Comparative genomics of the balanced lethal system in Triturus newts

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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 to fixation in an entire genus? We construct 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 duplication of the same region on the opposite chromosome, suggesting 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 sub-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 equally instantaneous 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 defy 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), but only heterokaryotypic individuals (possessing both versions of the chromosome) are viable (Macgregor & Horner 1980; Sims et al. 1984). Because the chromosomes are 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, accounting for the 50% of non-viable embryos (see also chapter 1: Fig. 1). Each version of the chromosome must contain unique mutations that are lethal when homozygous. 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; Steiner 1956; James et al. 1990).

To maintain the heteromorphism required for the balanced lethal system, the two versions of the chromosome 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 in 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 behavioural 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 they confer when heterozygous (Hallar et al. 2007; Küpper et al. 2016). A balanced lethal system could simply be considered a supergene system where 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* × *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 Sup. 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. 2025) and 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 (Fig. 1, 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; Stewart & Wiens 2025) synteny is also highly conserved between *Triturus* and *P. waltl*. 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.)

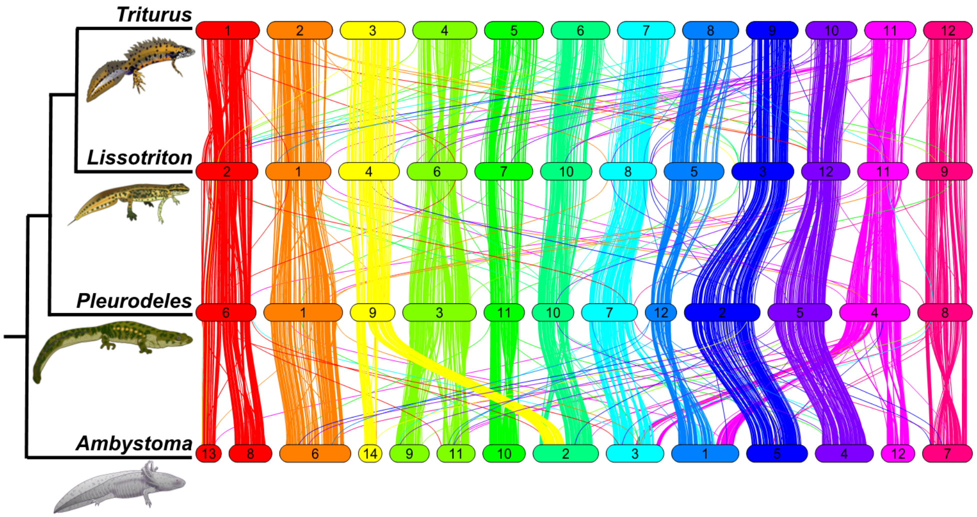

Figure 1: Comparison of the genomes of four salamander genera reveals striking conservation of synteny within newts. For *Triturus* and *Lissotriton* we construct linkage maps based on target capture data for ca. 4k coding sequences. For the more distantly related *Pleurodeles* and *Ambystoma* we incorporate data from published whole genome assemblies (Brown et al. 2025; Nowoshilow et al. 2018; Smith et al. 2019). We observe a striking conservation of synteny within the three newt genera (*Triturus*, *Lissotriton* and *Pleurodeles*), with all chromosomes showing one-to-one homology and little variation in gene order. When compared with *Ambystoma* we observe some fusions and translocations (Sup. Figs. S4-5)

Rather than SNPs specific to chromosome 1A or 1B, the variation between genotypes is primarily characterized by genes that are completely absent in one of the two categories of arrested embryos. We identify 30 markers 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. These two sets of genes are almost identical to those independently discovered by de Visser et al. (2024a) who also 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. de Visser et al. (2024a) also identify a third set of markers, which show presence/absence variation only in certain lineages of *Triturus*. We find that this set forms a third, smaller

(130 Mbp) non-recombing block, which we interpret as indicative of lineage specific post-establishment expansion of the original non-recombing region.

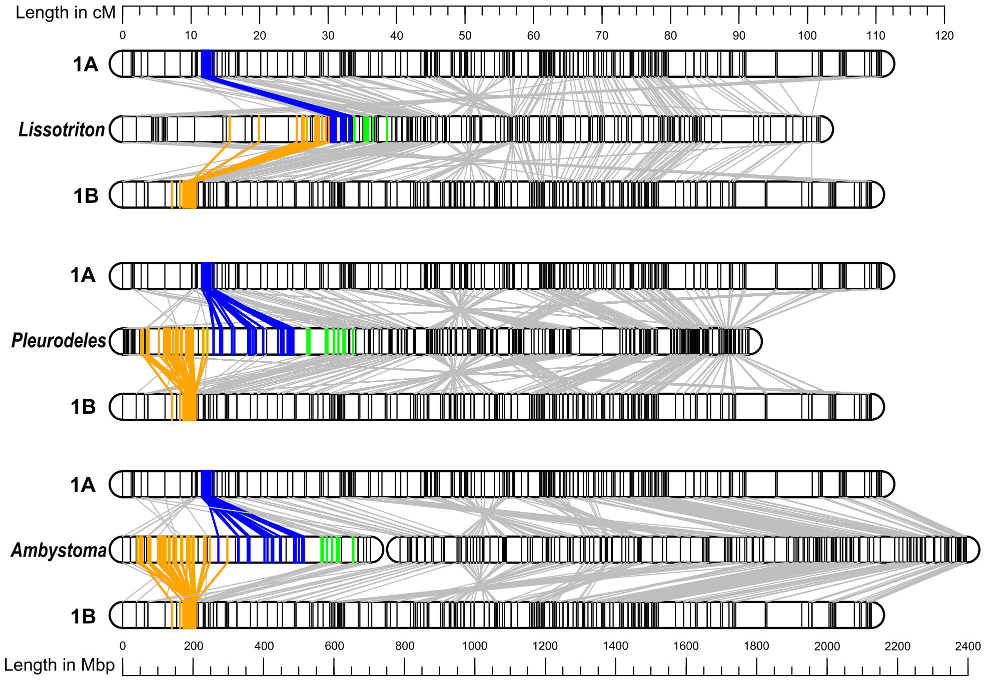

Figure 2: Groups 1A and 1B of the *Triturus* linkage map compared to the homologous group 2 of the *Lissotriton* linkage map, chromosome 6 of the *Pleurodeles waltl* genome assembly (Brown et al. 2025) and chromosomes 8 and 13 of the *Ambystoma mexicanum* genome assembly (Nowoshilow et al. 2018; Smith 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 et al. 2024a).

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 (Sessions et al. 1988; Kent et al. 2017).

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 would be insufficient to produce a normal phenotype – for reference, approximately 10% of human genes exhibit haploinsufficiency (Bartha et al. 2018). These dosage effects could 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, an unequal exchange between sister chromosomes (or possibly mitotic recombination between homologous chromosomes) would result in exactly this configuration, with the A-linked genes on one chromosome swapped for the B-linked genes on the other (Fig. 3).

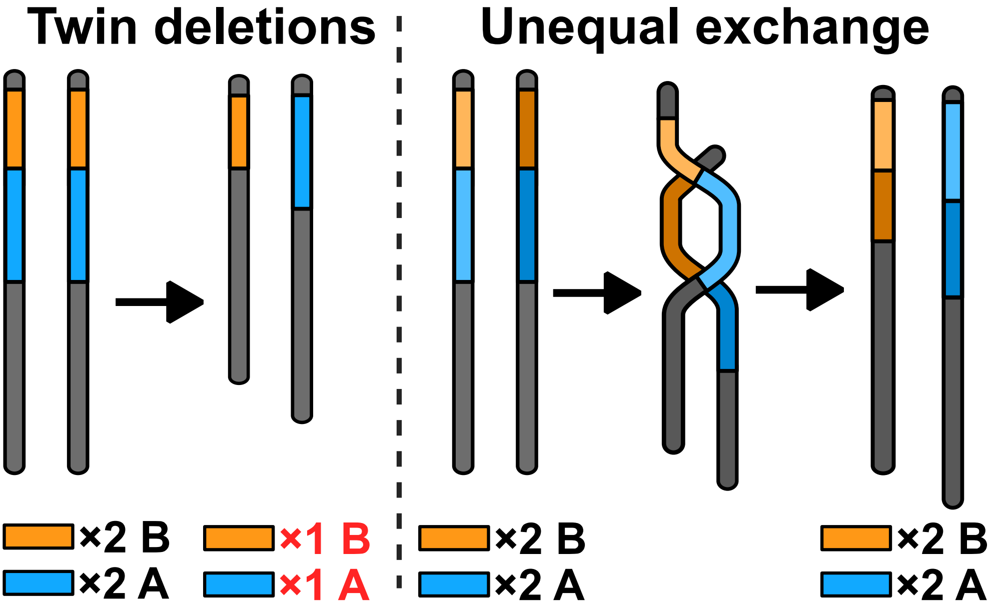

Figure 3: Contrasting scenarios of chromosome evolution in *Triturus* newts. If the absence of each set of balanced lethal system-related genes from one version of chromosome 1 is explained by simple uncompensated deletions (left) these will be left as single copy, which will reduce fitness if any of these exhibit haploinsufficiency. If the deletions are part of an unequal exchange (right), they will be compensated for by duplication of the same stretch of DNA on the opposite chromosome.

If the deleted regions in the *Triturus* balanced lethal system are compensated for by reciprocal duplications, then there will be 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) using data from a set of 30 F_1 *T. ivanbureschi* × *T. macedonicus* (10 for each genotype) (de Visser et al. 2024b). If there is only a single copy, then an allele can be present in either 0 or 100% of the reads 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%, strongly supporting diploidy for these genes and indicating that the A- and B-linked regions have indeed been duplicated.

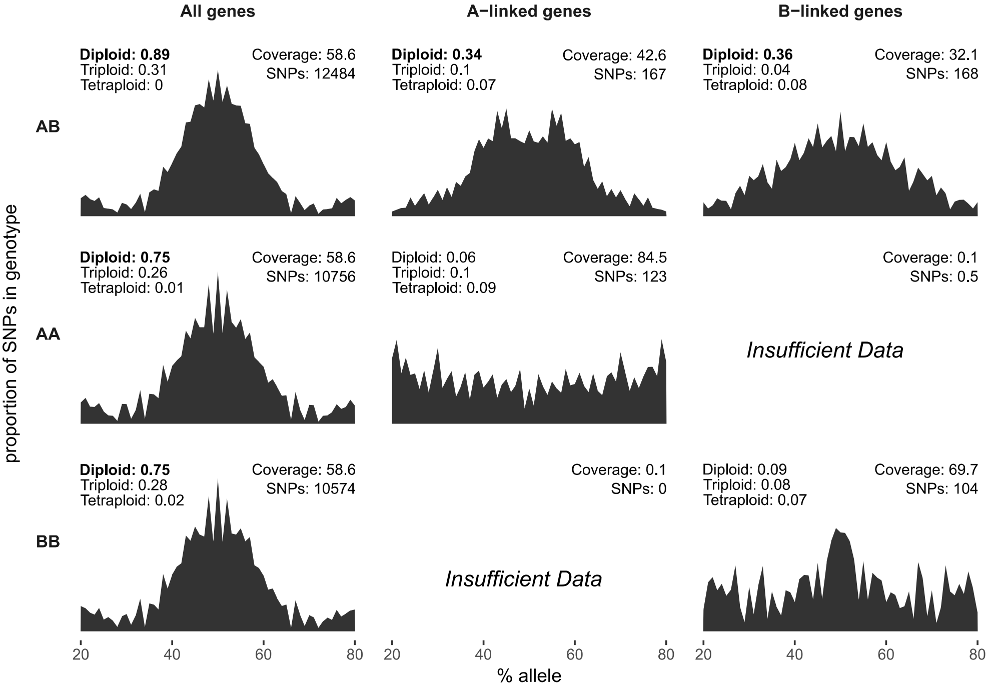

Figure 4: Profiles of allele ratios of three categories of genes (all 7,139 target sequences, 28 genes linked to chromosome 1A and 35 genes linked to chromosome 1B) based on target capture data of 30 F1 *Triturus ivanbureschi* × *T. macedonicus* F1 embryos, split evenly between three genotypes (AB, AA and BB). Coefficient of determination (R2) 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. 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.

In arrested embryos of genotype AA, there are two copies of chromosome 1A, so unless the duplication has occurred, we would expect a diploid distribution of SNP alleles in A-linked genes. 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 there are no copies of B-linked genes in the AA samples, we are unable to calculate allele ratios (Tables S3-5). The equivalent allele ratio distributions are found in reverse for the arrested embryos of genotype BB. These results match with the predictions of the reciprocal duplication. Accordingly, we conclude that the *Triturus* balanced lethal system arose in a single step, as the result of an unequal exchange.

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. Most 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, for *Triturus*, 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 favourable, 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.

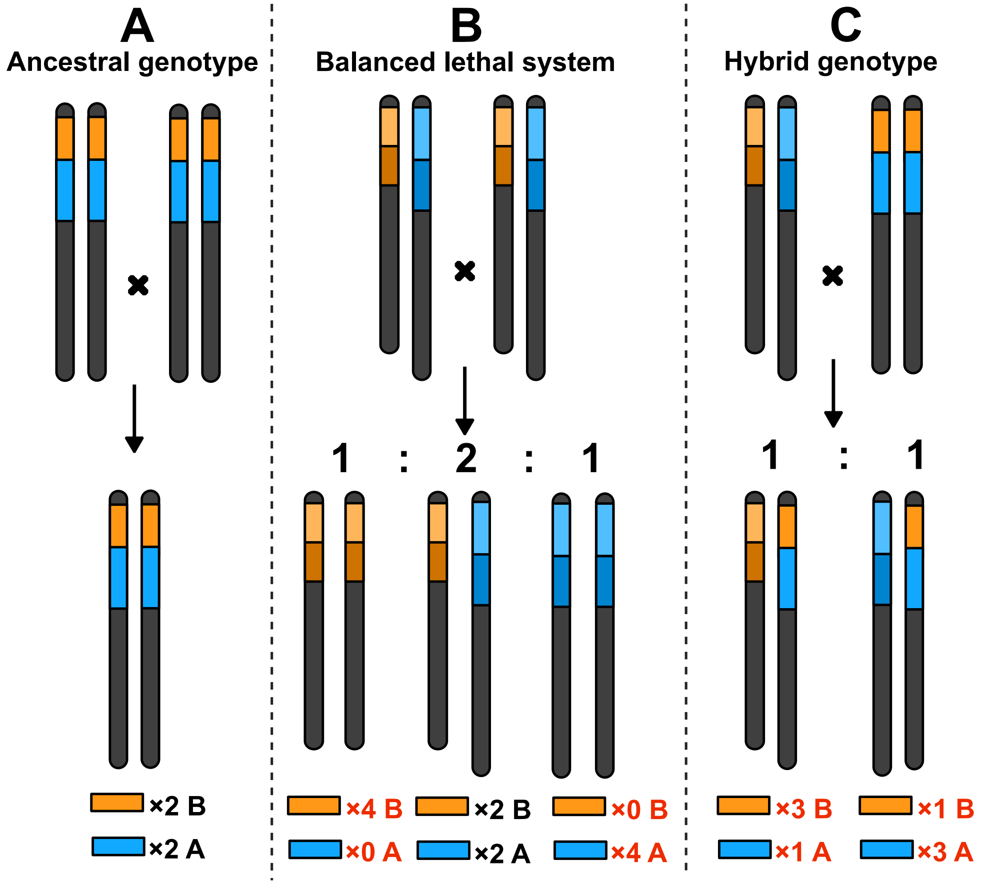

Fig. 4: Consequences of the possible combinations of rearranged and ancestral genotypes. (**A**) In the case of a cross between two individuals of the ancestral (pre-rearrangement) genotype, all offspring will inherit two copies of all genes. (**B**) If both parents are affected by the balanced lethal 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 will thus not be viable. (**C**) 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, likely significantly reducing fitness.

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. 6C). 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, 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).

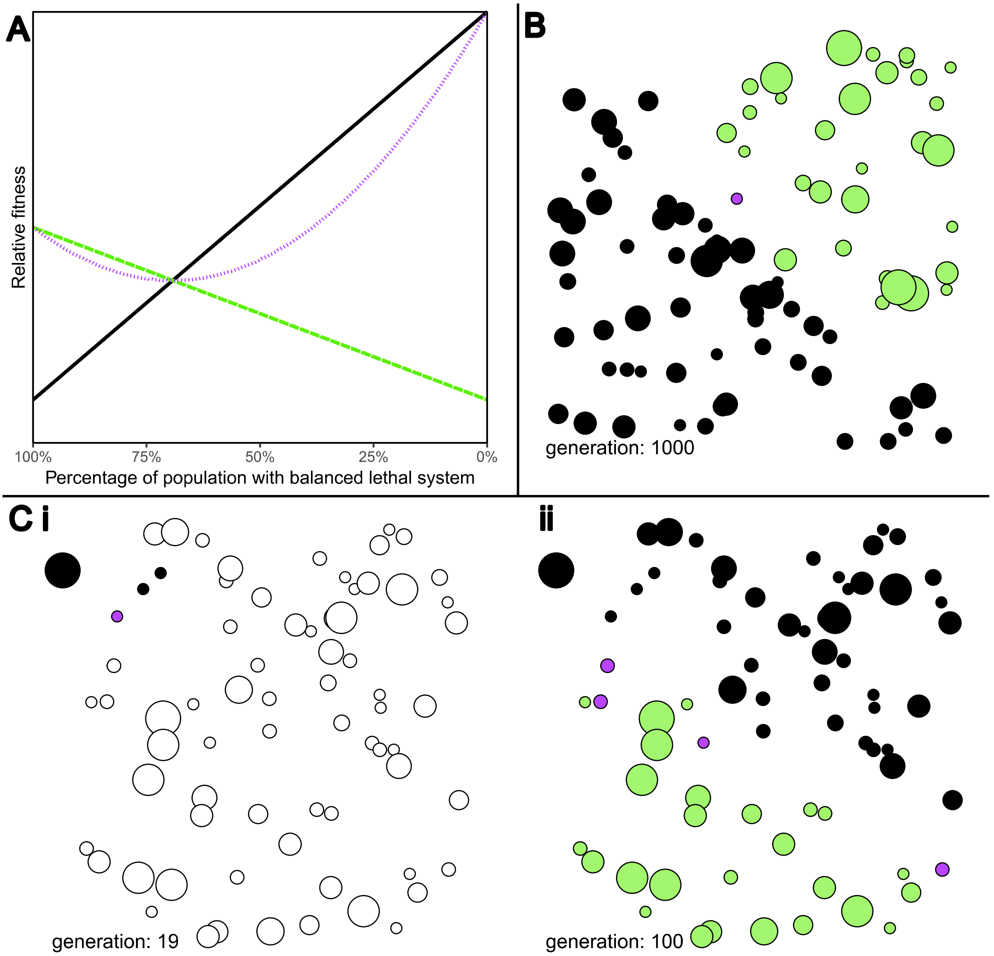

Figure 6: Simple and detailed models of outbreeding depression between the rearranged and ancestral genomes. **(A)** Plot showing the relative fitness of offspring from parents with the ancestral pre-rearrangement (black) and balanced lethal (green) genotypes against the proportion of the ancestral chromosome in the population. If 'mixed' offspring (with one rearranged and one ancestral chromosome) have an average fitness lower than 0.5, there is a threshold below 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 purple line with a minimum point, showing the effect of outbreeding depression. **(B)** Simulation of a persistent hybrid zone formed after secondary contact between populations carrying the balanced lethal system (green) and ancestral chromosome (black), 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 (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).

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 which 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 (Nag et al. 2004; Klein & O'Neill 2018) 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, Neuenschwander and Perrin, 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 unfavourable hybrid genotype, which requires that this genotype be 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. (2024a) 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.

Materials and Methods

Samples

For the *Triturus* linkage map a full-sibling family was bred at the University of Belgrade (Belgrade, Serbia). The experimental procedures were approved by the Ethics Committee of the Institute for Biological Research „Siniša Stanković", University of Belgrade (decisions no. 03-03/16 and 01-1949). The founder population consisted of two *T. macedonicus* males collected from Ceklin, Cetinje Municipality, Montenegro (42°21 N; 18°59 E) and two *T. ivanbureschi* females collect from Zli Dol, Pčinja District, Serbia (42°25 N; 22°27 E). Sampling from natural populations was approved by the Environmental Protection Agency of Montenegro (permit no. UPI-328/4) and Ministry of Energy, Development and Environmental Protection of the Republic of Serbia (permit no. 353-01-75/2014-08). From the F_0 founders a male-female (non-sibling) pair of F_1 *T. macedonicus* x *ivanbureschi* was raised to adulthood and mated producing 206 F_2 *T. macedonicus* x *ivanbureschi* offspring (73 hatchlings and 133 arrested embryos). For the *Lissotriton* linkage map an analogous family was bred at Jagiellonian University (Kraków, Poland). *Lissotriton* samples were collected in accordance with the Polish General and Regional Inspectorates of Environmental Protection permits DOP-OZGIZ.6401.02.25.2011.JRO, OP-I.6401.32.2020.GZ, GDOŚ DZP-WG.6401.24.2021.TŁ and all experiments were accepted by the I Local Ethical Committee for Animal Experiments in Kraków, permit 28/2011 and the II Local Ethical Committee for Animal Experiments in Kraków, permit 64/2020. A non-sibling male-female pair of F_1 *L. vulgaris* x *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. Full details of samples used in this study are available in Auxiliary Supplemental Table SA1 in the associated Zenodo repository (France et al. 2024a).

DNA extraction, library preparation and target capture sequencing

Laboratory protocols followed the NewtCap protocol (de Visser et al. 2024c). 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). Library concentration and fragment size distribution were measured via the Fragment Analyzer system (Agilent, Santa Clara, CA, USA) before the 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_0t-1 DNA to block the hybridization of repetitive sequences. Tissue to produce C_0t-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 B.V. (Leiden, the Netherlands).

Processing of sequence capture data

Bioinformatics and analyses were performed in the Academic Leiden Interdisciplinary Cluster Environment (ALICE) at Leiden University. The upstream data processing was performed via a custom Perl (version v3.38.0) script (Pipeline_1.pl). FASTQ files containing demultiplexed raw sequence data were trimmed with Trimmomatic version 0.39 (Bolger et al. 2014) and BBDuk version 38.96 (Bushnell et al. 2017) to remove adapter contamination. BWA-MEM version 0.7.17 (Li 2013) was used to map the trimmed reads against reference sequences previously assembled from *T. dobrogicus* (Wielstra et al. 2019). The resulting BAM files were processed, deduplicated and genotyped via the *AddOrReplaceReadGroups*, *MarkDuplicates* and *HaplotypeCaller* functions of GATK version 4.5.0.0 (McKenna et al. 2010) producing a VCF file for each sample. Sequencing depth was assessed with a custom R (version 4.4) script (Peakloop2.R) which processed the output of the SAMtools version 1.18 (Li et al. 2009) *depth* function of the deduplicated BAM file for each sample and evaluated the minimum depth of the best covered continuous 100 bp sequence within each target sequence of the reference assembly. All samples used for construction of the linkage maps were required to have a median best 100 bp sequencing depth of at least 10. After samples had been screened for coverage, each sample set was jointly genotyped with the *GenomicsDBImport* and *GenotypeGVCFs* function of GATK, producing a multi-sample GVCF file for each linkage family.

Lissotriton linkage map construction

To construct the *Lissotriton* linkage map, VCFtools version 0.1.16 (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 version 0.5 (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 22 and distortion LOD set to 1, unplaced markers were then incorporated with the *JoinSingles2All* module with a LOD limit of 15 (these settings were selected to maximize the numbers of included markers while yielding 12 linkage groups – the number of linkage groups increases rapidly as the LOD limit is raised until plateauing at 12 at LOD = 22, whereafter further increases only decrease the number of mapped markers). The markers were then ordered with the *OrderMarkers2* module, using 12 merge iterations and eight polish iterations, with the sexAveraged option enabled and the minError parameter set to 0.01. After construction the linkage groups were redesignated in order of decreasing length.

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

The per-marker 100 bp peak region sequencing depth was used to identify presence/absence of markers in arrested embryos using a custom R script (Select_presence_absence_1.R). This first calculates expected sequencing depth scores for all markers across the sample set (based on the product of the mean depth per sample and the mean depth per marker), before identifying markers in which at least 25% of samples show a depth of zero, while an equivalent number of samples show more than double the expected depth (the predicated behaviour of presence/absence markers associated with either chromosome 1A or 1B). The scripts then clusters candidate presence/absence markers into sets of where the same samples show zero coverage. As at this stage the genotype of the arrested embryos was unknown, used the findings of de Visser, et al. (2024a) – based on phenotyped embryos - as a guide to designate each cluster as A- or B-linked, following the terminology of Macgregor & Horner (Macgregor et al. 1980) where 1A1A embryos are dubbed "slim-tailed" and 1B1B embryos "fat-tailed".

Triturus linkage map construction

The *Triturus* linkage map was constructed via the methodology described above, modified to incorporate the presence/absence markers. To this end the coverage scores of these markers were converted into probabilistic pseudo-SNP calls using a custom R script (Add_presence_absence_to_call_table_4.R). 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 of LepMAP3 (Rastas 2017). From this point the LepMAP3 pipeline was used with the same settings as with *Lissotriton*, with the expected that the LOD limits for the SeparateChromosomes2 and JoinSingles2All modules were changed to 27 and 20 respectively (as with *Lissotriton* these settings were chosen as the point where the number of linkage groups plateaus at 12). 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 *Pleurodeles* and *Ambystoma* genome assemblies

The *Triturus* reference sequences placed on the linkage map were aligned against the against the genome assemblies published for the Iberian ribbed newt, *Pleurodeles waltl* (Brown et al. 2025) and the axolotl, *Ambystoma mexicanum* (Nowoshilow et al. 2018; Smith et al. 2019) using BLAST+ version v2.14.1 (Camacho et al. 2009). For *P. waltl* the default setting were used except for specifying a minimum E-value of 1e-20 for the more distantly related *A. mexicanum* a minimum E-value of 1e-10 and word size of 15 was used. 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 from a previous study (de Visser et al. 2024b) (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. The BAM files produced for each sample were subsetted 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 (de Visser et al. 2024a). These alignments were processed with nQuire (cloned from Git commit 8975c94) (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. The R^2 values comparing the fit of the observations to calculated allele distributions were averaged for each sample genotype and gene-category. Significant of agreement of best fit model was calculated with $p \leq 0.05$ requiring at least eight out the ten individuals in each genotype category to agree. For additional context mean coverage data from the same gene and genotypes categories of the F_2 *T. macedonicus* x *ivanbureschi* sample set calculated.

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 raw sequence data used in this study is available via at the Sequence Read Archive, associated with BioProject PRJNA1175462 (France et al. 2024b). All code used in this study as well as markdown documents detailing all commands used in the workflow are archived at this study's Zenodo repository, 10.5281/zenodo.14008529 (France et al. 2024a), together with detailed sample information and an .xlsx document detailing the sequences and positions of all markers located on the *Triturus* and *Lissotriton* linkage maps.

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Supporting Information

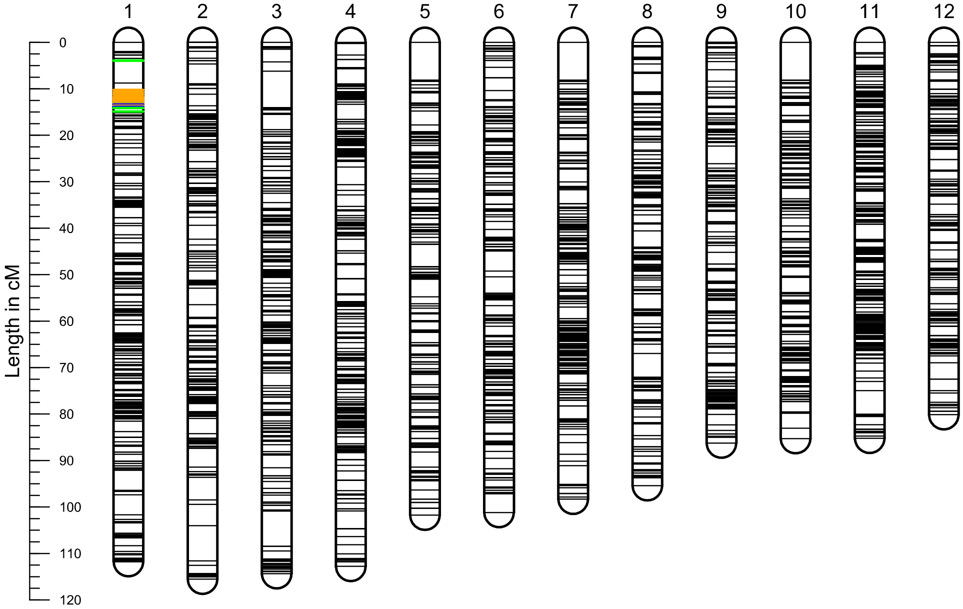

Figure S1: Linkage map based on target capture data from a full-sibling F_2 *Triturus ivanbureschi* × *macedonicus* family, consisting of four F_0 grandparents, two F_1 parents and 206 F_2 offspring. The map includes 4226 markers in 12 linkage groups spanning a total length of 1188 cM. Linkage groups are arranged by length, except for group 1, which is designated in accordance with the chromosome 1 linked presence/absence markers we located within it. Group 1 includes 29 A-linked markers, highlighted in blue and 33 B-linked makers highlighted in orange, 43 of these markers map to a single position at 13.659 cM from the group's origin, with the reminder deviating by up to 2 cM .This deviation is likely an artifact caused by translating the presence/absence data of low coverage markers into pseudo-SNP calls, with perfect data we would expect all markers to collapse to a single point. An additional 12 markers, highlighted in green, were independently discovered to be associated with the balanced lethal systems in other *Triturus* species in a separate study (de Visser et al. 2024a) – ten of these cluster within 1 cM of the presence/absence markers, while the other two are displaced by approximately 10 cM, possibly because they show presence/absence variation in *T. macedonicus* but not *T. ivanbureschi*.

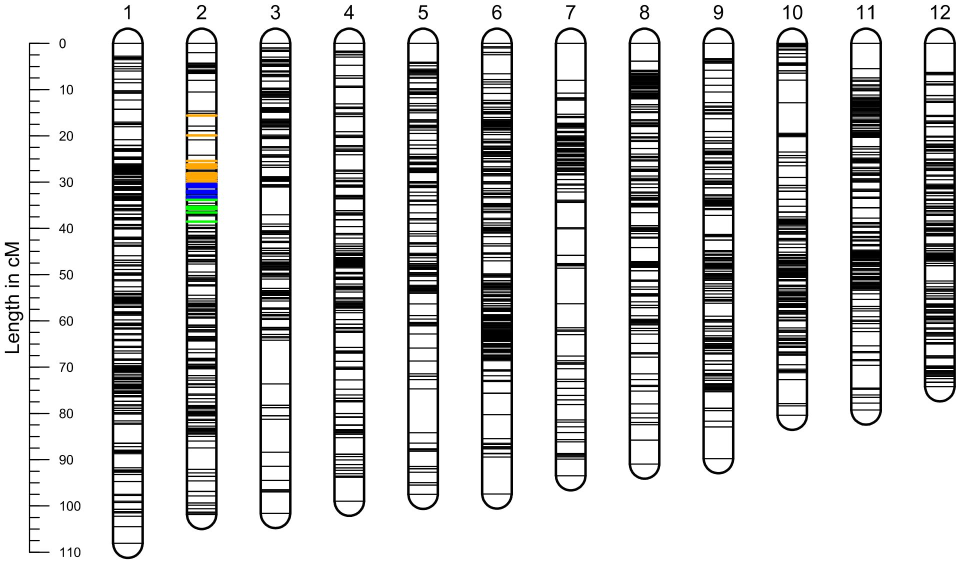

Figure S2: Linkage map based on target capture data from a full-sibling F_2 *Lissotriton vulgaris* × *montandoni* family, consisting of two F_1 parents and 203 F_2 offspring. The map includes 3693 markers in 12 linkage groups spanning a total length of 1113 cM. In *Lissotriton* the homologs of genes associated with the *Triturus* balanced lethal system are found in linkage group 2, where they form distinct blocks, corresponding to genes present only in *Triturus* chromosome 1A or 1B (highlighted in blue and orange respectively). A third block of genes (highlighted in green) show either species specific presence/absence variation within *Triturus* (de Visser et al. 2024a) or extreme heterozygosity specifically in viable embryos (indicating there are two distinct alleles, each associated with only one form of *Triturus* chromosome 1).

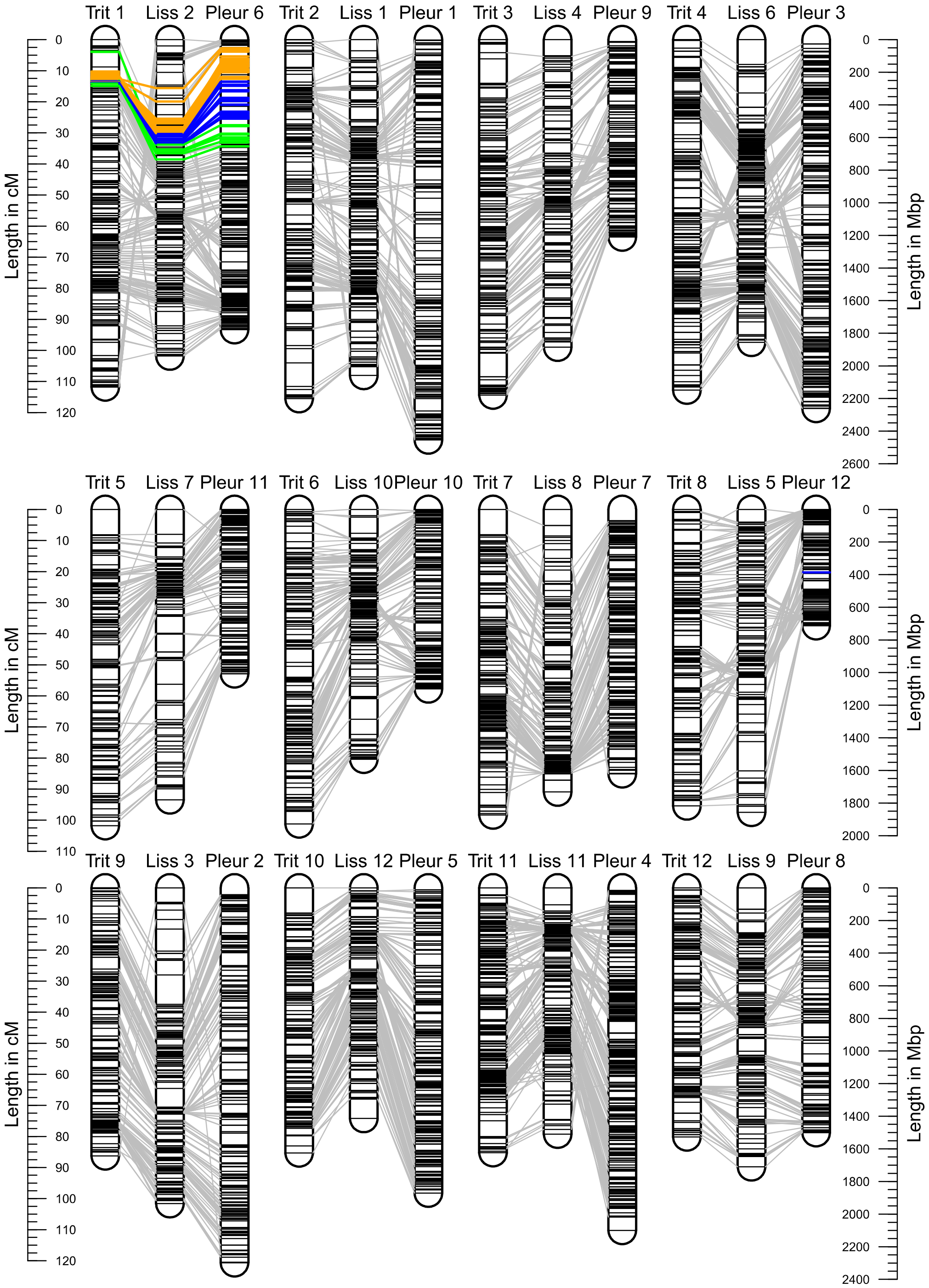

Figure S3: Homology between the *Triturus* and *Lissotriton* linkage maps and the *Pleurodeles waltl* genome assembly (Brown et al. 2025). *Triturus* chromosome 1 linked markers are highlighted: A-linked in blue, B-linked in orange and species specific in green.

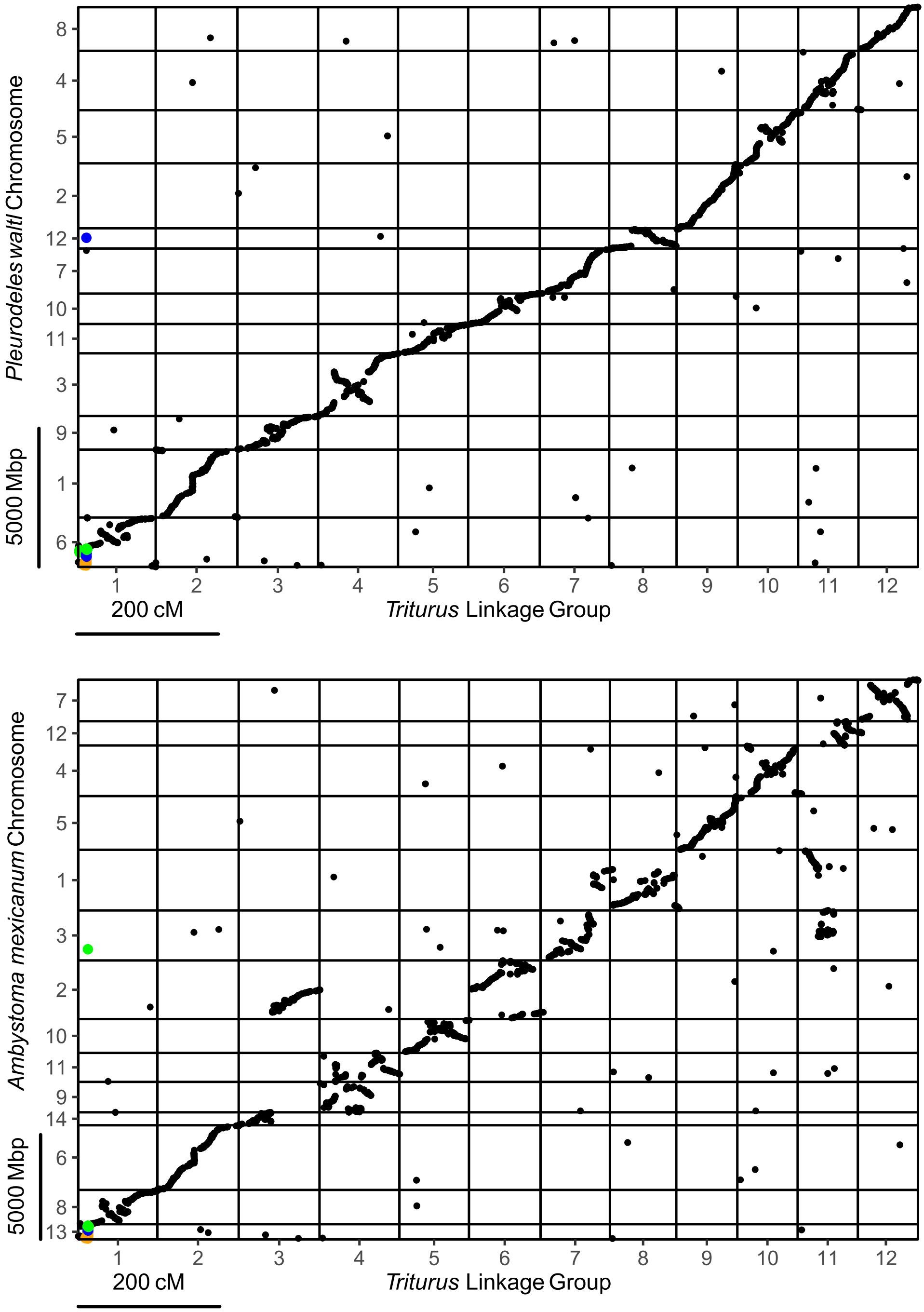

Figure S4: Oxford plots showing locations of markers within the *Triturus* linkage map and genome assemblies for *Pleurodeles waltl* (Brown et al. 2025) and *Ambystoma mexicanum* (Nowoshilow et al. 2018; Smith et al. 2019). Synteny is extremely tightly conserved between the two newt taxa, but less so in the more distantly related axolotl.

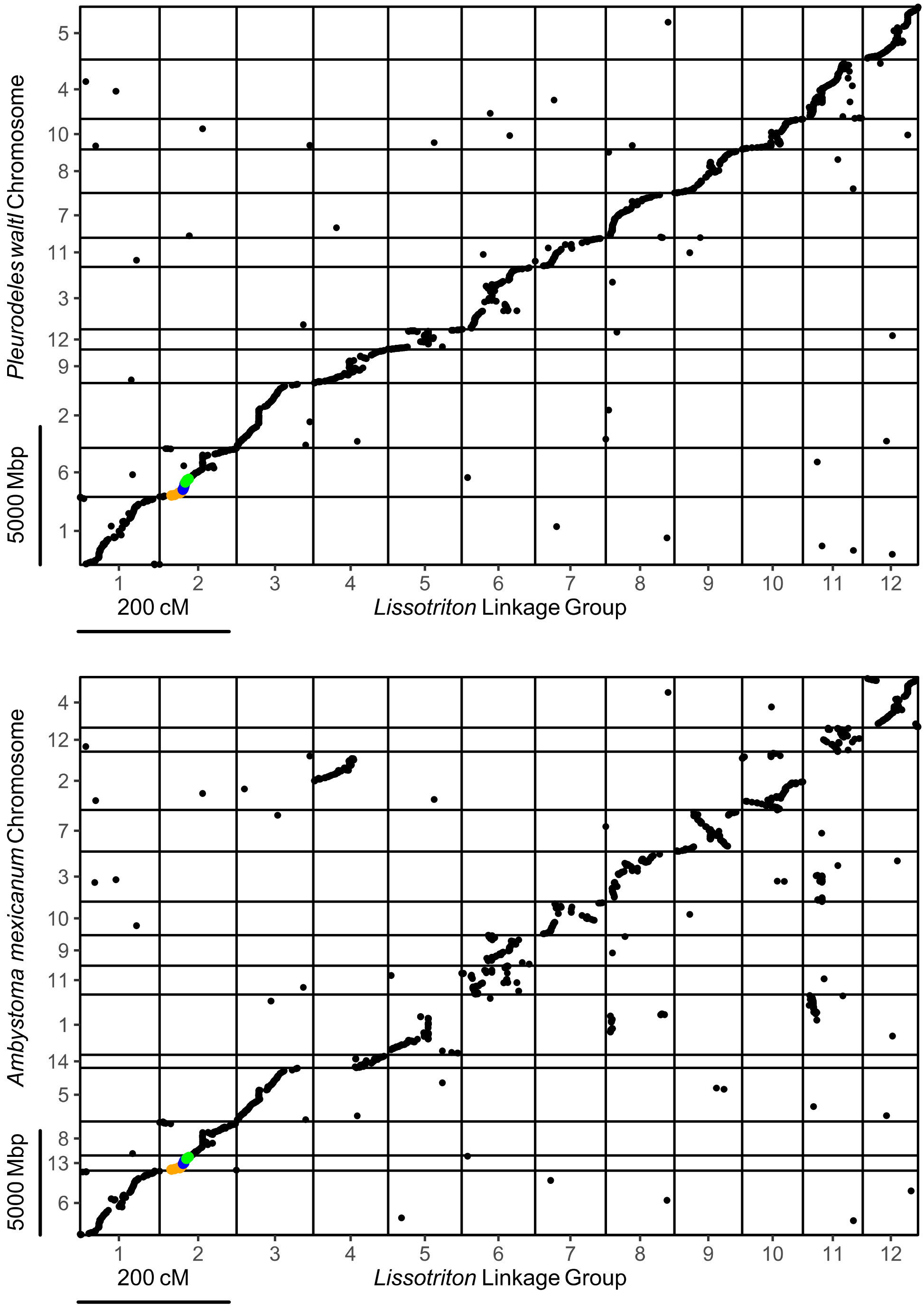

Figure S5: Oxford plots showing marker locations within the *Lissotriton* linkage maps compared to the *Pleurodeles waltl* (Brown et al. 2025) and *Ambystoma mexicanum* (Nowoshilow et al. 2018; Smith et al. 2019) genome assemblies.

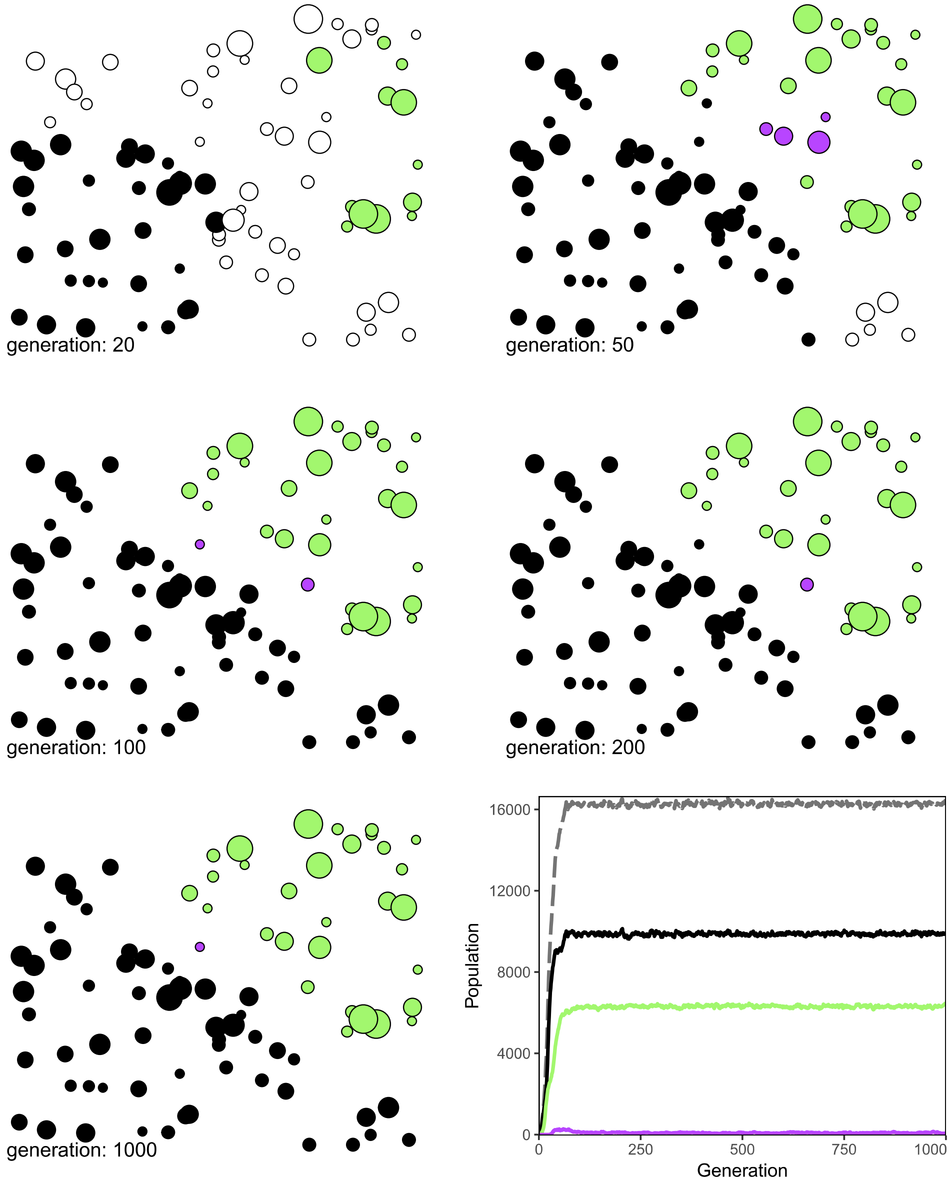

Figure S6: Simulation of secondary contact between the ancestral and balanced lethal population -shown in black and green respectively, with populations where less than 80% of individuals are of one genotype shown in purple. Despite the apparent fitness disadvantage of the balanced lethal population the effects of underdominance in chromosome 1 allows a persistent hybrid zone to form in an area that has low potential population density. Overall population quickly reaches a plateau after all ponds are colonised at approximately generation 100 (Total population shown in dashed grey, ancestral in black, balanced lethal in green and hybrid in purple).

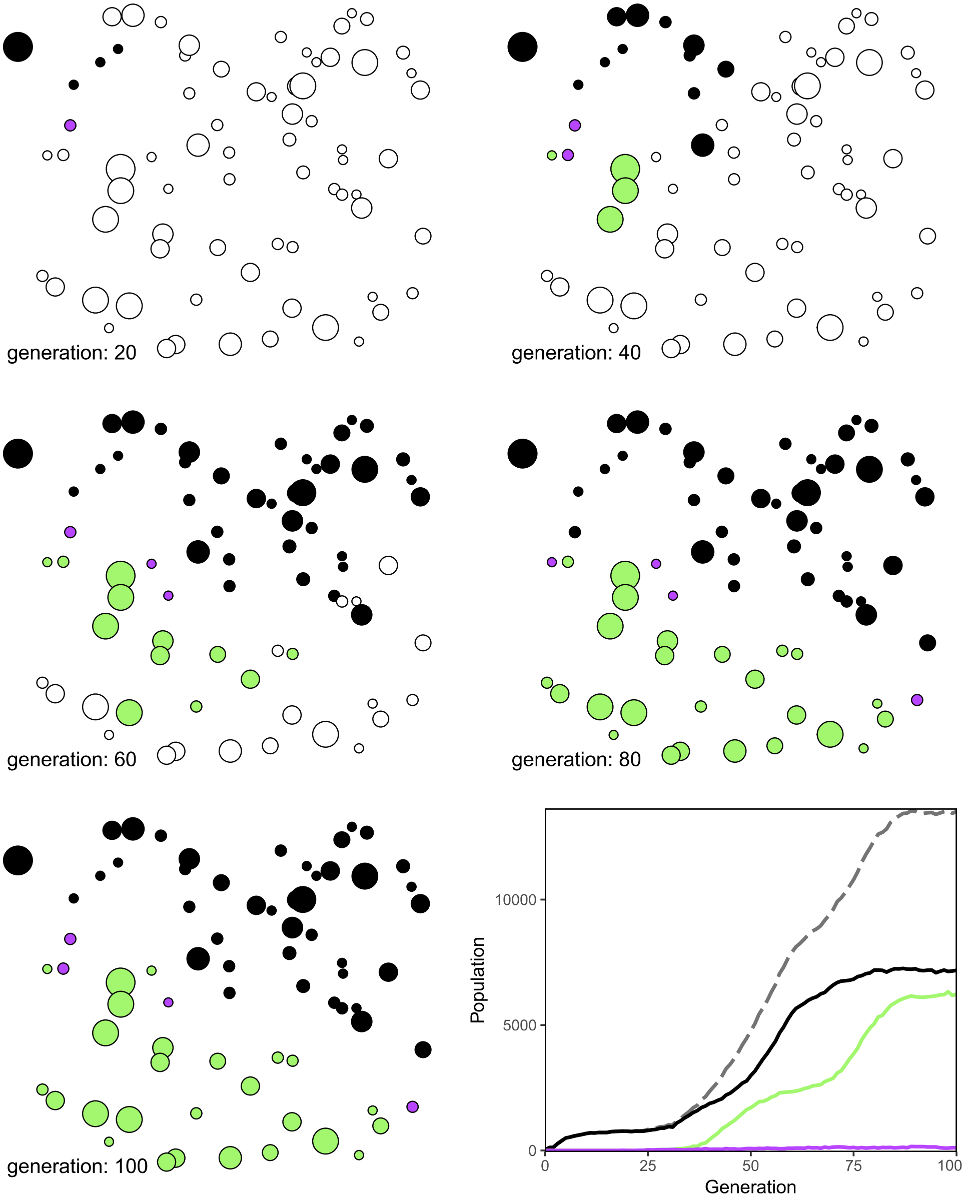

Figure S7: Simulation of evolution of a balanced lethal population from a single mutation at generation 19. By generation 100 all ponds have been colonised, and a hybrid zone has been formed between the ancestral and balanced lethal system population.

	Lissotriton		***Triturus***	
Group	**Number of markers**	**Length (cM)**	**Number of markers**	**Length (cM)**
1	410	108.1	399	111.8
2	343	101.8	335	115.5
3	257	101.6	339	114.4
4	259	99.0	471	112.8
5	239	97.5	290	101.8
6	465	97.4	299	101.2
7	167	93.5	388	98.3
8	302	90.9	284	95.4
9	263	89.8	322	86.2
10	305	80.4	309	85.3
11	409	79.3	518	85.2
12	274	74.2	272	80.1
Total	**3693**	**1113.5**	**4226**	**1188.1**

Table S1: Characteristics of the target capture linkage maps produced for *Triturus* and *Lissotriton*

	***Triturus* Linkage map**	
	Common Loci	**Loci on Homologous Chromosomes**
Lissotriton	2551	2497 (97.8%)
P. Waltl	3400	3359 (98.7%)
A. mexicanum	2816	2408 (85.5%)

Table S2: The number of loci identified on the *Triturus* linkage map compared to the number of homologs found within the *Lissotriton* linkage map and the *P. waltl* and *A. mexicanum* genome assemblies (out of a total of 4226 loci placed on the *Triturus* map). The proportion of loci mapping to homologous chromosomes is very high in *Lissotriton* and *P. Waltl*, but lower in *A. mexicanum* due to several rearrangements.

Sample and Genotype	***R^2* scores**								
	All Markers (n = 7139)			**A-linked Markers (n = 28)**			**B-linked Markers (n = 35)**		
	Diploid	**Triploid**	**Tetraploid**	**Diploid**	**Triploid**	**Tetraploid**	**Diploid**	**Triploid**	**Tetraploid**
BW_0024 **AB**	**0.94**	*0.36*	*0.01*	**0.26**	*0.01*	*0.20*	**0.48**	*0.03*	*0.05*
BW_0025 **AB**	**0.91**	*0.32*	*0.00*	**0.61**	*0.10*	*0.03*	**0.16**	*0.00*	*0.20*
BW_0026 **AB**	**0.93**	*0.36*	*0.01*	**0.40**	*0.19*	*0.06*	**0.27**	*0.00*	*0.08*
BW_0027 **AB**	**0.94**	*0.30*	*0.00*	**0.50**	*0.16*	*0.07*	**0.28**	*0.00*	*0.15*
BW_0028 **AB**	**0.88**	*0.29*	*0.00*	**0.26**	*0.03*	*0.13*	**0.23**	*0.02*	*0.08*
BW_0029 **AB**	**0.85**	*0.31*	*0.00*	*0.00*	*0.00*	**0.09**	**0.43**	*0.19*	*0.01*
BW_0030 **AB**	**0.78**	*0.20*	*0.01*	**0.35**	*0.17*	*0.02*	**0.46**	*0.09*	*0.02*
BW_0031 **AB**	**0.83**	*0.28*	*0.00*	**0.27**	*0.23*	*0.00*	**0.43**	*0.05*	*0.09*
BW_0051 **AB**	**0.92**	*0.34*	*0.00*	**0.61**	*0.07*	*0.01*	**0.38**	*0.02*	*0.01*
BW_0052 **AB**	**0.91**	*0.32*	*0.00*	*0.09*	*0.00*	**0.10**	**0.52**	*0.02*	*0.11*
BW_0040 **AA**	**0.45**	*0.13*	*0.01*	*0.04*	*0.02*	**0.07**	NA		
BW_0041 **AA**	**0.38**	*0.06*	*0.02*	*0.11*	**0.38**	*0.08*	NA		
BW_0042 **AA**	**0.46**	*0.16*	*0.01*	**0.03**	*0.01*	*0.01*	NA		
BW_0043 **AA**	**0.72**	*0.20*	*0.01*	**0.07**	*0.05*	*0.04*	NA		
BW_0044 **AA**	**0.94**	*0.37*	*0.01*	*0.02*	*0.13*	**0.22**	*0.03**	*0.00**	*0.01**
BW_0045 **AA**	**0.94**	*0.31*	*0.01*	**0.21**	*0.17*	*0.00*	NA		
BW_0046 **AA**	**0.91**	*0.32*	*0.00*	*0.01*	**0.02**	*0.01*	NA		
BW_0047 **AA**	**0.90**	*0.34*	*0.00*	**0.02**	*0.01*	*0.00*	NA		
BW_0064 **AA**	**0.90**	*0.35*	*0.00*	*0.04*	*0.15*	**0.22**	*0.02**	*0.03**	*0.09**
BW_0065 **AA**	**0.93**	*0.34*	*0.01*	*0.08*	*0.03*	**0.24**	NA		
BW_0032 **BB**	**0.53**	*0.22*	*0.01*	NA			**0.16**	*0.00*	*0.01*
BW_0033 **BB**	**0.47**	*0.10*	*0.05*	NA			*0.02*	*0.01*	**0.19**
BW_0034 **BB**	**0.57**	*0.18*	*0.00*	NA			*0.02*	*0.02*	**0.03**
BW_0035 **BB**	**0.42**	*0.11*	*0.03*	NA			**0.10**	*0.05*	*0.01*
BW_0036 **BB**	**0.83**	*0.31*	*0.00*	NA			**0.38**	*0.01*	*0.00*
BW_0037 **BB**	**0.93**	*0.42*	*0.03*	NA			*0.20*	*0.19*	**0.21**
BW_0038 **BB**	**0.91**	*0.33*	*0.00*	NA			*0.02*	**0.25**	*0.08*
BW_0039 **BB**	**0.93**	*0.36*	*0.01*	NA			*0.01*	**0.20**	*0.11*
BW_0056 **BB**	**0.92**	*0.35*	*0.00*	NA			*0.00*	*0.02*	**0.04**
BW_0057 **BB**	**0.95**	*0.37*	*0.02*	NA			*0.01*	**0.07**	*0.03*

Table S3: Per sample R^2 values showing the best fit ploidy model (highlighted in green with bold text) for all genes, A-linked genes and B-linked genes in 30 F_1 *T. ivanbureschi* × *macedonicus* samples (de Visser et al. 2024b), split evenly between the three chromosome 1 genotypes. For BB samples no A-linked loci whatsoever were available for analysis. In two AA samples R^2 values (marked with *) could be calculated for reads mapped to a single B-linked marker, but this is not sufficient data to produce a meaningful result, and these reads likely represent artifacts.

	All Markers		A-linked Markers		B-linked Markers	
Sample Genotype	**Total Genes**	**Total SNPs**	**Total Genes**	**Total SNPs**	**Total Genes**	**Total SNPs**
AB	4539.2	12484.5	15.9	166.6	19.1	168.4
AA	4130.4	10756.1	22.8	123.2	0.2	0.5
BB	4055.8	10574.1	0.0	0.0	22.4	104.0

Table S4: Average number of markers and SNPs (averaged across the 10 F_1 *T. ivanbureschi* × *macedonicus* samples of each genotype) used to calculate R^2 values for ploidy models for each of three categories of markers.

	Coverage					
Sample	**All Markers**		**A-linked Markers**		**B-linked Markers**	
Genotype	**Raw**	**Normalised**	**Raw**	**Normalised**	**Raw**	**Normalised**
AB	58.6	58.6	42.6	42.6	32.1	32.1
AA	74.4	58.6	107.3	84.5	0.1	0.1
BB	45.2	58.6	0.1	0.1	53.8	69.7

Table S5: Mean coverage (measured at the best covered 100 bp sequence within each marker) of the 206 F_2 *T. ivanbureschi* × *macedonicus* offspring of the linkage map family, aggregated by marker category and sample genotype. The ratio of mean raw coverage across all markers was used to normalise the mean coverage of the three genotypes. Coverage A-linked markers in samples of genotype AA is approximately double that of these markers in samples of genotype AB, and the same applies to B-linked markers in samples of genotype BB.

Outcome		**Number of replicates (n = 100)**
No secondary contact		5
Extinct before generation:	200	1
Extinct between generations	201 - 400	33
Extinct between generations	401 - 600	11
Extinct between generations	601 - 800	6
Extinct between generations	801 - 1000	4
Persisted till end		**40**

Table S6: Outcomes of 100 simulations (for 1000 generations) of secondary contact between the ancestral genotype and balanced lethal system. The balanced lethal system tends to either be driven to extinction within 400 generations (with contact typically occurring before generation 50) or form a persistent hybrid zone.

Outcome		Number of replicates (n = 10000)
Mutation does not occur		723
Extinct immediately		2106
Extinct between generations	1 - 10	5086
Extinct between generations	11 - 30	2072
Extinct between generations	31 - 50	11
Persisted till end (at least 90 generations)		**2**

Table S7: Outcomes for 10,000 simulations (for 100 generations) of the evolution of a balanced lethal system for a single mutation in an expanding population. The new genotype is almost always eliminated before it can become established. However, in both cases where the balanced lethal system survived for 50 generation a persistent hybrid zone remained until the end of the simulation.