilis*	Syria, Doura Europus, D. Frynta leg.	2n	1	111	33.483	36.000	

47	ZFMK 60946	DQ629726	*B. variabilis*	Libanon, Libanon mountains, above Bcharre, Cedrus forest, 2300 m a.s.I., Bischoff. J.F., H. Schmidtler. in den Bosch leg.	2n	1	153	34.250	36.016	2300
48	ZMB 58540, 58541, 58542	DQ629679, DQ629680, DQ629681	*B. viridis*	Russia, NW Caucasus, Dzhemete near Anapa, T. Kirschey leg.	2n	3	204, 205, 206	44.947	37.306	
49	ZMB 57384	DQ629682	*B. viridis*	Russia, NW of Caucacasus, Suko near Anapa T.Kirschev leg.	2n	1	203	44.883	37.317	
50	MVZ 218679	DQ629669	*B. viridis*	Tula region, Tula oblast, Russia, leg.	2n	1	271	54.117	37.367	
51	ZMB 64802, 64803	DQ629676, DQ629677	*B. viridis*	Russia, Abrau Peninsula, NW Caucasus, T. Kirschey leg.	2n	2	211,212	44.697	37.596	
52	ZMB 58562	DQ629683	*B. viridis*	Russia, NW Caucasus, Goverdovski near Maikop, T. Kirschey leg.	2n	1	209	44.608	40.106	
53	ZFMK 57912	—	*B. variabilis*	Syria, Dayr az Zawr, Hotel Al Waha, left Euphrat bank 1994, W. Bischoff leg.	2n	1	157	35.333	40.150	
54	MVZ 247493, 247505 (tissue), 247495-247503 (tadpoles)	DQ629631, DQ629633, DQ629649	*B. variabilis*	Turkey, Nemrut Dagh and E of Nemrut, L. Choleva leg.	2n	3	281, 284, 288	38.660	42.300	
55	MVZ 244345, 244346	DQ629628, DQ629629	*B. variabilis*	Russia, Caucasus, Terskol, L. Choleva leg.	2n	2	261,262	43.257	42.527	
56	MVZ 247494, 24704 (tissue)	DQ629634, DQ629635, DQ629650	*B. variabilis*	Turkey, Karahan-Kars lli, Van Golu (N), Karahan Koyu, 4 July 2004, L. Choleva leg.	2n	3	282, 286, 287	39.000	43.760	
57	—	DQ629701	*B. variabilis*	Iran, Kara Kelisa or Kare Kilise villiage, Urda, E. Gnidenko leg.	2n	1	95	38.950	44.467	
58	—	DQ629698	*B. viridis*	Kazakhstan, Beket-Ordinsky Rayon, village Urda, E. Gnidenko leg.	2n	1	337	48.770	47.434	
59	CAS 182891	DQ629653	*B. variabilis*	Russia, Dagestan Autonomous Republic, Sary Kum Sand Dunes, at Kumtorkala Railroad Station, T. Papenfuss/R. Macey leg.	2n	1	202	42.967	47.500	
60	MTD 45284	DQ629622	*B. variabilis*	Iran, Choqa Zanbil, Elamite zikkurat, Khuzestan province, D. Frynta leg.	2n	1	115	32.014	48.529	45
61	—	DQ629637, DQ629642	*B. variabilis*	Kazakhstan, Djangalinsky Rayon, village Djangala, E. Gnidenko leg.	2n	1	340, 341	49.213	50.307	
62	—	DQ629639	*B. variabilis*	Kazakhstan, Akjainskiy rayon, Kalmykovo, E. Gnidenko leg.	2n	1	335	49.031	51.825	
63	—	DQ629640	*B. variabilis*	Kazakhstan, Syrymski Rayon, village Djambeity, E. Gnidenko leg.	2n	1	338	50.254	52.605	
64	NME A 1038/03	DQ629723	*B. variabilis*	Iran, Central; Iran, Qasr-e-Sásán, D. Frynta leg.2000	2n	1	113	29.195	53.231	
65	MTKDD 43943	DQ629614, DQ629615, DQ629616, DQ629610	*B. luristanicus*	Iran, Posht Chenar, D. Frynta leg.	2n	3	58, 176, 177	29.200	53.333	1690
66	—	DQ629638	*B. variabilis*	Kazakhstan, W 80 km E of Uralsk city, Berezka river, M. Chirikova leg.	2n	1	334	51.000	53.354	
67	—	DQ629641	*B. variabilis*	Kazakhstan, Karatobinsky Rayon, village Karatobe, E. Gnidenko leg.	2n	1	339	49.692	53.549	
68	—	DQ629768, DQ629769	Unnamed	Turkmenistan, Bolshoi Balkhan, M. Stöck 1994 leg.	4n	2	5.9	39.717	54.483	500

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Appendix A (*continued*)

Map	Voucher (if available)	GenBank Acc. No.	Taxon	Locality	Ploidy	*N*	Sample-ID (see Figs. 3 and 4)	LAT	LONG	ELEVATION (if known)
69	—	DQ629690	*B.* cf. *turanensis*	Iran, NE,N-slope Elburz-Range, near Gorgan, M.Stöck 1994 leg.	2n	1	33	37.000	54.500	
70	—	DQ629690, DQ629688, DQ629689	*B.* cf. *turanensis*	Iran, NE, 50 km NE Gonbad-e-Kavus, M. Stöke leg.	2n	1	6.77	37.633	55.483	250
71	MVZ 249177, 249178	DQ629691, DQ629692, DQ629693, DQ629694, DQ629695	*B.* cf. *turanensis*	Iran, Marrave Tappe, Mazandaran province, Westernmost foothills ofKopet Dagh, D. Frynta leg.	2n	5	90–94	37.733	55.901	665
72	MVZ 245917, CAS 228604	DQ629702, DQ629703, DQ629605	*B.* cf. *turanensis*	Iran, Delbar Field Station, Touran Protected Area, T Papenfuss, O. Mozafari, H. Fahimi,S. Shafiei, K. Kamali 2005 leg.	2n	2	216,217	35.967	56.068	1196
73	MTD 39400, 39401	DQ629766, DQ629767	*B. oblongus danatensis*	Turkmenistan, Danata, M. Stöck 1994 leg.	4n	2	12, 13	38.617	56.633	
74	MTD 40730,40731	DQ629619, DQ629620	*B. viridis kermanensis*	Iran, Kerman, southern Central Iran, M. Stöck 1998 leg.	2n	2	43, 44	30.300	57.083	1860
75	CAS 228820–228823, MVZ 245911–245914	DQ629744, DQ629745, DQ629746, DQ629747, DQ629748, DQ629749, DQ629750	*B. oblongus*	Iran, Kharve, 23 km N Tabas, Yazd Prov., T. Papenfuss 2005 leg.	4n	7	228 to 234	33.641	57.162	
76	—	DQ629696	*B. turanensis*	Iran, Bik, Central Kopet Dagh, Khorasan province, D. Frynta leg.	2n	1	87	37.606	57.944	1467
77	MTD 44397	DQ629699	*B. turanensis*	Turkmenistan, Ashgabad, M. Stöck, A. Bischoff, K. Holländer 1994 leg.	2n	1	29	37.950	58.383	
78	MTKDD41347	DQ629778, DQ629779	*B. oblongus*	Iran, Birjand M. Stöck 1998 leg.	4n	2	45, 46	32.550	59.167	1500
79	CAS 228604, 228690–228694, 228699, MVZ 245904–24907	DQ629734, DQ629735, DQ629736, DQ629737, DQ629738, DQ629739, DQ629740, DQ629741, DQ629742, DQ629743	*B. oblongus*	Iran, Khorasan Province, Bande-dare Spring(Dam), ~4 km (by. road) S of Jaanbaazaan Square, Birjand, J.F. Parham, T. Papenfuss, O. Mozafari, H. Fahimi,S. Shafiei leg. 2005	4n	10	218–227	32.822	59.218	1655
80	MVZ 248372, 248373(tissue)	DQ629646, DQ629647	*B. variabilis*	Kazakhstan, Aral Sea, NW coast of Shevchenko Gulf, T.Duisebaveva leg.	2n?	2	305, 306	46.578	59.925	55
81	—	DQ629700	*B. turanensis*	Iran, Bazangan, Khorasan province, D. Frynta leg.	2n? 2n	1	104	36.280	60.548	750
82	MVZ 241548, 1241549, 248374	DQ629751, DQ629752, DQ629753, DQ629601	*B. oblongus*	Iran, Bazangan, Khorasan province, D. Frynta leg.	4n	3	96, 97, 98	36.280	60.548	750
83	MTD 39405, 39406, 40010	DQ629763, DQ629764, DQ629765	*B. pewzowi*	Uzbekistan, Nuratau range, M. Stöck 1996 leg.	4n	4	15, 16, 17	40.583	36.500	900-1600
84	MVZ 250382–250385, 250779	DQ629770, DQ629771, DQ629772, DQ629773, DQ629774,	*B. zugmayeri*	Pakistan, Pishin, T. Papenfuss leg. April 2005	3n	3	343, 344, 345, 346, 347, 348, 349, 350	30.580	67.000	

		DQ629775, DQ629776, DQ629777								
85	MVZ 248370(tissue),248371 (juv.)	DQ629791, DQ629792	*B.* cf. *turanensis*	Kazakhstan, environs of Taldy-Say village, T.Dujsebayeva leg.	2n?	2	303, 304	48.224	67.052	504
86	MVZ 249171, 249172	DQ629801, DQ629802	*B. viridis*	Kazakhstan,40 km NW of Kyzyldzhar, M. Stöck leg. 23 May 2005	2n	2	303, 304	48.542	69.283	504
87	ZMB 60364	DQ629652	*B.* cf. *variabilis*	Kazakhstan, Tengiz Lake, 12 km W of Abaya village. T. Dieterich	2n	1	143	50.667	69.667	
88	MVZ 249170	DQ629800	Supposed hybrid *B. variabilis*/*turanensis*	Kazakhstan, 45 km S of Kurgaldhinskiy, Kulanulpes-River, M. Stöck leg. 22 May 2005	2n	1	353	50.242	70.000	
89	MVZ 249168, 249169	DQ629643, DQ629644	*B.* cf. *variabilis*	Kazakhstan, SW of Astana, M. Stöck leg. 22 May 2005	2n	1	351, 352	51.125	71.267	
90	MVZ 249173	DQ629697	*B. viridis*	Kazakhstan, Karaganda, N of railway near city center, M. Stöck leg. 24 May 2005	2n	1	356	49.792	73.092	
91	—	DQ629780	*B. turanensis*	Kyrgyzstan, Bishkek, Botan. Garden, M. Stöck 1993 leg.	2n	1	14	42.900	74.600	
92	MVZ 249163, 259164	DQ629812, DQ629813	Supposed hybrids *B. pewzowi*/*B. turanensis*	Kyrgyzstan, S of Bishkek, loc. Point 4, M. Stöck leg. 15 May 2005	3n	2	365, 366	42.690	74.630	
93	—	DQ629814	Supposed hybrid *B. pewzowi*/*B. turanensis*	Kyrgyzstan, S of Bishkek, loc. Point 6, M. Stöck leg. 15 May 2005	3n	1	367	42.720	74.660	
94	MVZ 249174	DQ629809	*B. pewzowi*	Kyrgyzstan, S of Bishkek, loc. Point 1,M. Stöck leg. 15 May 2005	2n	1	362	42.780	74.660	
95	—	DQ629810, DQ629811	*B. pewzowi*	Kyrgyzstan, S of Bishkek, loc. Point 3, M. Stöck leg. 15 May 2005	2n	2	363, 364	42.790	74.760	
96	—	DQ629807, DQ629808	*B. turanensis*	Kyrgyzstan, N of Bishkek, Ala-Archinskoye Vodochranilishche, M. Stöck leg. 18 May 2005	2n	2	368, 369	43 300	75 000	
97	MTD 40012	DQ629783, DQ629784	*B. pewzowi*	Kyrgyzstan, Issyk-Kul, M. Stöck leg. 1995	4n	2	10, 11	42.467	76.200	
98	MVZ 249159–249162	DQ629803, DQ629804, DQ629805, DQ629806	*B. pewzowi*	Kazakhstan, Almaty, entrance Gorki park, M. Stöck leg. 27 May 2005	4n	4	357, 358, 359, 360	43.250	76.956	
99	MVZ 237418, 237419	DQ629846, DQ629847	*B. pseudoraddei*	Afghanistan, Kabul Prov., stream ca. 4 km above Paghman, T. Papenfuss leg.	3n	2	297, 298	34.610	68.920	2608
100	ZSM 106/1998, MTD 44393	DQ629843, DQ629844, DQ629845	*B. pseudoraddei pseudoraddei*	Pakistan, Swat-Valley, Kulalai, W-Himalaya, Pakistan, M. Stöck, M. Möller leg. 1996	3n	7	24, 125, 137	35.317	72.600	1750
101	MVZ 241553	DQ629815, DQ629816	*B. pseudoraddei baturae*	Pakistan, Chitral, Hinkukush, NW-Frontier Prov., M. Stöck, R. Dressel leg.	3n	11	71, 72	35.883	71.783	1480
102	—	DQ629837, DQ629838, DQ629839	*B. pseudoraddei baturae*	Pakistan, NW-Frontier Prov., Booni, M. Stöck, R. Dressel 2000 leg.	3n	3	B1, 2, 3	36.333	72.333	1900
103	MVZ 241554	DQ629817	*B. pseudoraddei baturae B. pseudoraddei*	Pakistan, Shandur-Pass, Hindukush, NW-Frontier Prov., M. Stöck, R. Dressel 2000 leg.	3n	8	73	36.066	72.517	3720

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Appendix A (*continued*)

Map	Voucher (if available)	GenBank Acc. No.	Taxon	Locality	Ploidy	*N*	Sample-ID (see Figs. 3 and 4)	LAT	LONG	ELEVATI ON (if known)
104	—	DQ629818, DQ629819	*B. pseudoraddei baturae*	Pakistan, Gupis, Karakoram, Northern Areas Prov., M. Stöck, R. Dressel 2000 leg.	3n	7	60, 61	36.233	73.450	2160
105	ZSM 111/1998, 112/1998	DQ629820, DQ629821, DQ629822, DQ629823, DQ629825, DQ629826, DQ629827	*B. pseudoraddei baturae*	Pakistan, Gilgit, Karakoram, Northern Areas Prov., M. Stöck, R. Dressel 2000 leg.	3n	8	32, 35–37, 48–50, 74	35.900	74.400	1550
106	—	DQ629842	*B. pseudoraddei baturae*	Pakistan, Northern Areas, Hunza-Valley, river bank, Karimabad near Ganesh, M. Stöck, R. Dressel 2000 leg.	3n	8	130	36.300	74.683	2060
107	MVZ 241552, ZMB 58769	DQ629828, DQ629829, DQ629830, DQ629831, DQ629832, DQ629833, DQ629834, DQ629835, DQ629836, DQ629604	*B. pseudoraddei baturae*	Pakistan, Pasu, Karakoram, Northern Areas Prov., M. Stöck, H. Veith,R. Dressel 1997 and 2000 leg.	3n	59	39, 40, 41, 62, 65–68, 117	36.500	74.867	2700
108	ZSM 101/1998, ZSM 102/1998	DQ629840, DQ629841	*B. pseudoraddei baturae*	Pakistan, Sust, Karakoram, Northern Areas Prov., M. Stöck, M. Möllerleg.	3n	2	27, 28	36.767	74.833	2950
109	ZSM 110/1998, CAS 197007–197010	DQ629756, DQ629757, DQ629758, DQ629759, DQ629760, DQ629603	*B. pewzowi taxkorensis*	China, NW-China, Taxkurgan, E-Pamir, M. Stöck, T. Papenfuss, J.R. Macey leg.	4n	5	25, 193–196	37.783	75.233	3350
110	ZSM 107/1998, 108/1998	DQ629781, DQ629782	*B. pewzowi*	China, Kashgar, Xinjiang, China, M. Stöck 1996 leg.	4n	2	22,23	39.483	76.033	1350
111	ZMB 62722, ZMB 62723	DQ629848, DQ629849, DQ629850, DQ629851, DQ629852, DQ629853, DQ629855, DQ629856, DQ629857, DQ629858, DQ629859, DQ629860, DQ629861,DQ629862, DQ629863, DQ629599	*B. latastii*	Pakistan, Northern Areas (Baltistan), Himalaya, Satpara river and Satpara lake SW of Skardu, M. Stöck, R. Dressel 2000 leg.	2n	12	78, 79, 81, 127, 128, 164	35.283	75.617	2300
112	—	DQ629796	*B. pewzowi*	Kazakhstan, SE, near Kokpak, M. Chirikova leg.	4n	1	328	42.810	79.872	1843

113	—	DQ629795, DQ629797	*B. pewzowi*	Kazakhstan, SE, Shalkudysu river, M. Chirikova leg.	4n	2	327, 329	43.231	80.349	2454
114	—	DQ629799	*B. pewzowi*	Kazakhstan, E Tarbagatai Tebiske river, M. Chirikova leg.	4n	1	333	47.433	82.872	971
115	CAS 171493	DQ629786	*B. pewzowi*	China Xinjiang Uygur Auto. Regionálli Kazak Auto. Prefecture Tacheng Dist., along Liu Su stream at Liu Su Gou, 29 km E of Miao'ergou Autonomous Region, B. Macey, T. Papenfuss leg.	4n	1	199	45.517	83.750	
116	—	DQ629798	*B. pewzowi*	Kazakhstan, E. Akzhar village, M. Chirikova leg.	4n	1	332	47.640	83.795	621
117	ZSM 109	DQ629785	*B. pewzowi*	China, E-Tien-Shan, NW-China, Xinjiang, M. Stöck 1996 leg.	4n	1	31	43.233	84.667	2145
118	—	DQ629789	*B. pewzowi*	Kazakhstan, S-Bukombay Mountains, (northern boundary of Zaissan Depression), T. Dujsebayeva leg.	4n	1	300	48.250	84.800	
119	—	DQ629790	*B. pewzowi*	Kazakhstan, S foothills of Altai range, Prirechnoye village, T. Dujsebajeva leg.	4n	1	301	48.450	85.150	
120	—	DQ629794	*B. pewzowi*	Kazakhstan, Altai range, environs of Terekti (formerly Alexeevka) village. T. Duisebaieva leg.	4n	1	302	48.450	85.733	
121	—	DQ629793	*B. pewzowi*	Kazakhstan, Altai, Pakhmanovskiye Klyuchi N boundaries of S Altay range. T. Dujsebayeva leg.	4n	1	307	49.550	86.516	
122	CAS167832, 167834	DQ629761, DQ629788	*B. pewzowi*	China, Xinjiang Uygur Autonomous Region, Bayingolin Mongol Macey, T. Papenfuss leg. 1988	4n	1	198, 201	40.700	87.633	
123	CAS171676	DQ629787	*B. pewzowi*	China, Xinjiang Uygur Auto. Region Changji Hui Auto. Prefecture, canyon above Dayou, 8.1 km S of Dayou, Tien Mountain, R. Macey and T.J. Papenfuss leg. 1988	4n	1	200	43.983	389.067	
124	CAS171053	DQ629762	*B. pewzowi*	China, Xinjiang Uygur Autonomous Region, sand dunes, Hami-Barkol Kazak Autonomous County (town), J.R. Macey, T. Papenfuss leg.	4n	1	197	43.317	93.600	2290
Laboratory crosses and outgroup taxa										
	—	DQ629754	Laboratory cross	Mother: *B. pewzowi*, Kyrgyzstan, lssyk-Kul $4n = 44$), father: *B.* cf. *oblongus*, Turkmenistan. Bolshoi Balkhan ($4n = 44$)	3n!	1	19C			
	—	DQ629755	Laboratory cross	Mother: *B. pewzowi*, Kyrgyzstan, Issyk-Kul ($4n = 44$), father: *B. oblongus*, Turkmenistan, Danata ($4n = 44$)	4n	1	20C			
	—	DQ629854	Laboratory cross	Mother: *B. latastii*, Pakistan, Skardu ($2n = 22$) × father *B. pseudoraddei baturae*, Pakistan. Karakoram. Pasu ($3n = 33$)	3n	1	Cr243			
	MVZ 186039	DQ629617, DQ629607	*B. calamita*	Spain, Cadiz Prov., Andalusia, 3.1 km S Benalup de Sidonia on road to Veier de La Frontera, J.A. Visnaw leg.	2n	1	169	36.333	5.817	

(*continued on next page*)

Appendix A (*continued*)

Map	Voucher (if available)	GenBank Acc. No.	Taxon	Locality	Ploidy	*N*	Sample-ID (see Figs. 3 and 4)	LAT	LONG	ELEVATION (if known)
	—	AJ584640	*B. melanostictus*	unknown, not provided by GenBank	2n	1	—			
	—	DQ629595, DQ629609	*B. regularis*	Egypt, Embaba, Giza, Abdul Karim leg.	2n	1	312	30.020	31.216	
	MVZ 241541	DQ629593, DQ629597	*B. brongersmai*	Morocco, close to type locality, details unknown	2n	1	172	—	—	
	MVZ 177905	DQ629612	*B. bufo*	Morocco, Marrakesh Prov., Oukaimeden, Stephen D. Busack, J. A. Visnaw	2n	1	177905	31.206	−7.864	2650
	MTD 45287	DQ629613	*B. raddei*	China Xinjiang, Kuku-Nor, J. Martens leg.	2n	1	116	37.000	100.333	
	MTD 44399	DQ629596	*B. stomaticus*	Pakistan, Mingorah, Swat valley, M. Stöck 1996 leg.	2n	1	30	34.783	72.367	
	MTD 45290	DQ629592	*B. arabicus*	Yemen Sara'a (road to Sada'a); C. Naumann, C. Klütsch leg.	2n	1	85	17.083	43.500	
	—	DQ629594	*B. mauritanicus*	Morocco, Sahrij, E. Recuero 2005 leg.	2n	1	370	31.794	−7.050	
	MTD 43944	DQ629618, DQ629611	*B. surdus*	Iran, Baluchestan, Deh Barez, D. Frynta leg.	2n	1	59	27.450	57.317	350

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