# Genomic evidence suggests the balanced lethal system in *Triturus* newts originated in an instantaneous speciation event.

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**Abstract**

The balanced lethal system found in crested and marbled newts of the genus *Triturus* presents an intriguing mystery. All adults possess two distinct forms of their largest chromosome, resulting in 50% of offspring inheriting two copies of one of these forms. These homomorphic individuals undergo fatal developmental arrest during embryogenesis. How could such an obviously maladaptive trait, destroying half of an organism's reproductive output, evolve? We construct a high-density linkage maps for *Triturus* and its sister genus *Lissotriton*, identifying genes involved in the balanced lethal system. We find that each of the two forms of *Triturus* chromosome 1 is characterized by a single massive deletion. Ploidy analysis shows that each deletion is compensated for by a duplication of the same region on the opposite chromosome. This suggests that the balanced lethal system was created suddenly, as the result of an unequal exchange between chromosomes in a single individual. We simulate the consequences of such a mutation, finding that, if the rearranged chromosomes exert a fitness penalty when combined with the ancestral version, a stable balanced lethal system can become fixed within a population. Counterintuitively, the deleterious nature of the rearranged chromosomes causes reproductive isolation that protects them from invasion by their fitter ancestor. We conclude

that the origin of the *Triturus* balanced lethal system is effectively an instantaneous speciation event, which resulted in the fixation of the balanced lethal system.

## Introduction

Evolution is a seemingly simple process that continually produces complex and counterintuitive outcomes. Organisms frequently evolve in ways that confound our expectations and occasionally even appear to contravene the principles of natural selection. Investigation of these paradoxical phenomena often leads to a deeper understanding of evolutionary processes. An extreme example of life apparently defying natural selection is found in crested and marbled newts (the genus *Triturus*). In these species, 50% of all embryos spontaneously die before hatching, resulting in a massive and uncompensated loss of reproductive output, due to a phenomenon known as a balanced lethal system (Rusconi, 1821; Sims et al., 1984; Wielstra, 2020).

The premature death of these embryos is due to the fact that *Triturus* chromosome 1 is heteromorphic, occurring in two distinct versions, named 1A and 1B. Only heterokaryotypic individuals, possessing both versions of the chromosome, are viable (Macgregor & Horner, 1980; Sims et al., 1984). Because chromosome 1 is inherited in a Mendelian fashion, and all adults must possess one copy of each version, it follows that half of all offspring will inherit two copies of the same version (fig. 1). The death of homomorphic embryos implies that each version of chromosome 1 must contain unique mutations that are recessively lethal. Normally these mutations would be selected against and eventually go extinct. However, because the alternative version of the chromosome also has its own lethal mutations, the selective forces are balanced and both chromosomes are maintained in the population, at the cost of half of the offspring (Muller, 1918; Wielstra, 2020). Artificial balanced lethal systems are commonly used in genetics to maintain stable stocks of deleterious mutations. However, given the massive fitness cost incurred, a naturally occurring balanced lethal system seems almost impossible. Yet, they have independently evolved in nature in widely divergent taxa, aside from *Triturus*, also in central American *Drosophila* and plants of the genera *Isotoma* and *Oenothera* (Dobzhansky & Pavlovsky, 1955; James et al., 1990; Steiner, 1956).

To maintain the heteromorphism required for the balanced lethal system, chromosomes 1A and 1B must be shielded from the blending effect of recombination, because otherwise a single,

healthy chromosome could be (re)created. Indeed, in *Triturus*, the long arm of chromosome 1 consists almost entirely of a non-recombining section along which chiasmata are not observed (Callan et al., 1960). This lack of recombination may have additional consequences: large stretches of the genome, containing multiple coding genes, would be consistently inherited together, and selected for or against as a single, giant gene (Dobzhansky, 1970). Such 'supergenes' have important evolutionary effects as they can lock together diverse morphological and behavioral traits, but can also lead to the accumulation of deleterious mutations. In some cases, such as in ruffs or fire ants, supergenes that are lethal when homozygous are maintained in the population due to the advantageous traits that they confer when heterozygous (Hallar et al., 2007; Küpper et al., 2016). A balanced lethal system could simply be considered a supergene system where not one but both versions are deadly in the homozygous state. How did the supergene underlying the *Triturus* balanced lethal system originate? We aim to unravel this mystery by mapping the architecture of the *Triturus* genome that suppresses recombination and reconstructing the evolution of the two distinct versions of chromosome 1.

**Genomic architecture**

Recombination is generally inhibited by chromosomal rearrangements, most commonly inversions. To identify any such rearrangement within the *Triturus* genome, we construct a high-density linkage map consisting of 4226 nuclear DNA markers sequenced in 206 full-sibling offspring of a *Triturus ivanbureschi* × *T. macedonicus* $F_2$ cross. These offspring include approximately equal numbers of developed (heterokaryotypic, designated AB) and arrested (homokaryotypic) embryos. After sequencing the homozygote embryos are further classified, depending on which forms of chromosome 1 they possess, designated AA and BB.

As a proxy for the ancestral state of the *Triturus* genome, we construct an analogous map (including 3693 markers, from the same bait set of c. 7k) for its sister genus *Lissotriton* (Rancilhac et al., 2021), which is unaffected by the balanced lethal system. (sup. figs. S1, S2 and table S1 contain full details of the linkage maps.) We compare these linkage maps to each other, and to the chromosome-scale genome assemblies of the Iberian ribbed newt (*Pleurodeles waltl*) (Brown et al., 2023) and Mexican axolotl (*Ambystoma mexicanum*) (Nowoshilow et al., 2018; Smith et al., 2019). Synteny between *Triturus* and *Lissotriton* is highly conserved, with 98% of genes placed in orthologous chromosomes (sup. table S2), and few disruptions of gene

order. This confirms that the rearrangement that led to the balanced lethal system is restricted to chromosome 1. Despite over 60 million years of divergence since their last common ancestor (Marjanović & Laurin, 2014), synteny is also highly conserved between *Triturus* and *Pleurodeles*. Beyond the family Salamandridae, we show that *Triturus* chromosome 1 is homologous to a fusion between *A. mexicanum* chromosomes 8 and 13. (for full details of synteny see sup. figs. S3-5).

We observe that rather than SNPs or other small variations specific to chromosome 1A and 1B, the variation between genotypes is largely characterized by genes that are completely absent in one of the two categories of arrested embryos. In the *Triturus* linkage map family we identify 30 genes which consistently fail to yield reads in embryos of genotype BB, and 35 are similarly missing in genotype AA – we designate these A-linked and B-linked genes respectively. De Visser et al. (2024) report that both sets of genes show highly consistent presence/absence variation across *Triturus* species, indicating that the balanced lethal system attained its modern form before the radiation of the genus. Remarkably, when the orthologs of the A- and B-linked genes are highlighted in the genomes of *Lissotriton*, *Pleurodeles* and *Ambystoma*, they are observed to form two distinct, adjacent, but non-overlapping blocks, each corresponding to genes present only in one form of chromosome 1 (fig. 2). This shows that the balanced lethal system results from a pair of large deletions - the sizes of the orthologous blocks in the *P. waltl* genome are 227 and 189 Mbp for the A- and B-linked genes, respectively. As each of the deleted blocks is only present on one form of chromosome 1, recombination between the two forms is impossible in this region. We also identify a third smaller (130 Mbp) non-recombing block, that de Visser et al. (2024) observe showing considerable interspecies variation in gene content, indicative of a post-establishment expansion of the original balanced lethal system.

Despite karyotypes showing that the non-recombining region occupying at least half of chromosome 1, with an estimated size of 1.3 Gbp (Sims et al., 1984), a large majority of genes on *Triturus* linkage group 1 are fully recombining. This does not appear to be an artifact of linkage map construction, as all regions of the homologous chromosomes of other genera are accounted for within the *Triturus* map. An explanation may be an accumulation of repetitive DNA swelling the size of the non-recombining region, which is unable to purge these sequences (Kent et al., 2017; Sessions et al., 1988).

**Twin deletions or unequal exchange?**

While deletions of the magnitude we observe in *Triturus* would almost certainly be lethal when homozygous, they should also be expected to be deleterious in heterozygous individuals. Given the number of genes involved, it is likely that at least some are haploinsufficient, meaning that a single copy is insufficient to produce a normal phenotype – for reference, approximately 10% of human genes exhibit haploinsufficiency (Bartha et al., 2018). Even if the combination of these effects was not fatal, the resultant fitness penalty would present an obstacle to the evolution of the balanced lethal system. However, these dosage effects would be compensated for if each region deleted from one version of the chromosome was duplicated on the opposite version, and vice versa. As Sessions et al. (1988) suggested, a single large mutation could result in exactly this configuration. The proposed mechanism is the unequal exchange between two homologous or sister chromosomes, where the section containing the A-linked genes on one chromosome is swapped for the section containing the B-linked genes on the other (fig. 3).

If the deleted regions in the *Triturus* balanced lethal system were compensated for by reciprocal duplications, then we should expect to find two copies of A- and B-linked genes in healthy (AB) *Triturus* embryos, even though they only possess a single copy of chromosome 1A and 1B. We test this prediction by analyzing the allele ratio of SNPs in A- and B-linked genes (fig. 4). For this purpose, we use a set of 30 $F_1$ *T. ivanbureschi* × *T. macedonicus* (10 for each genotype) and sequence the same set of marker genes as used for the linkage maps. If there is only a single copy, then an allele can be present in either 0 or 100% of the read covering each SNP locus within a sample. Instead, we observe that A- and B-linked genes in healthy (AB) embryos possess SNPs where the percentage of reads carrying each allele tends towards 50%. The distribution of allele ratios strongly supports diploidy for these genes, indicating that the A- and B-linked regions have indeed been duplicated. In arrested embryos of genotype AA, there are two copies of chromosome 1A, so we would expect a diploid distribution of SNP alleles in A-linked genes, unless the duplication has occurred. However, we observe a chaotic distribution, with no significant support for diploidy. Considering the results of the AB embryos, we suspect this is a tetraploid distribution of allele ratios (with peaks at 25, 50 and 75%) that has been obscured by noise. As expected, because there are zero copies of for B-linked genes in the AA samples we are unable to calculate allele ratios (sup. tables S3-5). The equivalent allele ratio distributions are found in reverse for the arrested embryos of genotype BB. These results match with the with the predictions of the scenario where each deletion is

compensated for by a reciprocal duplication. We accordingly conclude that the *Triturus* balanced lethal system arose in a single step, as the result of an unequal exchange between chromosomes.

**Evolutionary scenario**

Our investigation of the architecture of the *Triturus* genome gives a clear description of the rearrangement that underlies the balanced lethal system but does not explain how such a deleterious trait spread and became fixed in the entire population. Previous work seeking to model the evolution of the balanced lethal system posited a gradualistic scenario. Grossen et al. (2012) proposed that chromosomes 1A and 1B were originally two different lineages of a Y-chromosome, which evolved into the current system due to extinction of the X-chromosome. Berdan et al. (2022) model the origin of a balanced lethal system as an effect of a self-reinforcing heterozygote advantage, resulting in the gradual accumulation of lethal alleles. However, these models are incompatible with the new genomic evidence, which supports instantaneous evolution.

This result seems paradoxical. As Grossen et al. (2012) noted, even if the newly evolved chromosomes 1A and 1B exert no deleterious effects when present as a single copy, there will still be a strong negative frequency-dependent selection pressure against them, similar to any other lethal recessive allele. In fact, we may assume that the situation would appear even less favorable, as in the case where either chromosome 1A or 1B is combined with the ancestral, un-rearranged form of chromosome 1, then many genes will be present in single copy, resulting in a significant fitness penalty due to haploinsufficiency (fig. 5). There may be further deleterious effects due to the presence of a third copy of many other genes resulting in unbalanced dosage. This means that, even if chance allowed the balanced lethal system to become fixed in a small population, the ancestral version of chromosome 1 would invade and rapidly drive the rearrangements to extinction upon secondary contact.

Counterintuitively, a resolution to this paradox can be found precisely *because* of the haploinsufficiency predicted for the mixed ancestral/balanced system. If an individual carrying the ancestral chromosome disperses into a population where the balanced lethal system is fixed, then all its offspring will be of a mixed genotype, carrying one copy of the ancestral chromosome 1 and one copy of chromosome 1A or 1B (fig. 5C). As these offspring will possess

a single copy of one of the large chromosome segments otherwise deleted (and three copies of the other), they will likely suffer a significant fitness penalty. If this penalty exceeds 50%, then the offspring of the individual with the ancestral genotype will be less fit on average than the offspring of the parents carrying the balanced lethal system, and so, within this population, the ancestral un-rearranged form of chromosome 1 will be the one selected against most strongly. In this scenario, both the ancestral genotype and the balanced lethal system would experience positive frequency dependent selection, with a tipping point above which the balanced lethal system will be driven towards fixation (fig. 6A).

We explore whether this mechanism can shield a balanced lethal from invasion, by constructing a model in which the ancestral (NN) and heterokaryotic balanced lethal genotypes (AB) have equal fitness, the homokaryotic genotypes (AA and BB) are instantly lethal, and the mixed genotypes (AN and BN) have all fitness parameters (annual survival rate, female fecundity and male attractiveness) reduced by 25% compared to the ancestral state. We first simulate a scenario where two populations, one ancestral and one with the balanced lethal system fixed, colonize a region from opposite directions. In 40 of 100 replicates, we observe that after 1000 generations of secondary contact a persistent hybrid zone has formed at a region of low population density (fig. 6B, sup. fig. S6, sup. table S6).

Finally, we model the initiation of a balanced lethal system in a single individual. As Sessions et al. (1988) noted, if the chromosomal exchange occurred early enough in the germ line, all gametes produced by an individual would carry either chromosome 1A or 1B. We simulate an expanding population with the ancestral genotype. After the adult population size has reached >500, a single newt on the periphery of this population has its genotype changed to AB. Over 10,000 replicates we observe two (0.02%) instances of the formation of a persistent balanced lethal system after 100 generations (fig. 6C, sup. fig. S7, sup. table S7).

While the current structure of the *Triturus* genome closely matches the predicted outcome of an unequal exchange between homologous or sister chromosomes, we must consider whether any alternative sequence of rearrangements could produce the same outcome. The balanced lethal system may be viewed as composed of four major mutations, two deletions and two duplications. A scenario in which these each evolved independently is both less parsimonious than a single rearrangement and subject to mechanistic difficulties. Any deletion of the size we observe in *Triturus* is almost certain to be lethal when homozygous, as such it could never be

fixed (at both available loci) within any population and could only be maintained at high frequency under powerful balancing selection. This effectively requires that each deletion occurs within a pre-existing supergene system – for which we observe no evidence. Even in this case, each deletion would be significantly maladaptive compared to the ancestral version of the supergene with which it would be in competition. Evolution of the *Triturus* balanced lethal system in this scenario would require the sequential fixation of multiple highly deleterious mutations within the entire population – as there would be no analogue of the heterozygote disadvantage that protects the products of the unequal exchange.

## Conclusion

The balanced lethal system in *Triturus* newts is an anomaly of evolution in which an overwhelmingly deleterious trait persists despite apparently offering no advantage. Our mapping of the structure of the *Triturus* genome reveals that, instead of a genetic inversion, as typically used in artificial balancer chromosomes (Miller et al., 2019), the lack of recombination is caused by the total deletion of two large, adjacent sections of chromosome 1. We also show that both deletions are compensated for by a duplication of the same section on the opposite chromosome. This configuration is a predicted result of an unequal exchange between chromosomes, as presciently hypothesized by Sessions et al. (1988). Given that unequal recombination is often associated with repetitive elements (Klein & O'Neill, 2018; Nag et al., 2004), it is perhaps no coincidence that this system has evolved in *Triturus* which, like other salamanders, have extremely large and repetitive genomes (Sun et al., 2012).

At first glance, this genomic architecture provides no obvious mechanism to explain the seemingly impossible fixation of the balanced lethal system within *Triturus*. Indeed, because it implies an instantaneous origin, it is incompatible with models that proposed a gradual circumvention of natural selection, either via sex-chromosomes (Grossen et al., 2012) or supergenes (Berdan et al., 2022). However, when we consider a hybrid between the result of the unequal exchange and the ancestral genotype, we are left with a mixture of partial monosomy and trisomy over a significant stretch of the genome. These hybrids would be expected to suffer a substantial fitness penalty, potentially even larger than that incurred by the balanced lethal system. In this case the balanced lethal system does provide a relative fitness advantage in populations in which it is already dominant. Under simulations, this results in two genetically distinct populations, isolated by severe outbreeding depression, that can be

considered two species. Therefore, the balanced lethal system achieves fixation not just by spreading through an existing species, but by creating its own species.

Simulations suggest that the chance of an unequal exchange event progressing to a stable balanced lethal system, is low but not zero. It should be noted that this mechanism does not depend on the ancestral population having a low effective population size. In fact, an expanding population with a broad colonizing front maximizes the opportunities for initiation of the balanced lethal system. Because the unequal exchange event occurs in a single individual, the system must pass through at least one generation with the unfavorable hybrid genotype, which requires that this genotype is at least marginally viable. Evidence that this is the case can be found in the result of artificial crosses between *Triturus* and *Lissotriton*, which have been shown to be viable and would mimic this semi-balanced lethal genotype (Mancino et al., 1978). These hybrids would also allow for gene flow between the ancestral and novel species, which may mitigate the effects of inbreeding caused by the balanced lethal system originating in an extreme population bottleneck. An interesting prediction is that, as genetic linkage to the balanced lethal system would inhibit gene flow, the degree of introgression would vary across the genome. In support of this view, de Visser et al. (2024) show that the phylogeny of genes on *Triturus* chromosome 1 has a different topology to those on the other 11 chromosomes.

The architecture of the *Triturus* balanced lethal system provides an insight into how maladaptive traits can become fixed by reproductive isolation, through a newly described mechanism of instantaneous speciation by unequal sister chromosome exchange. It would be interesting to know whether this is a mechanism specific to *Triturus*, or if other naturally occurring balanced lethal systems have evolved in a similar way. Our work exemplifies that paradoxical evolutionary phenomena are worthy of special attention.

## Methods

### Samples:

For the *Triturus* linkage map a full sibling family was bred at the University of Belgrade (Belgrade, Serbia). The founder population consisted of two *T. macedonicus* males from Montenegro and two *T. ivanbureschi* females from Serbia. From these a male-female non-sibling pair of $F_1$ *T. macedonicus* x *T. ivanbureschi* was raised to adulthood and mated

producing 206 $F_2$ *T. macedonicus* x *T. ivanbureschi* offspring (73 hatchlings and 133 arrested embryos). An additional set of 30 $F_1$ *T. macedonicus* x *T. ivanbureschi* offspring was bred for ploidy determination (10 hatchlings and 20 arrested embryos). For the *Lissotriton* linkage map an analogous family was bred at Jagiellonian University (Kraków, Poland). A non-sibling male-female pair of $F_1$ *L. vulgaris* x *L. montandoni* was mated to produce 203 $F_2$ offspring. Samples consisted of tale-tips taken from adult newts (the $F_1$ parents of the *Triturus* and *Lissotriton* families, as well as the four $F_0$ grandparents for *Triturus*), and whole embryos or hatchlings from offspring. Details of samples are found in supplementary table S8

**DNA extraction, library preparation and target capture sequencing:**

Laboratory protocols followed the NewtCap protocol (de Visser, France, McCartney-Melstad, et al., 2024). Genomic DNA was extracted with the Promega Wizard™ Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the salt-based extraction protocol of Sambrook and Russel (2001). 1,000 µg of DNA from each sample was used for library preparation, performed using the NEBNext Ultra™ II FS DNA Library Prep Kit for Illumina (New England Biolabs, MA, USA) following the protocol provided by the manufacturer, with all volumes divided by 4 and an enzymatic fragmentation time of 6:30 minutes. Size selection was performed using NucleoMag™ magnetic separation beads (Macherey-Nagel, Düren, Germany) targeting an insert size of 300 bp. Libraries were indexed via eight cycles of PCR, using unique combinations of i5 and i7 indices from IDT (Integrated DNA Technologies, Leuven, Belgium). After concentration and fragment size distribution were measured via the Fragment Analyzer system (Agilent, Santa Clara, CA, USA), libraries were pooled equimolarly in batches of 16, with 250 ng of DNA per sample (4,000 ng total), and vacuum concentrated to 800 ng/µL.

Target enrichment was performed on the pooled libraries with the MyBaits-V4 kit (Arbor Biosciences, MI, USA). The bait set used (Ref# 170210-32) targets 7,139 genomic regions, based transcriptomes from multiple *Triturus* species (Wielstra et al., 2019). Enrichment followed the manufacturers protocol, with the following deviations: Blocks C and O were replaced with 30,000 ng of *Triturus* derived $C_0$t-1 DNA to block the hybridization of repetitive sequences. Tissue to produce $C_0$t-1 DNA was available from a removal action of an invasive population of *T. carnifex* (Meilink et al., 2015). A hybridization time of 30 hours and temperature of 63 °C were employed, and libraries were incubated with the blocking solution

for 30 minutes before addition of the RNA baits. After hybridization, the pools were amplified with 14 cycles of PCR before 150 bp paired-end sequencing, targeting a yield of 1 Gbp per sample, was performed on the NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA) by BaseClear (Leiden, the Netherlands).

**Processing of sequence capture data:**

Raw sequence data were trimmed with Skewer (Jiang et al., 2014) and BBDuk (Bushnell et al., 2017) to remove adapter contamination. The trimmed reads were then mapped against reference sequences previously assembled from *T. dobrogicus* (Wielstra et al., 2019) using BWA-MEM (Li & Durbin, 2010). Deduplication and initial genotyping were performed with GATK (McKenna et al., 2010) Coverage of each sample was assessed with a custom script which processed the output of the SAMtools (Li et al., 2009) depth function for each sample and evaluated the minimum depth of the best covered continuous 100 bp sequence within each target of the reference assembly. All samples used for construction of the linkage maps were required to have a median best 100 bp coverage of at least 10. After samples had been screened for coverage, each sample set was jointly genotyped with GATK, producing a multi-sample GVCF file.

***Lissotriton* linkage map construction:**

To construct the *Lissotriton* linkage map, VCFtools (Danecek et al., 2011) was used to filter the multi-sample GVCF file to exclude indels and SNPs with a mean sequencing depth less than 10, genotype quality lower than 20, minor allele frequency lower than 0.4, or missing data greater than 5%. Finally, a single SNP per reference target was selected. A linkage map was then constructed with LepMAP3 (Rastas, 2017). First the filtered GVCF was used as the input for the ParentCall2 module, then initial linkage groups were created with the SeparateChromosomes2 module, with a LOD limit of 28 and distortion LOD set to 1, unplaced markers were then incorporated with the JoinSingles2All module with a LOD limit of 24 (these settings were selected to maximize the numbers of included markers while yielding 12 linkage groups for both the *Lissotriton* and *Triturus* datasets). The markers were then ordered with the OrderMarkers2 module, using 20 merge iterations and eight polish iterations, with the sexaveraged option enabled. After construction the linkage map was ordered by linkage group length.

**Identification of chromosome 1 linked presence/absence markers in *Triturus*:**

The per-marker 100 bp peak region coverage was used to identify presence/absence markers in arrested embryos. Briefly, using a custom R script, expected coverage scores for all markers were calculated across the sample set. After correcting for markers with constitutive low coverage, markers where the measured value exceeded the expectation by greater than a factor of 2 in greater than 10% of samples, while having zero coverage in at least 25%, were selected as candidates. As at this stage the genotype of the arrested embryos was unknown, the candidate presence/absence makers clustered into two sets of markers where the same samples showed zero coverage. These clusters were designated as A- and B-linked in a manner consistent with other studies (de Visser, France, Paulouskaya, et al., 2024).

***Triturus* linkage map construction**:

The *Triturus* linkage map was constructed with the same methodology as *Lissotriton*, except that the presence/absence markers were also incorporated. To this end the coverage scores of these markers were converted into probabilistic pseudo-SNP calls. For example, where the measured coverage was close to expected, the genotype could be expressed as GT, where there was zero coverage GG, and where there was twice the expected coverage TT. These calls where then appended to the output of the ParentCall2 module. After construction of an initial map, the linkage group where the presence/absence markers clustered was assigned linkage group 1 and additional, separate maps for chromosome 1A and 1B (each including only one of the two sets of presence/absence markers) were constructed. The remaining linkage groups were ordered by length.

**Comparison with *P. waltl* and *A. mexicanum* genome assemblies:**

The *Triturus* reference sequences placed on the linkage map were BLASTed (Camacho et al., 2009) against the genome assemblies published for the Iberian ribbed newt, *Pleurodeles waltl* (Brown et al., 2023) and the Mexican axolotl, *Ambystoma mexicanum* (Nowoshilow et al., 2018; Smith et al., 2019) with a minimum E-value of 1e-20. Sequences that aligned outside of the main chromosomes of the assemblies or aligned in multiple locations were removed.

**Ploidy analysis:**

Data from thirty $F_1$ *T. macedonicus* x *T. ivanbureschi* samples (10 each of genotype AA, AB and BB) were used to examine SNP allele ratios of the A and B-linked genes. $F_1$ hybrids are desirable for this application as their use maximizes the number of heterozygous SNPs. These samples were processed with the same target capture methodology as the linkage map samples and taken from de Visser et al. (2024). The BAM files produced for each sample were sub-setted to produce one file with alignments for all 7,139 references sequences, and two each with alignment for 28 A-linked genes and 35 B-linked genes (selected as targets in which presence/absence variation has been validated across the genus. These alignments were processed with nQuire (Weiß et al., 2018), using the create function with -c set to 20 and -p to 10. The denoise function was then used on the resulting .bin files followed by ploidy model fitting with the histotest and view functions. The resulting allele ratio distributions were normalized and combined, and the $R^2$ values comparing the fit of the observations to calculated allele distributions were averaged for each sample genotype and gene-category.

**Simulation of balanced lethal system evolution:**

A custom non-Fisher-Wright model was developed in R, featuring overlapping generations and local colonisation and extinction. For each run, a landscape containing 50 to 150 breeding localities (i.e., ponds) was randomly generated in a 5×5 km area, with each pond given a size score (determining carrying capacity) between 500 and 5000, following a power law distribution. The simulation cycled through three phases. In the breeding phase, each adult female (of minimally two year of age) chose a male in the same pond as her mate randomly, weighed according to male attractiveness and produced a number of embryos equal to her fecundity. The embryos' genotype was a random combination of their parents and determined the chance of embryo hatching. The base survival rate of hatchlings was 5%, which was reduced if the number in a pond exceeds the pond's size so that the total number of juveniles never averaged more than 0.05 of the pond size. In the dispersal phase all newts of age 1 had a 50% chance of moving to another pond within 1 km, the destinations were selected randomly and weighted linearly with increasing size and decreasing distance. In the aging phase each individual had a survival chance determined by age and genotype, and survivor age was incremented by 1. All individuals had a genotype with three available alleles, N represented the ancestral chromosome where A and B are the heteromorphs of the balanced lethal system.

Genotypes NN and AB had values for embryonic survival, juvenile survival, adult survival, female fecundity and male attractiveness of 1.0, 0.2, 0.8, 200 and 1.0 respectively. The latter values are based on studies on *Triturus cristatus* by Arntzen and Teunis (1993) and Griffiths and Williams (2000). Genotypes BB and AA had values of zero for all parameters, whereas hybrid ancestral/balanced lethal system genotypes (AN and BN) were (arbitrarily) given values of $\frac{3}{4}$ that of genotype NN (0.75, 0.15, 0.6, 150 and 0.75) – note that as these parameters interact in a multiplicative manner this results in substantially greater deficit, with hybrids having a relative fitness of $\frac{27}{128} \approx 0.211$ compared to genotype NN.

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**Data availability**

All scripts used this study, as well as markdown document detailing all commands used in the workflow have been made available on the GitHub repository: Wielstra-

Lab/Triturus_Lethal_Evolution (https://github.com/Wielstra-Lab/Triturus_Lethal_Evolution). The raw sequence capture data is available via NCBI (bioproject - PRJNA1175462). The sequences and positions of all markers located on the *Triturus* and *Lissotriton* linkage maps are available as part of the supplementary data accompanying this study (Zenodo repository: Comparative Genomics of Triturus Balanced Lethal System - Linkage Maps and Marker Sequences Version 1.0 – DOI: 10.5281/zenodo.14008530)

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**Figures**

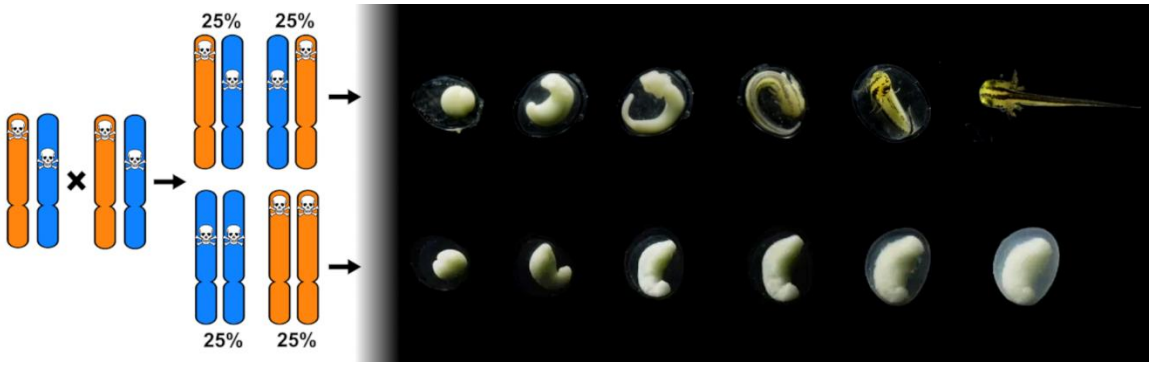


**Figure 1:** The balanced lethal system on *Triturus* chromosome 1. All adult crested and marbled newts carry two distinct versions of their largest chromosome (termed 1A and 1B, shown in orange and blue respectively). Each version carries unique, lethally recessive alleles. Mendelian segregation results in 50% of offspring inheriting two copies of the same version, but these embryos never hatch, instead suffering arrested embryogenesis and eventual death.

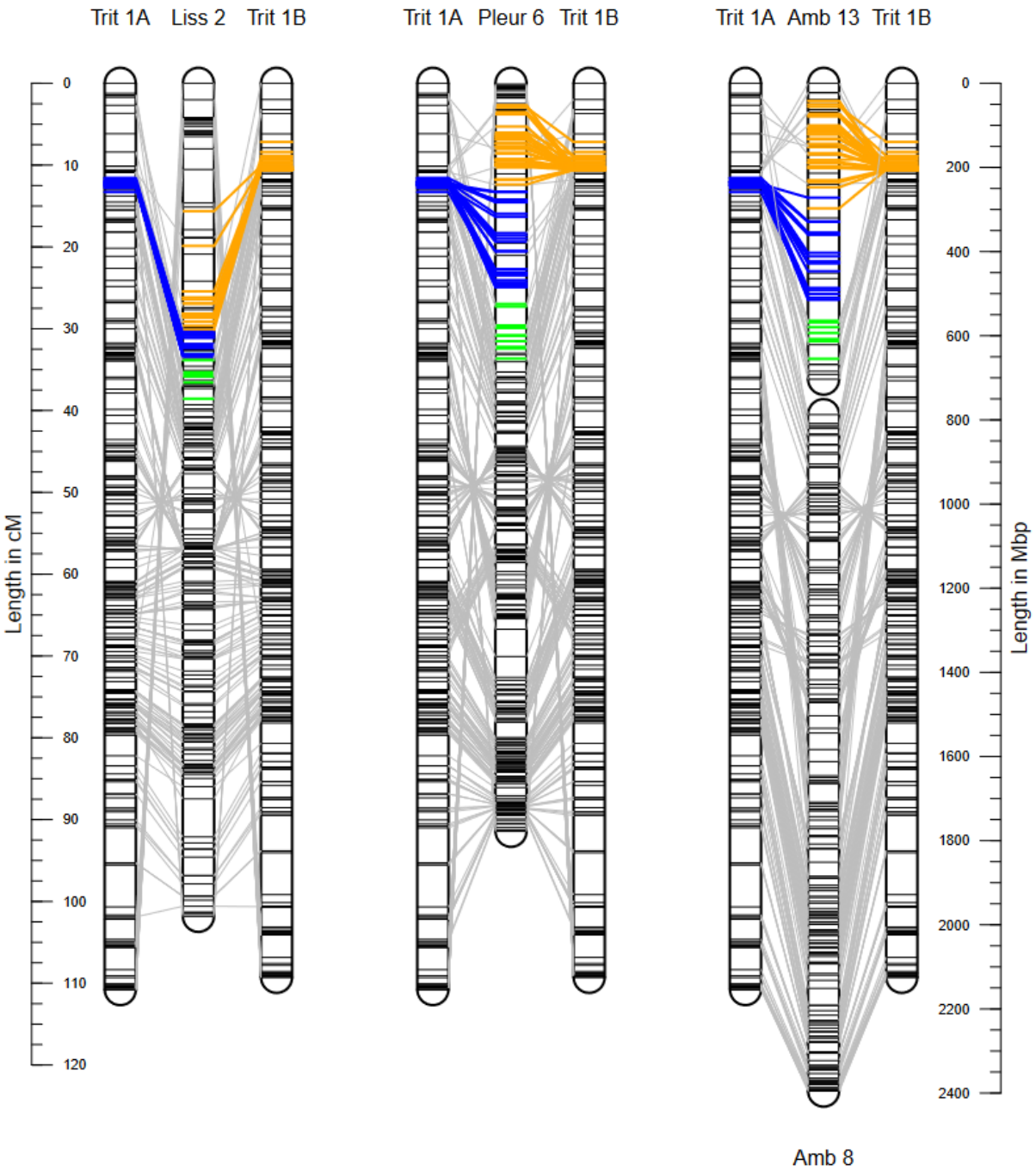


**Figure 2:** Groups 1A and 1B of the *Triturus* linkage map compared to the homologous group 2 of the *Lissotriton* linkage map, chromosome 6 of the *Pleurodeles waltl* genome assembly (Brown et al., 2023) and chromosomes 8 and 13 of the *Ambystoma mexicanum* genome assembly (Nowoshilow et al., 2018; Smith et al., 2019). Genes present only on chromosome 1A are highlighted in blue, those present only on 1B in orange. In all three comparisons the homologs of these genes present as two distinct adjacent blocks – which have each been entirely deleted from one of the two versions of chromosome 1 in *Triturus*. Genes shown in green form a third block, showing species specific chromosome 1 related variation (de Visser, France, Paulouskaya, et al., 2024).

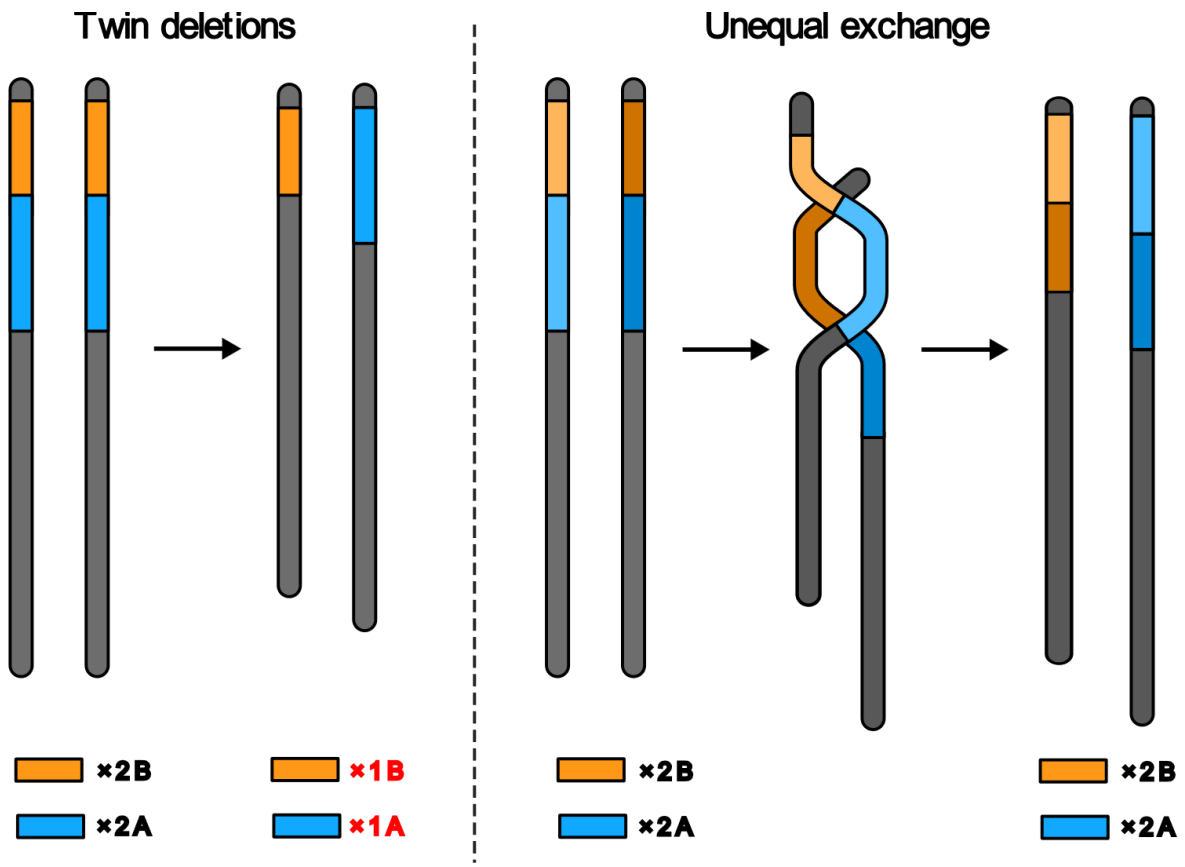


**Figure 3**: Contrasting scenarios of chromosome evolution in *Triturus* newts. If the absence of each set of balanced lethal system-related genes from one version of chromosome 1 is explained by a simple uncompensated deletion – as in the 'twin deletion' scenario to the left – the genes will be present in single copy, likely resulting in a significantly reduced fitness due to the presence of haploinsufficient genes (indicated by the 'x1' dosage effect in red). If, however, the deletions are part of an unequal exchange between chromosomes, as in the scenario shown to the right, they would be compensated for by duplication of the same stretch of DNA on the opposite chromosome, with no haploinsufficiency.

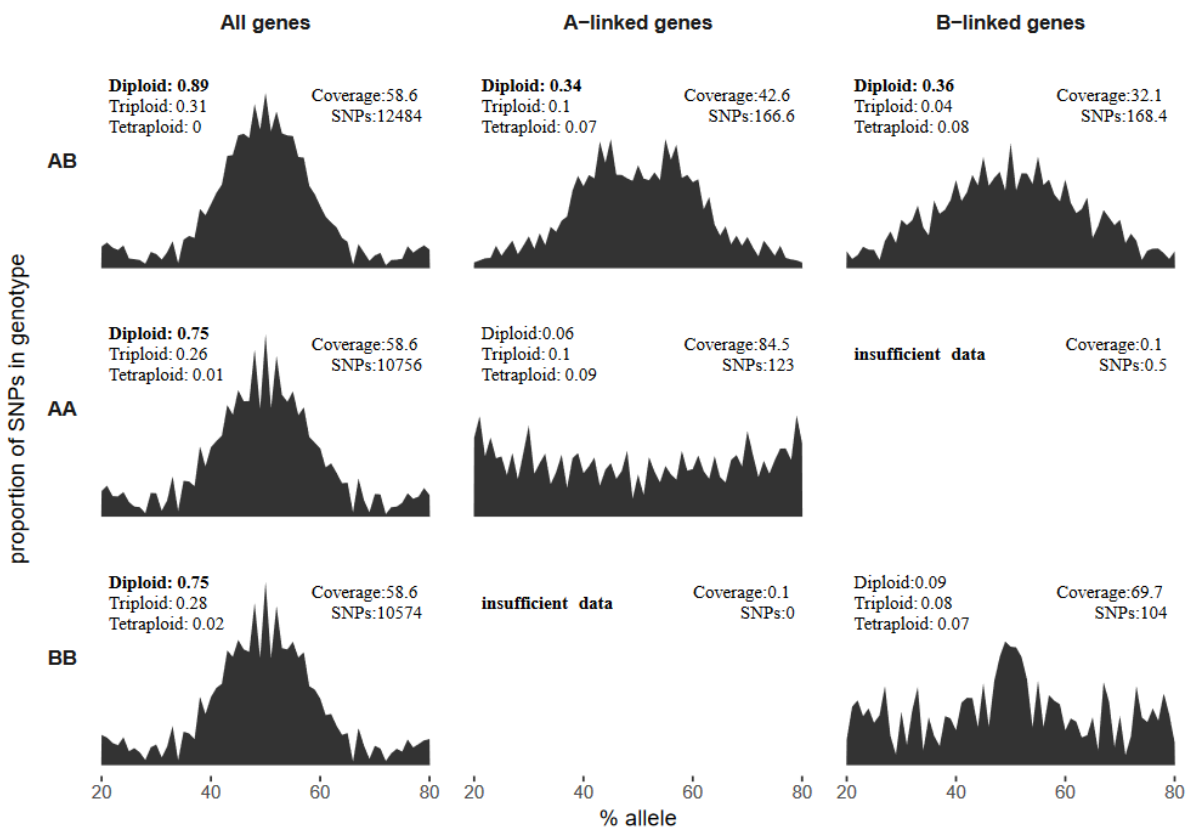


**Figure 4**: Profiles of allele ratios of three categories of genes (all 7,139 target sequences, 28 genes linked to chromosome 1A and 35 genes linked to chromosome 1B) based on target capture data of 30 $F_1$ *Triturus ivanbureschi* × *T. macedonicus* $F_1$ embryos, split evenly between three genotypes (AB, AA and BB), analysed with nQuire. Coefficient of determination ($R^2$) values comparing the observed results to idealised distributions for different copy numbers are displayed (bold where a significant ($p \leq 0.05$) majority (8/10) individual samples agree (sup table. S3). Normalised coverage and the average number of SNPs available for analysis per sample are also shown. In all three genotypes the overall genome showed a peak in allele ratio at 50%, as expected for a diploid genome and also as expected, in AA embryos insufficient data were available for analysis in B-linked genes and *vice versa*. However, in AB embryos, with only one copy each of chromosome 1A and 1B, SNPs that are A- and B-linked genes show allele ratio distributions consistent with diploidy, indicating that these genes are duplicated.

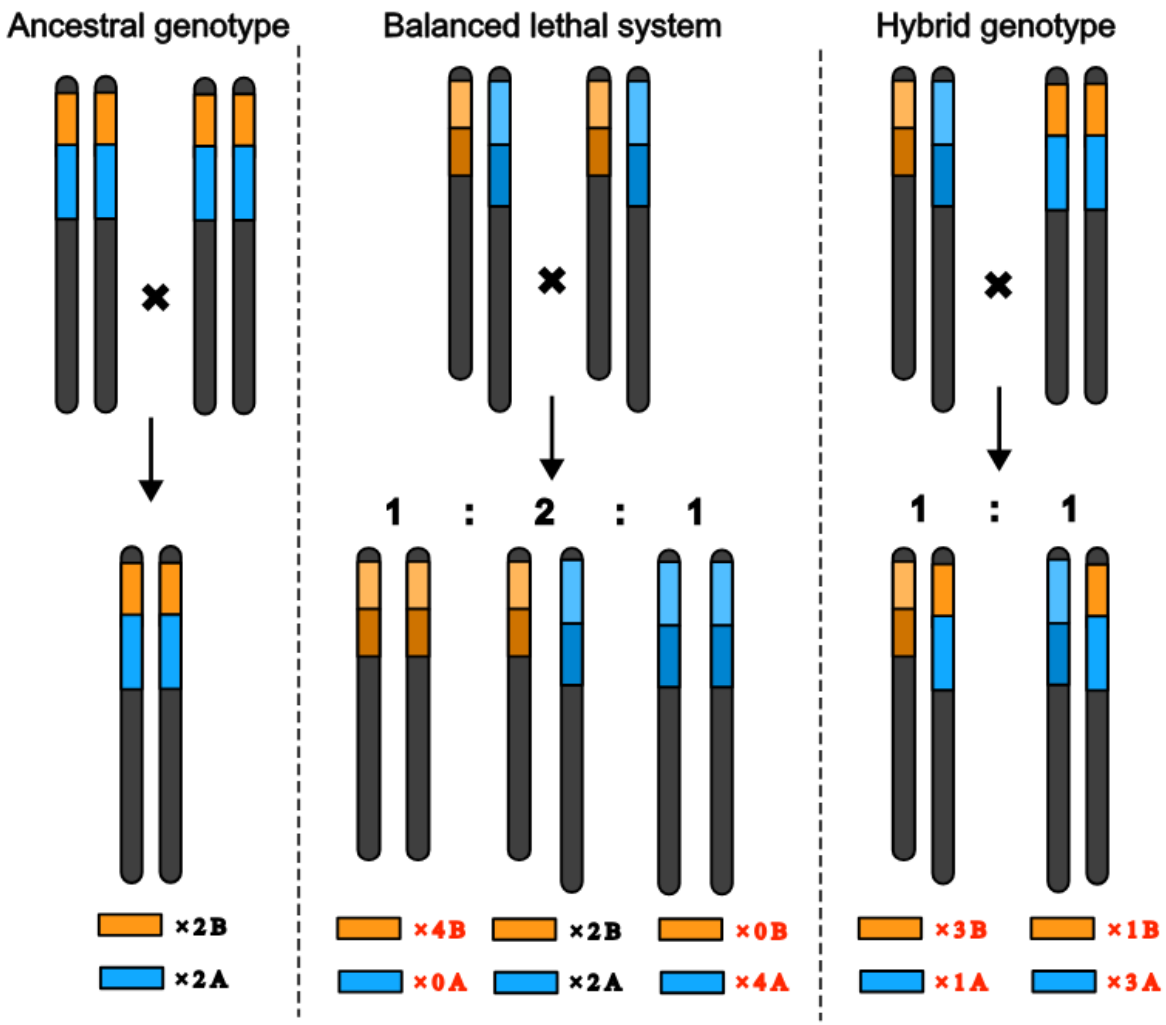


**Figure 5:** Consequences of the possible combinations of the rearranged and ancestral genotypes. Left: In the case of a cross between two individuals of the ancestral (pre-rearrangement) genotype, all offspring will inherit two copies of all genes. Middle: If both parents are affected by the balanced lethal system, then half of the offspring will inherit a full completement of genes, whereas the other half will have zero copies of either sets of genes involved and not be viable. Right: In a cross between a parent affected by the balanced lethal system and a parent with the ancestral genotype, no offspring will inherit two copies of any of the genes involved in the balanced lethal system. Instead, these genes will be present in a mixture of single and triple copies, as resulting from monosomy and trisomy, respectively. The resulting offspring may be viable, but will likely have reduced fitness.

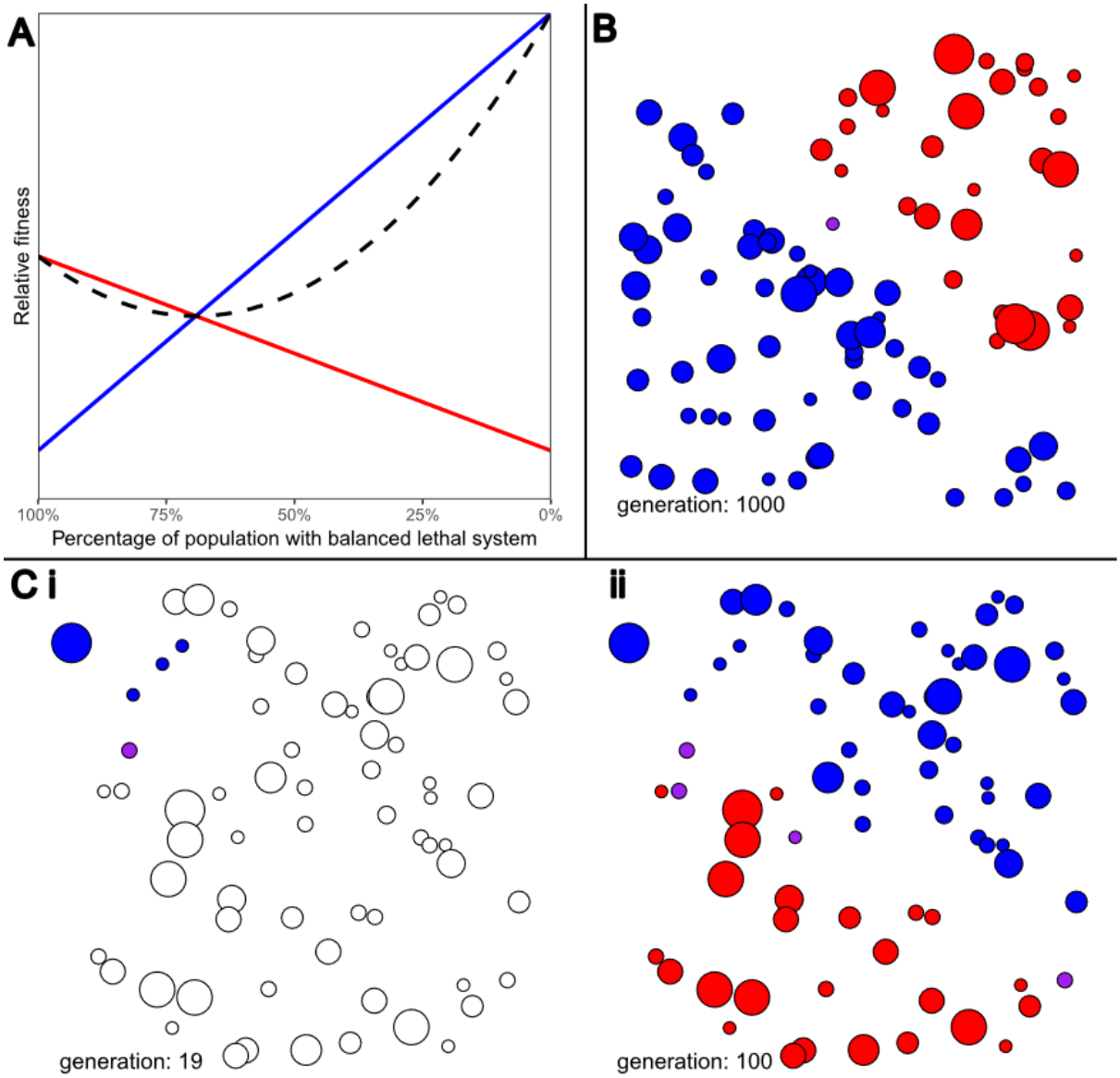


**Figure 6:**

Simple and detailed simulations of outbreeding depression between the rearranged and ancestral genomes. (A) plot showing the relative fitness of offspring from parents with the ancestral pre-rearrangement (blue) and (heterokaryotypic) balanced lethal (red) genotypes against the proportion of the ancestral chromosome in the population. If 'mixed' offspring (with one rearranged and one ancestral chromosome) have an average fitness lower than 0.5 there is a region in which the offspring of the parents of the balanced lethal system will have higher fitness than those of the ancestral chromosome. The average fitness of offspring is shown by the dashed black line with a minimum point, showing the effect of outbreeding depression. (B) Simulation of a landscape colonised by newts with the balanced lethal system (red) and ancestral chromosome (blue), with ponds where neither genotype represents at least

80% of the population shown in purple. Due to the lower fitness of the ‘mixed’ genotype, the ancestral chromosome is unable to displace the balanced lethal system and a stable hybrid zone has formed (sup. fig. S6). (C) Simulation of the initiation of a balanced lethal system (i) An initial population carrying the ancestral chromosome is founded at generation 0 in the large pond in the top-left of the region. After 19 generations, when this population has grown to 759, a single migrant mutates to the balanced lethal genotype (resulting in the small purple pond). (ii) 100 generations later the region is split between the ancestral and balanced lethal genotypes, with an apparently persistent hybrid zone formed (sup. fig. S7).