

ORIGINAL ARTICLE

Origin and genome evolution of polyploid green toads in Central Asia: evidence from microsatellite markers

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Polyploidization, which is expected to trigger major genomic reorganizations, occurs much less commonly in animals than in plants, possibly because of constraints imposed by sex-determination systems. We investigated the origins and consequences of allopolyploidization in Palearctic green toads (*Bufo viridis* subgroup) from Central Asia, with three ploidy levels and different modes of genome transmission (sexual versus clonal), to (i) establish a topology for the reticulate phylogeny in a species-rich radiation involving several closely related lineages and (ii) explore processes of genomic reorganization that may follow polyploidization. Sibship analyses based on 30 cross-amplifying microsatellite markers substantiated the maternal origins and revealed the paternal origins and relationships of subgenomes in allopolyploids. Analyses of the synteny of linkage groups identified three markers affected by translocation events, which occurred only within the paternally inherited subgenomes of allopolyploid toads and exclusively affected the linkage group that determines sex in several diploid species of the green toad radiation. Recombination rates did not differ between diploid and polyploid toad species, and were overall much reduced in males, independent of linkage group and ploidy levels. Clonally transmitted subgenomes in allotriploid toads provided support for strong genetic drift, presumably resulting from recombination arrest. The Palearctic green toad radiation seems to offer unique opportunities to investigate the consequences of polyploidization and clonal transmission on the dynamics of genomes in vertebrates.

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INTRODUCTION

New species arise in several different ways, which can be classified in two main categories: on one hand, an ancestral species may give rise to two new daughter species (e.g., via allopatric, parapatric or sympatric speciation); on the other hand, two ancestral species may give rise to a new daughter species, via hybridization. The outcome of such hybridization processes partly depends on the level of divergence between the parental species (e.g., Chapman and Burke, 2007). If weakly differentiated, genomes might merge and lead to a diploid hybrid (homoploid hybrid speciation; Buerkle *et al.*, 2000; Coyne and Orr, 2004; Mallet, 2007), with recombination taking place between homeologous chromosomes (i.e. partially homologous chromosomes derived from the same pair in the common ancestor). Alternatively, stronger differentiation may result in allopolyploidization (which may also occur between closely related species; Chapman and Burke, 2007): the two genomes will then coexist over evolutionary times within a tetraploid hybrid. Meiotic recombination will then take place between homologous and normally not between homeologous pairs of chromosomes (Otto and Whitton, 2000; Comai, 2005). The coexistence of homeologous pairs in allopolyploids generates a functional redundancy, which is expected to induce some re-diploidization via the elimination or silencing of redundant parts of the genome or their neo- or sub-functionalization (e.g., Ohno, 1970; Ferris and Whitt, 1977; Soltis and Soltis, 1999; Wendel, 2000; Ainouche and Jenczewski, 2010; Buggs *et al.*, 2011; Braasch and Postlethwait, 2012; Roulin *et al.*,

2012). In parallel to these changes in the function or expression of genes, chromosomal rearrangements, including deletions, inversions, intra- or inter-genomic translocations may occur rapidly and extensively after polyploidization (Soltis and Soltis, 1999; Adams, 2007; Gaeta *et al.*, 2007). Comparative genetic mapping between polyploid species and their diploid relatives allows a better understanding of the evolution of genome structure and associated rearrangements. Linkage analyses have uncovered further genomic consequences of polyploidization, including increased rates of recombination in polyploid plants relative to diploid ancestors (e.g., Leflon *et al.*, 2010; Pecinka *et al.*, 2011; reviewed in Grandont *et al.*, 2013) as well as homeologous (i.e., intergenomic) recombination, shown to occur in polyploid plants (Gaeta *et al.*, 2007) and salamanders (Bi and Bogart, 2006, 2010). Thus, provided one may identify the parental species and timing of hybridization events, allopolyploid lineages represent unique opportunities to address important questions regarding the reorganization of genomes that is expected to follow hybridization, and more generally to gain crucial insights on the evolutionary dynamics of genomes.

Polyploidy is much rarer in animals than in plants (Otto and Whitton, 2000), a pattern attributed by Muller (1925) to the disruption of sex determination in gonochoristic animals. Nevertheless, polyploidization has been documented across a wide range of animal taxa, including turbellarians, annelids, mollusks, insects and crustaceans (Gregory and Mable, 2005), teleost fishes (Schultz, 1980; Le Comber and Smith, 2004), reptiles (Gregory and Mable, 2005), and

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amphibians (Bogart, 1980). Natural polyploids are especially frequent in the latter class (Bogart, 1980; Schmid, 1980; Kawamura, 1984); they have evolved independently in multiple families (Mable *et al.*, 2011), with 50 polyploid anuran species and six polyploid salamander species described (Evans *et al.*, 2012). Palearctic green toads offer a particularly interesting system, forming a monophyletic radiation of at least 12 major mitochondrial DNA (mtDNA) haplotype groups (Stöck *et al.*, 2006) with several cases of range overlap and interactions through hybridization and polyploidization (Colliard *et al.*, 2010; Dufresnes *et al.*, 2014). This radiation includes in particular bisexually reproducing species of three ploidy levels (2n, 3n and 4n; Stöck *et al.*, 2010).

Here, we focused on five green-toad species of a diploid-polyploid complex from Central Asia, of which phylogenetic relationships are provided in Figure 1, based on mtDNA d-loop sequences. The diploid *Bufo latastii* (Boulenger, 1882; LL in Figure 2), spread in eastern Kashmir and Ladakh, constitutes an early-diverged lineage. The tetraploid *B. pewzowi* (Bedriaga, 1898), found in eastern Central Asia (from Uzbekistan to western Mongolia), presents two sets of chromosomes (P_mP_m ; termed ‘TT’ in Stöck *et al.*, 2010) from a maternal ancestor that, according to the mtDNA d-loop and three nuclear sequence markers (Stöck *et al.*, 2010), have been assumed to be related to the geographically proximate *B. turanensis* (Hemmer *et al.*, 1978; TT in Figure 1) whose range is restricted to the lowlands of Central Asia (from Eastern Iran to Kazakhstan). Genome-wide evidence for the maternal ancestry is missing, and the origin of the paternal sets (P_pP_p ; termed ‘LL’ in Stöck *et al.*, 2010) remains unidentified. The two sexes undergo a normal tetraploid meiosis (Stöck *et al.*, 2005), resulting in diploid sperm and ova with one P_p and one P_m set of chromosomes each (Stöck *et al.*, 2010). The triploid *B. baturae* (Stöck *et al.*, 1999), limited to Karakoram and Hindukush and possibly the High Pamirs, has a maternal ancestor that, according to mtDNA 16S rDNA (Litvinchuk *et al.*, 2011) is predicted to be closely related to *B. shaartusiensis* (Mezhzherin and Shcherbak, 1996; cf. Mezhzherin and Shcherbak, 1995; SS in Figure 2), found in southwestern Tajikistan. The ancestor of the two paternal sets (B_pB_p)

also remains unidentified (Figure 1). Triploidy is maintained by differential male and female forms of meioses: in the male germ line, the B_m set (referred to as NOR- in Stöck *et al.*, 2012) is eliminated, followed by a diploid meiosis of the B_p sets (referred to as NOR+ in Stöck *et al.*, 2012) that only produces recombinant haploid B_p sperm, whereas in the female germ line, the B_m set is first duplicated, followed by a tetraploid meiosis that produces diploid B_mB_p ova (Stöck *et al.*, 2012). Hence, the B_p set is sexually transmitted by both sexes, whereas the B_m set is only maternally and clonally transmitted, along with mtDNA. This unique mode of transmission (termed ‘pre-equalizing hybrid meiosis’ by Stöck *et al.*, 2012) is expected to bear important consequences on the evolution of the B_m set, by strongly enhancing genetic drift and drastically reducing the efficiency of selection. The effective population size of the B_m set is reduced to one quarter that of the B_p sets, and the absence of recombination over large genomic regions is expected to induce strong Hill-Robertson interferences between co-segregating loci (e.g., McVean and Charlesworth, 2000): deleterious mutations can get fixed by hitchhiking with beneficial ones during selective sweeps or, conversely, beneficial alleles can be eliminated by selection against co-segregating deleterious mutations (background selection; Charlesworth, 1994). In the absence of recombination, the class of individuals with the lowest genetic load cannot be restored once eliminated by drift (Muller’s ratchet; Haigh, 1978; Gabriel *et al.*, 1993). The small effective population size associated with strong Hill-Robertson interferences is expected to reduce the molecular adaptation of non-recombining genomic regions and lead to their rapid divergence (McVean and Charlesworth, 2000). The evolutionary forces acting on the non-recombining B_m set are similar to those of non-recombining Y or W sex chromosomes, and thus expected to induce a similar degeneration, but at the level of an entire genome.

Our study had two main objectives. The first one was to establish a topology for the reticulate phylogeny in a species-rich group that involves several lineages, some of which very closely related. Nothing was known regarding the origins of paternal subgenomes in polyploids; hypotheses had been formulated regarding the origin of maternal subgenomes, but these were primarily based on mtDNA similarities, and needed to be tested with fast-evolving multiple nuclear markers. To achieve this first goal, we sampled adults from the two polyploid species as well as from three diploid species (*turanensis*, *shaartusiensis* and *latastii*) that might have contributed to their ancestry, and genotyped them with co-amplifying nuclear microsatellite markers (Supplementary Text 1 for details). We expected the patterns of amplification and allelic size range similarities to shed light on the genetic relationships among these various genomes and subgenomes.

The second aim was to explore the potential processes of genomic reorganization that are expected to follow polyploidization. Specifically, we addressed the following questions: (i) Do markers always cluster into the same linkage groups in polyploids as in diploids, or have some translocation events occurred? (ii) Does recombination in polyploids only take place between homologous pairs, or can we find evidence for homeologous recombination? (iii) Do recombination rates increase following polyploidization events, as documented in other lineages? This second set of questions (i–iii) was addressed by rearing families from the same five species, and genotyping them for the same set of co-amplifying microsatellite markers to perform linkage analyses.

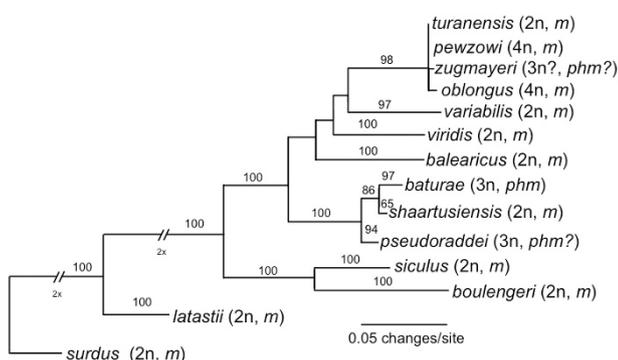


Figure 1 Maximum-Likelihood phylogram of the mitochondrial phylogenetic relationships of Palearctic green toads (*B. viridis* subgroup) based on ca. 880 bp of the mitochondrial control region (D-loop) with information on ploidy level (2n: diploid, 3n: triploid, 4n: tetraploid) and gametogenesis mode (m: meiotic, phm: pre-equalizing hybrid meiosis). Note that for some taxa ploidy and reproductive mode remain insecure (indicated by ‘?’). Numbers above branches show bootstrap support for 100 re-sampled datasets; missing numbers indicate less than 50% support. The most recent common ancestor of *B. balearicus*, *B. turanensis*, *B. siculus* and *B. shaartusiensis* was estimated to be of Pliocene age, that is, to have lived about 3.3 Mya (95% high probability density interval: 1.7–5.1 Mya) by Stöck *et al.* (2013).

MATERIALS AND METHODS

Animal sampling and DNA extraction

The available pedigrees consisted of 1045 individuals from 30 families, of which 9 were of *B. latastii*, 3 were of *B. turanensis*, 4 were of *B. shaartusiensis*, 6 were of *B. baturae* and 8 were of *B. pewzowi* (Table 1). Of these 30 families, 27 originated from mating pairs caught in amplexus during the 2005–2010 breeding seasons from one *B. pewzowi* population in Kazakhstan, three *B. turanensis* and three *B. pewzowi* populations in Kyrgyzstan, one *B. latastii* and two *B. baturae* populations in Pakistan, and one *B. shaartusiensis* population in Tajikistan (Table 1). The three other families stemmed from laboratory crosses between two females and three males of *B. baturae* (families B4, B5 and B6; Table 1) caught in a reproductive state during the 2009 spring breeding period). Females from these crosses were stimulated by injection of 0.1 ml of a 0.9% NaCl-solution containing 500–1000 IU of human chorionadotropin (Sigma-Aldrich, Inc.). Parts of the egg strings from each pair were reared, respectively, in the field or in the laboratory (Table 1) to sample whole embryos, or larvae

were fed with fish food (Tetramin) until tadpoles had grown enough to allow taitip sampling. All parents were sampled for buccal cells (Broquet *et al.*, 2007), finger tips or blood (using sterile syringes), except for two *B. shaartusiensis* families (S3, S4) of which only offspring embryos were collected. Two adult specimens of *B. latastii* and seven of *B. baturae* (buccal swabs) were added to our samples (Table 1). Buccal swabs were stored dry at -20°C ; blood and tissue samples in 70% ethanol at -20°C . DNA was extracted using the QIAGEN DNeasy Tissue Kit or the BioSprint robotic workstation (QIAGEN), both following the manufacturer's protocols with overnight sample incubation in proteinase K at 56°C . DNA was eluted in a 150 μl and a 50 μl volume (QIAGEN Buffer AE) and stored at -20°C .

Microsatellite primers, amplifications and scoring

All of the 30 microsatellite markers developed by Betto-Colliard *et al.* (2013) were used for analyses, using the same PCR protocols (for details of their cross-amplification success, see Supplementary Text 1). Allele sizes were scored with

Table 1 Sampling localities with geographic coordinates (degrees), cross origin and sample sizes per family

Species	Family	Locality	Coordinates	Offspring	Cross Origin
<i>B. latastii</i>	L1	Skardu, Pakistan	35.30°N, 75.37°E	40	natural
	L2	Skardu, Pakistan	35.30°N, 75.37°E	60	natural
	L3	Skardu, Pakistan	35.30°N, 75.37°E	39	natural
	L4	Skardu, Pakistan	35.30°N, 75.37°E	40	natural
	L5	Skardu, Pakistan	35.30°N, 75.37°E	28	natural
	L6	Skardu, Pakistan	35.30°N, 75.37°E	40	natural
	L7	Skardu, Pakistan	35.30°N, 75.37°E	40	natural
	L8	Skardu, Pakistan	35.30°N, 75.37°E	40	natural
	L9	Skardu, Pakistan	35.30°N, 75.37°E	40	natural
<i>B. turanensis</i>	T1	Bishkek, Kyrgyzstan	42.78°N, 74.66°E	15	natural
	T2	Bishkek, Kyrgyzstan	42.80°N, 74.69°E	14	natural
	T3	Bishkek, Kyrgyzstan	42.79°N, 74.68°E	40	natural
<i>B. shaartusiensis</i>	S1	Shaartuz, Tajikistan	37.29°N, 68.13°E	50	natural
	S2	Shaartuz, Tajikistan	37.29°N, 68.13°E	38	natural
	S3*	Shaartuz, Tajikistan	37.29°N, 68.13°E	20	natural
	S4*	Shaartuz, Tajikistan	37.29°N, 68.13°E	20	natural
<i>B. baturae</i>	B1	Pasu, Pakistan	36.50°N, 74.87°E	25	natural
	B2	Pasu, Pakistan	36.50°N, 74.87°E	25	natural
	B3	Gupis, Pakistan	36.23°N, 73.45°E	25	natural
	B4	Gilgit, Pakistan	35.90°N, 74.40°E	30	laboratory
	B5	Gilgit, Pakistan	35.90°N, 74.40°E	50	laboratory
	B6	Gilgit, Pakistan	35.90°N, 74.40°E	41	laboratory
<i>B. pewzowi</i>	P1	Almaty, Kazakhstan	43.26°N, 76.97°E	10	natural
	P2	Bishkek, Kyrgyzstan	♀: 42.68°N, 74.68°E ♂: 42.79°N, 74.65°E	41	natural
	P3	Bishkek, Kyrgyzstan	♀: 42.68°N, 74.68°E ♂: 42.64°N, 74.61°E	40	natural
	P4	Bishkek, Kyrgyzstan	♀: 42.68°N, 74.68°E ♂: 42.64°N, 74.61°E	39	natural
	P5	Bishkek, Kyrgyzstan	♀: 42.68°N, 74.68°E ♂: 42.64°N, 74.61°E	39	natural
	P6	Bishkek, Kyrgyzstan	42.68°N, 74.68°E	37	natural
	P7	Bishkek, Kyrgyzstan	42.68°N, 74.68°E	39	natural
	P8	Bishkek, Kyrgyzstan	♀: 42.68°N, 74.68°E ♂: 42.64°N, 74.61°E	40	natural
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Species	Adults	Locality	Coordinates	Females	Males
<i>B. latastii</i>	2	Skardu, Pakistan	35.30°N, 75.37°E	1	1
<i>B. baturae</i>	2	Gilgit, Pakistan	35.90°N, 74.40°E	1	1
	5	Pasu, Pakistan	36.50°N, 74.87°E	3	2

Sampling localities with geographic coordinates (degrees), sample sizes, number of females and males per species. In each family, the two parents have also been sampled, except for two *B. shaartusiensis* families (S3, S4).

GENEMAPPER v.4.0 (Applied Biosystems). Genotypic configurations for triploid and tetraploid individuals were easily inferred from segregation patterns among families in a majority of alleles (92.5%). In some cases (1.6%), dosage of the amplified products, based on quantitative values for microsatellite allele amplification (peak size) could be used to assign alleles in polyploid individuals (Esselink *et al.*, 2004 and Stöck *et al.*, 2010 for similar approaches). Whenever dosage interpretation was not trustworthy to identify allelic composition (5.9%), allele values were considered as 'missing data' to conserve a robust dataset. Null alleles were readily identified, given information on ploidy levels and pedigrees; for instance, in a diploid family where the father only expresses allele A and the mother allele B, the occurrence of offspring with allele A only reveals a null allele in the mother (genotype B0), while occurrence of offspring with allele B only reveals a null allele in the father (genotype A0). Deviations from Hardy-Weinberg equilibrium were tested using ARLEQUIN v. 3.5.1.3 (Excoffier and Lischer, 2010) in all subgenomes, except in the clonally and maternally transmitted B_m set.

Linkage analyses

To evaluate signs of genomic reorganization between polyploid species and their presumed diploid ancestors via homeologous recombination, changes in recombination rates or translocations events, linkage analyses were performed using CRIMAP v.5.0 (Green *et al.*, 1990) based on all families from the resource pedigree. Analyses in the triploid *B. baturoae* were limited to the recombining diploid B_p set, that is, excluding the clonally and maternally transmitted B_m set. In the tetraploid *B. pewzowi*, linkage analyses were achieved separately for the two homologous sets (P_pP_p and P_mP_m , respectively), identified from our pedigree analyses, thanks to similarity of allelic size ranges between these sets and their putative ancestors (namely, between P_pP_p and LL; P_mP_m and TT), and very low rates of male recombination (see Results). Pairwise linkage analyses, identifying linkages between any two loci, were performed using the TWOPOINT option, assuming sex-specific recombination rates. Pairwise linkage was considered significant if the logarithm of odds score exceeded 3.0 (i.e., the odds that the two loci are linked exceed 1000: 1). For linkage groups including more than two loci, we used the ALL option to select the order with the highest log likelihood. This likelihood was further compared with that of alternative orders by pairwise flipping of neighboring loci using the FLIPS option. Finally, we used the BUILD option to estimate recombination rates and Kosambi mapping distances for the sex-specific maps. Linkage maps were drawn using MAPCHART v.2.1 (Voorrips, 2002). Effects of sex, ploidy level and their interaction (explanatory variables) on the recombination rate (response variable) were tested with randomized ANOVAs over all data, in which recombination rates were randomly permuted 10^4 times between sexes and ploidy levels (Manly, 1997). Given the absence of ploidy effects (see Results), all genomes (LL, TT, SS, B_pB_p , P_mP_m , P_pP_p) were then independently tested for sex-specific differences in recombination rate, using Wilcoxon tests in R (R Development Core Team, 2009). Wilcoxon tests and randomized ANOVAs were performed on pairwise recombination rates of loci belonging to the same linkage group.

Clustering analyses

To investigate the genetic relationships between lineages, we performed a Bayesian clustering analyses using STRUCTURE v.2.3.4 (Pritchard *et al.*, 2000) based on the genotypes of 69 adults, comprising the 60 parents (those of families S3 and S4 were inferred from offspring genotypes) and the nine supplementary specimens (two *B. latastii* and seven *B. baturoae* individuals; Table 1), where the subgenomes of polyploid species (B_m , B_pB_p , P_mP_m , P_pP_p) were considered as distinct entities. Each run, replicated 10 times, comprised a 'burn-in' period of 10^4 and 10^5 iterations. The number of clusters (K) was varied from 2 to 7 (this maximal value corresponding to the expected number of distinct subgenomes: LL, TT, SS, B_m , B_pB_p , P_mP_m , P_pP_p), to establish a hierarchical clustering and provide insights on the depth of splitting among the genomes. The most likely number of clusters was inferred from the *ad hoc* ΔK statistic (Evanno *et al.*, 2005). Topology was also inferred from a Neighbor Net Tree, built with the program SPLITSTREE (v.4.13.1; Huson and Bryant, 2006) based on the F_{ST} -matrix of pairwise distances between genomes (computed with ARLEQUIN v.3.5.1.3; Excoffier and Lischer, 2010). A principal

component analysis was conducted to visualize the differentiation between genomes, using PCAGEN (Goudet, 1999) with 10^4 randomizations of genotypes to test for significance of axes.

RESULTS

Assignment of maternal and paternal sets of chromosomes

In the allotriploid *B. baturoae*, the single maternal set B_m was easily identified from our pedigree analyses because of its purely clonal and maternal transmission; it furthermore displayed the same patterns of amplification and range of allelic sizes as the putative maternal ancestor *B. shaartusiensis* (Supplementary file S1). In the allotetraploid *B. pewzowi*, the maternal P_m set also often displayed the same pattern of amplification and range of allelic sizes as in its putative maternal ancestor *B. turanensis*, while the P_p set displayed similarity with its putative paternal ancestor *B. latastii* (Supplementary file S1). In some ambiguous cases, identification was possible by the patterns of linkage with other already assigned markers, thanks to the extremely low levels of recombination in males (see below). In a few doubtful cases, markers were excluded; altogether, 4 out of 30 markers (*BaC111*, *BaC212*, *BaD5*, *BID114*) could not be assigned with certainty and were excluded from analyses. *BaD103* was also excluded from linkage analyses, being non-informative (monomorphic throughout).

Linkage groups

The patterns of marker amplification, polymorphism and assignments to different linkage groups based on the program CRIMAP are provided in Table 2. Depending on species and subgenomes, three to seven linkage groups could be formed (Supplementary Text 1). Some markers remained unlinked in several genomes (Table 2) because of a lack of polymorphism and statistical power. The linkage groups that could be established were largely consistent within and between species and subgenomes, pointing to a strong synteny across species and ploidy levels with no signs of intergenomic recombination. Loci orders, however, were not always conserved (Supplementary Figure S1a–c), possibly suggesting some inversions. More importantly, a few cases of potential translocations could be established (Table 2). Interestingly, these translocations affected only the paternal sets of the allotriploid *baturoae* (B_p) and the allotetraploid *pewzowi* (P_p) genomes, and consisted only of marker movement out of LG_1 , which had previously been identified as the sex chromosome in several diploid species of the *B. viridis* radiation (Stöck *et al.*, 2011, 2013). Another striking outcome was the heterogeneity found among the paternal sets of *B. baturoae* families: two markers (*BaD11* and *BIB223*) mapped to LG_1 in four families (families B1, B2, B3, B5; group 1, termed B_{p1}) but to different linkage groups (respectively, LG_6 and LG_2) in families B4 and B6 (group 2, termed B_{p2}).

Recombination rates

In all species, average recombination rates were much lower in males (0.02–0.16) than in females (0.34–0.46), a highly significant difference (Wilcoxon tests, $P < 0.001$ for all comparisons). These sex differences occurred on all linkage groups (Supplementary Table S1 and Supplementary Text 1). Accordingly, maps were always much shorter in males than in females (Supplementary Figure S1a–c). In contrast, recombination did not vary with ploidy level; there was no tendency for a larger recombination rate in polyploid species (Supplementary Table S1). The interaction between sex and ploidy level on recombination rate was not significant (Supplementary Table S1).

Table 2 Linkage-group association per microsatellite marker and chromosome set for all species

	<i>B. latastii</i> LL	<i>B. turanensis</i> TT	<i>B. shaartusiensis</i> SS	<i>B. baturae 1</i> <i>B_m</i> (= NOR- in Stöck <i>et al.</i> 2012)	<i>B. baturae 1</i> <i>B_{p1} B_{p1}</i> (= NOR+ in Stöck <i>et al.</i> 2012)	<i>B. baturae 2</i> <i>B_m</i> (= NOR- in Stöck <i>et al.</i> 2012)	<i>B. baturae 2</i> <i>B_{p2} B_{p2}</i> (= NOR+ in Stöck <i>et al.</i> 2012)	<i>B. pewzowi</i> <i>P_m P_m</i> (= TT in Stöck <i>et al.</i> 2010)	<i>B. pewzowi</i> <i>P_p P_p</i> (= LL in Stöck <i>et al.</i> 2010)
BaC212	LG6	U	U	NA	LG6	NA	LG6	U ^a	U ^a
BaC111	LG6	M	U	NA	LG6	NA	LG6	U ^a	U ^a
BID201	LG6	M	M	NA	U	NA	LG6	M	U
BaD11	NA	M	M	M*	LG1	M*	LG6	M	LG1
BaC115	NA	NA	NA	NA	LG1	NA	M	NA	LG1
BaC201	LG1	LG1	LG1	M*	LG1	M*	LG1	NA	U ^b
BID214	LG1	LG1	LG1	M*	LG1	M*	LG1	LG1	LG1
BaC223	M	LG1	LG1	M*	NA	M*	NA	LG1	LG1
BaD107	LG1	NA	NA	NA	LG1	NA	LG1	NA	M
BIB223	LG1	LG1	LG1	M*	LG1	M*	LG2	LG1	NA
BaC101	M	LG1	LG1	M*	LG2	M*	LG2	LG1	LG2
BID2	LG2	LG2	NA	NA	LG2	NA	LG2	LG2	NA
BID102	LG2	LG2	LG2	M*	LG2	M*	LG2	LG2	LG2
BIB7	LG2	LG2	LG2	M*	LG2	M*	LG2	LG2	LG2
BID118	LG2	U	LG2	M*	LG2	M*	LG2	LG2	LG2
BIB118	LG3	LG3	LG3	M*	LG3	M*	LG3	LG3	LG3
BaC224	LG3	LG3	LG3	M*	LG3	M*	LG3	LG3	U ^b
BaC105	M	M	M	M*	LG3	M*	U	M	LG3
BaC123	M	M	M	M*	LG3	M*	M	M	M
BaD124	LG4	NA	NA	NA	LG4	NA	LG4	LG4	NA
BID241	LG4	M	U	NA	LG4	NA	LG4	LG4	NA
BID115	LG4	U	U	M*	LG4	M*	LG4	LG4	LG4
BaD105	LG4	U	U	M*	NA	M*	NA	NA	LG4
BID103	LG5	M	M	NA	LG5	NA	U	NA	LG5
BID140	LG5	U	U	M*	LG5	M*	U	U	LG5
BID113	LG7	LG7	U	M*	NA	M*	NA	LG7	M ^b
BIB3	LG7	LG7	U	M*	U	M*	U	LG7	U
BaD5	U	U	U	M*	U	M*	U	U ^a	U ^a
BID114	U	U	U	M*	U	M*	U	U ^a	U ^a

Abbreviations: M, monomorphic in this species with the same allele fixed; M*, Monomorphic in all families of the species with different alleles fixed; NA, No amplification; U, unlinked.

^aInability to identify the chromosome set as either *P_p* or *P_m*-set.

^bPolymorphic only in the *P1* family, monomorphic in the other *B. pewzowi* families. BaD103 was excluded from linkage analyzes where it revealed to be non-informative.

A full color version of this colored table is available at the *Heredity* journal online.

Patterns of genome differentiation

Several microsatellite markers in the paternal subgenome *P_p* but none or only few markers in the other subgenomes (*L*, *T*, *S*, *B_p* and *P_m*) failed to meet Hardy-Weinberg expectations (Supplementary Text 1). Results from STRUCTURE analyses served to infer the relationships of genomes (Figure 2), together with a tree that summarizes the differential clustering obtained from our hierarchical approach for *K*, varying from 2 to 7. The first divergence (*K* = 2) occurred between the paternal (*L*, *B_p* and *P_p*) and the maternal (*B_m*, *S*, *P_m* and *T*) genomes or subgenomes. *K* = 3 separated *L* from *B_p* and *P_p* within the paternal branch. *K* = 4 (corresponding to a maximum in ΔK) separated the *batuarae* (*S* and *B_m*) from the *pewzowi* lineages (*T* and *P_m*) within the maternal branch. *K* = 5 separated the *batuarae* maternal lineage (*B_m*) from its presumptive ancestor (*S*), whereas *K* = 6 separated the two paternal lineages of *pewzowi* and *batuarae*. Finally, fixing *K* to 7 generated a split among several clonal lineages of *batuarae* maternal subgenome (*B_m*). It is worth noting that the *pewzowi* maternal lineages always clustered together with *turanensis*, and that one copy of *batuarae*'s maternal subgenome always clustered with *shaartusiensis*.

The neighbor net tree (Supplementary Figure S2) provided a very similar topology, with the deepest split between the paternal (*L*, *P_p*, *B_p*) and maternal (*T*, *P_m*, *S*, *B_m*) lineages, and the shallowest one between the *pewzowi* maternal subgenome and the *turanensis* genome. The only deviation from the STRUCTURE clustering corresponded to an earlier branching of *B_m* from the other maternal lineages in the *F_{ST}*-based tree, possibly caused by strong drift and Hill-Robertson interferences affecting this clonal lineage.

These patterns were also highly consistent with the outcomes of the principal component analyses (Figure 3). The seven genomes were best discriminated along the first and fourth axes; the first axis (accounting for 13.67% of the variance) separated the paternal (left) from the maternal (right) genomes, while the fourth axis (accounting for 4.63% of the variance) separated the maternal *pewzowi* (*P_m*, *T*; top) from the maternal *batuarae* (*B_m*, *S*; bottom) lineages.

DISCUSSION

Allopolyploid origins and genome topologies

Clustering analyses based on our multi-locus genotype data, the vast majority of which represent newly established, cross-amplifying microsatellite loci, showed that the maternal ancestor of *B. pewzowi* (*P_m*) was closely related to *B. turanensis*, in accordance with previous suggestions that had been based on limited mtDNA and nuDNA sequence evidence (Stöck *et al.*, 2006, 2010). The strong genetic similarity between these two genomes pointed to a very recent hybridization event: for all *K* values investigated in the STRUCTURE analyses, the *P_m* and *T* genomes clustered together (Figure 2). We also confirmed with nuclear markers that the maternal ancestor (*B_m*) of *B. batuarae* was related to *B. shaartusiensis*. These new data represent the first nuclear and multilocus evidence for the maternal ancestry of *B. batuarae*, which is one of the few gonochoristic all-triploid vertebrates (Stöck *et al.*, 2012), for which previous hints on this hybrid species' maternal ancestor came only from mtDNA (Litvinchuk *et al.*, 2011). The close relationship between *B. batuarae* and *B. shaartusiensis*, that had been inferred from identical mtDNA sequences (Litvinchuk *et al.*, 2011; Figure 1), contrasts with the deeper

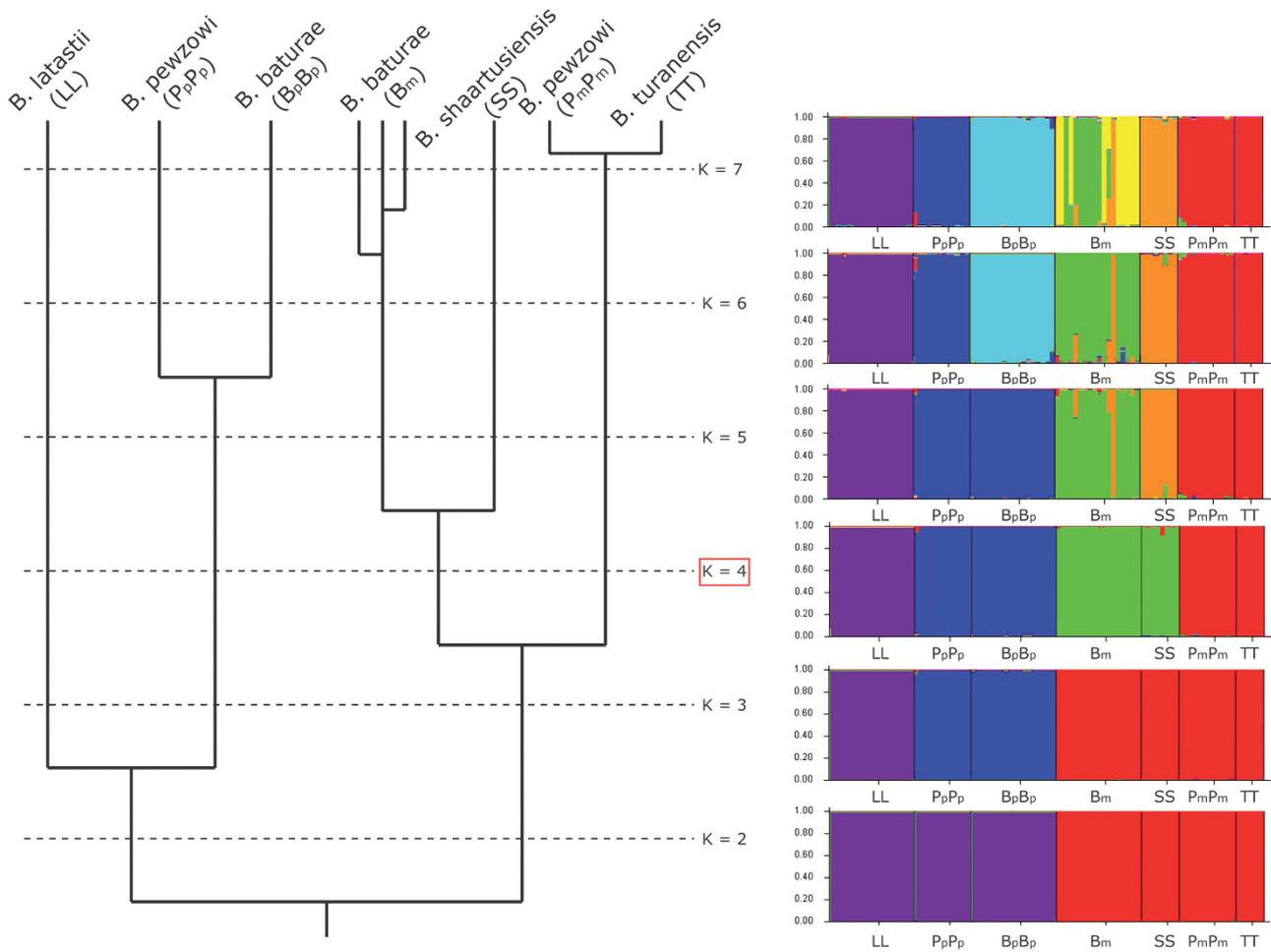


Figure 2 Right: Bar plots from the Bayesian STRUCTURE clustering, hierarchically ordered from $K=2$ (bottom) to $K=7$ (top). Left: associated topology for genome phylogenies. A maximum in ΔK was found at $K=4$.

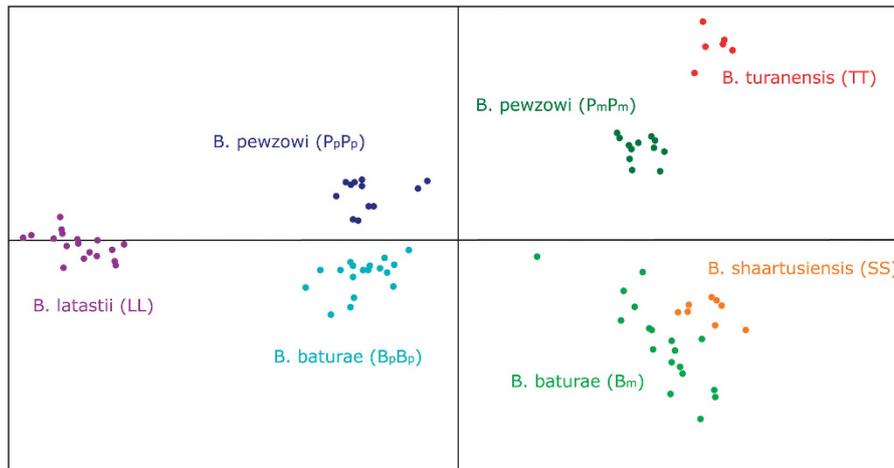


Figure 3 Principal component analysis based on adult and parental samples; results plotted along the first (horizontal) and fourth (vertical) axes.

divergence that we found between B_m and S based on nuclear data (Figure 2 and Supplementary Figure S2). This latter pattern might result from the haploid and clonal transmission of the B_m subgenome in *B. baturae*: the small effective population size and strong Hill-Robertson interferences affecting this non-recombining subgenome is

bound to induce large genetic drift and rapid divergence. This affects not only the differentiation from other lineages, but also among different clonal B_m lineages, as revealed by several sub-clusters of B_m occurring for STRUCTURE setting $K=7$ (Figure 2). Interestingly, one of the B_m lineages of *B. baturae* always clustered with S in STRUCTURE

analyses (Figure 2), for any given K value, indeed supporting the very recent hybridization history suggested by the strong mtDNA similarity (Litvinchuk *et al.*, 2011).

Our data also provided a completely new line of evidence regarding the paternal ancestries of both allotriploid *B. baturnae* and allotetraploid *B. pewzowi*. In contrast to the maternal origins, for which mtDNA had provided hints, nothing was known about the paternal origins of these hybrid polyploids. Our data suggest deeper divergence between the paternal subgenomes (B_p and P_p) and the lineage represented by the *latastii* genome (Figure 2 and Supplementary Figure S2). Given the strong similarities of the maternal lines B_m and P_m with the S and T genomes, respectively, we assume that the paternal genomes B_p and P_p stem from two unsampled (and/or possibly extinct) species that had already largely diverged from *B. latastii* when hybridization events occurred. At this time, these two paternal species had also likely diverged from each other: the origin of *pewzowi* cannot predate the P_m -T divergence, which our results suggested to be very recent (Figure 2), in line with previous mtDNA evidence (Stöck *et al.*, 2006, 2010).

The clonal transmission of the maternal subgenome B_m combined with the departure from Hardy-Weinberg equilibrium in several markers from the paternal subgenome P_p could have affected the topology arising from our STRUCTURE analyses. In the future, gene sequencing should provide alternative and powerful tools to investigate our working hypotheses, and confirm or falsify the genome relationships proposed here (Figures 2 and 3). In particular, comparative investigations of series of genes from the maternal lineages (B_m , S, P_m and T) would offer a fascinating opportunity to compare the evolution of a given genome put in distinct evolutionary settings: namely, in either a recombining diploid state (S and T), a recombining polyploid state (P_m) or a non-recombining polyploid state (B_m). Such comparisons should allow disentangling the consequences of polyploidization from those of Hill-Robertson interferences on the evolutionary dynamics of genomes.

Sex, but not ploidy level, affects recombination rates

Our data also provided indications that sex had a major impact on recombination rates: all linkage groups and species investigated displayed strongly female-biased recombination. Heterochiasmy (i.e., sex differences in recombination rates) seems widespread throughout animals, with a trend for strongly reduced male recombination across amphibians (e.g., Sumida and Nishioka, 2000; Berset-Brandli *et al.*, 2008; Rodrigues *et al.*, 2013) and fishes (Moens *et al.*, 2004). Heterochiasmy might have evolved as a pleiotropic consequence of selection on sex chromosomes (Haldane, 1922; Huxley, 1928; Nei, 1969): the easiest or quickest way to reduce sex-chromosome recombination in the heterogametic sex might be to reduce recombination over the whole genome in this sex. Accordingly, several diploid species of the *B. viridis* radiation have been shown to be male heterogametic (Stöck *et al.*, 2011, 2013). If this correlation also holds for polyploids as our data support, the strongly female-biased recombination documented here for *B. baturnae* and *B. pewzowi* suggests maintenance of the initial male heterogamety during polyploidization events. This point obviously deserves further investigation, given that sex chromosomes and sex-determination systems have been seen as major obstacles to polyploidization (Muller, 1925; Orr, 1990).

In contrast, recombination rates did not vary with ploidy level. These patterns differ from those recently documented from plants (*Arabidopsis* and *Brassica*), where auto- and allopolyploids were shown to display higher recombination rates than their diploid ancestors (Leflon *et al.*, 2010; Pecincka *et al.*, 2011; Grandont *et al.*, 2013).

However, those polyploid plant lineages resulted from recent laboratory crosses, and thus presumably did not have time to evolve advanced states of re-diploidization of genomes, a process that follows polyploidization (e.g., Soltis and Soltis, 1999; Wendel, 2000; Tayalé and Parisod, 2013). Despite their relatively young evolutionary ages, polyploids from the *B. viridis* subgroup already show some signs of re-diploidization, as illustrated by the physical deletion of entire rDNA clusters (NORs; Stöck *et al.*, 2002, 2005). Thus, the increased recombination rates documented from laboratory-produced plant polyploids might only constitute a transient state, with a return towards lower rates during the process of re-diploidization.

Genome synteny and reorganization

Our linkage group maps (Supplementary Figure S1a–c) pointed to a generally strong synteny among the genomes investigated, with however some evidence for a few reorganization events. A relocation of *BaC101* from LG_1 to LG_2 was found both in *pewzowi* (P_p) and in the two *baturnae* groups (B_{p1} and B_{p2}), and thus likely occurred early in the evolution of these subgenomes, before their divergence. The relocation of *B1B223* and *BaD11* from this same LG_1 to LG_2 and LG_6 , respectively, was only observed in the B_{p2} group, and thus likely occurred much more recently. Although still anecdotal, these events point to patterns that might be more than coincidental: all three translocations occurred in the paternal ancestors' subgenomes of polyploid species, and all affected the same linkage group (LG_1), identified in the studies by Stöck *et al.* (2011, 2013) as the sex chromosome in several diploid species from the *B. viridis* radiation.

Extensive chromosomal rearrangements are expected following polyploidization, as repeatedly documented in polyploid plant species (Soltis and Soltis, 1999; Wendel, 2000; Bento *et al.*, 2011; Tayalé and Parisod, 2013). Accordingly, the translocation events documented here in polyploid green toads contrast with the strong synteny that otherwise characterizes diploid species from this group, and amphibians in general (e.g., Miura, 1995; Sumida and Nishioka, 2000; Brelsford *et al.*, 2013). It might also be meaningful that such translocations were limited not only to the paternal subgenomes, but also to the supposed sex-chromosome pairs. Assuming parental species to be male heterogametic (as are other diploid species from this radiation; Stöck *et al.*, 2011, 2013), one would expect sex in polyploid green toads to be determined by paternal subgenomes (B_p and P_p , respectively). Similarly, assuming sex-determination systems to be conserved during polyploidization, then sex-determining genes with functional X and Y alleles should localize on the paternal LG_1 in both *B. baturnae* and *B. pewzowi*. Given that sex-determination systems have been proposed to constitute major barriers to polyploidization in animals (Muller, 1925; Otto and Whitton, 2000), the reorganization of sex chromosomes that may follow polyploidization is an important issue, which deserves further investigation by sequence analyses of paternal subgenomes in polyploid species from the *B. viridis* radiation. Very strong mtDNA similarity in the D-loop (Stöck *et al.*, 2006 and our still unpublished data) suggests a single maternal origin of allotriploid *B. baturnae* sampled here in two isolated high mountain valleys in the Karakoram. However, the observed LG_1 differences interpreted here as translocations, could alternatively stem from, so far undocumented, multiple paternal contributions to the allotriploid genome.

Conclusions and perspectives

The multiple and independent events of hybrid-origin polyploidization that characterize Palearctic green toads (*B. viridis* subgroup) offer remarkable opportunities to investigate the rise and consequences of polyploidization and thus polyploid hybrid speciation in animals. Our

sibship analyses of microsatellite markers allowed us to generate working hypotheses regarding ancestry relationships and genome topologies, to identify translocation events presumably associated with the reorganization of sex chromosomes, and to suggest Hill-Robertson effects on clonally transmitted subgenomes. Sequence analyses of autosomal and sex-linked genes will allow further tests of the manifold consequences of polyploidization and clonal transmission on the reshaping of genomes on general, and that of sex chromosomes in particular.

DATA ARCHIVING

Genotype data available from the Dryad Digital Repository: doi:10.5061/dryad.ljc02.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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