Simultaneous Mendelian and clonal genome transmission in a sexually reproducing, all-triploid vertebrate

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Meiosis in triploids faces the seemingly insuperable difficulty of dividing an odd number of chromosome sets by two. Triploid vertebrates usually circumvent this problem through either asexuality or some forms of hybridogenesis, including meiotic hybridogenesis that involve a reproductive community of different ploidy levels and genome composition. Batura toads (Bufo baturae; 3n = 33 chromosomes), however, present an all-triploid sexual reproduction. This hybrid species has two genome copies carrying a nucleolus-organizing region (NOR+) on chromosome 6, and a third copy without it (NOR−). Males only produce haploid NOR+ sperm, while ova are diploid, containing one NOR+ and one NOR− set. Here, we conduct sibship analyses with co-dominant microsatellite markers so as (i) to confirm the purely clonal and maternal transmission of the NOR− set, and (ii) to demonstrate Mendelian segregation and recombination of the NOR+ sets in both sexes. This new reproductive mode in vertebrates (‘pre-equalizing hybrid meiosis’) offers an ideal opportunity to study the evolution of non-recombinating genomes. Elucidating the mechanisms that allow simultaneous transmission of two genomes, one of Mendelian, the other of clonal inheritance, might shed light on the general processes that regulate meiosis in vertebrates.

Keywords: biased genome transmission; clonal; Mendelian segregation; recombination; triploid vertebrate; Bufo viridis subgroup

1. INTRODUCTION

According to Mendel’s second Law [1], alleles of different genes assort independently of one another during gamete formation. In sexually reproducing species, random transmission of paternal and maternal genomes is achieved by the independent segregation of chromosomes during meiosis. Some animal genomes, however, display transmission biases, often according to parent of origin, and usually as a consequence of ancient hybridization [2]. Hybrid lineages of ants, for instance, carry two independently evolving genomes, which are transmitted either meiotically [3] or clonally [4,5]. Similar processes occur in hybridogenetic vertebrates: in diploid hybridogenesis, one genome is transmitted clonally through the hybrid lineage, while the other is transmitted sexually by one of its parental species [6]. In meiotic hybridogenesis, both of the hybridizing genomes can be transmitted sexually, through crosses between diploid and triploid hybrids of different genomic compositions (figure 1; [14,16]).

Bisexual reproduction of pure triploids is constrained because of the problem of equally distributing three chromosome sets in meiosis [17], for a review see [18]. Hybrids in Peciliopsis, for instance, are hybridogenetic in their diploid forms, but become gynogenetic as triploids [19,20]. As an alternative to gynogenesis or parthenogenesis (figure 1; [7–9,21]), some triploid vertebrates combine clonal and sexual elements in their reproductive modes—e.g. kleptogenesis or different forms of hybridogenesis [10,22–24], including meiotic hybridogenesis, which requires a reproductive community of different ploidy levels and genome composition.

In this context, Batura toads (Bufo baturae) are exceptional in being sexually reproducing triploids [25]. This species of hybrid origin inhabits the high mountains of northern Pakistan (greater than 1500 m a.s.l.). Its genome (3n = 33; [25,26]) is composed of two chromosome sets carrying a nucleolus-organizing region (NOR+) on chromosome 6, and another set without such a region (NOR−; figure 2). Males only produce haploid NOR+ sperm, suggesting elimination of the NOR− set (11 chromosomes) before the onset of meiosis. In contrast, ova are diploid (2n = 22) with one NOR+ and one NOR− sets. Immature oocytes exhibit 22 lampbrush chromosome bivalents [25].

Therefore, it has been assumed that the NOR− set undergoes purely maternal and clonal transmission. However, it remained unknown, whether and how the
NOR+ sets recombine. For females, two hypotheses (figure 2) can be envisaged: (a) One NOR+ set is eliminated (either randomly or depending on parent of origin), followed by endomitotic auto-duplication of the remaining two sets. Meiosis thus only occurs between pseudo-bivalents [27], and produces one or at most two classes of otherwise clonal diploid ova. Alternatively (b), the NOR− set is auto-duplicated before meiosis, during which the two NOR+ sets recombine normally.

For males, similarly, a first hypothesis (figure 2c) is that the whole maternal complement (NOR+, NOR−) is eliminated, followed by paternal NOR+ duplication through pre-meiotic endomitosis. Meiotic pairings (NOR+/NOR+; [25]) would thus represent pseudo-bivalents, implying clonal transmission of one NOR+ set. Spermatocytes would comprise a single multi-locus genotype (or at most two, if NOR+ elimination were random). Alternatively (figure 2d), only the NOR− set is eliminated, and the two NOR+ sets undergo normal meiosis and recombination.

From multi-locus fingerprint data, Stöck et al. [25] identified several genotypes among the offspring from a single family. However, dominant multi-locus markers are not always straightforward to interpret, and thus shed little light on the underlying mechanisms. In the present paper, we performed sibship analyses with 15 co-dominant microsatellite loci to evaluate genome-wide
patterns of transmission and segregation. Our results clearly confirm the purely clonal and maternal transmission of one set of chromosomes (NOR−), and show independent segregation and recombination of the other sets (NOR+) in both males and females. This reshuffling of genetic material should allow efficient purging of the two sets of NOR+ chromosomes as in normal sexual reproduction. This is the first example of parallel clonal and meiotic transmission of chromosome sets within the same lineage of vertebrates.

2. MATERIAL AND METHODS

Animals used in controlled breeding experiments were collected from three localities in northern Pakistan (electronic supplementary material, table S1) during three periods of fieldwork (June–July 1996, 1997 and 2000). We performed five breeding experiments involving triploid Batura toads. In addition, we crossed one Batura female with both a diploid *Bufo variabilis* male from Syria (2n = 22, with two NOR− sets) and a tetraploid *Bufo oblongus* male from Iran (4n = 44, including two NOR+ and two NOR− sets; [28]). Twenty to 100 offspring were raised in tanks up to a larval length of 2–3 cm (Gosner-stages 30 to 38, [29]). A total of 85 tadpoles from the seven crosses were sampled for genetic analyses. Tadpoles were either karyotyped or their ploidy level determined by flow cytometry; DNA was extracted as described in Stock et al. [30].

We tested a series of microsatellites markers from a genomic library enriched for repetitive elements from the Batura toad, some of which were previously used in other species (electronic supplementary material, table S2). Alleles were amplified, scored with GeneMapper v. 3.7 (Applied Biosystems), and named according to their lengths in base pairs as described [30]. Alleles from the NOR+ and NOR− sets, as well as null alleles (0), were identified from inheritance patterns (see §3). Linkage analyses were performed with GenePop (http://genepop.curtin.edu.au/; [31,32]) under default parameters, and potential linkage groups were checked by visual inspection. Given the manageable size of the dataset, progeny genotypes were also visually inspected for cases of recombination. The number of recombination events was normalized to the number of informative events per family, and departures from random segregation were tested for significance (using a $\chi^2$-test).

3. RESULTS

(a) Inheritance patterns in Batura toads

A total of 15 microsatellites primer pairs amplified products in one or more families. Five of them (D103, D105, D5, C224 and C203; electronic supplementary material, dataset S1) displayed up to three alleles per individual, which implies product amplification from both the NOR− and the two NOR+ sets. NOR− alleles were easily identified, being always homomorphic among offspring from a family, identical to the maternal copy, and different from the paternal one whenever parental copies differed (table 1 and electronic supplementary material, table S3 and dataset S1). Both NOR+ sets, by contrast, displayed biparental inheritance and Mendelian segregation, following expectations from meiotic NOR+ sets (electronic supplementary material, dataset S3 and dataset S1). Each heterozygous parent transmitted its two alleles with equal probability (binomial tests). The 10 other markers presented a maximum of two alleles per individual, with biparental inheritance and Mendelian segregation (electronic supplementary material, dataset S1).

Four linkage groups could be identified, involving two markers each (table 2). Out of 199 informative events, we detected a total of 25 cases of recombination (table 2), occurring in both sexes (electronic supplementary material, dataset S1 and table S3). All other pairs of markers were transmitted independently, generating a high diversity of multi-locus genotypes per family. Interestingly, the five markers amplifying a NOR− product were assigned to different linkage groups in the NOR+ genome (table 2 and electronic supplementary material, dataset S1), supporting genome-wide distribution of the NOR− markers.

(b) Inter-ploidy crosses

Locus D105 could also be amplified from the progeny of a female *B. baturae* with (i) a diploid *B. variabilis* male (2n = 22, comprising two NOR+ sets) and (ii) a tetraploid *B. oblongus* male (4n = 44 including two NOR+ and two NOR− sets). All offspring sired by the *B. variabilis* father were triploid and inherited the maternal NOR− allele at locus D105, while the two NOR+ sets displayed biparental inheritance with Mendelian segregation in both parents. The offspring sired by the *B. oblongus* father were tetraploid, and presented four allelic copies at locus D105, corresponding, respectively, to two NOR− and two NOR+ sets. One NOR− allele was identical to the maternal copy, while the other was randomly inherited from the two paternal NOR− copies. The two NOR+ sets also showed biparental inheritance, with Mendelian segregation in both parents. Hence in both crossings, the Batura toad mother produced 2n oocytes with a clonally transmitted NOR− and a recombined
The NOR+ allele, which is clonally duplicated, and another that undergoes normal meiosis. The closest system seems to be found in plants, such as heathers and dog roses, in which pollen only transmits clonally reproducing genomes in addition to a recombined genome. The hybrid between Rana ridibunda (RR) and Rana lessonae (LL) [33,34]. When associated with R. ridibunda, R. esculenta females (RL) drop their paternal genome (R) from the germ line while doubling their L genome by pre-miotic endomitosis. The ensuing meiosis thus involves completely homozygous pseudo-bivalents (LL), and produces non-recombined haploid (L) oocytes [35]. Mating with a R. ridibunda male then restores the RL genome. Hence, one set of chromosomes (R) recombines in the parental species, while the other set (L) is transmitted clonally by the hybrid.

Both sets of chromosomes recombine during meiotic hybridogenesis [15,36], which involves a reproductive community of hybrids of different ploidy levels and genomic compositions (RRL, LLR and RL). Triploids RRL drop their L genome from the germ line, and produce recombined haploid R gametes by normal meiosis. Triploids LLR similarly drop their R genome before meiosis, producing recombinant haploid L gametes. Finally, RL diploids form clonal diploid RL gametes after endomitosis [11]. Combining these gametes restores the original diploid and triploid genomes [36–38]. Similar mechanisms have been documented in several hybridogenetic teleost fishes [39–44].

Thus, the pre-meiotic elimination of NOR− in male Batura toads, followed by normal diploid meiosis of the two NOR+ sets, shares similarities with some processes occurring during meiotic hybridogenesis and other forms of hybridogenetic or kleptogenetic reproductive modes known from triploid vertebrates (figure 1). Similarly, the duplication of the NOR− in female Batura toads occurs through a gametogenetic mechanism (premeiotic endomitosis) that is well known from parthenogenetic and hybridogenetic vertebrates [23,45,46]. However, the Batura toad system seems unique among vertebrates in that (i) meiotic processes differ between sexes, (ii) females simultaneously transmit one genome that is clonally duplicated, and another that undergoes normal meiosis. The closest system seems to be found in plants, such as heathers and dog roses, in which pollen only transmits a sexually reproducing genome, while ovaules transmit clonally reproducing genomes in addition [47–49]. There is, however, no pre-miotic duplication of
conjugate forces of enhanced drift, selective sweeps, counter-balanced by masculinizing factors evolving on genomes. Maternally transmitted mitochondrial or nuclear mutations that are deleterious only in males are not counted in genomes only. Similarly, where the maternally transmitted clonal R hypothesis for old lineages of gynogenetic fish [50]. Rhodominic processes and evolutionary fate. Polyploid (3n, 4n) lineages of green toads, which evolved several times independently, are clearly associated with harsh habitats [50]. Batura toads, in particular, live in extreme conditions of altitude and xericity [52]. One might speculate that the clonal reproduction of the NOR-genome allows preserving epistatic components of fitness, which may matter when selection stems from abiotic and predictably harsh environmental factors [53]. As a matter of fact, asexual lineages often occur in marginal habitats with more extreme conditions (colder, dryer, higher altitude and higher UV-radiation) than their sexual relatives [54–57]. An important question in this context is whether both genomes are expressed, and, if so, whether expression is differential (tissue-specific) as observed in allopolyploid fishes [58] and plants [59].

This exceptional mode of NOR-inheritance should also have detrimental evolutionary consequences. First, its purely maternal transmission opens opportunities for genomic conflicts. We expect, in particular, feminizing factors to evolve on the matrilineal NOR-, to be then counter-balanced by masculinizing factors evolving on the biparentally transmitted NOR+. This might result in sex-ratio biases, as observed in the hybridogenetic R. esculenta, where the maternally transmitted clonal R genome harbours feminizing factors only [60]. Similarly, mutations that are deleterious only in males are not counter-selected and might accumulate, as found in other maternally transmitted mitochondrial [61] or nuclear [62] genomes.

Second, the non-recombining NOR-set should progressively accumulate deleterious mutations, under the conjugate forces of enhanced drift, selective sweeps, background selection and Muller’s ratchet [63–65], as happens to sex chromosomes (Y or W), and to the non-recombining genomes of hemiclone vertebrates [66]. Batura toads, however, may have arisen too recently for such mutational meltdown or genomic conflict over sex determination to be detectable [25,50].

By contrast, the Mendelian segregation and recombination found in the NOR+ genome should prevent its evolutionary decay, ensuring the long-term evolutionary potential of Batura toads, as found in sexually reproducing vertebrates with normal meiosis.

Comparing gene sequences of the NOR-genome of B. baturae with those of its parental species, as well as those of tetraploid green toad lineages as B. oblongus and B. petrozowii, where the NOR-genomes also recombine according to cytological [28] and microsatellite (this study) evidence, might help gaining information, not only on the phylogenetic history of the NOR-set, but also on the patterns of selection occurring in this non-recombining genome. This might also allow investigating potential conflicts in sex-determination pathways, as well as possible intergenomic recombinations, such as observed in kleptogenetically reproducing Ambystoma [67].

5. CONCLUSIONS
The reproductive mode of B. baturae differs from those known so far in other vertebrates (figure 1) not only because the meiotic processes differ between sexes, but also because females display clonal and sexual reproduction simultaneously (pre-metiotic auto-duplication affects one chromosome set, while the others undergo normal meiosis). We hereby name this process ‘pre-equalizing hybrid meiosis’. Elucidating the mechanisms underlying these peculiarities might shed much light on the general processes that regulate meiosis in vertebrates. Batura toads also offer intriguing opportunities to compare evolutionary forces in recombining and non-recombining genomes within the same organism.

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