A bisexually reproducing all-triploid vertebrate

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Green toads are common in the Palaearctic region, where they have differentiated into several taxa[1,2]. The toads exist with variable amounts of ploidy, similar to other anuran species[3] or reptiles[4]. In vertebrate biology, the very rare occurrence of triploidy is coupled with infertility or unisexuality, or requires the coexistence of individuals of different ploidy in a reproductive community. The reproduction of naturally occurring triploids has been reported to occur only through parthenogenesis, gynogenesis or hybridogenesis. The bisexual reproduction of pure triploids has been considered to be impossible because of the problem of equally distributing three chromosome sets in meiosis. Here we report geographically isolated populations of green toads (*Bufo viridis* complex) that are all-triploid and reproduce bisexually.

Tetraploid green toads reproduce through diploid eggs and sperm cells that are the result of a 'normal meiosis'[5,6]. Triploid specimens have been found in northern Central Asia that seem to result from the hybridization of diploid and tetraploid toads with whom they form mixed breeding complexes[7–10]. A new taxon (Batura toads, *Bufo pseudoraddei baturae*[11]; Fig. 1a) was discovered at an isolated site in the Karakoram mountain range. These animals inhabit the high Hunza valley, which is surrounded by steep rocky mountain desert ranges. Every wild toad caught (n=131) from eight localities[11] was triploid (Fig. 1b). In one population (Pasu), we analyzed a total of 82 triploid individuals. In extensive sampling, we did not find a single diploid or tetraploid Batura toad. Both males and females were found, and the sex ratio did not deviate from that expected from observations of diploid and tetraploid populations (χ^2 test, $P>0.05$; n=259).

We mated three pairs of triploid Batura toads, which produced viable offspring that underwent metamorphosis normally. Cytogenetic and flow cytometric analyses showed that all offspring (n=62)

were triploid like their parents. To determine whether the offspring result from parthenogenesis or gynogenesis, we carried out multilocus fingerprinting[12]. For clonal offspring, the banding patterns of mothers and their offspring should be identical with no paternal bands. In two independent matings, however, the offspring showed individual banding patterns with bands originating from both parents (Fig. 1c), which indicates that the mode of reproduction is not asexual.

Closer inspection of the banding pattern shows that some paternal bands are not found in any offspring (Fig. 1c), whereas all of the maternal bands are present in some siblings. The band-sharing index (twice the number of bands shared by one parent and its offspring divided by the sum of individual bands of both)[13] of the offspring averaged 0.54 with their mother, but only 0.23 with their father—a significant deviation from the expected 1:1 ratio ($P<0.001$, *t*-test; n=31). This observation prompted us to consider a possible mechanism for sexual reproduction in an all-triploid vertebrate that includes an unequal contribution of both parents to their offspring.

Comparative DNA image cytometry (Fig. 2)[14] on stages of spermatogenesis from monolayers of testis nuclei (tissue imprints) and from mature sperm cells of diploid, triploid and
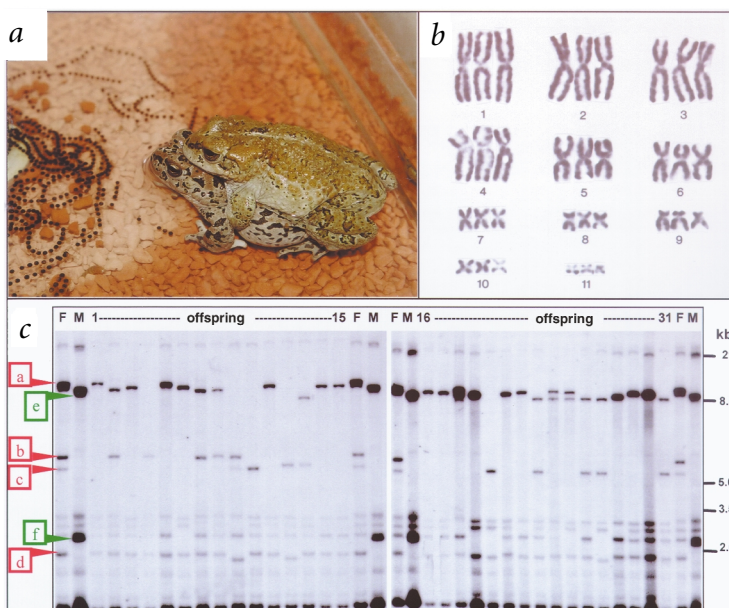
Fig. 1 All-triploid, bisexually reproducing green toads (*B. pseudoraddei baturae*) from the Hunza Valley, Karakoram, Pakistan. *a*, Reproducing pair from Pasu, with clutch string. *b*, Giemsa-stained karyotype with 3*n* (33) chromosomes. *c*, Example of multilocus fingerprints of DNA samples from a somatic triploid pair and its triploid offspring, digested with *Hin*fI and hybridized to oligonucleotide (TCT)₆. Bands from 31 F1 tadpoles are shown: (a–d) are maternal bands, and (e) a paternal band, showing segregation among F1 specimens; band (f) is a paternal band transmitted to none of the 31 F1 specimens. M, male; F, female.

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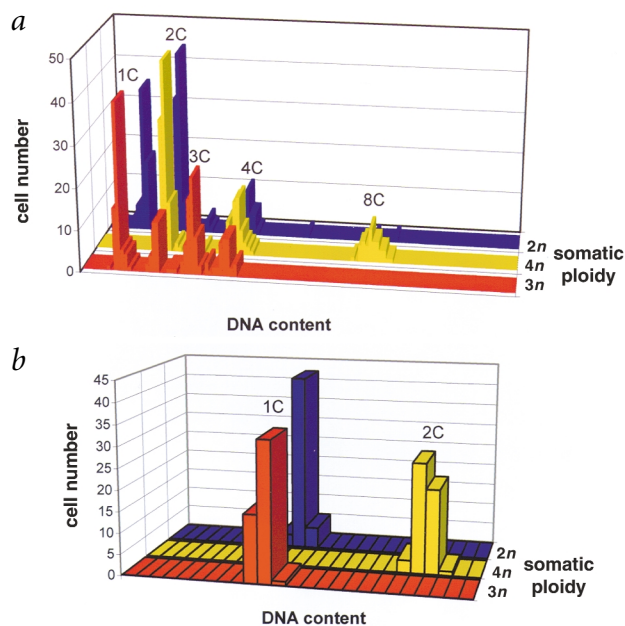
Fig. 2 DNA content of testis cells in green toads of different ploidy. The histograms show results obtained by DNA image cytometry from testis imprints of a somatic triploid (3*n*), a tetraploid (4*n*) and a diploid (2*n*) male, prepared on the same slide. ***a***, Two hundred Feulgen-stained nonsperm nuclei. ***b***, Fifty Feulgen-stained sperm nuclei.

artificially produced allotriploid anuran males[19,20]. The cells of these species contain two plus one chromosome sets from two parental species and eliminate the chromosome set that is single; these cytogenetic mechanisms seem to be modifications of hybridogenesis. The situation in the Batura toads is different from that in *Rana* and *Leuciscus*, however, because the triploidy in the latter species occurs only in mixed breeding systems with 2*n* or 2*n* and 4*n* individuals, and triploids in these populations can be maintained only by interbreeding these particular forms. In addition, crosses of triploid parents in all reported triploid vertebrates have never revealed triploid offspring.

Lampbrush chromosomes of female Batura toads contain 22 bivalents, in accordance with the meiosis of a tetraploid oocyte. Both chromosomes of only one bivalent carry the NORs (Fig. 3*d*). This indicates that crossing over takes place between homologous rather than between homeologous chromosomes. Recombination nodules in the G2 stage show the occurrence of crossing over. This situation can be explained by two hypothetical mechanisms (Fig. 4), both of which are formally possible.

First, premeiotic elimination of one of the two NOR-carrying chromosome sets, NOR$^+$, is followed by a whole-genome duplication (endomitosis), which leads to the observed onset of meiosis (G2). Depending on the eliminated NOR$^+$, only two classes of genetically identical eggs can result. The DNA fingerprint analysis shows, however, the reappearance of all maternal bands among the offspring and more than two patterns derived from the mother. Assuming that this mechanism is correct, patterns such as this seem possible only if some recombination occurs between the NOR$^+$ and NOR$^-$ sets, even if there is no suggestion of recombination from chromosome 6.

Second, the chromosome set without the NOR marker, NOR$^-$, is duplicated premeiotically, which also leads to the observed G2/lampbrush stage of meiosis. This mechanism will generate genetically different ova, in accordance with the DNA fingerprint data.
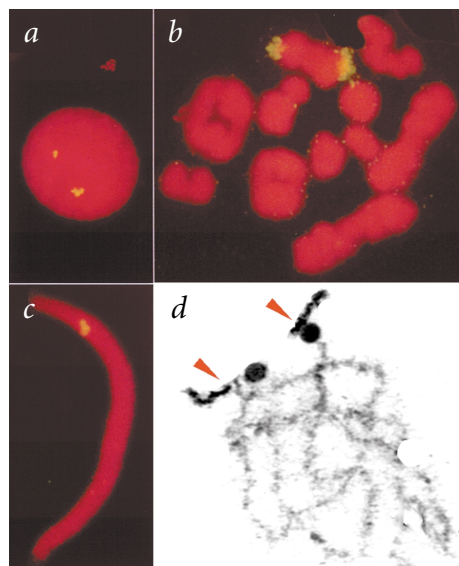
tetraploid males shows that, first, the sperm cells of 4*n* males are diploid, whereas 3*n* males produce haploid sperm like normal diploids; second, both 2*n* and 4*n* males have three main DNA peaks of nonsperm nuclei that represent, respectively, the G2 stage of somatic and premeiotic mitosis and of meiosis I (4C in diploid/8C in tetraploid), the somatic nuclei and primary spermatocytes (2C/4C) and the secondary spermatocytes (1C/2C); and last, the germline of triploid males is similar to that of diploids, except for an additional 3C peak from somatic and spermatogonial nuclei.

In other preparations of all-triploid specimens, we found some 6C cells representing the expected somatic, mitotic G2 phases. The low observation of 6C cells may be due to occurrence of 6C cells only in somatic tissue that does not participate in the massive cohort cell division of spermatogenesis. Karyotype analyses of more than 60 metaphase cells (I and II) from the testes of triploid toads show that they have a normal chromosome number and a morphology characteristic of meiosis of a diploid toad (Fig. 3*b*). Together, these data indicate that premeiotic elimination occurs in males for one set of the 3*n* (33) chromosomes.

Additional support for such a mechanism comes from studying chromosomes carrying the nucleolus organizer region (NOR). In the Batura toad, chromosome 6 is heteromorphic for the presence of the NOR; that is, only two of three chromosomes 6 have NORs. Notably, all male meiotic stages (Fig. 3*a,b*) contain two NOR-carrying chromosomes 6, and consequently all haploid stages including sperm have one NOR (Fig. 3*c*). This shows that the chromosome set lacking the NOR is eliminated.

The situation is reminiscent of, for example, fertile triploid males of the hybrid form of the *Rana esculenta* complex[15,16], of triploid females of the *Leuciscus alburnoides* complex[17,18] and of

Fig. 3 NOR signals in triploid toads. *a–c*, *In situ* hybridization with 18S + 28S rDNA. A triploid somatic nucleus (*a*) shows two NOR signals; male meiotic diakinesis in testis tissue (*b*) with 11 bivalents shows two NOR signals in the paired chromosomes 6; a haploid sperm nucleus (*c*) shows one NOR signal. *d*, Distamycin A/mithramycin counterstaining in the diplotene oocyte reveals part of the only lampbrush bivalent of chromosomes 6, which both show fluorescing NORs (arrows), and also two laterally attached nucleoli.
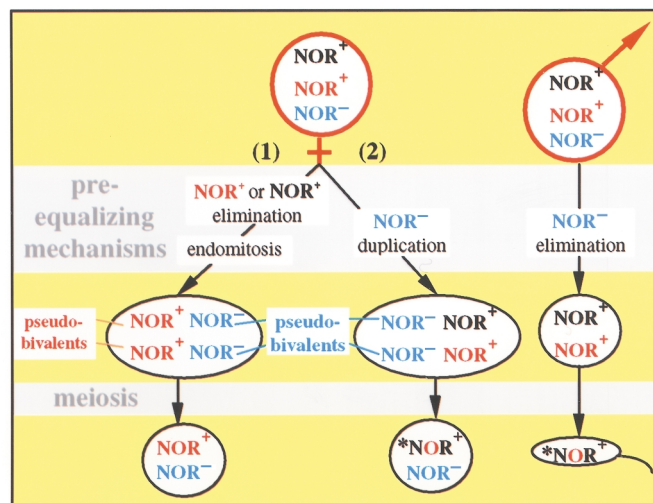
Fig. 4 Diagram of the reproductive system in triploid Batura toads with two possible mechanisms for oogenesis. NOR⁺ (orange or black) indicates different heterozygous NOR-carrying chromosome sets; NOR⁻ indicates an unrecombined chromosome set without NORs; *NOR⁺ indicates recombined NOR-carrying sets.

To explain how $3n$ females reach the observed meiotic G2 stage, we favor the second mechanism in which a separate duplication of the chromosome set without NOR (NOR⁻) results in 11 heterozygous bivalents of NOR-carrying sets (NOR⁺) plus 11 'pseudobivalents'[21] of auto-reduplicated NOR⁻. Recombination only occurs between the NOR⁺ sets, however, because recombination is ineffective in the homozygous NOR⁻ sets. Thus, females transmit a recombined NOR⁺ and probably a clonally inherited NOR⁻ chromosome set to the offspring.

This hypothesis is supported by an analysis of length variation in the NORs and microsatellite loci, which are informative in outcrosses of a $3n$ female with $2n$ and $4n$ males from populations of closely related taxa. The triploid female, having two chromosomes 6 with long NORs, produces diploid eggs containing one NOR⁺ (long) and one NOR⁻. When crossed with a $4n$ male, which transmits diploid sperm of NOR⁺ (short) and NOR⁻, all-tetraploid progeny result in which short paternal and long maternal NORs are always identified (Fig. 5a). A microsatellite marker that represents the maternal NOR⁺ set shows a reduced intensity in every $4n$ F1 specimen, whereas the NOR⁻ microsatellite allele always reoccurs unchanged (Fig. 5b).

When the triploid female is mated with a conspecific triploid male, both parents and their $3n$ F1 progeny show the same pattern of intensities for the microsatellite marker (Fig. 5c), because the maternally reduced NOR⁺ marker intensity is regularly complemented by the paternal one, as triploid males only transmit a NOR⁺ set. Together these observations confirm that in female meiosis the recombining chromosome sets that contain NOR are reduced. When the same triploid female is crossed with a diploid male transmitting only NOR⁺ (short), all-triploid offspring containing NOR⁺ (long), NOR⁺ (short) and NOR⁻ result (data not shown). In this case, a polymorphic microsatellite product of Bcal 7 (which is not informative for NOR⁺ or NOR⁻) reconfirms, by the lower intensity of paternal bands, that the paternal contribution is one-third, whereas the maternal contribution is two-thirds (Fig. 5d).

Bisexual reproduction in Batura toads could hardly be termed 'triploid hybridogenesis', because in retaining some of the advantages of the meiosis they are neither 'normally hybridogenetic' nor 'normally sexually reproducing'. The occurrence of meiotic stages and recombination in both sexes, at least in two-thirds of the whole genome, allows the perpetuation of all-triploidy in a closed breeding system and protects these animals in the long term from the deleterious effects of accumulating mutations, as has been proposed for ameiotic naturally triploid populations[22]. To our knowledge, this represents the first case of naturally occurring gonochoristic, bisexually reproducing triploid animals. It unites characteristics of sexual (genetic recombination) with those of asexual reproduction (probably unrecombined inheritance of the NOR⁻). Another notable feature of this reproductive system is that the NOR⁻ set behaves similarly to a complete set of B chromosomes. The fact that euploid gametes can be produced regularly on the basis of uneven ploidy in the soma encourages speculation that other bisexually reproducing all-triploid species exist. It also raises the intriguing issue of how and when a whole set of chromosomes may be duplicated (in females) or eliminated (in males).

Methods

Animals. We collected $3n$ toads from north Pakistan during three expeditions (13 June to 27 July 1996; 6 June 1997 to 29 June 1997; 22 June to 17 July 2000) from eight localities in the Hunza, Gilgit and Chitral valleys. The parental animals originated from the Karakoram Range: Pasu (36° 30′ N; 74° 52′ E; 2,600–2,800 m) and Gilgit (35° 54′ N; 74° 24′ E, 1,550 m). We chose offspring specimens randomly from two breeding pairs and crossed
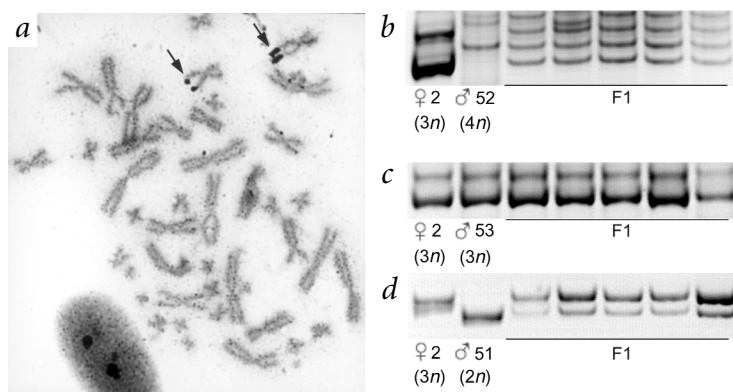

Fig. 5 Inheritance of polymorphic markers in the offspring of a triploid female. *a*, Mitotic metaphase with $4n$ (44) chromosomes of an F1 tadpole from the cross with a tetraploid male: the chromosome 6 with long NORs is maternal (right arrow); the chromosome 6 with short NORs is paternal (left arrow). *b*, Microsatellite markers amplified with primer BM 224 in $4n$ F1 individuals from the cross with the tetraploid father. The intensities of the bands imply that both parents contribute one-half (two chromosome sets) of the genes in every $4n$ F1 tadpole. It is also inferred that the lower maternal band (~135 bp) represents both NOR⁺ sets because it is reduced in intensity in all F1 individuals; in contrast, the higher maternal band (~145 bp), which represents the NOR⁻ set, is transmitted at the same intensity. *c*, Products of BM 224 in $3n$ F1 individuals from matings with a conspecific triploid male. The pattern is consistent with triploid males only transmitting NOR⁺ (135-bp allele) sets to the F1 individual, the maternally reduced intensity of the NOR⁺ marker being complemented by the paternal one. If the NOR⁻ set were represented by a 135-bp allele, then offspring with two alleles of 145 bp and one allele of 135 bp would be expected, and this was not observed. *d*, Products of Bcal 7 in $3n$ F1 individuals from the cross with a diploid male. The lower intensity of the paternal bands shows that the female contributes two alleles (representative of two sets of chromosomes) to the F1 individual.

one triploid female from Pasu with both a naturally diploid (Syria, Barqash, 33° 29′ N, 36° 0′ E) and a tetraploid male (Iran, Birjand, 32° 33′ N, 59° 10′ E, 1,500 m above sea level).

Ploidy determination. For ploidy determination, we counted chromosomes from bone marrow and intestine as described previously[23], or we carried flow cytometry of blood cells stained with the fluorochromes Hoechst 33258 or DAPI. We prepared fresh imprints of testis cross-sections of diploid, triploid and tetraploid males as monolayers of nuclei on the same microslide and fixed them for 24 h with 5% formaldehyde solution, followed by the Feulgen reaction. We made comparative DNA measurements of 200 nonsperm nuclei or 50–100 mature sperm cells with the CYDOK image analysis system (wavelength 546 nm), using haploid sperm of diploid males as an internal (slide-specific) standard. The proportionality factor[24] between spermatids (before) and sperm cells (that is, after spermiohistogenesis) of the same DNA content was determined to be 1.45. This factor compensates for the known optical effect leading to lower extinction values for small nuclei with highly condensed chromatin than for nuclei with a greater nuclear area having the same DNA content.

Chromosome analyses. We used *in situ* hybridization[25] with 18S + 28S rDNA to visualize somatic and testis chromosomes and testis imprints, and distamycin A/mithramycin counterstaining to visualize lampbrush chromosomes. We removed a small part of one ovary of an anesthetized triploid female and prepared lampbrush chromosomes[26,27] from oocyte nuclei. Photographs were taken with phase contrast or after DNA staining (Hoechst 33258).

Multilocus fingerprinting. Standard protocols were used to extract DNA from pooled organs, and then 6 μg of DNA from individual parental toads and their offspring was digested with restriction enzymes (*Hin*fI, *Sau*IIIa, *Hae*III, *Alu*I), separated using 1.5 V/cm for 45 h on 0.8% agarose gels and hybridized to simple repeat synthetic oligonucleotides. The most informative banding patterns were obtained with *Hin*fI and hybridization with $(TCT)_6$, $(TAC)_5$, $(TC)_8$, $(TTTC)_4$, $(CT)_4$ and $(CA)_5$, with *Hae*III and $(TAC)_5$ and $(TC)_8$, and with *Alu*I and $(TTTC)_4$ and $(TC)_8$. For the statistical analysis, we examined the banding patterns from eight gels.

Microsatellites. Among 11 tested PCR primers (Bcal1–Bcal8, BM 121 and BM 224, Bbμμ 1) developed for other species of *Bufo*[28–30], only two (BM 224, Bcal 7) amplified polymorphic microsatellite loci in both Batura toads and taxa used for crosses.

Competing interests statement

The authors declare that they have no competing financial interests.

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