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Genetic and Genomic Interactions of Animals with Different Ploidy Levels

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Key Words

Diploid-polyploid interaction · Genomic in situ hybridization · Gynogenesis · Hybridogenesis · Kleptogenesis · Microsatellites · Mitochondrial DNA · Parthenogenesis · Whole genome duplication

Abstract

Polyploid animals have independently evolved from diploids in diverse taxa across the tree of life. We review a few polyploid animal species or biotypes where recently developed molecular and cytogenetic methods have significantly improved our understanding of their genetics, reproduction and evolution. Mitochondrial sequences that target the maternal ancestor of a polyploid show that polyploids may have single (e.g. unisexual salamanders in the genus *Ambystoma*) or multiple (e.g. parthenogenetic polyploid lizards in the genus *Aspidoscelis*) origins. Microsatellites are nuclear markers that can be used to analyze genetic recombinations, reproductive modes (e.g. *Ambystoma*) and recombination events (e.g. polyploid frogs such as *Pelophylax esculentus*). Hom(e)ologous chromosomes and rare intergenomic exchanges in allopolyploids have been distinguished by applying genome-specific fluorescent probes to chromosome spreads. Polyploids arise, and are maintained, through perturbations of the 'normal' meiotic program that would include pre-meiotic chromosome replication and genomic integrity of homologs. When possible, asexual, unisexual and bisexual polyploid species or biotypes interact with diploid relatives, and genes are passed from diploid to polyploid gene pools, which increase genetic diversity and ultimately evolutionary flexibility in the polyploid. When diploid relatives do not exist, polyploids can interact with another polyploid (e.g. species of African Clawed Frogs in the genus *Xenopus*). Some polyploid fish (e.g. salmonids) and frogs *(Xenopus)* represent independent lineages whose ancestors experienced whole genome duplication events. Some tetraploid frogs *(P. esculentus)* and fish *(Squalius alburnoides)* may be in the process of becoming independent species, but diploid and triploid forms of these 'species' continue to genetically interact with the comparatively few tetraploid populations. Genetic and genomic interaction between polyploids and diploids is a complex and dynamic process that likely plays a crucial role for the evolution and persistence of polyploid animals. See also other articles in this themed issue.

The number of known polyploid animals continues to increase as additional individuals and populations are investigated using a battery of genetic and cytogenetic methods [Schmid et al., 2010; Mable et al., 2011; Neiman et al., 2011; Evans et al., 2012]. It is obvious, based on the diversity of animal and plant polyploids, that polyploidy has independently arisen multiple times across a wide range of taxonomic groups. Polyploids can be bisexual,

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unisexual, asexual, or even a combination of these reproductive modes. They may arise from one species (autopolyploids) or, most commonly, from interspecific hybridization events (allopolyploids), and sometimes it may be difficult to tell if a polyploid fits either grouping. In animals, polyploidy occurs most frequently in populations of asexual or unisexuals, which seem to have a higher propensity for ploidy elevation events than bisexually reproducing species. The relative frequency of autopolyploid versus allopolyploid origins remains uncertain, but in both plants and animals, there is a strong association of polyploidy with hybridization. Polyploids arise in diverse lineages of diploids, and we believe that we are presently observing a relatively small number that exist in our current snapshot in time. Assessing possible advantages or disadvantages of polyploidy or of inter-ploidy interaction that involve related diploids and polyploids is complicated without knowledge of evolutionary events that were responsible for the origination of a polyploid and the intrinsic and extrinsic factors that such lineages experienced through time and space. This is complicated by the fact that most polyploid animals cannot be easily distinguished from their diploid relatives by morphology or even by using genetic data, so the frequency of polyploidy has likely been under-estimated in animals. Polyploid evolution and persistence relies on genetic variation that may, initially, be conferred through the redundancies and duplication of genomes. It seems logical that genetic and epigenetic mechanisms must co-evolve with polyploidization to manage the interactions of duplicated or novel linkage groups and their resident genes.

In this review, we wish to explore the different mechanisms that some polyploid animals use to genetically interact with diploid species. Our contention is that animal polyploids can persist over time and derive an evolutionary advantage by sampling, or 'dipping into', diploid gene pools. Our focus will be limited to a relatively few animal polyploids where molecular methods have been applied and have provided insight with respect to the evolution and/or the persistence of a polyploid lineage.

Unisexual Salamanders in the Genus *Ambystoma*

Diploid (2n) gene pool 'dipping' reaches a high level of sophistication in the North American salamanders of the unisexual *Ambystoma* complex. From an origination from a putative hybridization about 5 Mya [Bi and Bogart, 2010a], unisexuals have spread through north eastern North America and are especially abundant around the Great Lakes. Over their range, they share nuclear genomes with as many as 5 distinctly different bisexual species of *Ambystoma (A. barberi, A. jeffersonianum, A. laterale, A. texanum*, and *A. tigrinum)* [Bogart, 2003; Bogart et al., 2009]. To facilitate discussion, respective genomes for these species may be coded as BB, JJ, LL, TT, and TiTi. For example, LJJ would signify a triploid unisexual salamander that possesses 1 *A. laterale* and 2 *A. jeffersonianum* nuclear genomes and would be one 'genomotype' [Lowcock, 1994] of more than 20 [Bogart, 2003; Bogart et al., 2009] diploid, triploid (3n), tetraploid (4n), or even pentaploid (5n) nuclear genomic combinations that have so far been identified. All unisexual genomotypes have at least one L nuclear genome and very similar mitochondrial genomes that distinctly differ from mitochondrial sequences in all 5 species [Hedges et al., 1992; Bogart, 2003; Bi and Bogart, 2010a]. Unisexual salamanders normally outnumber individuals of the sympatric, bisexual species in this complex [Bogart and Klemens, 1997; 2008]. Their reproductive mode has been termed kleptogenesis [Bogart et al., 2007] because they steal sperm cells from sympatric males. Sperm is most often used only to initiate the development of unisexuals' eggs by gynogenesis [Elinson et al., 1992], but sperm cells can also be incorporated in some eggs to elevate ploidy [Bogart et al., 1989] or to replace one of the original genomes in the resulting offspring [Bogart, 2003; Bi et al., 2008].

Ploidy Elevation

The modal ploidy level in unisexual *Ambystoma* is triploid, but diploid, tetraploid and pentaploid individuals have been encountered over the extensive unisexual range. Pentaploids are very rare [Lowcock and Murphy, 1991], and tetraploids are more common than diploid unisexuals [Phillips et al., 1997; Bogart and Klemens, 1997; 2008]. So far, no entirely tetraploid population has been encountered, but all 36 individuals sampled from one southern Quebec population were diploid unisexuals [Nöel et al., 2011]. Bogart and Licht [1986] examined offspring of diploid, triploid and tetraploid unisexuals from Pelee Island, Ontario and found that diploid females commonly produced both diploid and triploid offspring, triploid females commonly produced triploid and tetraploid offspring, and tetraploids produced tetraploid and, very rarely, triploid offspring. In laboratory crosses [Bogart et al., 1989; Elinson et al., 1992], triploid females produced triploid and tetraploid offspring, and tetraploid females produced tetraploid and pentaploid offspring. There was a significant increase in the number of tetraploid offspring from triploid mothers if the temperature

was increased to 15°C from 6°C [Bogart et al., 1989]. These data suggest that triploidy is the most successful polyploid level but also show that ploidy elevation is a common phenomenon in unisexual *Ambystoma* and may be related to extrinsic factors, such as temperature.

The assessment of ploidy level has been facilitated with the use of microsatellite DNA alleles at polymorphic loci [Ramsden et al., 2006]. When offspring from a unisexual female are sampled for several microsatellite loci, gynogenetic offspring have the same multi-locus genotype as their mother. Ploidy-elevated offspring have an additional allele at loci where the sperm-derived genome possesses alternate alleles. Indeed, it is possible to genotype a male sperm donor of a unisexual triploid female from the tetraploid offspring that are produced (fig. 1). In a population of bisexual and unisexual *Ambystoma*, a unisexual would be expected to have a selective advantage by stealing a genome from males. Because unisexuals share genes with their sperm donors, it might be difficult for males to evolve pre-mating reproductive isolating mechanisms that could be used to distinguish between bisexual and unisexual females. It is also expected that the genes residing in bisexual individuals have been sorted over many generations of natural selection and represent the 'best' genes for the contemporary environment. By stealing such genes, syntopic unisexuals could by-pass long periods of natural selection and be able to successfully compete with bisexuals. Subsequent gynogenetic reproduction would avoid the expected 50% loss of 'good genes' that would be expected to occur during normal meiosis used by bisexuals. Although empirical data show that genome elevation exists in most unisexual salamander populations, ploidy elevation appears to have an upper limit that is likely to be pentaploid because rare pentaploids have been encountered in nature [Lowcock and Murphy, 1991] and in the laboratory [Bogart et al., 1989]. Based on range and population densities, triploidy is the most successful ploidy level in unisexual salamanders.

Genome Exchange

The costs and benefits of polyploidy have been outlined by Comai [2005]. Assuming that polyploid unisexual or asexual animals derive an evolutionary advantage through heterosis, especially for allopolyploids, and through the incorporation of additional and perhaps 'better' genes from a diploid ancestor in a contemporary environment, they must also have some mechanism to purge 'bad' genes to circumvent the accumulation of deleterious alleles [Müller, 1964]. Genome replacement in unisexual *Ambystoma* could be such a mechanism.

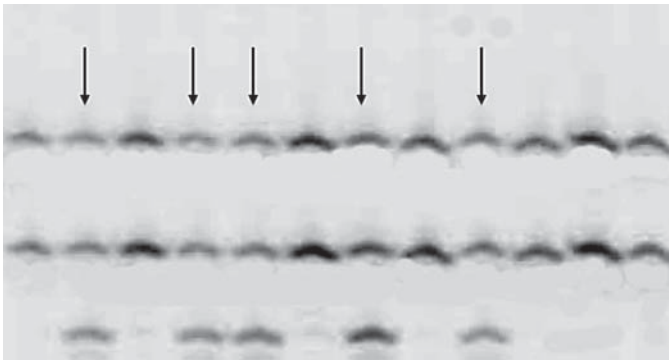

Fig. 1. Polyacrylamide gel that demonstrates ploidy elevation. Microsatellite DNA fragments at locus AjeD378 for 12 unisexual LJJ and LJJJ larvae that developed from eggs laid by the same LJJ female are shown. Only *Ambystoma jeffersonianum* (J) fragments are amplified for this locus. Seven triploid individuals possessing only the top 2 fragments are the same as their mother and were produced by gynogenesis. The third (smallest = lowest) fragment in 5 tetraploid larvae (arrows) came from a male *A. jeffersonianum*.

The problem of ever increasing ploidy levels through ploidy elevation could be solved if genomes can be discarded as well as added. From an evolutionary perspective, it would be advantageous for a unisexual to keep a genome that confers a selective advantage in a contemporary population and discard a genome that would confer a lower fitness. Previous isozyme [e.g. Bogart and Klemens, 2008] and microsatellite [Bogart et al., 2007, 2009; Ramsden, 2008; Nöel et al., 2011] data show that diploid, triploid and tetraploid unisexuals share alleles with available sympatric sperm donors. It is of interest, and perhaps significant, that all of the more than 20 unisexual genomotypes [Bogart, 2003; Bogart et al., 2009] contain at least one *A. laterale* genome. The *A. laterale* genome was targeted by Bi et al. [2008], who constructed a genealogy of that genome using sequences from an *A. laterale*-specific, variable, nuclear DNA marker (L-G1C12). They found that unisexuals share the same haplotype with *A. laterale* in sympatric populations over much of the unisexual range and rejected the hypothesis that an 'ancient' L genome persists in all of the various unisexual genome combinations and is not involved in intergenomic exchange.

Using simulation models, Charney [2012] tracked expected theoretical progressions through time of unisexuals that engaged in ploidy elevation and genome exchange. He concluded that the nuclear genomes would be expected to eventually be indistinguishable from those found in host populations, so unisexuals living with *A. jeffersonianum* would lose *A. laterale* genomes, and uni-

sexuals living with *A. laterale* would lose *A. jeffersonianum* genomes. Both *A. laterale* and *A. jeffersonianum* genomes could only be maintained in unisexuals by strong positive selection for hybrid genomes. The time taken for such homogenization would be related to the frequency of genome exchange that was calculated by multiplying the rates of ploidy elevation and ploidy reduction, which were considered equivalent. The logical outcome of Charney's simulations would be populations of *A. laterale*, *A. jeffersonianum* and other sperm donor species possessing triploid (e.g. LLL or JJJ) individuals with unisexual mitochondrial DNA (mtDNA) haplotypes. The fact that such populations have not been discovered is, according to Charney, the result of inadequate sampling effort. Finding one triploid *A. laterale* [Lowcock et al., 1991] and one triploid *A. texanum* [Bogart and Licht, 1986] would only support Charney's [2012] model if those individuals possessed a unisexual mtDNA, which was not sequenced in these triploids. Surveys of natural populations often reveal the presence of rare autotriploids in many species of amphibians [Lowcock and Licht, 1990; Borkin et al., 1996; Litvinchuk et al., 2001, 2012; and earlier field and laboratory observations reviewed by Kawamura, 1984]. We suspect that the triploid *A. laterale* and *A. texanum* are autotriploids and are not derived from unisexuals.

We propose an alternate hypothesis that would be more compatible with recent microsatellite, ploidy and sequence data. Our proposal hinges on symmetrical tetraploid unisexuals, which, unlike other unisexuals, would not have a pre-meiotic endoduplication event. Eggs produced by such females would be diploid, possess 1 copy of the 2 different maternal genomes, and resulting female offspring would reproduce mostly by gynogenesis. Symmetrical tetraploids are rarely found in unisexual populations but have been encountered in many different localities. When *A. laterale* and another potential sperm donor co-exist with unisexuals in a breeding pond, symmetrical tetraploids can be produced by ploidy elevation. Although *A. jeffersonianum* is the most common sexual species co-occurring with *A. laterale* across the range, there are populations where other sexuals are found instead (for example, *A. texanum* on Pelee Island), and the unisexuals in these cases display a combination of those (L and T) genomes [Bogart and Licht 2004]. Although rare, surveys that include symmetrical unisexual tetraploids are consistent with the presence of 2 possible sperm donors. For example, in their eastern North American surveys of *A. laterale*, *A. jeffersonianum*, and unisexuals [Bogart and Klemens, 1997, 2008] that included a combined total of more than 2,000 individuals, 7 *A.* 2 *laterale* – 2 *jeffersonianum* (LLJJ) were found. With the exception of a Hillsborough County, New Hampshire population where 2 LLJJ were found, single LLJJ individuals were found with *A. laterale*, *A. jeffersonianum* and other unisexuals with varying combinations of the sexual genomes (e.g. LJ, LLJ, LLLJ, LJJ, LJJJ) in populations in Dutchess County, New York; Danbury and Litchfield Counties, Connecticut; and Addison County, Vermont. Seventeen of more than 1,200 salamanders on Pelee Island in Lake Erie were symmetrical *A.* 2 *laterale* – 2 *texanum* tetraploids or LLTT [Bogart and Licht, 2004]. In a microsatellite study of breeding adults and larvae in a southern Ontario pond, Bogart et al. [2007] found LLJJ and LJJ larvae that hatched from the same egg mass. The egg mass was from a pond that had both *A. laterale* and *A. jeffersonianum* males. Microsatellites of the LJJ and LLJJ sisters clearly showed that the only difference were single microsatellite alleles in the tetraploids that were contributed by *A. laterale*. An LJJ female must have stolen sperm from *A. laterale*: her gynogenetic offspring were LJJ and symmetrical tetraploids resulted from sperm incorporation and ploidy elevation. *A. jeffersonianum* is not found on Pelee Island, and the unisexuals have genomes with combinations of *A. laterale* and *A. texanum* [Bogart and Licht, 2004]. Although ploidy elevation is well documented in unisexual salamanders [Bogart and Licht, 1986; Bogart et al., 2007], very little is known about ploidy reductional events. Figure 2 provides a flow chart of possible reproductive events that a symmetrical LLTT tetraploid could initiate. This scenario explains how diploid unisexuals, which have been found in several populations [Bogart and Klemens 1997, 2008], could be produced. The events outlined in figure 2 would explain the consistent presence of an *A. laterale* genome and would also explain genome replacement because new triploids could be produced from unisexual diploids that would have a new genome in comparison with gynogenetically produced triploids in the population. Testing our hypothesis would require the examination of offspring produced by symmetrical tetraploids, which are not easily obtained. The rarity of symmetrical tetraploids could be evidence that symmetrical unisexuals are single generational events. In an examination of Pelee Island *Ambystoma* [Bogart and Licht, 1986], none of the 121 eggs laid by 6 LLTT unisexuals hatched. Sperm was not detected in cloacal examinations of the LLTT females, which meant that these females did not pick up a male's spermatophore. Failure of the LLTT females' eggs to develop was consistent with many other 'sperm negative' females in that study. In order to explain the presence of an *A. laterale* genome in all unisexuals, the

symmetrical unisexual would be required to contain 2 *A. laterale* genomes, which would limit the geographical range of symmetrical unisexuals to areas where *A. laterale* could be found in sympatric association with another sperm donor (such as *A. texanum* on Pelee Island).

Intergenomic Recombinations and Translocations

Chromosomes of unisexual *Ambystoma* have traditionally been used to confirm ploidy level [e.g. Bogart and Licht, 1986; Bogart and Klemens, 1997; Ramsden et al., 2006], but could not be used to identify genomotypes because of the conservative nature of the karyotypes in species of *Ambystoma* [Taylor and Bogart, 1990]. To circumvent this problem, Bi and Bogart [2006] employed genomic in situ hybridization (GISH). For example, extracted DNA from *A. laterale* was used to make fluorescent probes, and DNA from *A. jeffersonianum* was used as unlabeled blocking DNA. When the probes and excessive blocking DNA are applied to chromosome spreads of unisexuals that contained genomes of both species, homeologous chromosomes have a distinctly different fluorescence (fig. 3). GISH can also identify the chromosomes in unisexuals that contain more than 2 distinct genomes by making different probes from species whose genomes are included in a unisexual [Bi and Bogart, 2006; Bogart et al., 2009] (fig. 4). Unexpected recombinations and translocations were found in some of the GISH experiments [Bi and Bogart, 2006; Bi et al., 2009] that were clearly visible as blocks of the 'wrong' fluorescence in the homeologous linkage groups (arrows, fig. 3). These mutations were consistent in populations and were heritable. Offspring possessed the same mutations as their mothers, so they must have originated meiotically and not mitotically. Offspring would be expected to have variable and/or mosaic mutations if they arose during mitotic division. Unisexual meiotic lampbrush bivalents are expected to be sister chromosomes that were duplicated in a pre-meiotic endoduplication event so observed chiasmata in bivalents would not provide any recombination involving homeologous chromosomes. Occasionally, lampbrush quadrivalents have been observed in lampbrush chromosomes during the unisexual meiotic prophase. Rare unisexual lampbrush quadrivalents have been found by Macgregor and Uzzell [1964] and Bogart [2003]. GISH was applied to lampbrush chromosomes [Bi and Bogart, 2010b] to examine associations of homologous and homeologous chromosomes in unisexuals. As expected, all bivalents involved homologous chromosomes, and the recombined segments observed in mitotic chromosomes were duplicated in the sister chromosome

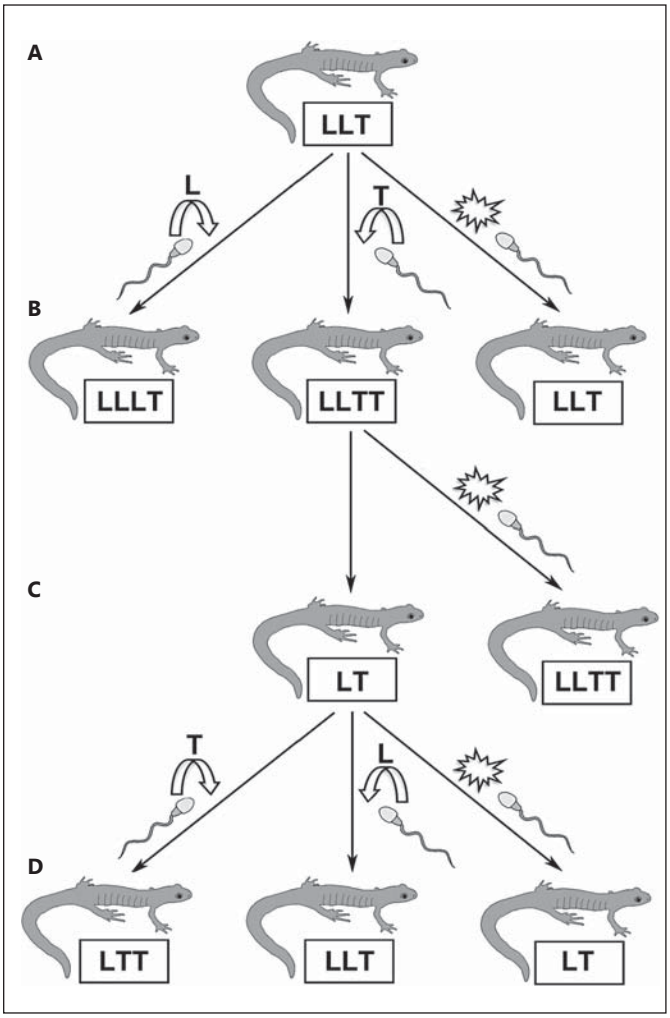

Fig. 2. Hypothetical reproductive events to explain the possible reproductive system used by unisexual salamanders on Pelee Island, Ont., Canada. *Ambystoma laterale* (LL) and *A. texanum* (TT) are the only bisexual species of *Ambystoma* found on the island. A triploid LLT female (level A) lays unreduced LLT eggs that can produce LLT larvae by gynogenesis (sperm is rejected). If the sperm from an *A. laterale* male is incorporated, the offspring are tetraploid LLLT, and if a sperm cell from *A. texanum* is incorporated, the offspring are symmetrical tetraploid LLTT (level B). Eggs from a symmetrical tetraploid could be unreduced and develop by gynogenesis or be reduced during meiosis to produce LT eggs (level C). Diploid LT eggs can develop by gynogenesis or be fertilized by *A. laterale* sperm to produce LLT or by *A. texanum* sperm to produce LTT (level D). All these biotypes are found on Pelee Island, and such a system could explain the formation of diploid LT unisexuals. Sperm incorporation elevates the ploidy to a triploid level and explains genome replacement. One L genome (in level A female) is replaced with a new L or a new T genome in level D individuals.

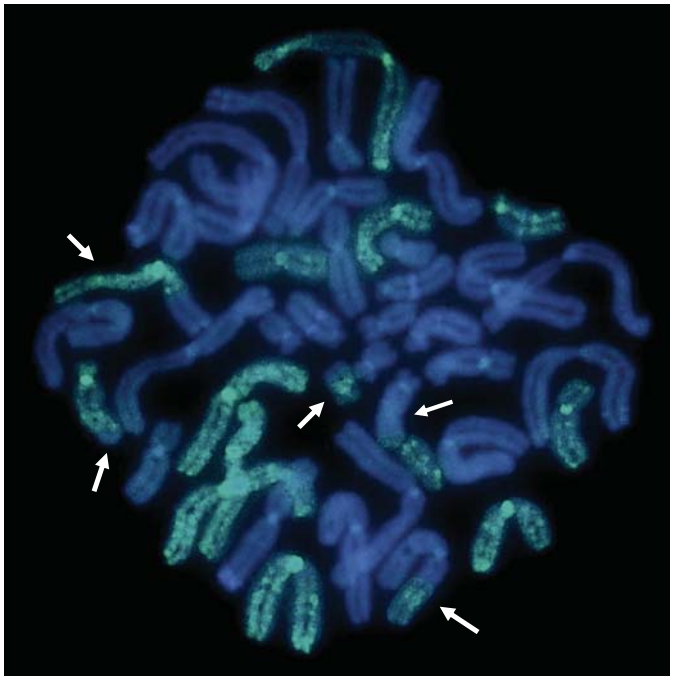

Fig. 3. Chromosome spread from a triploid (3n = 42) LJJ larva that was used in a GISH experiment. *Ambystoma laterale* probe (green fluorescence) DNA and *A. jeffersonianum* blocking DNA were applied. The preparation was counterstained using DAPI (blue). There is a clear distinction between the 14 *A. laterale* (green) chromosomes and the 28 *A. jeffersonianum* (blue) chromosomes. Arrows point to exchanged chromosome segments.

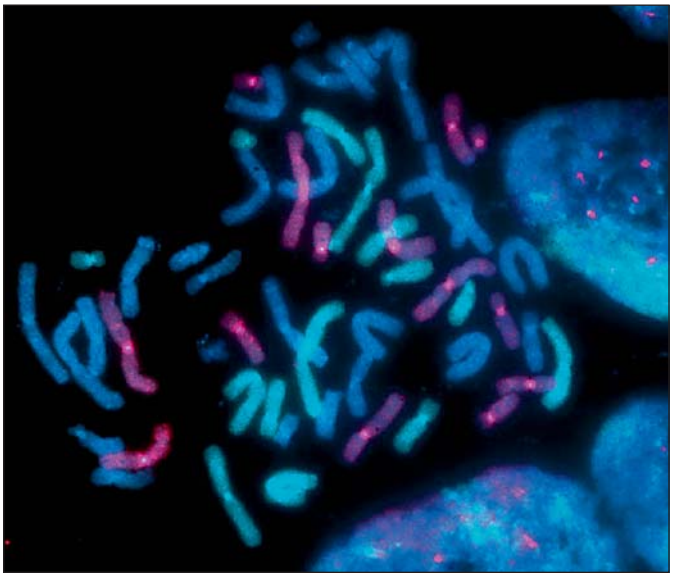

Fig. 4. Chromosome spread from a tetraploid (4n = 56) tri-hybrid *Ambystoma laterale* – 2 *jeffersonianum* – *barbouri* (LJJB). This GISH experiment used fluorescent probes for *A. laterale* (14 light blue-green chromosomes) and *A. barbouri* (14 pink chromosomes). Blocking DNA was from *A. jeffersonianum*. A DAPI counterstain revealed 28 dark blue *A. jeffersonianum* chromosomes.

bivalents. Applying GISH, all of the quadrivalents that were observed were also combinations of homologous chromosomes (fig. 5). These observations support premeiotic endoduplication, the pairing of the duplicated sister chromosomes in bivalents, and the heritability of recombined linkage groups. Because homeologous associations were not observed in lampbrush quadrivalents, it was not possible to document recombination events that could give rise to mitotic chromosomes that carried blocks of recombined or translocated chromosome segments (fig. 3). We believe that intergenomic recombination is a rare event because if it was very common, genomic integrity would be at risk and this could have an impact on a unisexual for such genetic processes as genome replacement. GISH has only been applied to lampbrush chromosome preparation from 16 females of 3 genomotypes (LLJ, LLLJ and LJJ) [Bi and Bogart, 2010b]. Thus, empirical evidence is lacking to support our hypothesis that chromosome recombinations and translocations, which are observed in unisexual salamanders, are derived from homeologous pairing in meiosis.

Parthenogenetic Polyploids

Parthenogenetic species do not require males, but many still interact with, and derive genes from, diploid bisexual species. Genetic variation that is observed in some clones can often be tracked from extant bisexuals using cytogenetic, nuclear and mitochondrial markers. Multiple hybrid origins of polyploid asexual parthenogenetic lizards from diploid progenitors are well documented in the genus *Aspidoscelis* (formerly *Cnemidophorus*) [Wright and Lowe, 1967; Neaves, 1971; Dessauer and Cole, 1989], *Heteronotia binoei* [Moritz 1984; Moritz et al., 1989; Strasburg et al., 2007] and in the genus *Darevskia* (formerly *Lacerta*) [Moritz et al., 1992]. A hybrid origin for some parthenogenetic lizards is less clear or disputed. Molecular and cytogenetic data were used to ascribe a hybrid origin for *Gymnophthalmus underwoodi* from Trinidad and Suriname by Kizirian and Cole [1999] but, using the same methodology, a hybrid origin could not be confirmed for this same parthenogenetic species from Rorama in Brazil [Benozzati and Rodrigues, 2003]. DNA se-

quences and microsatellites did not support a hybrid origin for the parthenogenetic xantusid lizards *Lepidophyma flavimaculatum* and *L. reticulatum* [Sinclair et al., 2010]. Some problems involved in distinguishing between a hybrid or a spontaneous origin for parthenogenetic animals was discussed by Pellegrino et al. [2011] in their molecular and cytogenetic study of Brazilian diploid and triploid parthenogenetic lizards *(Leposoma percarinatum)*. No possible ancestors were recovered in their mitochondrial analysis, and the largely allopatric diploid and triploid clones were genetically divergent. They likely represent 2 distinct parthenogenetic clones that diverged 2.8–5.7 Mya. Apparently, parthenogenesis arose spontaneously a very long time ago or the extant descendants of possible hybridizing progenitors could be genetically unrecognizable or extinct. Multiple diagnostic nuclear markers and a wider sampling of populations that include additional species are required to understand the origin of parthenogenesis in *L. percarinatum* [Pellegrino et al., 2011].

Most parthenogenetic lizards in the genera *Aspidoscelis* and *Darevskia* are diploid, but ploidy elevation produces triploid clones. Triploids are derived from sperm incorporation when a diploid parthenogen crosses with a male of one of the progenitor bisexual species of the diploid parthenogen or from a third species. Triploid male and female hybrid *Darevskia* exist and are normally sterile [Darevsky et al., 1989; Danielyan et al., 2008] but, in a zone of hybridization in central Armenia, 2 parthenogenetic diploid species *(D. armeniaca* and *D. unisexualis)* co-exist and hybridize with bisexual *D. valentini* [Danielyan et al., 2008]. Finding a male tetraploid lizard in this hybridization zone was used as evidence that a fertile triploid mated with *D. valentini*. Based on population densities and ranges, triploid clones of *Aspidoscelis* and *H. binoei* are successful, but tetraploid *Aspidoscelis* and *H. binoei* are rare [Moritz et al., 1989; Manning et al., 2005]. Using an enclosure, Lutes et al. [2011] mated parthenogenetic triploid *A. exsanguis* with a diploid bisexual male *A. inornatus*. Both male and female tetraploids were produced. The tetraploid females were viable and, through parthenogenesis, gave rise to tetraploid daughters and granddaughters. No diploid clones of *H. binoei* have been identified; the 2 major triploid clones (3N1 and 3N2) that arose from reciprocal hybridization of 2 bisexual populations of *H. binoei* have spread over much of Australia and are assumed to have independently arisen 240,000 (3N1) and 70,000 (3N2) years ago from possible diploid races [Strasburg et al., 2007]. Observed chromosomal variation within the triploid clones is attributed to past hybridization of bisexual individuals with the, presumably extinct,

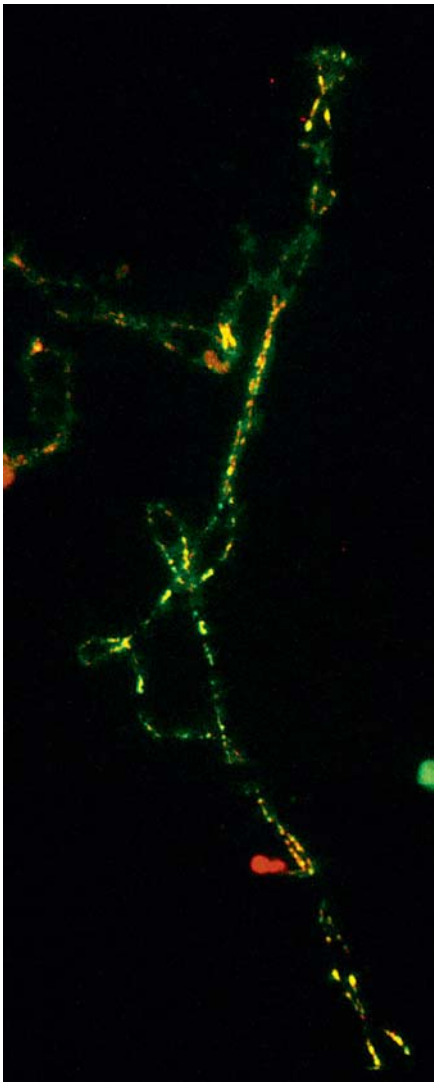

Fig. 5. A prophase meiotic quadrivalent from a lampbrush chromosome preparation using eggs from a unisexual LJJ. The same GISH treatment was applied in figure 3. This quadrivalent consists only of homologous (green *A. laterale* fluorescence) chromosomes.

allodiploid races of *H. binoei* [Moritz et al., 1989; Strasburg et al., 2007]. Although few meiotic data are available for the parthenogenetic lizards, Cuellar [1971] and Moritz [1984] speculated that meiosis in all of the parthenogenetic lizards involves a pre-meiotic endomitosis that is similar to meiosis in unisexual *Ambystoma*. Lutes et al. [2010] used fluorescent in situ hybridization (FISH) probes to examine meiosis in diploid parthenogenetic *A. tessalatus*. The probes, derived from one of the diploid bisexual progenitors *(A. gularis)*, identified homologous

chromosomes in parthenogens and they confirmed that synapses in bivalents involved sister chromosomes, from a pre-meiotic endoreplication event, and were not homeologous chromosomes.

There are also examples of diploid/polyploid interactions in invertebrate parthenogens. In the genus *Daphnia* (water fleas), there are diploid bisexual populations, diploid cyclical parthenogenetic populations, diploid and triploid obligately parthenogenetic populations. *D. pulex* and *D. pullicaria* hybridize to produce diploid clones in temperate regions and triploid clones (designated as *D. middendorffiana*) in subarctic and arctic regions [Crease et al., 1989]. The maternal parents of *D. middendorffiana* can be *D. pullicaria* or *D. pulex*, which are repeatedly formed from different mitochondrial lineages of these 2 species [Vergilino et al., 2009]. Evidently, triploid clones of *D. middendorffiana* have arisen several times. Parthenogenetic weevils can be diploid, triploid, tetraploid, pentaploid, hexaploid (6n), and even decaploid (10n) [Saura et al., 1993]. Using a mtDNA phylogeny, Stenberg et al. [2003] studied weevils that make up the *Otiorynchus scaber* 'system'. From their results, they concluded that diploid parthenogens independently arise within bisexual populations. Parthenogenetic weevils, with elevated ploidy levels, are derived through ploidy elevation using sympatric bisexual males [see also Stenberg and Saura, this issue]. Triploid male and female snails of the *Potamopyrgus antepodarum* 'system' have arisen multiple times from sexual diploids [Neiman et al., 2011]. Triploid female snails are asexual, and it is speculated that triploid males can mate with asexual triploid females, which could explain the genome size variation observed in snail populations. These few examples demonstrate that parthenogenetic polyploids maintain an association with diploid bisexuals and likely derive an evolutionary advantage through this interaction.

Polyploid European Water Frogs (*Pelophylax esculentus* Complex)

The European Water Frog, *Pelophylax esculentus* (formerly *Rana esculenta*) complex is a wide-ranging hybrid 'species' in central Europe that combines genomes of *P. lessonae* (or LL) and *P. ridibundus* (or RR). Reproduction of *P. esculentus* (LR) is hybridogenetic or hemi-clonal because, in nature, when *P. esculentus* mates with either *P. lessonae* or *P. ridibundus*, hybrid offspring *(P. esculentus)* are regenerated. This interesting genetic system has been the subject of a large number of investigations. The early history of the complex is reviewed by Graf and Pelaz [1989]. All hybrid populations of male and female diploid and triploid *P. esculentus* exist and can reproduce without either parental species in some northern portions of their range [Borkin et al., 2004]. Some of these populations have been the subject of more recent investigations by Christiansen [2009] and Christiansen and Reyer [2009]. Microsatellites were used to identify genome combinations in adults and in tadpoles derived from crosses performed using combinations of male and female triploid LLR, female LRR, and male and female diploid LR *P. esculentus*. LLR males and females produce L gametes, LRR females produce R gametes, LR females produce LR and R gametes, and LR males produce R gametes. Non-hybrid tadpoles (LL, RR, LLL, RRR) were found to be lethal. Christiansen [2009] modeled the equilibrium frequency for the all hybrid populations using the observed frequencies of current parental biotypes and the frequency of the gametes that they would be expected to produce. The model worked well when applied to observations in many Swedish ponds. The models also pointed out factors that could dramatically alter genotype frequencies. For example, if symmetrical male and female tetraploids (LLRR) had a higher survival rate than was observed in the examined ponds, this could increase the output of LR gametes, so tetraploid LLRR could eventually displace diploid and triploid *P. esculentus*.

Triploid hybridogenesis may be a rather convoluted genetic path leading to polyploid speciation, as was suggested by Christiansen [2009] and Christiansen and Reyer [2009]. Significant recombination occurs in the L genomes of triploid LLR as well as in the R genomes of LRR triploids [Christiansen and Reyer, 2009], and they speculate that non-hybrid homogenomic individuals (LL, RR) are produced but rarely survive because they probably carry deleterious alleles, derived from recombination events in the triploids, that are not balanced with alleles provided by a heterogenome such as R in LLR and L in LRR. There is evidence that hybridogenesis can be a pathway for selective gene exchange between species through diploid hybridogenetic individuals. mtDNA of *P. lessonae* was found to have introgressed into several populations of *P. ridibundus*, but mtDNA of *P. ridibundus* was not found in *P. lessonae* [Plötner et al., 2008]. Using allozymes, Schmeller et al. [2005] demonstrated that alleles can be exchanged between *P. ridibundus* and *P. perezi* through their hybridogen, *P. grafi*. *Pelophylax grafi* is a diploid hybridogenetic 'species' that combines genomes of *P. ridibundus* and *P. perezi* and is known as the PG *('perezi – grafi')* system. All of the known hybridogenetic

'species' include (a) genome(s) of *P. ridibundus*. More gene flow was observed from *P. perezi* into *P. ridibundus* than from *P. ridibundus* into *P. prezi* [Schmeller et al., 2005]. *P. ridibundus* likely derives a selective advantage and is able to increase its distributional range by incorporating mtDNA [Plötner et al., 2008] and nuclear genes [Schmeller et al., 2005] from other species via hybridogens. In order for gene flow to occur between the species through a hybridogen, there must be some intergenomic recombination. Recombination was not observed in GISH experiments on Central European diploid *P. esculentus* chromosomes [Zaleśna et al., 2011], so the recombination may be too rare to be easily observed or only involves smaller portions of the linkage groups than would be detected by GISH.

Polyploid European Fish in the *Squalius alburnoides* Complex

S. alburnoides represents a complex of diploid and polyploid fish that were derived from (a) hybridization event(s) of a female *S. pyrenaicus* (P haplotype) and a, presumably extinct, male of a species in the genus *Anaecypris* (A haplotype). All *S. alburnoides* have *S. pyrenaicus* mtDNA and contain one or more A nuclear genomes [see also Collares-Pereira et al., this issue]. In the southern part of the range, *S. alburnoides* interacts with diploid bisexual *S. pyrenaicus*, and the complex comprises diploid (PA), triploid (PPA, PAA), tetraploid (PAAA, PPAA) and non-hybrid, reconstituted, diploid AA and triploid AAA male individuals. With the exception of the all-male AA or AAA biotypes, *S. alburnoides* can be male or female. In the northern part of the range, *S. alburnoides* interacts with diploid bisexual *S. caroliterti* (C haplotype) and comprises diploid (CA), triploid (CAA, CCA) and tetraploid (CCAA) biotypes [Cunha et al., 2011]. A variety of reproductive modes have been described for different populations of *S. alburnoides* based on breeding experiments, isozyme analyses, mtDNA, and microsatellite DNA loci [Crespo-López et al., 2006; Sousa-Santos et al., 2007; Cunha et al., 2008]. Ploidy has been verified by chromosome analyses [Gromicho et al., 2006] and flow cytometry [Cunha et al., 2008]. In most populations of *S. alburnoides*, symmetrical tetraploids (PPAA or CCAA) are either not found or are very rare, but in 2 northern populations, symmetrical CCAA tetraploids make up 71 and 86% of *S. alburnoides* and function as normally bisexual interbreeding male and female allotetraploids that produce reduced (CA) gametes [Cunha et al., 2011].

Genetic diversity in *S. alburnoides* is assured because alleles from the diploid bisexual species *S. pyreniacus* consistently cycle through *S. alburnoides* in the southern range of *S. alburnoides* as do alleles from *S. caroliterti* in northern populations. The A genome that is present in all *S. alburnoides* has distinctly different microsatellite sizes from those found in P or C genomes [Boto et al., 2011; Cunha et al., 2011], which suggests that recombinations do not occur between homeologous chromosomes. However, Rampin et al. [2012], using GISH, documented a possible incident of genome exchange between P and A chromosomes. Higher genetic diversity was found in southern populations where AA and AAA males exist with *S. pyreniacus* and *S. alburnoides* than in northern populations of *S. caroliterti* and *S. alburnoides* where such males are absent. Northern CCAA tetraploids, that could represent a tetraploid speciation event, have a comparatively low level of genetic diversity that may be indicative of relatively recent founder events [Cunha et al., 2011].

Bisexual Polyploids

Salmonid Fishes

Current models of chromosome evolution [Nakatani et al., 2007] posit that ancestors of vertebrates experienced 2 rounds (2R) of whole genome duplication (WGD), Ray-finned fishes (Actinopterygii) experienced an additional round (3R) [Meyer and Van de Peer, 2005] and salmonid fishes, recognized to be polyploid actinopterygians by Svärdson as early as 1945, represent a 4R WGD lineage [Moghadam et al., 2005]. According to Allendorf and Thorgaard [1984], the genome duplication event in salmonids occurred as an autopolyploid event 25–100 Mya (Late Cretaceous to Early Tertiary time periods). Unlike many polyploid animals that can be traced to relatively recent diploid ancestors, there are no diploid salmonids. Salmonids still maintain polyploid signatures, such as meiotic quadrivalent formation [Ohno et al., 1968] and retention of gene duplications [Market et al., 1975; Moghadam et al., 2005]. Assigning ploidy level to a taxon becomes complex when invoking WGD because all vertebrates would be tetraploid (4n), actinopterygians would have a basic octoploid (8n) ploidy level and salmonids would be hexadecaploid (16n). Traditionally, salmonids are considered to be a tetraploid linage that diverged from a diploid ancestor whose diploid descendants are currently represented by herrings (Clupeidae), anchovies (Engraulidae) and smelt (Osmeridae) [Schultz, 1980]. Salmonids are not the only ancient tetraploid fish lineage.

The suckers (Catostomidae) are also all tetraploid and are believed to be derived from a diploid minnow (family Cyprinidae) more than 50 Mya [Uyeno and Smith, 1972]. Because these polyploidy lineages are speciose, obviously more successful than their extinct diploid progenitors, and have a lengthy evolutionary history as polyploids, lessons can be learned about polyploid genetics that might be applied to diverse polyploids. As well, recent molecular data for salmonids are significant. Genetic maps have been established by employing several molecular markers (amplified fragment length polymorphisms, expressed sequence tags, simple sequence repeats or microsatellites, and Type I genes) in family breeding experiments [Garbi et al., 2006; Danzmann et al., 2008]. Mapped linkage groups have been assigned to individual chromosomes using FISH employing bacterial artificial chromosome (BAC) probes that contained markers that were used in the construction of the linkage maps [Phillips et al., 2006]. These data support the retention of syntenic blocks of genes and chromosome arm regions that have been conserved for millions of years, and homologous linkage group affinities are observed between the 3R and 4R WGD lineages [Danzmann et al., 2008]. Construction of linkage maps has been facilitated using next generation sequencing (NGS). Everett et al. [2012] used more than 1,000 genetic markers to rapidly constructed male and female linkage maps of the sockeye salmon *(Onchorhynus nerka)* that could be compared with more conventionally constructed maps of other salmonids.

African Clawed Frogs (Xenopus)

Bisexual polyploid frogs are found in many different families and in different regions of the world [Mable et al., 2011; Evans et al., 2012]. As bisexual polyploids, it may be assumed that they would follow a similar evolutionary path as salmon or suckers. As randomly breeding bisexual individuals, they should have all the advantages that diploid bisexuals experience and would be expected to adhere to the same standard parameters of population genetics. The costs that unisexual or asexual organisms face would be alleviated and they are endowed with more genes. In some ways, the WGD of clawed frogs in the genus *Xenopus* (family Pipidae) parallel salmonid and catostomid fish evolution, but unlike these polyploid fishes, species of *Xenopus* exploit different levels of polyploidy from tetraploid to dodecaploid (12n), and they are all considered to be allopolyploid. Speciation in *Xenopus* is accomplished through 'normal' bifurcation as well as hybridization that can result in ploidy elevation [Evans, 2008]. There is no documentation for ploidy reduction, and there is also no indication that polyploid *Xenopus* utilize, or incorporate, genes or genomes from any diploid species [Evans, 2007, 2008]. *Silurana* is a sister pipid genus to *Xenopus* that contains diploid *(S. tropicalis)* as well as tetraploid *(S. epitropicalis)* species. Tetraploidization in *Xenopus* is estimated to have occurred, independently from tetraploidization in *Silurana*, 21–41 Mya [Evans et al., 2004, 2012]. To compare chromosome duplications, restructuring and *S. tropicalis* homeologous chromosomes in *X. laevis*, Krylov et al. [2010] developed whole chromosome painting probes from all 10 (haploid) chromosomes of *S. tropicalis*. Cross-hybridization (Zoo-FISH) was performed by applying the *S. tropicalis* fluorescent probes to chromosome spreads of *X. laevis* (4n = 36). The technique identified some chromosomal quartets in *X. laevis* that were homologous to *S. tropicalis* linkage groups and supported a previous hypothesis [Schmid and Steinlein, 1991] that the smallest chromosome in a *S. tropicalis* – like karyotype was lost through translocation in the hypothetical 18 chromosome diploid ancestors of tetraploid *Xenopus*. Clear distinction of all *S. tropicalis* homologous chromosome regions in *X. laevis* was not achieved, which is probably a reflection of their phylogenetic distance. Divergence estimates for *Silurana* and *Xenopus* are 50–65 Mya [Bewick et al., 2012]. Even though the complete *S. tropicalis* genome has been sequenced [Hellsten et al., 2010], assessing duplicate gene expression of orthologous *S. tropicalis* genes in *X. laevis* is hampered by the fact that orthologs have evolved since their divergence from the most recent common ancestor(s) [Chain et al., 2011]. Genetic variation in polyploid *Xenopus* can not be attributed to 'dipping' into diploid gene pools because extant diploid species are not available. Variability can, however, be achieved through allopolyploidization and genome elevation, so genetic variation and evolution in this lineage has been accomplished by polyploids 'dipping' into other polyploid rather than diploid gene pools.

Eurasian Toads of the Bufo viridis Subgroup

Molecular techniques have significantly improved our understanding of the evolution and interaction of diploid, triploid and tetraploid populations of Palaearctic Green Toads that are found in often remote areas in the Middle East and Central Asia. Stöck et al. [2005] used data derived from mitochondrial sequences and chromosomes as evidence to support a multiple origin for the tetraploids, and those data suggested that tetraploids can be allopolyploid *(B. oblongus)* or possibly autopolyploid *(B. pewzowi)*. Based on homogenous chromosome banding patterns [Stöck et al., 2005], *B. pewzowi* appears to be

an autopolyploid, but the application of molecular sequence markers revealed *B. pewzowi* to also be of hybrid (allopolyploid) origin [Stöck et al. 2009]. Triploid specimens are found in several populations [Stöck et al., 2005, 2006, 2009]. Microsatellite, mtDNA and nuclear sequence analyses clearly show that triploids can result from reciprocal hybridization events involving diploid *B. turanensis* and allotetraploid *B. pewzowi* [Stöck et al., 2009]. But triploid *B. baturae* has a different mtDNA that is almost identical to that found in the poorly studied *B. shaartusiensis* [Litvinchuk et al., 2011]. So far, *B. baturae* is the only known bisexually reproducing triploid (3n = 33) vertebrate [Stöck et al., 2002]. Nucleolar organizer regions (NORs) are present on 2 of the 3 chromosome 6 copies in both sexes (NOR^+, NOR^+, NOR^-): there are 22 female lampbrush bivalents, and the male sperm is haploid and always NOR^+ [Stöck et al., 2002]. Using microsatellite analyses and laboratory hybridization experiments that involved several families of *B. baturae* and a female *B. baturae* that was crossed with diploid and tetraploid males of related species, Stöck et al. [2002, 2011] proposed a novel breeding system, pre-equalizing hybrid meiosis, that best fit the resulting data. In *B. baturae* females, prior to meiosis, there is a duplication of one set of chromosomes (the NOR^- set), and the, now tetraploid, oocyte (NOR^+, NOR^+, NOR^-, NOR^-) follows normal reduction in meiosis to produce diploid (NOR^+, NOR^-) eggs. The male eliminates a set of chromosomes (the NOR^- set) prior to meiosis, and his, then diploid (NOR^+, NOR^+), primary spermatocyte undergoes meiosis to produce haploid (NOR^+) sperm. Mating restores the triploid male and female NOR^+, NOR^+, NOR^- genotypes. Under this mode, the NOR^+ sets recombine in both sexes in a Mendelian manner, while the NOR^- set is clonally transmitted only by females [Stöck et al. 2011]. The origin and the evolution of bizarre meiotic systems such as that used by *B. baturae* are not known. It has been hypothesized [Stöck et al., 2005, 2009] that an intermediate allo- or autopolyploid triploid gave rise to *B. baturae* and was an intermediate, or bridging, step in the evolution of the tetraploids.

A low frequency of autotriploid individuals is likely to be encountered in diverse anurans when the second polar body in female meiosis is not eliminated. This frequency can easily be elevated by temperature (hot or cold) or pressure [reviewed by Kawamura, 1984]. The resulting triploids can be both male and female and the females can be fertile. Nishioka and Ueda [1983] crossed such triploid *Hyla arborea* females with *H. arborea* males to produce abundant male and female autotetraploids. First generation offspring of male and female tetraploid *H. arborea* were 3:1 female biased, but a 1:1 sex ratio was achieved in the second generation. No tetraploid *H. arborea* have been found in nature, but based on this experiment, the possibility certainly exists for the evolution of tetraploid *H. arborea* and many other species of amphibians. If a male from some species, other than *H. arborea*, was crossed with the triploid *H. arborea* females, allotetraploids could be produced that would have 3 *H. arborea* genomes and 1 non-*H. arborea* genome. Unless the hybridizing male was very closely related to *H. arborea*, meiosis would likely be disrupted by the single foreign genome in such asymmetrical tetraploids.

Tetraploids must initially arise in sympatry with a diploid progenitor, and it is likely that diploids and tetraploids would hybridize. It would be difficult to distinguish triploids, especially autotriploids that arose from polar body suppression from triploids that resulted from 2n × 4n crosses. Allotetraploids, derived from genetically distinctive species, may also arise through a triploid bridge. Not a single tetraploid was observed in Bogart's [1972] chromosomal study that included 50 bufonid species and tadpoles from 175 experimentally produced hybrid combinations. Triploid and pentaploid tadpoles were found in several of those cross-combinations and were especially common, or the only viable tadpoles, when distantly related species were crossed. These ploidy classes represent fertilization of eggs, derived from suppression of the first (tetraploid egg) or second (diploid egg) polar body during meiosis. If an allotriploid female produced unreduced triploid eggs, in a similar manner to autotriploid *H. arborea*, a backcross could give rise to symmetrical allotetraploids. Such artificial breeding experiments have been performed in ranid frogs and are discussed by Kawamura [1984]. Of course, in a sympatric diploid/tetraploid population, asymmetrical allotriploids could be relatively common and could constantly be produced through 4n × 2n mating [e.g. Stöck et al., 2011]. When such triploids backcross to the diploid parent, the resulting tetraploids would again be symmetrical allopolyploids. It is expected that reproductive isolating mechanisms would rapidly evolve if triploids had a reduced fitness, but even a low level of hybridization would be sufficient to pass genes from the diploid gene pool into the tetraploid gene pool.

North American H. versicolor and H. chrysoscelis

Tetraploid *H. versicolor* and diploid *H. chrysoscelis* are widely distributed in eastern North America, and there are allopatric and sympatric populations of both species. The 2 species cannot be distinguished by morphology, but they

have distinctly different vocalizations that were originally used to differentiate these species [Johnson, 1966] prior to their identification as a diploid and tetraploid cryptic species pair [Wasserman, 1970; Bogart and Wasserman, 1972]. Their morphological similarity, combined with duplicated isozyme loci [Ralin and Selander, 1979], and observed quadrivalent formation in male meiosis [Bogart and Wasserman, 1972] suggested an autopolyploid origin for *H. versicolor* from *H. chrysoscelis*. The acoustic differences between the 2 species serve as a reproductive isolating mechanism, so in sympatric populations, female diploids and tetraploids are able to recognize their respective calling males [Littlejohn et al., 1960; Gerhardt 2005]. Immunological [Maxson et al., 1977], acoustic [Gerhardt, 1974] and chromosomal [Wiley, 1983] differences were observed between 'eastern' and 'western' populations of *H. chrysoscelis*, and those data were used to suggest that *H. versicolor* is an allopolyploid that arose from (a) mating(s) of genetically distinctive populations of *H. chrysoscelis*. More recently, Ptecek et al. [1994] sequenced mitochondrial cytochrome b (Cytb) from several populations of *H. versicolor* and *H. chrysoscelis*. They found that some *H. versicolor* individuals were clustered within different *H. chrysoscelis* lineages and more closely align with one or the other *H. chrysoscelis* lineages than they do with other *H. versicolor* individuals. From their data, Ptecek et al. [1994] concluded that *H. versicolor* arose at least 3 times, twice from *H. chrysoscelis* and once from an unknown maternal ancestor. Holloway et al. [2006] suggested that *H. versicolor* or 'tetraploids' arose multiple times from *H. chrysoscelis* and from 2 other extinct lineages. In addition to Cytb, their study included 3 nuclear markers.

These data, for *H. versicolor* and *H. chrysoscelis*, would be consistent with the same triploid bridge hypothesis outlined for the European Green Toads and was an hypothesis that was proposed by Ralin and Selander [1979] to address the phenomenon that genetic similarity was greatest where the species co-occur: 'If triploids were formed in nature by hybridization and produced un-reduced eggs, fertilization by haploid sperm could reconstitute the tetraploid condition. Thus, triploid hybrids could serve as a one-way bridge for gene flow from the diploid to the tetraploid' [Ralin and Selander, 1979, p 605]. They [Ralin and Selander, 1979] rejected that hypothesis because they believed that the female's exceptional abilities to discriminate vocalizations of diploid and tetraploid males, the absence of triploids in nature, and the reduced viability of laboratory produced F1 hybrids [Johnson, 1963] would preclude such a(n) event(s).

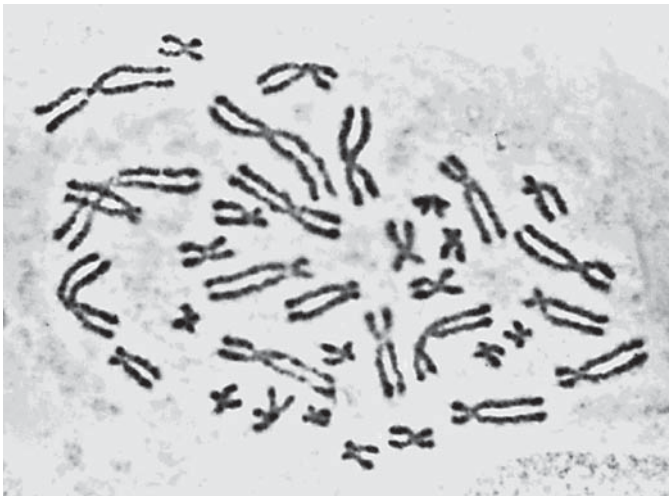

Fig. 6. Chromosome spread from a triploid (3n = 36) *Hyla* '*versicolor*' collected in a sympatric population of *H. versicolor* and *H. chrysoscelis* in Oklahoma, USA. This is the only documented evidence that *H. versicolor/H. chrysoscelis* triploids exist in nature.

Identification of the species in most *H. versicolor*/*H. chrysoscelis* investigations has relied on acoustic differentiation of males [Ralin and Selander, 1979; Ralin et al., 1983; Romano et al., 1987; Ptacek et al., 1994; Holloway et al., 2006]. Ptacek et al. [1994] also used flow cytometry to confirm ploidy in females and non-calling males, and chromosomes were examined from several of the individuals used by Ralin and Selander [1979], but ploidy has only been confirmed in relatively few individuals from a limited number of populations of either species. During a road trip in southern Oklahoma in April 1991, one of us (J.P.B.) came across a sympatric population of 'fast-trilling' *H. chrysoscelis* and 'slow-trilling' *H. versicolor*. It is especially easy to distinguish these different calls in sympatric association, and a small collection of males was made of each call type. The specimens were eventually karyotyped, and one male, that had a 'slow-trilling' call, was determined to be triploid (fig. 6). We sequenced and compared the Cytb sequence of that triploid with the 2 sequences deposited in GenBank from the study of Ptacek et al. [1994]: *H. chrysoscelis* from Kentucky (accession L22017) and *H. versicolor* from Oklahoma (accession L22018). The triploid was found to have the same sequence as *H. versicolor* so was most likely a *H. versicolor* × *H. chrysoscelis* hybrid rather than an autotriploid derived entirely from *H. chrysoscelis*. Other individuals of both *H. versicolor* and *H. chrysoscelis* were karyotyped from that same Oklahoma population [Bogart, unpubl.

data]. Ralin and Selander's [1979] 'one-way bridge' hypothesis relied on similar triploids that were derived from 2n × 4n mating and a subsequent F1 backcross with *H. chrysoscelis*. They did not consider a bridge structured on unreduced eggs that are likely produced in some low frequency by diploid female *H. chrysoscelis*. Such a system was mentioned by Romano et al. [1987] as one of a number of alternate hypotheses in their attempt to explain the remarkable similarity of observed isozyme alleles and their frequencies in *H. versicolor* and *H. chrysoscelis* from a number of widely separated sympatric populations.

Mitochondrial sequences and allozyme data support the hypothesis that *H. versicolor* dips into the *H. chrysoscelis* gene pool when these species are in contact. Gene flow occurs from *H. chrysoscelis* into *H. versicolor* and acts to increase genetic variability and heterozygosity in *H. versicolor*. The average genetic heterozygosity in *H. versicolor* is 5 times greater than that of *H. chrysoscelis*, and with a mean of 0.332, *H. versicolor* ranks among the most heterozygous bisexual animal species [Ralin and Selander, 1979]. It is difficult to understand how 2 possibly extinct species could have hybridized with themselves and with *H. chrysoscelis* to produce the observed *H. versicolor* lineages [Holloway et al., 2006]. We think that Holloway et al.'s alternate, and rejected, hypothesis that various populations of a wide spread diploid species *(H. chrysoscelis)* gave rise to independent lineages of tetraploids would be more realistic and is supported by most available data with respect to mitochondrial sequences, isozyme data and the possible developmental events that would be required to produce a polyploid. They consider the 2.0–3.5% Cytb pair-wise sequence divergence between populations of *H. versicolor* to be sufficiently high to support species status for the unknown, and possibly extinct, diploid ancestral lineages based on unpublished data for populations of 2 other hylid frogs *(H. andersonii* and *H. femoralis)* that have much smaller ranges than *H. versicolor* or *H. chrysoscelis*. The sequence divergence between 'eastern' and 'western' *H. chrysoscelis* is 2.3% [Ptacek et al., 1994], and an interpopulational sequence divergence rate of 3.5% is not especially high for wide-ranging species in North America [Burbrink et al., 2000; Zamudio and Savage, 2003]. Pair-wise sequence divergence ranged from 0.3–6.62% in another hylid frog, *Pseudacris crucifer*, which is widely sympatric with *H. versicolor* and *H. chrysoscelis* [Austin et al., 2004]. Holloway et al.'s [2006] alternate hypothesis could be merged if the, possibly extinct, diploid species were, in fact, genetically somewhat isolated populations of *H. chrysoscelis* that may or may not be extant and *H. versicolor* 'dipped' into those diploid gene pools at various points in time. Mitochondrial genes have only been sequenced from a very few individuals in relatively few populations of *H. versicolor* or *H. chrysoscelis*. The most interesting populations to address diploid/polyploid interactions, and the frequency of such interactions, would be sympatric populations of *H. versicolor* and *H. chrysoscelis*, which was the focus of Romano et al.'s [1987] isozyme study, but which only examined those types of nuclear markers. No comparable genetic data are available for sympatric populations in New Jersey [Zweifel, 1979], Wisconsin [Jaslow and Vogt, 1977], or Michigan [Bogart and Jaslow, 1979] in USA, Manitoba in Canada [D.M. Green and J.P. Bogart, pers. observations], and elsewhere in the extensive range of these 2 frogs. Microsatellite DNA alleles would be an obvious choice of nuclear markers that could test recent interactions of diploids and tetraploids. Microsatellites were designed for *H. chrysoscelis*, but of 41 possible microsatellite loci [Krenz et al. 1999], only 3 have been used, with limited success, to examine gene flow between populations of *H. versicolor* [Espinoza and Noor, 2002].

South American, African, and Australian Polyploid Frogs

Several of the diploid/polyploid 'cryptic species' of anurans in South America and Africa [Bogart and Wasserman, 1972; Bogart and Tandy, 1976; Bogart, 1980] are now recognized to be diploid and tetraploid species [Tandy et al., 1982; Channing and Bogart, 1996; Martino and Sinsch, 2002; Vieira et al., 2006], and several additional bisexual polyploid species of frogs have been found in South America whose present interaction with diploids is not yet known. In the South American horned frog genus *Ceratophrys* (family Ceratophryidae), 3 of 8 species are octaploid and at least 3 others are diploid. It is suspected that the octaploids arose independently from some diploid progenitors [Vieira et al., 2006]. In the South American family Leiuperidae, several species of *Pleurodema* are diploid, but *P. bibroni* and *P. kriegi* are tetraploid and *P. cordobae* is octaploid [Valetti et al., 2009]. The evolution of the polyploid species of *Pleurodema* was discussed by Faivovich et al. [2012] in their mitochondrial sequence analysis of *Pleurodema*. All 3 polyploidy species of *Pleurodema* are the only species in a monophyletic clade and there is an extremely low level of sequence divergence among the 3 species, so no possible diploid progenitor could be identified. Previously, Barrio and Rinaldo de Chieri [1970] hypothesized that tetraploidy arose in *Pleurodema* by allopolyploidization because the chromosomes could be grouped in pairs, rather than quartets,

and no quadrivalents were observed in meiosis. Meiotic data for the octaploid was not reported by Valetti et al. [2009]. The sequence data would be most consistent with an autopolyploid origin for polyploid *Pleurodema* and subsequent speciation, but it is possible that (a) hypothetical diploid ancestor(s) were not sampled or are extinct [Faivovich et al., 2012]. Unlike the tetraploid *Pleurodema*, mitochondrial ND2 sequences show that the South American tetraploid tree frog *Phyllomedusa tetraploidea* is paraphyletic with diploid *P. distincta* [Brunes et al., 2010], and mitochondrial genome sharing (tetraploid with diploid and vice versa) was found in zones of hybridization that were defined by Haddad et al. [1994]. Brunes et al. [2010] also sequenced 2 nuclear genes *(P-fibint7* and *C-myc2)* and found both nuclear genes were shared among *P tetraploidea*, *P. distincta* and *P. ingeringii*. Thus, the evolution of polyploidy in *P. tetraploidea* is not clear. The mitochondrial data suggest an autopolyploid event from *P. distincta* or a close relative, and the nuclear sequences could be used to support the contention that *P. tetaploidea* is an allopolyploid that arose from the hybridization of a female *P. distincta* and a male *P. ingeringii*. At the present time, the distributional range of *P. ingeringii* does not overlap the ranges of *P. distincta* or *P. tetraploidea* [Haddad et al., 1994].

In Africa, *Tompoterna tandyi* was described as an allopolyploid species [Channing and Bogart, 1996] whose ancestors were male *T. delalandii* and female *T. cryptotis*. Evidence for possible multiple origins of this tetraploid comes from a mitochondrial DNA sequence study of several populations of tetraploid *T. tandyi* [Dawood et al., 2002]. All South African populations of *T. tandy*i possess the same mtDNA haplotype, but 2 different Cytb haplotypes were found in separate populations of *T. tandyi* in Namibia.

Four tetraploid and 6 diploid species are recognized in the Australian burrowing frog genus *Neobatrachus*. The species are morphologically very similar and are identified using a combination of behavioral, cytogenetic, morphological patterns, and geographic location [Mahoney and Robinson, 1980]. Some tetraploids and diploids are sympatric and hybridization may occur on occasions among diploids and tetraploids [Mable and Roberts, 1997]. This very complex system is not resolved, but despite low bootstrap support for some nodes, there are interesting mitochondrial (CO1) haplotype associations (fig. 7) that might relate to similar patterns that we proposed for other diploid/polyploid systems. The tetraploids that are largely allopatric from diploids and parapatric with each other *(N. sudelli* and *N. centralis)* all cluster together. It would be difficult for these species to interact with extant diploids or 'dip' into any diploid gene pools. Other tetraploids (e.g. *N. aquilonius* from the Northern Territory) cluster with those tetraploids *(N. sudelli* and *N. centralis)*, but *N. aquilonius* from Western Australia clusters with diploid, parapatric *N. fulvus*. Tetraploid *N. kunapalari* clusters with diploid *N. albipes* and is also a single member taxon on a very divergent branch. If a diploid female *N. fulvus* produced diploid (unreduced) eggs and mated with sympatric, tetraploid *N. aquilonius*, the tetraploid offspring would have a *N. fulvus* mtDNA. Tetraploid *N. kunapalari* could end up in a *N. albipes* cluster if it mated with a sympatric, diploid female *N. albipes* that produced unreduced eggs. The original influx of diploid genes into the tetraploid gene pool would be sorted and diluted in subsequent gen-

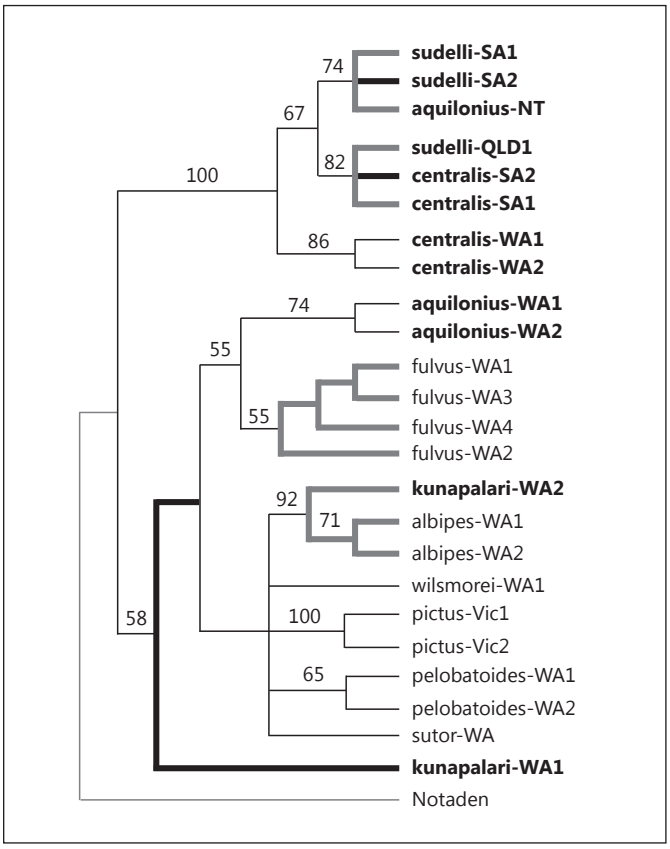

Fig. 7. Phylogeny of Australian diploid and tetraploid (bold) taxa of *Neobatrachus*. The strict consensus tree was produced from mitochondrial (CO1) sequence analyses and was rooted with diploid *Notadon melanoscaphus*. The numbers at each node are bootstrap support values (100 replicates). Mitochondrial haplotype sharing between diploids and tetraploids demonstrates hybridization events (see text) [from Mable and Roberts, 1997].

erations of random mating. Distinctive mitochondrial haplotypes in the same nominal taxon is likely a signature of past hybridization. If tetraploids bred with each other, the resulting hybrids would carry the hybridizing female's CO1 sequence. In figure 7, *N. kunapalari* – WA2 has a diploid *N. albipes* – WA1 CO1 haplotype that was probably derived relatively recently from that diploid species. *N. kunapalari* – WA1 also has a tetraploid *N. aquilonius* –WA1 or WA2 CO1 haplotype that was probably derived less recently from that tetraploid species. Such simplistic speculation does not do justice to this intriguing diploid and tetraploid Australian frog complex.

The Promise of NGS in Genomic Research of Polyploidy

Obtaining genome-wide sequence data is indispensable to understand the evolution and maintenance of polyploids from both genomic and transcriptomic levels. Molecular phylogenetic and population genetic studies of polyploids have been a challenging task because of the difficulties in sequencing homeologous nuclear markers using Sanger sequencing of traditional PCR products. In this context, polyploid genomes contain multiple copies of each nuclear gene (homeologues) that are often associated with complex structural and epigenetic changes during evolutionary time [Buggs et al., 2012]. Such genomic complexity, often difficult to predict, has impeded our understanding of the origin, diversification, genome evolution, and adaptation from populations to entire clades of various polyploid systems [Griffin et al., 2011].

Recent developments of NGS techniques have provided a significant boost in many molecular and evolutionary applications. NGS fundamentally changes the scale of DNA sequence data that can be collected and does not require prior genome knowledge of the organism to be sequenced. Furthermore, the same NGS associated genomic and bioinformatic tools that are applied in 'model' species can be applied in most non-model systems that lack a preexisting reference genome [Bi et al., 2012]. The integration of various genome reduction [Miller et al. 2007] and targeted enrichment techniques [Hodges et al., 2009; Lemmon et al., 2012; McCormack et al., 2012] further reduces the cost of NGS with increased sequencing quality and coverage of selected regions of interest. These tools reduce the level of complexity of sequenced genomes and are especially suitable for studying organisms with complex genomes, such as polyploids. Thus, recent technical advances provide an unprecedented opportunity to tackle various evolutionary questions that remain difficult to answer using traditional molecular approaches. Despite these merits, the application of NGS in polyploid animals has largely lagged compared to diploids or even polyploidy plants [Bundock et al., 2009; Brenchley et al., 2012; Buggs et al., 2012; Trick et al., 2012]. Combined with transcriptome sequencing (RNA-seq) and genome reduction methods such as restriction site associated DNA tags (RAD-tags) [Miller et al. 2007], NGS has been used to discover genome-wide SNPs in rainbow trout *(O. mykiss)* and westslope cutthroat trout *(O. clarkii lewisi)* [Hohenlohe et al., 2011] as well as in sockeye salmon *(O. nerka)* [Everett et al., 2011, 2012]. NGS has also been successfully used to study gene expression in the polyploid Lake Sturgeon *(Acipenser fulvescens)* [Hale et al., 2009].

We believe that the continued reduction of cost and technical improvements can accelerate the application of NGS and associated genomic tools in polyploid animals. This research will facilitate our understanding of basic genetic/epigenetic phenomena as well as revealing evolutionary consequences of genomic interactions in polyploids. For example, NGS can significantly enhance our understanding of evolution and persistence of unisexual polyploidy vertebrates. GISH can only provide a rough picture for large-scale inter-chromosomal exchanges (e.g. in unisexual salamanders): minor interactions cannot be detected. NGS can provide precise information regarding the distribution, pattern and rate of intergenomic as well as intragenomic translocations and recombinations as well as other type of mutations (e.g. gene loss, gene conversion or new activities of transposable elements) [Fujita and Moritz 2009; Chester et al., 2013]. NGS can be applied to study genome-specific gene expression and epigenetic patterns that may provide important insights with respect to genome replacement, gene regulation, phenotypic consequences, and the persistence of genes in a given environment. NGS should also enable us to compare patterns of nucleotide substitutions (e.g. genome wide dn/ds) to investigate the efficiency of natural selection (e.g. detecting mutation load/Muller's ratchet) in different genome combinations of polyploids with different reproductive modes or different rates of genome replacement and intergenomic exchanges.

Conclusions

In our brief overview of polyploid organisms, we have focused on a few 'systems' where advances in DNA technology have improved our understanding of the evolu-

tion and persistence of animal polyploids as well as diploid/polyploid interaction. In general, compared with related diploid species, polyploids have elevated levels of genetic diversity that has, in many cases, been accomplished by sampling and re-sampling diploid gene pools. Polyploids accumulate genes from such diploid associations by having additional linkage groups. Meiotic aberrations are necessary for the initial evolution of polyploids and persist, especially in allopolyploids, to maintain genomic integrity. Clonality may have a short-term benefit to maintain heterozygosity and may also avoid the cost of meiosis, but genetic diversity that is normally derived through syngamy is necessary to extend a clone's evolutionary time frame. Historically, WGD may be viewed as successful when diploid progenitors are replaced by polyploid lineages (e.g. salmonid fish and *Xenopus*), but interploidy interaction and reticulate evolution is common. Polyploids often retain genetic associations with diploids and/or other polyploid biotypes, and this includes several bisexual polyploid frogs (species of *Neobatrachus*, *B. viridis* subgroup, *P. tetraploidea*, *P. esculentus* complex (LLRR), and *H. versicolor*) as well as the neotetraploid fish, *S. alburnoides* (CCAA and PPAA).

Little is known or understood concerning meiotic programs that guide hybridogenesis or maintain genomic integrity in polyploid males and females. Genomic integrity is important for meiotic processes such as hybridogenesis. Although intergenomic translocations and recombinations do occur in unisexual *Ambystoma* [Bi and Bogart, 2006], and this mechanism would allow for the observed introgression of genes that flow from *P. perezi* to *P. ridibundus* through hybridogenetic *P. grafi* [Smeller et al., 2005], most data show that the majority of recombinations occur between homogenomic genomes in allopolyploids. Sex chromosomes or sex genes and their interactions do not seem to result in expected imbalances or in the production of intersexual individuals. It is expected that natural selection acts to eliminate sterile individuals or even individuals with lower fitness in heterogeneous gene pools, but in a laboratory experiment, neotetraploid *H. arborea* whose offspring were initially female biased, produced a 50:50 male to female ratio in the second generation [Nishioka and Ueda, 1983]. Thus, it would appear that some other factor(s) than natural selection may be operating to balance the sex ratio in these artificially produced autotetraploids.

The triploid bisexual toad, *B. baturae*, could provide answers to some interesting genetic and evolutionary questions. Prior to meiosis, females add a genome and males lose a genome, so, after meiosis, diploid eggs are fertilized by haploid sperm [Stöck et al., 2002]. Individual haploid genomes (gained in females and lost in males) are not randomly chosen. Meiosis in this toad demonstrates aspects of hybridogenesis and pre-meiotic endoduplication that target specific genomes in different sexes. It would also be of interest to investigate the reproductive strategy of these toads. Resulting offspring would have twice as many, including some duplicated, genes derived from their mother than they do from their father. Males eliminate more than half their genes prior to, and during meiosis. Thus, female *B. baturae* should win in an evolutionary competition unless males mated with several females.

Continuing advancements in DNA technology has significantly improved our understanding of many diploid – polyploid 'systems' but much can still be gained by incorporating data based on older techniques. The maternal parent, the number of hybridization events, and the time of lineage origins can be estimated by sequencing mitochondrial genes. Isozyme analysis is still a very useful technique that has traditionally been used to distinguish genomes in polyploids and has been instrumental in identifying male genome contributions or examining clonal or hemiclonal reproduction in diploids and polyploids: in most cases, this same information can be derived through the examination of microsatellite DNA. Microsatellite loci are neutral genetic markers that have higher mutation rates than isozymes and are generally very polymorphic, so microsatellites can reveal more recent genetic events in extant populations than can be obtained with isozymes. DNA can be extracted from small tissue samples for both mitochondrial and nuclear (including microsatellite) gene sequences and does not normally require sacrificing individuals, which is especially important when studying rare and protected populations and species. Massively parallel, NGS has recently, and fundamentally, changed the scale of DNA sequence data that can be collected. It is now possible to inexpensively sequence genomes and transcriptomes with much deeper coverage and reliability. NGS and associated genomic tools provide an unprecedented opportunity to tackle many questions that remain unanswered by using traditional molecular techniques, and NGS will facilitate our understanding of basic genetic/epigenetic phenomena as well as revealing evolutionary consequences of genomic interactions in polyploids. Molecular cytogenetics is a powerful technique that facilitates the examination of chromosome segregation, mutational events in mitosis and meiosis, genomic interactions and genomic stability in polyploid organisms. GISH is especially useful for

studying chromosomes in allopolyploids, and it is now possible to locate genes of interest on homologous or homeologous linkage groups using FISH painting probes and BACs. Molecular technology has dramatically improved our understanding of the evolution and persistence of diploid/polyploid 'systems'. The interaction of diploids and polyploids can be viewed as a complex and dynamic process that varies with environmental and genomic associations over time and space. It is very possible that populations of polyploids, which can provide answers to known, and even unknown, evolutionary and genetic questions, currently exist but are yet to be sampled.

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