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-recombining 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 hybridogenic 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 Poeciliopsis, 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 lambrush 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
pre-meiotic endomitosis. Meiotic pairings (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 two remaining 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

**Figure 1. Reproductive modes of triploid vertebrates.** Shown are the parental, gametic and offspring genomes (rows) under different reproductive modes (columns). A, B: genomes of different parental species. Bold coloured symbols indicate clonally transmitted copies, while thin black symbols with superscripts indicate different (recombined) copies. True parthenogenesis: clonal (males absent), restricted to reptiles [7,8]; Sperm-dependent parthenogenesis (i.e. gynogenesis): clonal, embryogenesis requires trigger from allospecific sperm that is not incorporated (rare 'paternal leakage' might incorporate subgenomic amounts of paternal DNA), occurs in teleost fishes and urodelan amphibians [9]; Kleptogenesis: females acquire full or partial genomes from their mates by a not fully understood mechanism, allowing them to purge genomes from deleterious alleles (here BB); described from urodelan amphibians [10]; Unnamed form of hybridogenesis: clonal diploid eggs are fertilized by sperm from a recombining sexual species that can be diploid or triploid (as in meiotic hybridogenesis); occurs in anuran amphibians and teleost fishes [11–13]; Meiotic hybridogenesis: may occur in triploid males and/or females; found in teleost fishes and anuran amphibians [14,15]; ploidy elevation of the diploid offspring, which might produce diploid hybrid gametes, can occur in the next generation (becoming then e.g. ABB′ to restore triploidy (similar to preceding form of hybridogenesis); Pre-equalizing hybrid meiosis: occurring in Batura toads: Both sexes are triploid and exhibit Mendelian segregation and recombination in the B genomes (equivalent to NOR+; this paper), while the A genome (i.e. NOR−) is clonally transmitted by the mother.

<table>
<thead>
<tr>
<th>Mode</th>
<th>True Parthenogenesis</th>
<th>Sperm-dependent Parthenogenesis (= gynogenesis)</th>
<th>Kleptogenesis</th>
<th>Unnamed Form of Hybridogenesis</th>
<th>Meiotic Hybridogenesis</th>
<th>Pre-equalizing Hybrid Meiosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parents</td>
<td>ABB</td>
<td>ABB</td>
<td>ABB′</td>
<td>ABB</td>
<td>ABB′</td>
<td>ABB′</td>
</tr>
<tr>
<td>Gametes</td>
<td>ABB</td>
<td>B′B′</td>
<td>B′B′</td>
<td>B′B′</td>
<td>A′B′</td>
<td>A′B′</td>
</tr>
<tr>
<td>Offspring</td>
<td>ABB</td>
<td>ABB+</td>
<td>ABB+</td>
<td>ABB+</td>
<td>ABB+</td>
<td>ABB+</td>
</tr>
</tbody>
</table>

**Figure 2. Scheme of the reproductive system in triploid Batura toads with hypothetical mechanisms (a) and (b) for oogenesis and (c) and (d) for spermatogenesis.** Blue NOR− symbol: unrecombined (clonal) chromosome set without NORs. Red or magenta NOR+ symbols: different NOR-carrying chromosome sets. Mixed red and magenta NOR+ symbols: recombined NOR-carrying sets. The mechanisms confirmed in the present study are framed.
patterns of transmission and segregation. Our results clearly confirm the purely clonal and maternal transmission of one set of chromosomes (NOR\textsuperscript{−}), and show independent segregation and recombination of the other sets (NOR\textsuperscript{+}) in both males and females. This reshuffling of genetic material should allow efficient purging of the two sets of NOR\textsuperscript{+} 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\textsuperscript{−} sets) and a tetraploid Bufo oblongus male from Iran (4n = 44, including two NOR\textsuperscript{+} and two NOR\textsuperscript{−} 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 Stöck 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\textsuperscript{−} and NOR\textsuperscript{−} sets, as well as null alleles (0), were identified from inheritance patterns (see S3). 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 χ\textsuperscript{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\textsuperscript{−} and the two NOR\textsuperscript{+} sets. NOR\textsuperscript{−} 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\textsuperscript{+} sets, by contrast, displayed biparental inheritance and Mendelian segregation, following expectations from meiotic NOR\textsuperscript{+} sets (electronic supplementary material, 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, table 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, table 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, table 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, table 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, table S3 and dataset S1). Each heterozygous parent transmitted its two alleles with equal probability (binomial tests).
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Table 2. Recombination patterns at four linkage groups. Rec., recombination observed; non-rec., no recombination observed. Expected values assuming independence are provided in italics. Total indicates total number of informative events over families and sexes.

<table>
<thead>
<tr>
<th>linkage group</th>
<th>rec.</th>
<th>non-rec.</th>
<th>total</th>
<th>rate</th>
<th>$\chi^2$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>D106/D124</td>
<td>16</td>
<td>53</td>
<td>69</td>
<td>0.23</td>
<td>19.84</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>34.5</td>
<td>34.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C224/C123</td>
<td>2</td>
<td>38</td>
<td>40</td>
<td>0.05</td>
<td>32.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C111/D103</td>
<td>5</td>
<td>27</td>
<td>32</td>
<td>0.16</td>
<td>15.12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D11/D107</td>
<td>2</td>
<td>56</td>
<td>58</td>
<td>0.03</td>
<td>50.27</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>25</td>
<td>174</td>
<td>199</td>
<td></td>
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</tbody>
</table>

NOR+. The B. variabilis male produced haploid sperm with a recombined NOR+, while the B. oblongus male produced diploid sperm with recombined NOR+ and NOR− sets (see also [25,33]).

4. DISCUSSION

Our results show purely maternal and clonal transmission of all NOR− markers. Offspring inherited only the maternal copy at the five markers (D5, D103, D105, C224 and C203) that amplified a NOR− allele. As we show, these five markers are localized on different linkage groups on the NOR+ genome (electronic supplementary material, dataset S1), supporting the view that the whole NOR− set of chromosomes undergoes clonal and maternal transmission.

Furthermore, our results provide evidence for NOR+ recombination in both sexes. The 15 markers that amplified NOR+ alleles were assigned to 11 different linkage groups (corresponding to the haploid number of chromosomes in Batura toads), which displayed random and independent segregation in both sexes (electronic supplementary material, dataset S1). Heterozygous adults always transmitted their two alleles with equal probability, indicating random segregation of paternal and maternal NOR+ chromosome sets in both sexes (figure 2: pathways (b), (d)). In addition, recombination also occurred in both sexes, among loci from the same linkage groups (table 2).

Altogether, our results rule out hypothesis (a) for oogenesis (which assumes clonal production of NOR+/NOR− oocytes) and (c) for spermatogenesis (which assumes clonal production of NOR+ sperm). These findings raise important issues with respect to both the proximate mechanisms and evolutionary consequences associated with this unusual mode of reproduction.

(a) Proximate mechanisms

The NOR− genome of Batura toads is eliminated in males, but duplicated in females before meiosis. Premesiotic elimination and/or duplication of genomes have already been documented in hybridogenetic vertebrates, such as in the water frog Rana (Pelophylax) esculenta, a 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-meiotic 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, and (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 ovules transmit clonally reproducing genomes in addition [47–49]. There is, however, no pre-meiotic duplication of
clonal genomes in these cases. To our knowledge, auto-
duplication of one entire chromosome set (NOR−) in the
presence of a foreign diploid genome (NOR+/NOR−); which
remains pre-meiotically unchanged and is later
transmitted in a Mendelian manner) has not been shown
so far (figure 1).

(b) Evolutionary aspects
Batura toads display remarkably homogeneous (within-
species) mitochondrial sequences [50], suggesting a
unique and recent origin (though none of its potential par-
etal species occurs within the species range, which
encompasses three river drainages from northern Paki-
stan). Although triploids may directly arise from a cross
between a diploid and a tetraploid parental species, the
complex meiotic processes documented here (including
the duplication of a whole genome in females and its
elimination in males) might not have evolved right at the
initial hybridization event. Intermediate steps may have
included a period of hybrid interactions between lineages
of different ploidy levels and genomic compositions, simi-
lar to the situation occurring now in northern Kyrgyzstan,
where some triploid males, resulting from natural crosses
between 2n Bufo turanensis mothers and 4n Bufo petzovii
fathers, backcross with females from either parental
species [30]. Such mechanisms must be rare, however,
owing to the low probability of meeting the complex genetic
requirements necessary to achieve stable hybrid combi-
nations of clonal and Mendelian genomes, as recently also
hypothesized for old lineages of gynogenetic fish [51].

This reproductive mode also raises questions regarding
selective 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 condi-
tions 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
allo-polyploid fishes [58] and plants [59].

This exceptional mode of NOR− inheritance should
do 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.
escalenta, where the maternally transmitted clonal R
genome harbours feminizing factors only [60]. Similarly,
mutations that are deleterious only in males are not coun-
ter-selected and might accumulate, such as found in other
maternally transmitted mitochondrial [61] or nuclear

Second, the non-recombining NOR− set should pro-
gressively 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 hemiclonal 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 recombi-
nation found in the NOR+ genome should prevent
its evolutionary decay, ensuring the long-term evolution-
ary potential of Batura toads, as found in sexually
reproducing vertebrates with normal meiosis.

Comparing gene sequences of the NOR− genome of B.
batureae with those of its parental species, as well as
those of tetraploid green toad lineages as B. oblongus
and B. petzovii, 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. batureae 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 reproduc-
tion simultaneously (pre-meiotic 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 evolu-
tional forces in recombining and non-recombining
genomes within the same organism.

Research was carried out according to approved guidelines
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veterinaires, Canton de Vaud, Epalinges, Switzerland.

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