Comparative genomics of the balanced lethal system in Triturus newts

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Introduction & Outline of Thesis

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General Background

Biological evolution can appear a simple, even obvious idea. Thomas Huxley recalls his initial reflection upon reading Darwin's 'On the Origin of Species' (1859) as "How extremely stupid of me not to have thought of that!" (Huxley 1888). Yet in nature this apparently simple concept plays out as a multitude of complex interactions between subtle processes, frequently producing results that seem counterintuitive, or even paradoxical. For example, Darwin initially found it difficult to explain the evolution of costly traits that seemed to reduce the chance of surviving or producing offspring, such as the sterility of many eusocial insects, or the cumbersome tail feathers of male peacocks (Burkhardt et al. 1993). Later researchers puzzled over questions such as how evolution might allow animals to aid unrelated individuals at cost to themselves? (Hamilton 1963) Or the 'C-value paradox' - why some species had evolved genomes many times larger and more expensive to replicate than other, similarly complex species (Thomas 1971). These paradoxical phenomena proved more than mere curiosities. Darwin's confusion gave way to insights that prompted the development of theories of kin selection and sexual selection (Darwin 1859, 1871). The later examples led to entire subfields studying reciprocal altruism (Trivers 1971) and selfish genetic elements (Orgel & Crick 1980). The potential for unexpected insight makes the investigation of evolution's most counterintuitive outcomes valuable.

Amongst the most important principles of evolutionary theory is natural selection, a concept so simple and self-evident that it has been described as a tautology (Waddington 1959) – organisms with traits that make them efficient at surviving and producing numerous offspring tend to survive and produce numerous offspring. It follows that, if those beneficial traits are inheritable, they will become more common, and opposingly, traits which inhibit reproduction or survival will become less common and eventually go extinct (Darwin 1859). Despite this incontrovertible logic, some organisms appear to defy natural selection. A notorious example of this are the newts of the genus *Triturus*. In these species 50% of all offspring spontaneously die during embryogenesis, cutting their reproductive potential in half. This trait is clearly massively disadvantageous, provides no known benefit, and is not shared by any other newt taxa – clearly it should be heavily selected against. However, it has persisted for over 20 million years and is fixed in every species in the genus.

Triturus – The Crested and Marbled Newts

Newts (subfamily Pleurodelinae) are salamanders in the family Salamandridae, which also includes the true salamanders. Newts are characterised by a semiaquatic lifestyle, where adults alternate between terrestrial and aquatic habitats, with breeding occurring in the water during spring and summer. All salamanders possess extremely large genomes and in newts the haploid genome consists of around 30 billion base pairs (30 Gbp). The newt genome is arranged into twelve pairs of chromosomes, except for the two new world genera, which have eleven pairs (Sessions 2008).

Triturus is a genus of newt distributed throughout Europe and western Asia. As of 2024 it is recognised to include three species of marbled newt and seven species of crested newt (Arntzen 2024). All *Triturus* species are capable of breeding together to produce viable offspring, however hybrids between the crested and marbled newt lineages have very low fertility (Arntzen et al. 2009, 2018). *Triturus* is part of a clade sometimes termed 'modern European newts', which also includes genera such as *Ichthyosaura* (the alpine newt) and *Ommatotriton* (banded newts) (Veith et al. 2018). The phylogenetic relationships within this lineage have been obscured by extensive ancient introgression, but recent publications place *Triturus* as the sister genus of *Lissotriton* (Rancilhac et al. 2021), which occupies a similar distribution. Europe is also home to 'primitive newts' of the genus *Pleurodeles*, which are much more distantly related, having diverged 60 mya (Marjanović & Laurin 2014). Compared to their close relatives, *Triturus* newts are notably larger and occupy different ecological niches. For example, the northern crested newt *Triturus cristatus* is approximately twice overall length and five times the total mass of the common newt *Lissotriton vulgaris*, with which it co-occurs across most of northern Europe and opportunistically predates upon (Cicort-Lucaciu et al. 2005; Sparreboom 2014).

The most striking difference between *Triturus* and its relatives concerns reproduction. Similar to other newt taxa, fertilisation occurs internally and the females lay clutches of 150-500 eggs, with approximately 200 being typical for most crested newt species (Sparreboom 2014). However, whereas in related genera close to 100% of fertilised eggs will hatch as viable larvae (Sessions et al. 1988), in *Triturus* only 50% will. The other 50% will develop normally until they reach the late tailbud stage, which occurs at day 6-7 in embryos developing at 20 °C, and then will spontaneously cease to develop and eventually die (Horner & Macgregor 1985).

The death of half of *Triturus* embryos had been noted over two centuries ago, by Italian naturalist Mauro Rusconi, in his treatise '*Amours des salamandres aquatiques*' - The love life of newts (Rusconi 1821). However, the mechanism behind this trait and its evolutionary implications remained uninvestigated until the second half of the 20th century. Superficially this phenomenon might appear similar to phenomena observed in other amphibians. Frogs of the genus *Oophaga* lay unfertilized eggs, which are eaten

by their tadpoles (Brust 1993). In fire salamanders, *Salamandra salamandra*, intrauterine cannibalism is observed in viviparous populations (Buckley et al. 2007). However, in these examples, the sacrifice of a mother's resources is balanced by a clear benefit to her surviving offspring. In contrast, *Triturus* larvae do not consume their unhatched brood mates (Grossen et al. 2012), and the result is *Triturus* losing half of their reproductive capacity, which must be considered strictly maladaptive.

Chromosome 1 in *Triturus*

The logic of natural selection suggests that trait as deleterious as the spontaneous loss of half of all offspring should be swiftly eliminated (Darwin 1859), but a peculiarity of the *Triturus* genome prevents this. The largest chromosome pair (chromosome 1) is unusual, while clearly a matched pair, the chromosomes are heteromorphic – the long arm of one chromosome is extended in comparison to the other (Callan et al. 1960). The larger of these forms is designated 1A and the smaller 1B. Under Giemsa staining, the long arms of chromosome 1 show an extreme amount of banding, in a pattern that differs between 1A and 1B (Sims et al. 1984). This implies that the long arms are a heterochromatic region that does not undergo homologous recombination, a suggestion supported by the lack of chiasmata observed in this region (Morgan 1978).

Initially chromosome 1A and 1B were proposed to be sex chromosomes, as the lack of recombination and accumulation of heterochromatin would indicate (Callan et al. 1960; Mancino & Nardi 1971). As many early investigations into the karyology of salamanders relied on lampbrush chromosomes prepared from female oocytes, and female heteromorphism (Z- and W-chromosomes) was already known in *Pleurodeles* (Lacroix 1970), this determination was accepted until it was realized that chiasmata were absent from the long arm in both males and females (Morgan 1978; Macgregor 1979) and chromosome 1 is heterozygous in both sexes. As all *Triturus* adults carry both chromosome 1A and 1B, Mendelian genetics would suggest that half of all offspring will be homozygous (1A,1A or 1B,1B). However, these genotypes are never observed in adults, because it is these homozygote embryos that undergo premature arrest (Macgregor & Horner 1980).

The requirement of both 1A and 1B for survival in *Triturus* is likely due to each form of the chromosome carrying genes that are essential for viability, that are either not functional or not present on the other form. This phenomenon, where heterozygosity is persevered in a population because homozygosity is invariably lethal, is termed a balanced lethal system (Muller 1918).

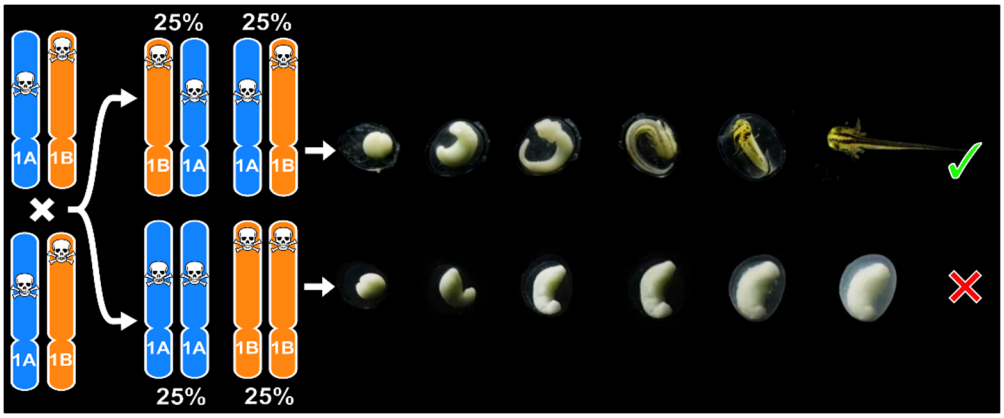

Figure 1: The balanced lethal system in *Triturus* chromosome 1. Each form of chromosome 1 has (recessively) lethal alleles at different locations. Consequently the 50% of offspring that inherit two copies of either version are unable to survive embryogenesis, and their development arrests, followed by eventual death. The 50% of offspring that inherit one copy of both versions of chromosome 1 survive and hatch as viable larvae. (Photographs courtesy of Micheal Fahrbach).

Balanced Lethal Systems

For over a century, artificial balanced lethal systems have been used to maintain stocks of organisms which carry alleles that are recessively lethal (Muller 1917). Most of these systems have been created in *Drosophila*, but they have also been engineered in nematodes (Herman et al. 1976) and mice (Zheng et al. 1999). Balanced lethal systems preserve recessively lethal alleles in a population by pairing them with a 'balancer chromosome' (Miller et al. 2019). This is a homologous chromosome which also carries a gene that is lethal when homozygous. This enforces heterozygosity, ensuring all that all individuals possess the allele of interest.

Genetic recombination is a problem for balanced lethal systems. If the balancer chromosome exchanges its lethal allele for a functional gene, it will become viable when homozygous and displace the allele of interest from the population. To prevent this, balancer chromosomes are developed with one or more genetic inversions (Miller et al. 2019). Recombination between the inverted and normal chromosome is inhibited within the inverted region both because the formation of chiasmata is often suppressed and because the chiasmata that do occur tend to result in inviable gametes (Coyne et al. 1993; Navarro & Ruiz 1997; del Priore & Pigozzi 2015).

The naturally occurring balanced lethal system in *Triturus* accounts for the inability of natural selection to purge what has been termed 'chromosome 1 syndrome' (Wallace 1994). However, it remains difficult to explain the origin of such a disastrously maladaptive trait. While extreme artificial selection has occasionally forced a balance lethal system into existence, as Dawson (1967) demonstrated with *Tribolium* beetles, it is improbable these conditions could occur in nature. However, the *Triturus* balanced

lethal system cannot be dismissed as an evolutionary fluke; similar systems have been described in widely divergent branches of the tree of life, including *Oenothera* (Steiner 1956), *Isotoma* (James et al. 1990) and *Drosophila Tropicalis* (Dobzhansky & Pavlovsky 1955).

Suppressed Recombination

For the balanced lethal system to remain stable, recombination between the two forms of chromosome 1 must be inhibited. This implies that the region of suppressed recombination we observe in *Triturus* chromosome 1 must have evolved before or concurrently with the balanced lethal system. The non-recombing region may have expanded afterwards, forming strata similar to those observed on sex chromosomes (Lahn & Page 1999).

Suppression of recombination is often primarily discussed in relation to genetic inversions, but other chromosomal rearrangements such as deletions and translocations can also inhibit homologous recombination, although these are much more likely to be immediately deleterious and hence are observed less frequently. Certain DNA sequences, for example the PRDM9 binding site (Paigen & Petkov 2018), also affect the local frequency of recombination and transposable elements have a bidirectional association with regions of suppressed recombination (Kent et al. 2017).

When initially describing the *Triturus* balanced lethal system Macgregor and Horner (1980) briefly posited that both chromosome 1A and 1B could have been created in a single event, during an unequal exchange between sister chromosomes. Sessions et al. (1988) elaborated upon this, suggesting that a pair of reciprocal deletions and duplications resulting from such an exchange would explain the non-viability of the homozygotes as well as suppressing recombination. However, this concept was dismissed by later authors as it provided no mechanism by which such deleterious mutations could achieve fixation (Grossen et al. 2012).

Recombination is a process of immense importance in evolution and so its suppression has significant consequences. Recombination is vital to the efficient purging of deleterious alleles, and its absence results in an accumulation of such alleles in an effect termed 'Muller's ratchet' (Felsenstein 1974), which may gradually reduce the fitness of the population. This effect is responsible for the accumulation of transposons and other repetitive elements in non-recombing regions resulting in the build-up of heterochromatin commonly observed (Charlesworth et al. 1994). As alleles for different loci within a non-recombining region will be inherited together for many generations the synergistic effects between them become more visible to selective pressures, which contributes to the evolution of phenomena such as sex chromosomes and supergenes (Charlesworth et al. 2005; Thompson & Jiggins 2014).

Supergenes

A supergene is a group of genes located on the same chromosome and consistently inherited together (Dobzhansky 1970). Supergenes can have profound effects on the evolution of a species (Thompson et al. 2014). By locking together a large number of genes a supergene can produce highly distinctive, complex phenotypes (Schwander et al. 2014). Among the most studied systems are the Müllerian mimicry supergenes in Heliconius butterflies (Joron et al. 2006) and the supergenes that control social organization in fire ants of the genus Solenopsis (Wang et al. 2013). Example in vertebrates include the supergenes that give rise to the spectacular mating morphs in the ruff (Küpper et al. 2016) and white throated sparrow (Tuttle et al. 2016).

Supergenes can also promote speciation, via several mechanisms. A lack of recombination can cause a supergene to rapidly accumulate mutations (Muller 1964), resulting in faster divergence between populations. Rigid genetic linkage also facilitates the evolution of meiotic drive systems, which selfishly destroy gametes that do not possess the supergene (Larracuente & Presgraves 2012). Drive systems typically impose a fertility penalty, favouring the evolution of genes which suppress them (Zanders & Unckless 2019). If a hybridization event introduces the drive system into a population that lacks the suppressor, then less fertile offspring will be produced (McDermott & Noor 2010). This will result in reproductive isolation and may explain some of the hybrid infertility often observed in nature (Patten 2018). Ironically, while supergenes can separate species by preventing hybridization, some supergenes are created by hybridization between two species (Tuttle et al. 2016; Jay et al. 2018). In addition, like any gene, supergenes can introgress between species, sometimes carrying over entire complex phenotypes with them (Corcoran et al. 2016).

As supergenes can only exist in regions of suppressed recombination, they often originate as a chromosomal inversion (Kirkpatrick 2010). If the inversion is not immediately deleterious, it may spread through the population and start to acquire mutations that cannot be passed back to the original chromosome, the beginnings of a supergene. With the inverted chromosome now in competition with the original, selection or genetic drift will eventually eliminate one of the variants. If the inversion is adaptive, it may become fixed in the population (Lande 1985). It must be noted that typically, an inverted chromosome will be capable of recombining with itself. Consequently, if the inversion becomes fully fixed, there will be no suppressed recombination, and the chromosome will cease to evolve as a supergene system.

For a supergene system to be stable in the long term, it must exist in a balanced polymorphism, where some selective mechanism actively maintains multiple alleles in a population (Charlesworth 2006). One possible mechanism is negative frequency dependent selection, where a trait is advantageous when rare, but disadvantageous when common (Ayala & Campbell 1974). This may be the case in grove snails *Cepaea*

nemoralis, which exist in several distinct morphs, coded for by a supergene (Gonzalez et al. 2019). It is suggested that the rarer a morph is, the less likely it is to be recognized by the snail's main predator, the song thrush (Clarke 1969).

Balanced polymorphism may also be sustained by heterozygote advantage. This occurs when possession of a single copy of an allele is more advantageous than having two (or zero) (Birchler et al. 2003). In severe cases an otherwise advantageous allele may be lethal when homozygous, which is the case in many supergene systems, such as those found in fire ants, ruffs and fruit flies (Kenvon 1972; Wang et al. 2013; Küpper et al. 2016). Recessive lethality may result from the accumulation of deleterious mutations, or from the disruption of an essential gene by the original inversion (Albornoz & Domínguez 1994).

The non-recombining region of *Triturus* chromosome 1, can be viewed as a supergene system exhibiting the most extreme form of heterozygote advantage possible. However, this prompts the question of how the original, un-inverted (or otherwise rearranged) form of the chromosome also loses viability when homozygous. A possible mechanism is that of a self-reinforcing heterozygote advantage: An increased proportion of a population heterozygous for a supergene leads to fewer opportunities for recombination within the affection section of the chromosome. With less recombination deleterious alleles are purged less efficiently, which in turn increases the relative heterozygote advantage. Berdan et al. (2021) model this scenario, showing that under strict conditions symmetric degeneration within a supergene locus can eventually result a balanced lethal system.

Alternatively, chromosomes 1A and 1B may be two incompatible descendants of the same supergene, with the original arrangement driven extinct. This would be the case if the balanced lethal system evolved from the most ubiquitous class of supergene: a sex chromosome.

Sex Chromosomes as Supergenes

The human Y-chromosome may be the most familiar example of a supergene (Charlesworth 2016). It displays all the typical characteristics of a supergene: it gives rise to a complex phenotype, it does not undergo homologous recombination over a portion of its length, and it is maintained in balanced polymorphism with the X-chromosome (Charlesworth 2017).

Sex chromosomes can also demonstrate some of the more diverse aspects of supergene biology. Haldane's rule summarizes how sex chromosomes decrease the fertility of hybrids and promote speciation (Haldane 1922). In some species highly divergent Y chromosomes can code for distinct morphs. This is the case in the guppy, *Poecilia reticulate*, where the polymorphism is maintained by females which preferentially mate with rare male morphs (Hughes et al. 2013). Interestingly it is

possible to breed viable YY guppies (by using temperature dependent sex reversal to produce an XY female), but only if the Y-chromosomes are heterozygous. This indicates that each Y chromosome linage possesses a unique set of recessively lethal alleles (Haskins et al. 1970).

In contrast to mammals and birds, where the XY and ZW systems have been relatively stable for hundreds of millions of years (Cortez et al. 2014; Zhou et al. 2014), amphibians exhibit a far more diverse array of sex determination systems (Nakamura 2009; Miura 2017). For example, the Iberian ribbed newt, *Pleurodeles waltl*, possesses a ZW system (Cayrol et al. 1983), but in *Triturus* chromosome 4 is identified an XY pair (Sims et al. 1984). In many Salamander species sex chromosomes are difficult to identify with a karyotype, as they are relatively homomorphic (Keinath et al. 2018), which is characteristic of evolutionarily young chromosomes (Stöck et al. 2011). This suggests the Salamanders experience rapid turnover of sex determination systems (Hillis & Green 1990).

Did Chromosome 1 Evolve from a Sex Chromosome System?

Wallace (1987) suggested that this rapid turnover explained the origin of the balanced lethal system - chromosome 1A and 1B were the remnants of an ancient sex determination system. This model relied upon the X-chromosome becoming self-incompatible but provided no mechanism by which this mutation might become fixed. A similar, but more developed hypothesis was later proposed by Grossen et al. (2012) where it was given the imaginative title "A Ghost of Sex Chromosomes Past?". This model is based on the following sequence of events:

1) Firstly, *Triturus* chromosome 1 was previously the Y-chromosome of a XY sex determination system.

2) Within this system the Y-chromosome diverged into two lineages, which were maintained in balanced polymorphism. The authors propose that, after divergence, each Y-chromosome lost several (different) essential genes which were maintained on the X-chromosome, creating a unique set of recessively lethal alleles on each lineage of the Y-chromosome. This created a system much like that observed in guppies.

3) The climate became colder. The sex determination mechanisms of salamanders are affected by temperature, with lower temperatures having a feminizing effect (Wallace & Wallace 2000). The environmental shift resulted in a proportion of XY *Triturus* developing as female.

4) When XY females bred with XY males, YY offspring resulted. These behaved similarly to sex reversed guppies: they were viable, but only when heterozygous for the two Y-chromosome linages. To preserve these linages, they must have been so divergent that they were incapable of homologous recombination with each other.

5) The climate became even colder resulting in all XY individuals developing as female. However, the YY individuals had two copies of the masculinizing chromosome, so a proportion of them remained male.

6) The temperature induced sex reversal resulted in a large excess of females over males. In this situation the X-chromosome (which was always in females) was at a disadvantage and was eventually lost from the gene pool. This resulted in a population that was purely YY, with temperature dependent sex determination.

7) The balanced lethal system was now fully established. All individuals carry two copies of the chromosome, but only those which had each of the two lineages were viable. This resulted in the 50% embryonic mortality rate now characteristic of *Triturus*.

8) Finally, a masculinizing mutation evolved on chromosome 4. As the sex ratio was still biased in favour of females, this mutation spread rapidly and eventually evolved into the XY sex determination system observed today on chromosome 4.

As each step in this sequence is plausible under the conditions proposed it presents a viable theoretical route to the fixation balanced lethal system – which is supported by the simulations performed by the authors. However, the pre-requisites are exacting. The Y-chromosome lineages must have diverged early enough that they share no common lethal factors and then been maintained in polymorphism, likely requiring some degree of balancing selection. They must also have evolved separate chromosomal rearrangements to prevent recombination between them in the YY individuals. The mechanism relies on specific patterns of climate alteration and depends on the final masculinizing mutation being delayed until after the balanced lethal system has been fixed, despite a strong selection pressure in its favour from the beginning of the scenario.

A major virtue of the "Ghost of Sex Chromosomes Past" model is that it is not simply an evolutionary "Just So Story" (Smith 2016), but a falsifiable hypothesis that implies testable predictions. If *Triturus* chromosome 1 is a former Y-chromosome, the most parsimonious scenario is that it descended from (and hence is homologous with) the same common ancestor as the current XY systems found in related European newt genera such as *Lissotriton*. Furthermore, because the model involves sex-chromosome turnover specific to the *Triturus* lineage, it implies that the current *Triturus* Y-chromosome is different to that of any common ancestor, and so is extremely unlikely to be homologous to those found its relatives.

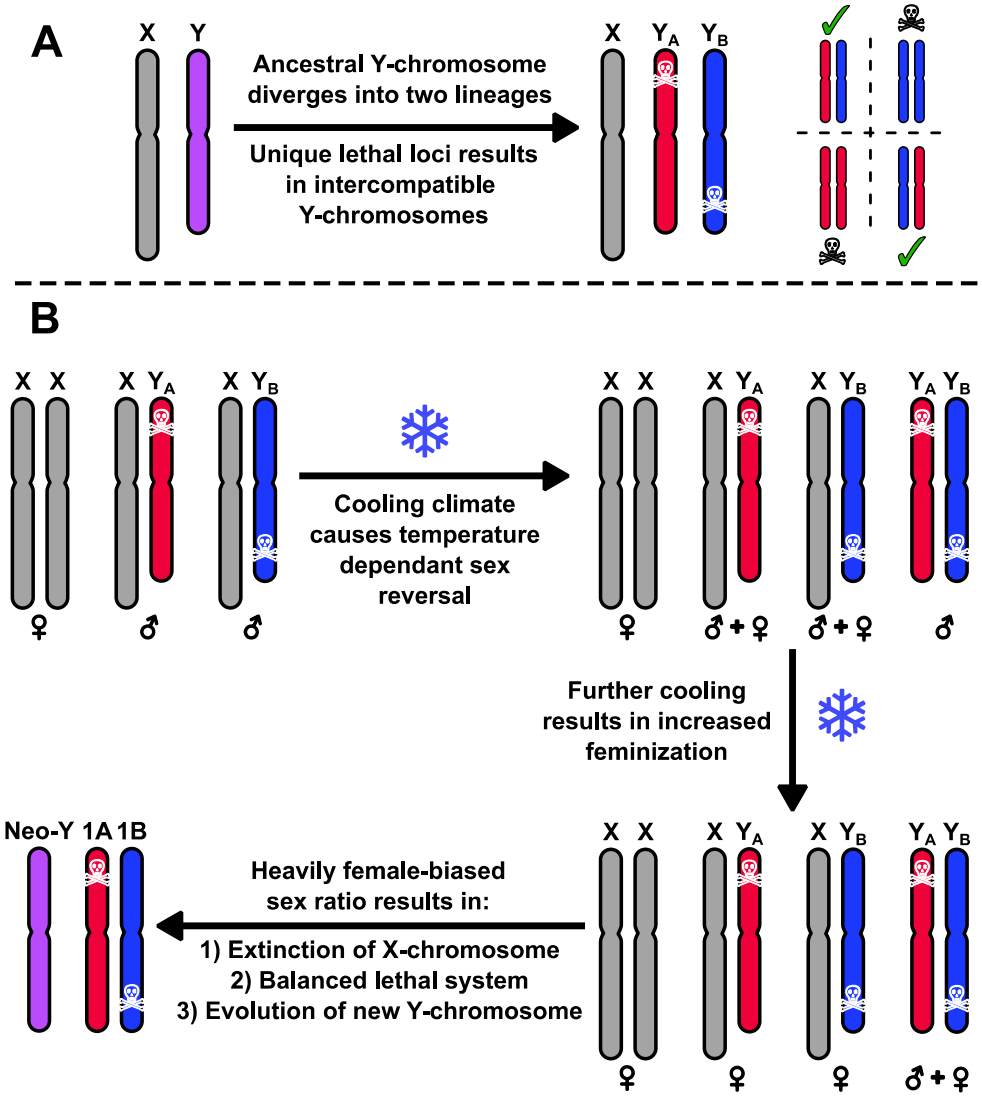

Figure 2: The hypothesized evolution of the *Triturus* balanced lethal system from a Y-chromosome (Grossen et al. 2012). A) The ancestral Y-chromosome diverges into two lineages, each other which possesses its own lethal alleles. Normally the Y-chromosomes cannot meet, but if they did only individuals with one copy of each of the two different lineages would be viable. **B)** As the climate cooled, temperature dependent sex reversal would result in XY females, producing the possibility of YY individuals. With drastic climate change even a YY genotype may not always be sufficient for masculinization, resulting in a sufficiently female biased sex-ratio that the X-chromosome is driven extinct, even though this results in a balanced lethal system.

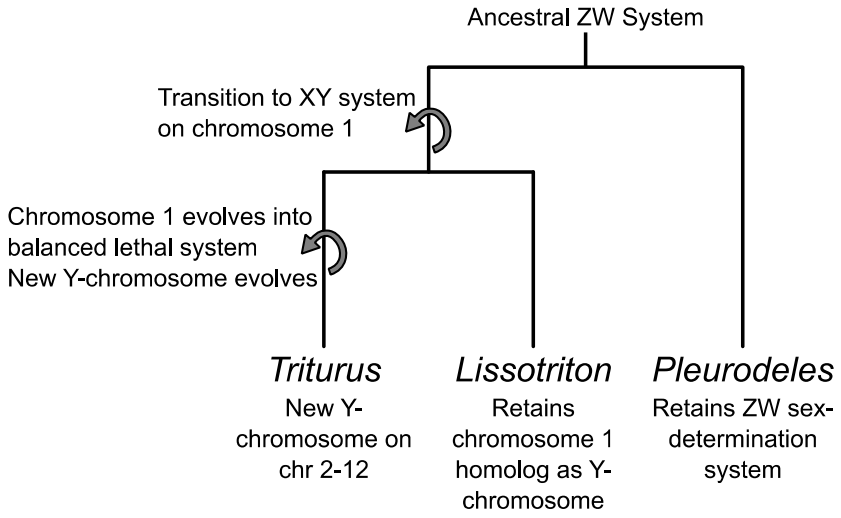

Figure 3: Sex chromosomes turnover events in newts required by the Y-chromosome origin hypothesis. A transition between an ancestral male-determining region on chromosome 1 to a new Y-chromosome is an essential feature of this mechanism. This implies that the Y-chromosome of *Lissotriton* cannot be homologous with that of *Triturus* and will instead likely be homologous with the *Triturus* balanced lethal system. Also indicated is transition from the ancestral ZW system of 'primitive newts' to the XY system in 'modern' newts.

A Degenerate Supergene?

While Grossen et al. (2012) presented the first detailed simulation of the evolution of a balanced lethal system the alternative hypothesis of a runaway heterozygote advantage has recently been modelled by Berdan et al. (2022) These simulations concern different arrangements of a supergene that are initially mildly overdominant with respect to each other (i.e. the supergene locus drives heterozygote advantage) which can be either inherent to the arrangement, or a consequence of genetic linkage to other overdominant alleles (associative overdominance). As individuals carrying two different arrangements of the supergene have a fitness advantage in this scenario, they will experience disproportionate reproductive success and may constitute the majority of the breeding population.

The supergene arrangements can only undergo recombination in homozygous individuals, and so if these homozygotes reproduce less often, there will be less frequent recombination within the supergenes. This will reduce the efficiency of purifying selection within these regions and therefore cause an accumulation of deleterious mutations within each supergene arrangement. As most deleterious mutations are recessive, and the mutations will be different in each supergene arrangement, they will have little impact on individuals that are heterozygote for the supergene. However, homozygotes will suffer reduced fitness due to the additional mutation burden and so will make up even less of the breeding population. This forms the basis of a vicious cycle where there is ever less recombination and purifying selection within the supergene

arrangements, resulting in an ever-increasing mutation burden which eventually drives the fitness of the homozygotes to zero, producing a balanced lethal system.

Berdan et al. (2022) show that this is a plausible mechanism for the formation of a balanced lethal system, but in common with the degenerate sex-chromosome scenario, its viability depends on specific conditions, especially a small effective population size and low rates of gene conversion. An interesting aspect of this mechanism is that the degeneration must be symmetric. If the mutation burden in one of the supergene arrangements results in significantly lower fitness of its homozygotes than the other arrangement, then its frequency within the population will decrease. Any imbalance in frequency will reduce purifying selection within the less common supergene arrangement (and enhance its efficiency in the more common arrangement) resulting in this arrangement experiencing an even greater mutation burden and exacerbating the original imbalance. This cycle would continue until the less common arrangement was either driven extinct, or, if the heterozygote advantage was very high, evolved into a 'half-lethal' supergene – like those observed in ruffs or fire ants (Kenvon 1972; Wang et al. 2013; Küpper et al. 2016).

The genomic signature that this mechanism would leave in modern *Triturus* requirement is less obvious than evolution from a 'ghost' sex chromosome. However, the requirement for steady, symmetric degeneration of both supergene arrangements suggests that we would not expect to find evidence of macro-mutations (such as multi-gene deletions) that would have abruptly incurred major fitness penalties. This is a stark contrast to the first mechanism proposed for the evolution of the *Triturus* balanced lethal system, the unequal exchange described by Sessions et al. (1988) which likely would leave clear evidence of large-scale deletions.

Exploring the Balanced Lethal System and the Giant *Triturus* Genome

There has been considerable investigation of the *Triturus* balanced lethal system at the embryonic and cytological level. Multiple mechanisms have been proposed, and in some cases simulated, to explain the evolution of naturally occurring balanced lethal systems in general (Berdan et al. 2021) and the *Triturus* system in particular (Wallace 1987; Sessions 2008; Grossen et al. 2012). Despite this interest, essentially nothing is known about the system at the genetic or genomic level. The specific mechanism of lethality remains a mystery. Not a single gene has been shown to be associated with the non-recombing regions of either chromosome 1A or 1B. No structural rearrangements have been definitively identified with the *Triturus* genome. This is unfortunate, as understanding the evolution of the balanced lethal system may be almost impossible without knowledge of the structure and content of this part of the genome and so far, any predictions implied by the proposed hypotheses have remained untested.

The relative lack of progress has been typical of salamander genetics/genomics in the first two decades of the 21st century (for example no male-linked markers have been identified on the Y-chromosome of any salamander) and may be due to the intimidating size of the genomes involved. Other than lungfish (Metcalfe et al. 2012) (Meyer et al. 2021), salamanders have the largest genome of any vertebrate. In *Triturus*, estimates of the haploid length varies between 27 GB and 32 GB depending on species (Litvinchuk et al. 2007) and this does not account for the heteromorphy observed in the largest chromosome. This size complicates sequencing and genome assembly. Chromosome scale assemblies have been published for the axolotl and Iberian ribbed newt (Brown et al. 2025; Nowoshilow et al. 2018; Smith et al. 2019), but while more are sure to follow, these remain very resource intensive projects.

An alternative to whole genome sequencing is reduced representation sequencing (Hirsch et al. 2014). This is diverse category of techniques that aim to interrogate subset of loci. Importantly, if the techniques are applied consistently, the same loci are sequenced across multiple samples. Compared to whole genome sequencing, these approaches have several advantages. Sequencing a single sample is much cheaper, large sample sizes become viable, very high coverage is possible (making it easier to identify rare polymorphisms) and useful data can be quickly generated even from the largest of genomes.

Two techniques of particular interest are RADseq and target capture sequencing. RADseq sequences sections of DNA flanked by the binding sites of selected restricted enzymes (Davey & Blaxter 2010). Though restriction sites will be scattered randomly throughout the genome, their position will be conserved between samples of the same species. RADseq may yield hundreds of thousands of loci with little preliminary work, but these are rarely coding sequences, and often poorly conserved between species. Target capture relies on the hybridization of synthetic complementary RNA probes to preselected target sequences (Mertes et al. 2011). The targets chosen are generally coding genes, and often highly conserved between species. However, one limitation is that the general sequences of the targeted loci must be known in advance. Often transcriptome data is used to design the probes, but this is not helpful for non-transcribed sequences, making investigation of gene-poor regions, such as sex chromosomes, difficult. For *Triturus* a probe set targeting ca. 7000 genes has already been designed and used to construct a phylogeny of the genus (Wielstra et al. 2019).

Unlike a whole genome assembly, reduced representation sequencing does not directly give information about the position of any marker within a genome. However, if a family of related individuals is sequenced, the relative position of any two markers can be inferred by calculating the frequency of recombination between them. With enough markers a linkage map covering all chromosomes within the genome can be constructed (Kai et al. 2014).

Aims and Structure of the Thesis

The primary objectives of this PhD project are to map *Triturus* genome, to compare the structure of the *Triturus* genome to that of related genera and identify regions of shared synteny, to identify any chromosomal rearrangements which may have been involved in the evolution of the non-recombing region of *Triturus* chromosome 1, and to test the "A Ghost of Sex Chromosomes Past" hypothesis by determining whether or not chromosome 1 ever has acted as a sex chromosome.

Chapter 2

To verify whether *Triturus* chromosome 1 has ever acted as a sex chromosome it will be necessary to identify the sex-linked regions of the genome of its close relatives (on the assumption that these still retain the sex determination system of their common ancestor). To this end, a RADseq based linkage map will be constructed for the common newt *Lissotriton vulgaris*, which, like *Triturus*, also possess a small heteromorphic Y-linked region close to the telomere of one of its middle-ranked chromosomes.

Other researchers working in amphibians have shown that a sex-linked region may be identified directly from such a map without any previously known sex-associated sequence and without knowledge of the sex any samples other than the parents of a family (Brelsford et al. 2016). If this is also possible in newts it would negate the need for a separate study searching for sex-associated markers. However, the large genomes and apparently small sex-linked regions observed in the karyotypes of both *Triturus* and *L. vulgaris* make it questionable whether this approach is reliable. Consequently, for *L. vulgaris*, an association based RADseq approach is also employed, and the markers identified used to benchmark the suitability of a purely linkage mapping based study. The sex-specific performance of any Y-linked markers identified in *L. vulgaris* will also be verified across the genus *Lissotriton*.

Chapter 3

With the Y-linked region of the *Lissotriton* genome identified, the same must be achieved for *Triturus*. The same RADseq methodology used in chapter 2 will be employed for the Balkan crested newt *T. ivanbureschi*. Sex association will be used to identify male-linked RAD markers in sequences from samples of adults of known sex. These markers will then be located a on RADseq-based linkage map. Bioinformatic approaches will be used to determine whether the *Lissotriton* and *Triturus* Y-chromosomes are homologous. Additionally, the Y-linked regions of both genera will be placed on target capture-based linkage which also include genes involved in the *Triturus* balanced lethal system. Finally, the performance of Y-linked markers developed in *T.*

ivanbureschi will be assessed throughout the genus *Triturus*, as multispecies sex-specific markers may be valuable for many future research projects.

Chapter 4

This chapter will focus directly on the genes involved in the *Triturus* balanced lethal system and the identification of structural rearrangements. Target capture sequencing of F2 hybrid families will be used to construct linkage maps for both *Triturus* and *Lissotriton*. As the *Triturus* dataset will include both viable and arrested embryos it will become possible to distinguish alleles associated with each of the chromosome 1 heteromorphs. Via the linkage maps any structural rearrangements between *Triturus* and *Lissotriton* involving these loci will become apparent. Additionally, allele ratio analysis will be used to investigate any change in the copy number of the genes. Finally, the viability of the various hypotheses for the evolution of the *Triturus* balanced lethal system will be discussed in light of the results, and an evolutionary scenario will be modelled.

Chapter 5

In the final chapter of this thesis will summarize and synthesize the findings of the previous three chapters and place them in the context of the surrounding literature. Topics of discussion will include: Which, if any, the different proposed models for the evolution of balanced lethal system best fit the observations made in *Triturus*? Whether the evolutionary processes experienced by *Triturus* are relevant to other naturally occurring balanced lethal systems? What are the broader relationships between the origin of the balanced lethal system and other evolutionary processes, such as genetic surfing and speciation? And, what is the outlook for future research?

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Linkage Mapping vs Association

A Comparison of Two RADseq-based Approaches to Identify Markers for Homomorphic Sex Chromosomes in Large Genomes

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Abstract

Reliable tools for the identification of genetic sex are invaluable in many fields of biology, but their design requires knowledge of sex-linked sequences, which is lacking in many taxa. Restriction-site associated DNA sequencing (RADseq) is widely used to identify sex-linked markers, but multiple distinct strategies are employed, and it is often not obvious which is most suitable. In this study we compare two approaches for using RADseq to identify sex-linked markers. We use the common newt, *Lissotriton vulgaris*, as our study system, providing a challenging combination of homomorphic sex chromosomes and an exceptionally large genome. We attempt an associative approach, sequencing 60 adult newts of known-sex individuals, and compare this to a linkage mapping approach utilizing a family of 146 offspring with unknown sex. After optimization for a highly paralogous genome, the associative approach identifies five Y-chromosome linked markers in *L. vulgaris* and we design a robust PCR protocol for molecular sexing of four more related species. Via the linkage approach we construct a high-density map featuring 10,763 markers, matching the observed karyotype of *L. vulgaris* and showing broad synteny with the Iberian ribbed newt (*Pleurodeles waltl*). However, without incorporating the markers identified via the association-based approach, we cannot confidently distinguish a sex-determining region in the linkage map, either by analysing marker density or by identifying clusters of paternal markers. We conclude that linkage mapping alone is unlikely to yield sex-linked markers in organisms with very small sex-determining regions, however association-based RADseq can still be effective under these conditions.

Introduction

Sex-linked genetic markers are vital to both applied and fundamental biology. In agriculture and aquiculture molecular markers boost productivity by, for instance, enabling the early selection of fruit bearing female date palms (Intha & Chaiprasart 2020) or aiding the maintenance of all-male stocks of tilapia (Curzon et al. 2021). Molecular sex identification is viable even on small and degraded samples, making it invaluable for ecology, conservation and forensic biology. Examples include determining the sex of tiger prey from hairs recovered from scat (De et al. 2019), monitoring elephant sex ratios by genotyping dung (Vidya et al. 2003) and identifying the illegal poaching of female pheasants (An et al. 2007). As sex determining regions of the genome have been identified as drivers of speciation (Dufresnes & Crochet 2022; Johnson & Lachance 2012; Payseur et al. 2018), their identification and study is of particular importance to evolutionary biology.

Many taxa have highly conserved sex determination systems (Cortez et al. 2014; Ellegren 2010) enabling a single method of molecular sex identification to be used across an enormous range of species with little modification. For example, all birds possess a ZW chromosome system, and a single primer pair based on the CHD1 gene allows for sex identification across the neognathae (which includes over 99% of bird species) (Fridolfsson & Ellegren 1999). Similarly, amplification of the SRY gene identifies the presence of the Y-chromosome in a wide range of eutherian mammals (Hrovatin & Kunej 2018).

However, such conservation is far from universal, and other branches of the tree of life have experienced frequent turnover of sex chromosomes (Ma & Rovatsos 2022), often made obvious by transitions between male and female heterogametey (Bachtrog et al. 2014; van Doorn & Kirkpatrick 2010). Groups notable for rapid evolution in sex determination systems include fish (Kitano & Peichel 2012), squamate reptiles (Ezaz et al. 2010) and amphibians (Miura 2017). In addition, while the majority of plants are hermaphroditic (or monoecious), dioecy (two fully separate sexes) has independently evolved on numerous occasions (Renner 2014). Rapid turnover complicates molecular sex identification, as new markers may have to be identified on a lineage-by-lineage basis. Exacerbating this, evolutionally young sex chromosomes are typically not highly differentiated, resulting in homomorphic chromosomes with only a small region in which sex-linked markers may be found (Charlesworth et al. 2005; Wright et al. 2016).

Several sequencing approaches are employed for the identification of sex-linked markers. Recent studies have tended to employ either whole genome sequencing (WGS) (Darolti et al. 2019; Keinath et al. 2018; Rafati et al. 2020) or restriction-site associated DNA sequencing (RADseq) (Gamble et al. 2015; Hime et al. 2019; Hu et al. 2019). WGS is a powerful technique, but the cost of sequencing the entire genome of

multiple individuals of both sexes may be prohibitive. This is particularly the case for organisms with exceptionally large genomes, such as salamanders, lungfish and many genera of dioecious plants such as gingko, mistletoe and yew, that all have genome sizes in excess of 10 Gbp (Gregory 2024; Pellicer & Leitch 2020). For such gigantic genomes, RADseq may be a superior technique. RADseq targets restriction site-bounded sequences scattered randomly throughout the genome (Miller et al. 2007), giving genome-wide data, while requiring orders of magnitude less sequencing than WGS.

RADseq is employed to identify sex-linked makers via multiple methodologies (Gamble 2016). The most conceptually simple approach is association-based (Gamble & Zarkower 2014). A number of individuals from both sexes are sequenced, and the recovered RAD markers are screened for those present in one sex and absent in another. The RAD marker set can also be screened for SNPs present only in one sex, and markers present at twice the copy number in one sex than the other (Brelsford et al. 2017; Trenkel et al. 2020). This approach typically requires 10-30 individuals per sex, with decreasing sample sizes increasing the risk of generating false positives.

As RADseq involves many thousands of markers scattered randomly across the genome it is ideal for building high density linkage maps. Sex-linked regions can then be identified by quantitative trait locus analysis (QTL) (Peng et al. 2016), detecting clusters of SNPs unique to the heterogametic parent (Hu et al. 2021) or by locating regions of reduced recombination (Brelsford et al. 2016). The map may also be a valuable resource for questions beyond that of sex determination, for example, anchoring scaffolds of a whole genome assembly (Lee et al. 2019). A linkage map requires more investment than an association-based approach, as a linkage family (or families) must be bred and typically at least 100 individuals must be sequenced. However, as the sex of the offspring does not have to be known (Brelsford et al. 2016), a linkage mapping approach may be more feasible in cases where large numbers of adults are not available to be morphologically sexed and juveniles are readily bred but difficult to sex.

Little literature is available on the relative performance of different approaches for using RADseq to identify sex-linked markers. Most studies employ a single methodology, and while this does provide a list of strategies that have proved successful in at least one situation, the publication bias against negative results means that the limitations of these approaches remain obscure. The publications that do compare different tools for sex-linked maker discovery tend to either be reviews, which aggregate results generated in wildly different contexts (Palmer et al. 2019), or comparisons of bioinformatic approaches (Trenkel et al. 2020). This presents an issue for researchers designing such studies, as it is not clear which sequencing strategy is more likely to yield useful sex-linked markers. In this study we aim to contrast two approaches to sex-linked marker discovery using RADseq (linkage mapping, and presence/absence association)

by applying both to a single, challenging species, the common newt (*Lissotriton vulgaris*).

Lissotriton vulgaris one of the most widely distributed amphibian species in Europe, ranging from Ireland to Siberia (Sparreboom 2014). It is part of the smooth newt species complex, which includes six closely related newt species found in Europe and western Asia (Pabijan et al. 2017; Wielstra et al. 2018). The wider genus *Lissotriton* includes four additional species more distantly related to *L. vulgaris* (Babik et al. 2005). Like all salamanders, *Lissotriton* have gigantic genomes, estimated at 27.7-32.0 Gbp (Gregory 2024; Litvinchuk et al. 2007). *Lissotriton* possess XY sex-determination systems with little to no heteromorphism (Schmid et al. 1979; Zbożeń & Rafiński 1993). No Y-linked marker has previously been reported in any Salamander, however RADseq studies have identified the ancestral amphibian ZW system in the family Cryptobranchidae (Hime et al. 2019; Hu et al. 2019, 2021), and the first salamander whole genome assembly revealed a tiny 300 Kbp W-linked region in the axolotl (Keinath et al. 2018).

We first attempt to identify Y-linked markers via the associate approach by performing RADseq on a group of known sex *L. vulgaris*. We then test the linkage mapping approach to identify a Y-linked region, gathering RADseq data from a full-sibling *L. vulgaris* family with offspring of unknown sex. Finally, we validate candidate markers by PCR amplification in multiple taxa within the genus *Lissotriton*.

Methods & Materials

Sample acquisition

For identification of Y-linked markers by association, samples from 60 sexed adult *L. vulgaris* (30 male, 30 female) were collected from the Kraków metropolitan area in Poland, these samples are also reported in Babik et al. (in press). For construction of the linkage map an *L. vulgaris* family was bred consisting of 2 parents (1 adult male and 1 adult female, collected in Kraków, Poland) and 146 offspring of unknown sex. For validation of candidate markers via PCR, 12 samples (6 male, 6 female) of both *L. vulgaris* and *L. montandoni* and an additional 2 samples (1 male, 1 female) of multiple taxa belonging to the smooth newt complex (*L. v. ampelensis*, *L. v. meridionalis*, *L. graecus, L. kosswigi* and *L. schmidtleri*) as well as more distantly related *Lissotriton* species (*L. boscai*, *L. helveticus* and *L. italicus*,) were obtained from localities across Europe and Anatolia (see online supporting information for a full details of samples). Samples from adults consisted of tail tips and for offspring the freshly hatched larvae were collected whole. Samples were stored in 96% ethanol.

DNA extraction, library preparation and RAD-sequencing

Whole genomic DNA was extracted from the selected tissue samples with the Promega Wizard™ Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the salt-based extraction protocol of Sambrook and Russel (2001). Double digest RADseq libraries were prepared according to the Adapterama III High-Throughput 3RAD protocol (Bayona-Vásquez et al. 2019) from 100 ng of genomic DNA, using restriction enzymes *Eco*RI, *Xba*I, and *Nhe*I. Fragments in the range of 490-600 bp were excised using Pippin Prep, the libraries were pooled equimolarly and 150 bp paired-end sequencing was performed by Novogene (Cambridge, UK) on the Illumina NovaSeq 6000 (Illumina Inc., San Diego, CA, USA) platform, targeting a yield of 1 Gbp per sample for the linkage map family and 2 Gbp for the known-sex adults.

RADseq data processing

The Stacks package v2.54 (Catchen et al. 2013; Rochette et al. 2019) was employed to process raw reads from all RADseq samples. Reads were demultiplexed and trimmed via the process_radtags program. The denovo_map.pl pipeline was then used to group reads into putative loci. Default settings were used except for the parameter M, which controls how many mismatched bases two read-pairs may have and still be assigned to the same locus. If M is too low, Stacks may not correctly group reads from the same locus together (especially if samples are genetically divergent), however if M is too high it may result in reads from paralogous loci being inappropriately aggregated together (Paris et al. 2017). As our RADseq analysis was based on two sample-sets which would not be expected to exhibit great genetic diversity (a captive bred linkage map family and a group of wild-caught newts from a relatively small area) a low value of M would seem appropriate. Accordingly, we selected the default value of M=2 for the linkage family, as this low value will minimise the distortions caused by mapping paralogs together as falsely heterozygous loci.

However, we hypothesised that an overly high value of M may actually be helpful when selecting markers for molecular sex identification. This increases the chance sex-linked loci with autosomal paralogs will be assigned reads even in the opposite sex, and so filtered out in subsequent analysis. This is desirable as these loci would likely give false positives in PCR based genotyping (due to amplification of the paralog). Consequently, we ran our analysis of the known-sex RADseq data three times, with values of M=2, M=6 and M=10.

Sex-associated presence/absence marker discovery

The bam files produced from each of the three runs of denovo_map.pl were processed with the depth function of SAMtools (Danecek et al. 2021) to produce a table of the number of reads of each marker in each of the sexed-adult samples. A custom R script was then used to identify candidate Y-linked markers which had reads in at least 90% of male samples, and less than 10% of female samples.

We aimed to minimise the likelihood of candidate Y-linked markers failing in PCR validation by avoiding candidates with a large number of paralogous sequences present in the genome. Primers designed for such markers would have a high chance of amplifying products from autosomal paralogs, resulting in false positives in female samples. Therefore, a BLAST (Camacho et al. 2009) search was then conducted for each candidate against the catalogue of all RAD markers found in the run of the same M value, and the number of potential paralogous hits (> 80% sequence similarity with a query coverage of > 25%) recorded. Candidate markers were ranked based on absence of residual reads in females, number of potential paralogs and average read depth in males. The ten highest ranked candidate markers from each run were selected for PCR screening.

After removing any duplicate markers (where the same sequence was selected from multiple runs of the pipeline), primers were designed with Primer 3 (Untergasser et al. 2012) targeting an optimal primer length of 20 bp and melting temperature of 60°C. To facilitate the design of a multiplex PCR, for each marker we attempted to design two primer pairs, amplifying both a shorter (ca. 100 bp) and a longer (ca. 200 bp) product. For candidate markers that did not consist of a continuous sequence, as the RAD fragments were longer than 2 x 150 read, the primer pairs amplifying the shorter products were derived entirely from the forward read, whereas the longer product would incorporate an additional sequence of unknown length between the forward and reverse reads. Sequences of all primers are found in Supplementary Table S2.

Sex associated marker validation

The primers designed for candidate sex-associated markers were tested via PCR amplification with 2x QIAGEN multiplex master mix (QIAGEN B.V, Venlo, Netherlands). After optimisation a final PCR protocol was designed consisting of a 95°C hot start for 10 minutes, followed by 35 cycles of denaturation for 30 seconds at 95°C, 60 seconds annealing at 61°C and 45 seconds extension at 72°C, with a final extension of 10 minutes at 72°C. All primers were used at a final concentration of 0.1 µM. Initial screening was against a single male/female pair of *L. vulgaris*. Markers showing male specificity were validated against a panel of six male and six female *L. vulgaris*. Validated makers were then tested against a male/female pairs of both *L. montandoni* and *L. helveticus* (as representatives of the *L. vulgaris* complex, and the wider *Lissotriton*

genus, respectively). Any markers with multispecies sex specificity were tested in male/female pairs of all available species of *Lissotriton*. Finally, a multiplex PCR was designed, combining the most broadly male-specific markers with an autosomal control marker, CDK-17, which amplifies a product of 537 bp.

Linkage map construction

The joint VCF file produced by Stacks was filtered with VCFtools (Danecek et al. 2011) to exclude indels and SNPs with greater than 5% missing data, a mean depth of less than 10, or a minor allele frequency of less than 0.2. The thin function of VCFtools was used to select a single SNP per marker. Lep-MAP 3 (Rastas 2017) was then used to construct paternal, maternal and sex averaged linkage maps. To incorporate the candidate Y-linked markers identified in the known-sex adults into the linkage map, a custom R script was used to translate the presence/absence of reads for these markers into pseudo-SNP genotype calls for all samples (markers with no reads were assigned an artificial AA genotype whereas markers with reads were assigned as AT). These calls were appended to the call file produced by the first stage of the Lep-MAP 3 pipeline (the ParentCall2 module) and incorporated into all subsequent steps. Initial linkage groups were created with the SeparateChromosomes2 module, with a LOD limit of 20 and distortion LOD set to 1. Unplaced markers were then added with the JoinSingles2All module with a LOD limit of 15. The markers were then ordered with the OrderMarkers2 module, using 20 merge iterations, 8 polish iterations, a minError value of 0.02 and the scale setting M/N 2. The informative mask options 23 and 13 were used for the paternal and maternal maps respectively.

Linkage map comparison with *Pleurodeles waltl* genome

Initial validation of the linkage map was performed by BLASTing the sequences of the mapped makers against the genome assembly of the Iberian ribbed newt (*Pleurodeles waltl*) (Brown et al. 2025), using a word size of 11 and requiring a minimum E value of 1e-20. To account for the high degree of paralogy typical of newt genomes, the blast results were filtered to include only hits that exceeded the significance of the next best hit by five orders of magnitude, following the methodology of Purcell et al. (2014). The filtered blast hits were then used to create an Oxford plot via a custom R script.

Marker density analysis

In an XY system, the sexed-linked region is not expected to undergo recombination in males, and so markers within this region should exhibit extreme genetic linkage when transmitted from father to offspring. When a paternal linkage map is constructed, these markers will form a region of high marker density. However, as the sex-linked region does undergo recombination in females, these markers should be spread over a wider region when the corresponding maternal map is constructed. We attempted to locate the sex-linked region by plotting marker density in both paternal and maternal linkage maps via a custom R script, and identifying a peak which is exclusive to the paternal map.

Analysis of paternal specific markers and SNPs

To be placed on the linkage map a marker must have a SNP that is heterozygous in at least one the parents. SNPs which are heterozygous in the father but homozygous in the mother are termed paternal specific SNPs. RAD loci which include only paternal specific SNPs are termed paternal specific markers. While a large number of paternal (and maternal) specific SNPs and markers will be randomly scattered across the genome, the Y-linked region is expected to be particularly enriched in paternal specific SNPs and markers, as in an XY system this region is, by definition, heterozygous in males. To identify this enriched region, we first plotted the number of paternal SNPs and markers against the total number of markers per linkage group. We then used a custom R script to divide each linkage group into bins of 2 cM and plot the probability (assuming the markers were randomly and independently distributed) of obtaining the measured number of paternal specific markers and SNPs, using a binomial distribution for the markers and a Poisson distribution for the SNPs.

Results

Sex association in known-sex adults

After demultiplexing and filtering, the 60 known-sex individuals yielded a total of 901 million reads (per sample median: 13.7 M, interquartile range: 11.5-16.5 M). The three runs of the denovo_map.pl pipeline yielded differing results as parameter M was varied. Increasing M decreased the total number of loci identified in the dataset (M=2: 1,541,940, M=6: 1,026,619, M=10: 911,354) and increased the mean adjusted sequencing depth per sample (M=2: 28.2, M=6: 30.6, M=10: 31.7) and the proportion of loci found in at least 50% of samples (M=2: 8.61%, M=6: 12.56%, M=10: 13.31%).

As expected, in the initial sets of candidate markers (M=2: 32, M=6: 26, M=10: 19, after duplicates removed, 35 unique sequences in total) selected by screening for presence in males and absence in females a high degree of paralogy was observed, with the number of BLAST hits per marker varying from 1 to 500. The proportion of candidate markers without paralogous hits increased with parameter M (M=2: 19%, M=6: 38%, M=10: 53%). After the 10 highest ranked candidate markers from each run were selected, the majority of candidates appeared in multiple runs. Removing duplicates left 14 unique candidate markers for PCR validation. In total 25 primers pairs were designed (Supplementary Table S2), as for three markers Primer3 failed to generate a valid primer pair for the 'short' product under the given conditions.

Sex-associated marker validation

Of the 14 candidate markers, six show validated male-specific amplification in *L. vulgaris*, with an additional marker showing initial male-specificity screening but failing in the 12 individual validation panel. Three markers retain sex-specificity in *L. montandoni* (Table 1). Two markers, LvY-79267 and LvY-51393 show broad male-specificity within the smooth newt species complex. However, both markers fail to amplify in males in at least one taxon within this group, with LvY-79267 failing in *L. v. meridionalis* and LvY-51393 failing in *L. v. ampelensis*. In the more distantly related species (*L. helveticus*, *L. italicus* and *L. boscai)* no sex-specific amplification is observed with any primer pair, with markers failing to amplify in either sex or amplifying in both sexes (all gels resulting from screening and validation found in Supplementary Figures 1-4). If the candidate markers from each run are considered separately, higher values of parameter M give more useful results. Five out of ten candidates selected from M=10 are sex-specific in *L. vulgaris* compared to four out of ten from M=6 and two out of ten selected from M=2. A final multiplex mix (Table 2), amplifying fragments from both LvY-79267 and LvY-51393 as well as the autosomal control marker CDK-17, shows robust sex-identification across all taxa within the *L. vulgaris* species complex included in this study (Fig. 1).

Marker	Ranked in Stacks run M=			Primer pair	Male specific amplification in *Lissotriton* taxa									
					L. vulgaris species complex							Other		
	2	6	10		*L. vulgaris*	*L. montandoni*	*L. v. ampelensis*	*L. v. meridionalis*	*L. schmidtleri*	*L. graecus*	*L. kosswigi*	*L. helveticus*	*L. italicus*	*L. boscai*
lvY-36220	yes	yes	yes	short	○									
				long	×									
lvY-65590	yes	yes	yes	short	○									
				long	○									
lvY-81842	yes	yes	yes	short	○†	○						×		
				long	○†	×						×		
lvY-123701	yes	yes	yes	short	+	×						×		
				long	+	×						×		
lvY-138925	yes	yes	yes	short	○									
				long	○									
lvY-143365	yes	yes	yes	short	○									
				long	○									
lvY-79267		yes	yes	short	+	+	+	×	+	+	+	×	×	×‡
				long	+	+	+	×	+	+	+	×	×	×‡
lvY-115632		yes	yes	short	+	+	×	×	○	+	+	×	○	×
				long	+	+	○	○	○	○	○	×	×	×‡
lvY-128014		yes	yes	short	+	×‡						×		
				long	○									
lvY-51393	yes		yes	short	+	+	×	+	+	+	+	○	○	×‡
				long	+	×	×	+	×	+	+	○	○	×‡
lvY-99941		yes		long	○									
lvY-11521	yes			long	○									
lvY-28978	yes			long	○									
lvY-102891	yes			short	○									
				long	○									

Table 1: Summary of results of PCR screening of primer pairs designed for candidate Y-linked markers in *Lissotriton* newts. Results are indicated as: + amplification only in male samples, ○ amplification in both male and female samples, × no amplification in either sex. Twenty-five primer pairs were tested in a male-female pair of *L. vulgaris*, followed by validation of successful markers in a 12 individual panel. Nine primer pairs, covering five candidate markers, show confirmed male-specific amplification in *L. vulgaris*, with a further two primer pairs, marked with †, showing initial male-specificity but failing in the wider panel. The successful primer sets were then tested in male-female pairs of *L. montandoni* and *L. helveticus*, and the three markers which show sex-specificity in *L. montandoni* were then tested in male-female pairs of all available *Lissotriton* taxa. While markers lvY-79267 and lvY-51393 demonstrate broad male-specificity within the *L. vulgaris* species complex no marker shows any sex-specificity in more distantly related *Lissotriton* taxa. In several cases, marked with ‡, the PCR results are difficult to interpret due to faint amplification of multiple off-target bands.

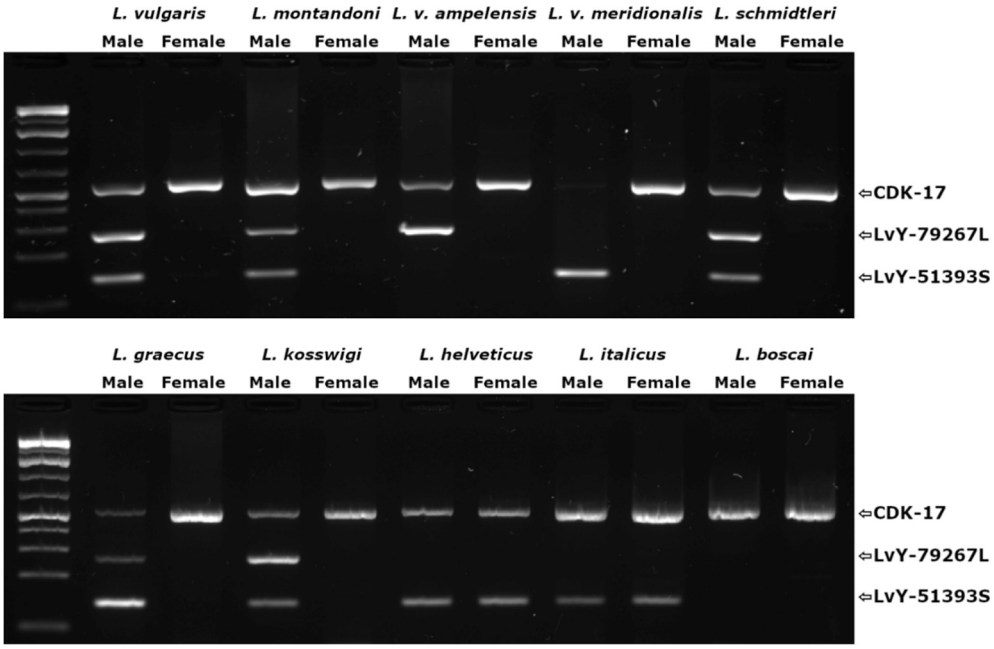

Figure 1: The results of the multiplex PCR designed for molecular sex identification in the *L. vulgaris* species complex. The three primer sets amplify a control marker CDK-17 (537 bp) and two Y-linked markers LvY-79267-Long (ca. 240 bp) and LvY-51393-Short (124 bp). Within the *L. vulgaris* complex (*L. vulgaris, L. montandoni, L. schmidtleri, L. graecus* and *L. kosswigi*) amplification of the Y-linked markers is observed only in male samples. In more distantly related *Lissotriton* species (*L. helveticus, L. italicus* and *L. boscai)* sex-specific amplification is not observed. As male amplification of one of the Y-linked markers is not observed in each of the non-nominate subspecies of *L. vulgaris* (LvY-51393-Short *in L. v. ampelensis* and LvY-79267-Long in *L. v. meridionalis*) we recommend using both diagnostic primer pairs for reliable sex-identification.

Primer Pair	Forward Sequence	Reverse Sequence	Product (bp)
CDK-17	GGCATGGGAAGAACAGAAGA	CCATCTGCTTGGACTGTTGA	537
lvY-51393-short	GACCACTGTAGAGGAGGTTGG	GCTGCCTGTTTCTGGATGTC	124
lvY-79267-long	CAAGGCCAAAATGATCCCGC	TGTGCATTGACCATAAAGCCC	ca. 240

Table 2: Primer sequences used for the sex diagnostic multiplex PCR for use within the *L. vulgaris* species complex, as demonstrated in Fig. 1. CDK-17 is a control marker which amplifies in all species, lvY-51393-short and lvY-79267-long amplify only in males, however inclusion of both is recommended as some taxa may fail to amplify one the markers.

Linkage map construction

The 148 individuals of the linkage map family yielded a total of 1.34 billion reads (per sample median: 7.91 M, interquartile range: 7.04-8.92 M). The denovo_map.pl pipeline produced a total of 414,146 RAD loci of which 137,538 (33.2%) were present in at least 50% of individuals. After filtering with VCFtools a total of 16,738 markers (each with 1 representative SNP) were available for linkage map construction. The final linkage maps consist of 12 linkage groups. The sex-averaged map contains 10,763 markers and has total length of 1,366 cM (Fig. 2). Respectively, the paternal and maternal maps) contain 7,484 and 7,452 markers and have total lengths of 1,300 and 1,688 cM (Sup. Figs. 5-6, Sup. Table S1). The sex-averaged and paternal maps include 32 Y-linked presence/absence markers. In both maps these form a tight cluster, spanning less than 2 cM, located at one end of linkage group 5.

Figure 2: Sex-averaged linkage map for *L. vulgaris* based on a full-sib family of 146 offspring. The linkage map is composed of 10,763 RAD markers in 12 linkage groups, ordered by length in centimorgans. Thirty-two Y-linked presence/absence markers (highlighted in red), first identified in known-sex adult *L. vulgaris* are located within a 2 cM region of Group 5, identifying this as the Y-chromosome.

Comparison with *Pleurodeles waltl* genome

Five hundred and seventy-nine (5.4%) of the markers placed on the linkage map, including two Y-linked markers, can be aligned with sequences within the *P. waltl* genome assembly (Fig. 3). Synteny between the taxa appears strongly conserved, with each linkage group reciprocally matching a single *P. waltl* chromosome, 472 (82%) *L. vulgaris* markers mapping to their orthologous chromosome, and large blocks of conserved synteny are observed within each chromosome/linkage group pair. Evidence of a large inversion is also seen on linkage group 10. We observe no clear pattern in the 18% of markers mapping to non-orthologous chromosomes, indicating that these are a likely a result of misalignment of paralogous sequences, rather than evidence of any large-scale genomic rearrangements. Linkage group 5 is clearly orthologous to *P. waltl* chromosome 5. The two Y-linked presence/absence markers that have identifiable orthologs both align with sequences close to one end of chromosome 5, with start co-ordinates of 35.2 and 62.5 Mbp (the overall length of chromosome 5 is 1.91 Gbp).

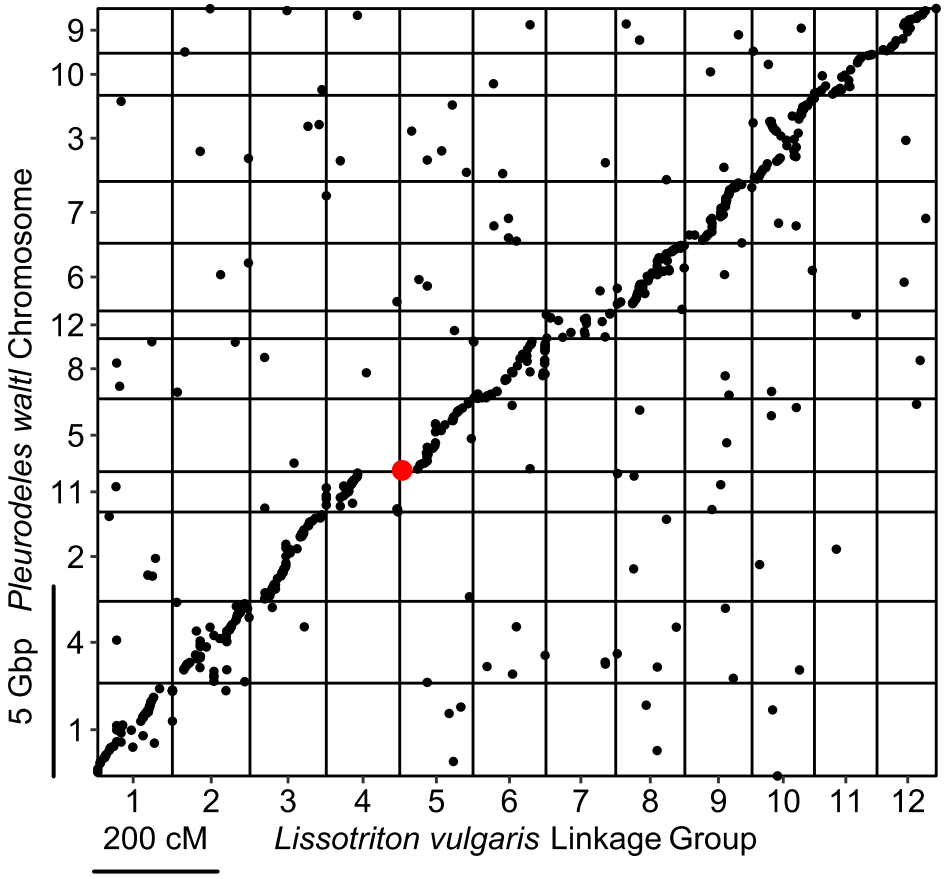

Figure 3: Oxford plot comparing the locations of 579 RAD markers in the *L. vulgaris* linkage map with their orthologs within the *P. waltl* genome, as assembled by (Brown et al. 2025). Two Y-linked presence/absence markers are highlighted in red. Four hundred and seventy-two markers (82%) map to orthologous chromosomes, demonstrating broad synteny between the two newt genera.

Marker density analysis

Marker density differs significantly between paternal and maternal linkage maps (Fig. 4). In the male map each linkage group is dominated by a single, tight cluster of markers, indicating large regions of reduced recombination. The clusters are usually in the centre of the group, suggesting that most recombination events occur near the ends of the chromosomes. In the maternal map marker density is more uniform, although areas of increased marker density are observed towards the end of some linkage groups. As expected, the Y-linked presence/absence markers are found in a region that shows high marker density in the paternal map but not in the maternal map. However such regions can be observed across the linkage map, which is an expected consequence of the differing rates of recombination in male and female meiosis. While

linkage group 5 does show the greatest difference in length and average marker density between the paternal and maternal map of any of the groups, the peak of marker density six other paternal linkage groups exceeds that of the Y-linked region. Peak marker density in the paternal map is found in linkage group 10, where 337 markers map to a single point.

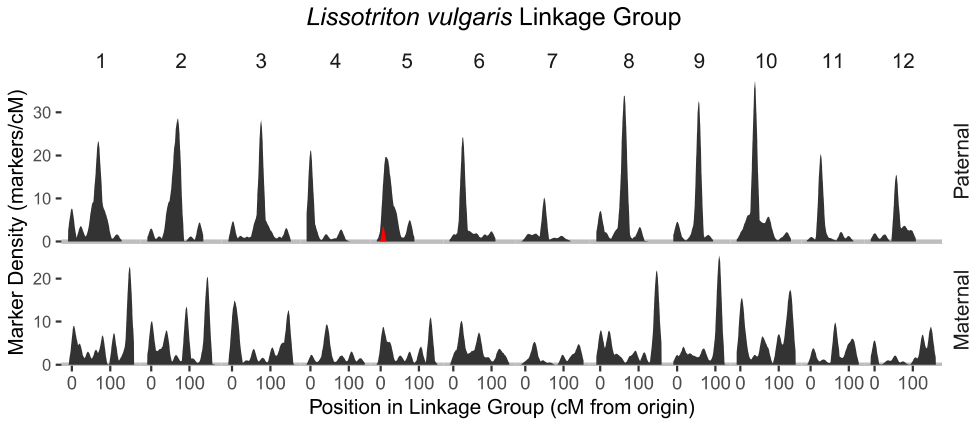

Figure 4: Marker density in the paternal and maternal *L. vulgaris* linkage maps. Total maker density is shown in black, and density of Y-linked presence/absence markers is shown in red, highlighting the Y-linked region on linkage group 5. Markers were aggregated into 2 cM bins and a Gaussian smoothing function with a 10 cM range was applied.

Paternal specific markers and SNPs

3,389 paternal specific markers and 9,414 paternal specific SNPs were located within the sex-averaged linkage map. Distribution across linkage groups was extremely uniform, with a linear trend observed between the number of total markers and paternal specific markers/SNPs within each linkage group (Fig. 5). Neither linkage group 5 nor any other group deviated significantly from this trend. The region containing the majority of the Y-linked presence absence markers has a significantly elevated concentration of both paternal specific markers ($P = 1.38 \times 10^{-4}$) and SNPs ($P = 7.77 \times 10^{-10}$), however more significant concentrations are present at multiple locations throughout the linkage map (Fig. 6). Eighteen 2 cM bins have a more significant concentration of paternal specific markers and eight have a more significant concentration of paternal SNPs.

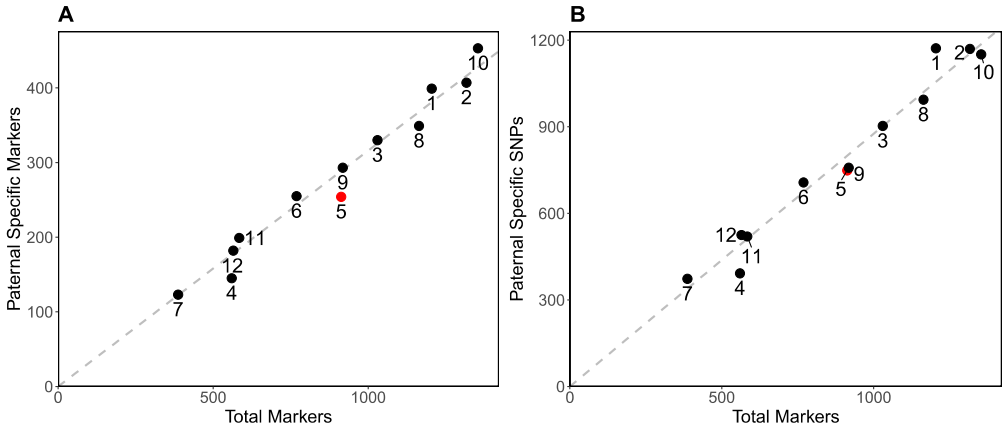

Figure 5: Plots showing the number of the paternal specific markers (A) and SNPs **(B)** against the total number of markers in each group of the sex-averaged *L. vulgaris* linkage map. Linkage group 5, baring the Y-linked presence/absence markers is highlighted in red.

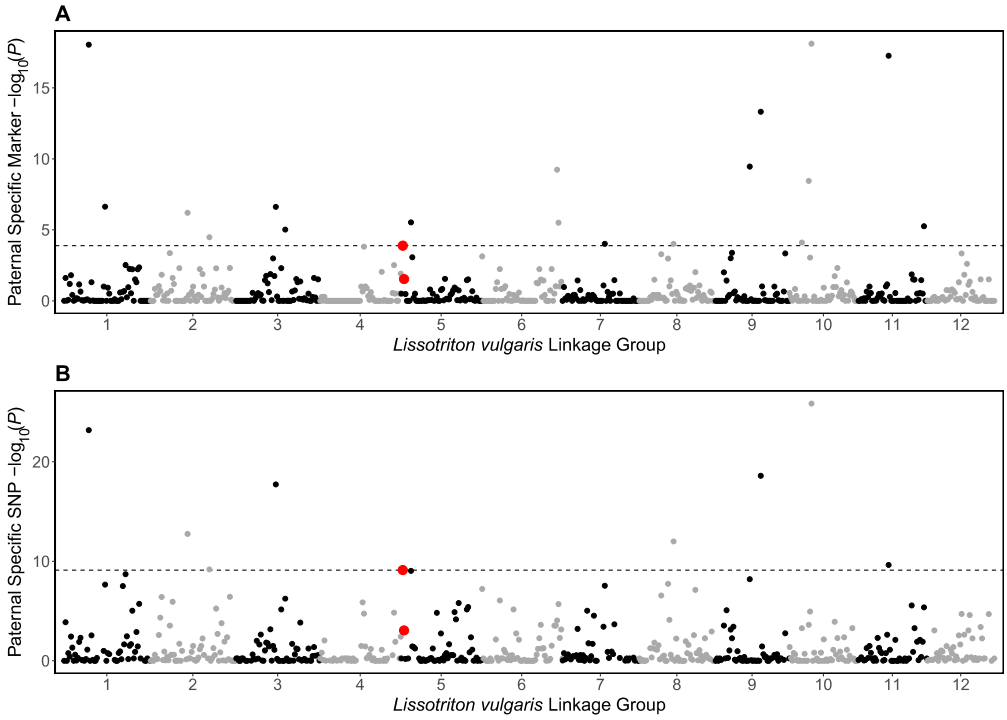

Figure 6: Manhattan style plot showing enrichment in paternal specific markers (A) and SNPs **(B)** in the sex-averaged *L. vulgaris* linkage map, divided into 689 bins of length 2 cM. Enrichment is shown as $-\log_{10}$ of the probability of a bin containing the observed number of paternal specific markers and SNPs. The 32 Y-linked presence absence markers are located in two adjacent bins highlighted in red. As expected, these bins show significant enrichment in paternal-specific markers ($p = 1.38 \times 10^{-4}$) and SNPs ($p = 7.77 \times 10^{-10}$). However other regions of the genome show even greater enrichment, with 18 and 8 bins exceeding the significance of the Y-linked region (shown as the dashed horizontal line) in paternal specific markers and SNPs respectively.

Discussion

We successfully identify sex-linked presence/absence markers in the smooth newt, *L. vulgaris* via an associative RADseq approach, confirming that this method remains effective even in exceptionally large genomes, such as those of Salamanders. As such genomic gigantism is often the result of accumulation of repetitive elements (Lee & Kim 2014; Sun et al. 2012) there is high chance that any given sequence will have multiple paralogs throughout the genome. Our results indicate that the efficiency of the discovery process can be significantly enhanced by aggressively filtering out candidate markers showing such paralogy. Out of 14 markers we test via PCR, five show validated sex association in *L. vulgaris*. Compared to the two previous studies using a similar methodology in salamanders, this is a notably high success rate. Hime et al. (2019) screened 43 loci for sex association in the hellbender, *Cryptobranchus alleganiensis*, with four successful in PCR. Hu et al. (2019) designed 100 candidate primer pairs to yield four reliable W-linked markers in the Chinese giant salamander, *Andrias davidianus*. In addition, our experience indicates that thoughtful optimisation of the upstream bioinformatics increases the chance of identifying a useful marker. Increasing the value parameter M in the denovo_map.pl/ustacks programs of the Stacks package from 2 to 10 vastly reduced the chance of a marker with paralogs being selected as a sex-linked candidate and doubled the number of candidates that gave sex-specific PCR amplification.

To our knowledge these presence/absence markers are the first tool for genetic sex identification described in any species of newt (subfamily Pleurodelinae) or salamander within the family Salamandridae. Two markers, LvY-79267 and LvY-51393, are particularly notable, as we show that in combination they allow for molecular sex identification across the smooth newt species complex with a simple a multiplex PCR protocol. As *Lissotriton* take 2-3 years to reach sexual maturity, and are difficult to morphologically sex as juveniles (Sparreboom 2014), a robust genetic assay of sex will be of significant benefit for researchers interested in the conservation, ecology and behaviour of these species.

The sex-specificity of the markers decreases with phylogenetic distance. Five markers are male-specific in *L. vulgaris*. Three of these retain specificity in *L. montandoni* – the most basal species within the smooth newt complex, which is notable as previous cytological studies were unable to identify any sex-chromosome in this species (Zbożeń et al. 1993). No marker is found to amplify sex-specifically in more distantly related *Lissotriton* species. Variation in the Y-chromosome may contribute to the differing degrees of reproductive isolation within the genus (Johnson et al. 2012; Yoshida et al. 2014) – while species within the smooth newt complex hybridise readily,

L. vulgaris and *L. helveticus* (the palmate newt) show almost complete reproductive isolation, despite co-occurring over a large area of western Europe (Miralles et al. 2024).

We were unable to obtain known-sex samples of two *Lissotriton* taxa. The Caucasian smooth newt *L. lantzi* is a member of the smooth newt complex (Wielstra et al. 2018) and so we predict that the identified markers will also be sex-specific in this species. *L. maltzani* has recently been recognised as a separate species from its close relative the Iberian newt, *L. boscai* (Sequeira et al. 2020; Speybroeck et al. 2020), but can be expected to show a similar, non-specific result with the markers described above.

We construct a high-density linkage map for *L. vulgaris* and identify a Y-determining region at the end of linkage group 5 via the incorporation of the markers identified above. The resulting map matches the observed karyotype of *L. vulgaris* (Wickbom 1945), and the number and density of markers is significantly increased compared to *L. vulgaris* x *L. montandoni* linkage maps published by (Niedzicka et al. 2017). A disadvantage of RADseq-based linkage maps is that the information they provide typically lacks context; it is difficult to directly relate a given linkage group to a particular chromosome, and the mapped sequences are unlikely to have any known function. To provide useful context, we attempt to align the *L. vulgaris* linkage map with the *P. waltl* genome assembly (Brown et al. 2025). Surprisingly, given the challenges of aligning short, non-coding sequences with a highly paralogous genome that diverged over 60 million years ago (Marjanović & Laurin 2014; Zhang & Wake 2009) a useful comparison is possible and shows that genome-level synteny between the two genera is highly conserved.

However, we are unable to confidently identify a sex-determining region of this linkage map without using additional information. We do observe considerable variation in intra-group marker density between the paternal and maternal maps, but this is not restricted to any one linkage group. In general, we show that *L. vulgaris* conforms to an extremely widespread pattern where male and female meiosis differ significantly, with males experiencing relatively more recombination near the telomeres and less recombination closer to the centromere of chromosomes (Sardell & Kirkpatrick 2020). Linkage group 5 varies slightly, as the telomeric region in which the Y-linked markers cluster shows reduced paternal recombination. This could be interpreted as restricted recombination between the X and Y chromosomes, however a similar phenomenon can also be observed in the autosomal linkage group 4.

Brelsford et al. (2016) reported a significant excess of paternal specific SNPs on the linkage group corresponding to the Y-chromosome of the European tree frog, *Hyla arborea*. In our study however, we do not observe RAD markers baring paternal specific SNPs to be more prevalent on a particular linkage group or form an obvious cluster within any linkage group. The likely explanation is that the sex-determining region of *L. vulgaris* is simply too small to be observable with this methodology - especially given

the enormous size of the overall genome and the consequently low per base pair recombination rate.

As recombination frequency, and the density of RAD markers will vary over the length of each chromosome, it is not possible to estimate the size of the Y-linked region in *L. vulgaris* with any accuracy. However, we can identify orthologous loci for two Y-linked presence/absence sequences within *P. waltl* chromosome 5, which corresponds to our linkage group 5. The orthologs are separated by just 27.3 Mbp, approximately 1.4% of the overall chromosome length. If this is reflective of the situation in the *L. vulgaris* Y-linked region, it would explain the absence of an obvious cluster of paternal SNPs. A region this small would contain too few RAD markers to stand out against the genetic background, which will contain many other clusters of paternal (and maternal) specific SNPs.

It is somewhat unexpected that the non-recombing region is not more prominent. A cytological study by Schmid et al. (1979) reported that, in male *L. vulgaris*, no chiasmata were observed in any region of the long arm of chromosome 5, which was hence identified as the Y-chromosome, despite being largely homomorphic. While we do observe reduced paternal recombination at the end of linkage group 5 where the Y-linked markers are placed, there is still a significant degree of recombination occurring within this region, the markers have not been collapsed to a single point on the paternal map. In addition, if the non-recombing Y-linked region covered the entirety of the long arm, we would expect a far great greater density of paternal specific SNPs and markers than we observe. It is possible that *L. vulgaris* shows regional diversity in the structure and recombination frequency of the Y-chromosome, as is noted in other amphibians, including *Hyla arborea* (Dufresnes et al. 2014) and the alpine newt *Ichthyosaura alpestris* (Herrero & López-Fernández 1986). Such diversity may explain the discrepancy between the karyology, performed on a population gathered near Ulm, Germany, and our linkage map, generated from a Polish population collected in the vicinity of Kraków.

We conclude that, while linkage maps are of great benefit for locating previously discovered sex-linked markers within a genome, their utility for identifying sex-linked regions without *a priori* knowledge is strongly dependent on the size of the region of supressed recombination. In species with very large genomes and small sex-linked regions, the technique is unlikely to be successful. However, we show that an associative RADseq approach can still be highly effective even in these situations, especially when measures are taken to suppress the selection of markers with autosomal paralogs.

Data Availability

All raw reads can be found as a part of the NCBI accession associated with Bioproject: PRJNA1118769. (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1118769) (France et al. 2024a). Information on samples and the positions and sequences of all markers in the *Lissotriton* RADseq linkage map can be found in a .xlsx file hosted together with scripts and bioinformatic pipelines used for analysis in the associated Zenodo repository (https://doi.org/10.5281/zenodo.13870462) (France et al. 2024b). Scripts are also available at an associated GitHub repository (https://github.com/Wielstra-Lab/Lissotriton_RADseq_Y).

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Zhang P, Wake DB (2009) Higher-level salamander relationships and divergence dates inferred from complete mitochondrial genomes. *Molecular Phylogenetics and Evolution*. 53: 492–508. DOI: 10.1016/j.ympev.2009.07.010.

Supporting Information

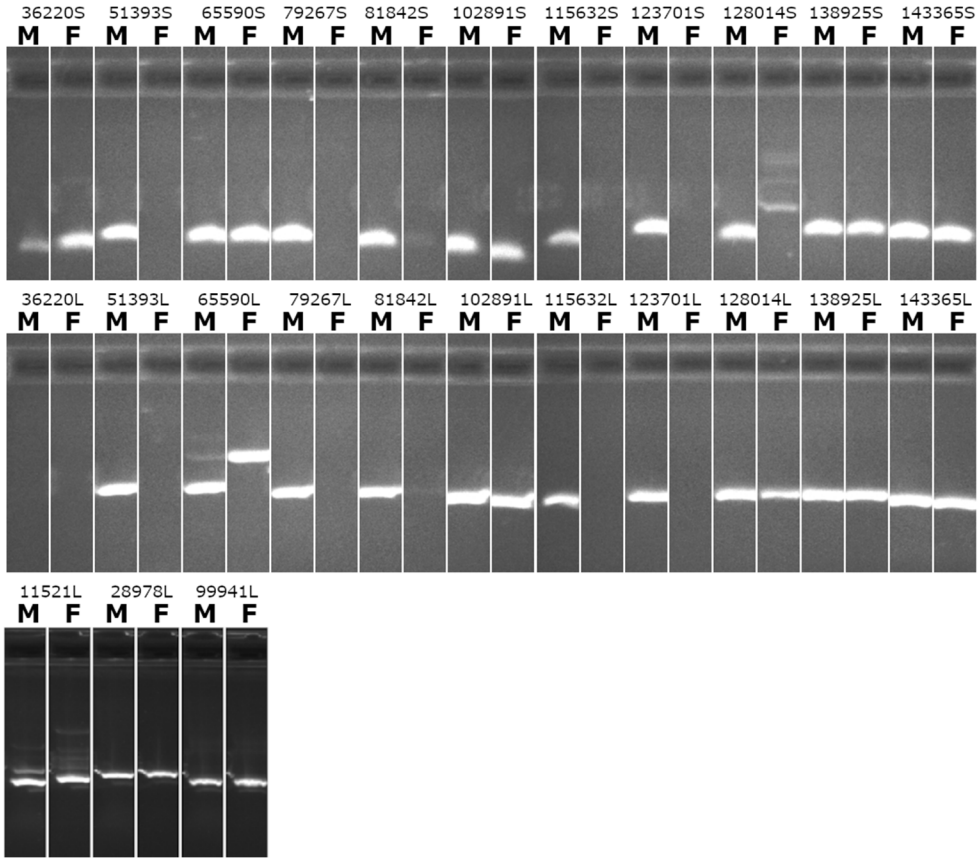

Figure S1: PCR screening of 25 primer pairs designed for candidate Y-linked markers for male specific amplification in *L. vulgaris*. Label M indicates the male sample and label F indicates female. Markers are indicated by number followed by either S (for primer pairs designed for the short product – c.a. 100 bp) or L (for primer pairs designed for the long product – c.a. 200 bp). 10 primer pairs, and 5 markers showed amplification only in the male sample. One further pair, LvY-128014-short showed strong amplification in the male and only weak amplification in the female. A single primer pair, LvY-36220-long, failed to amplify in either sample. The other 25 primer pairs amplified in both the male and female samples.

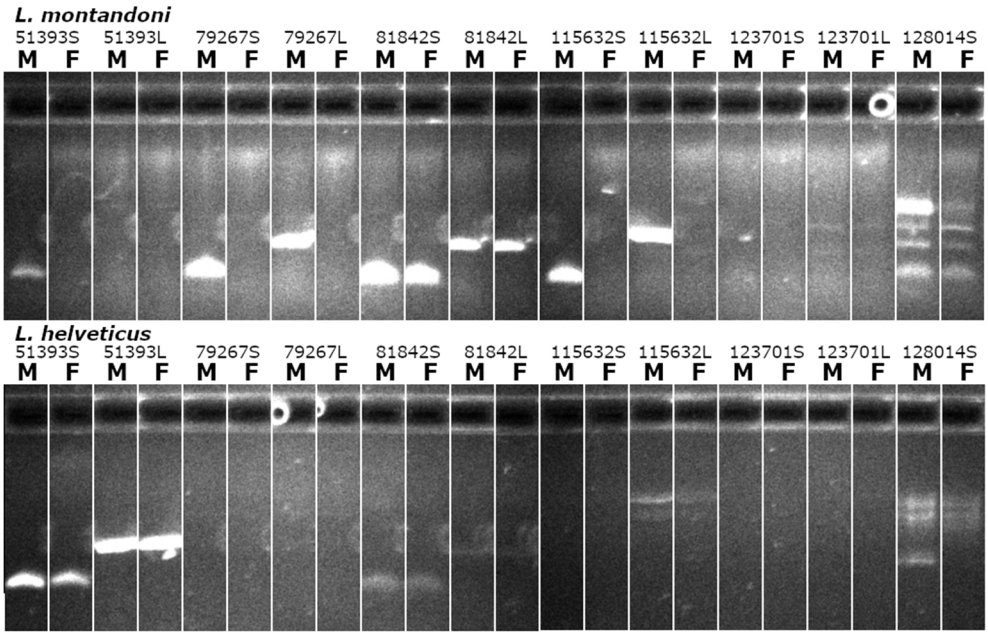

Figure S2: PCR screening of the 11 primer pairs showing successful male-specific amplification in *L. vulgaris*, in *L. montandoni* and *L. helveticus*. Label M indicates the male sample and label F indicates female. Markers are indicated by number followed by either S (for primer pairs designed for the short product – c.a. 100 bp) or L (for primer pairs designed for the long product – c.a. 200 bp). Five primer pairs, designed for three marker sequences, show male specific amplification in *L. montandoni*. Only two primer pairs show strong amplification in L. helveticus, and no male specificity is observed.

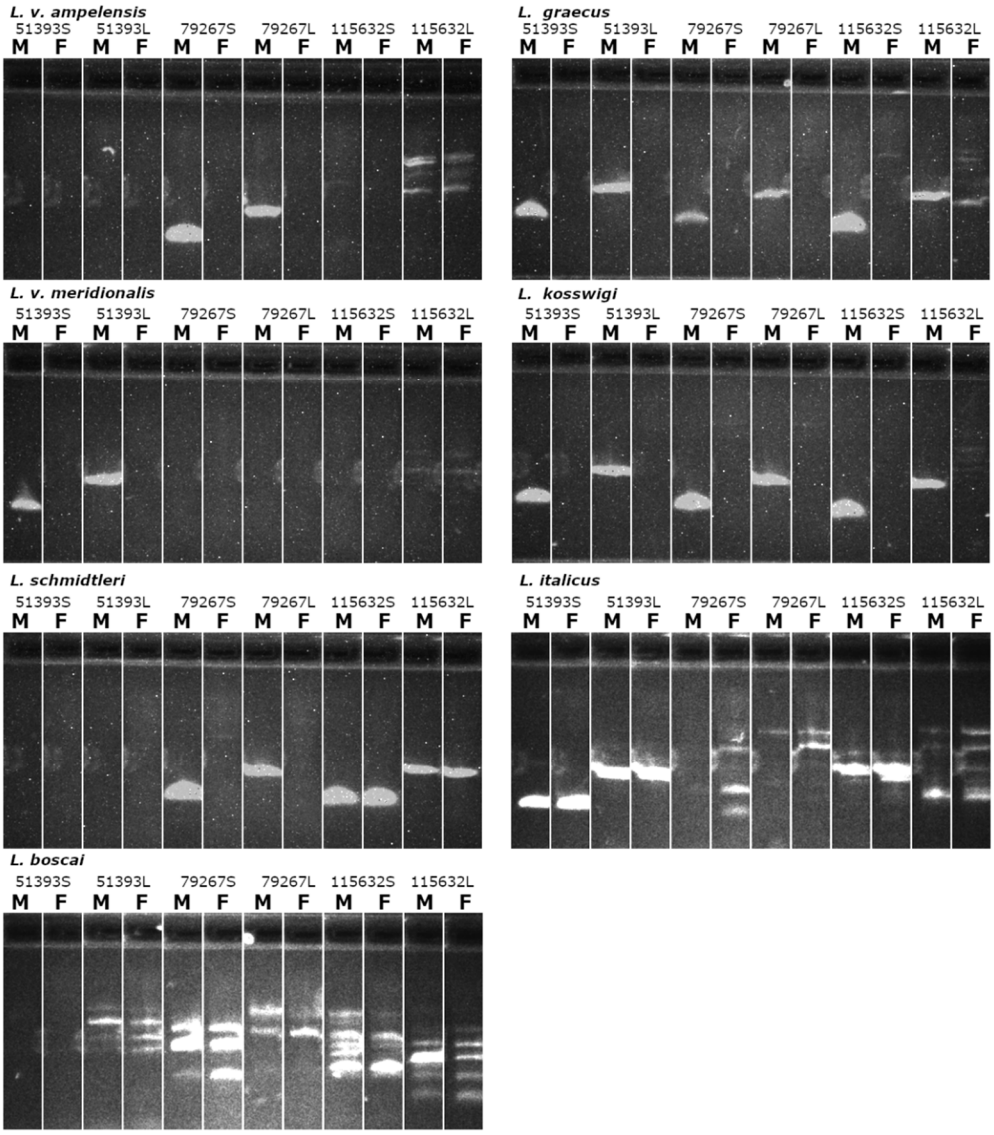

Figure S3: PCR screening in seven further *Lissotriton* taxa, using the primer pairs for the three markers that show male specificity in *L. montandoni*. For the five taxa in the *L. vulgaris* species complex, at least one marker shows male-specific amplification. In the two more distantly related species (*L. italicus* and *L. boscai*) no male-specific amplification is observed, and multiple non-target bands appear.

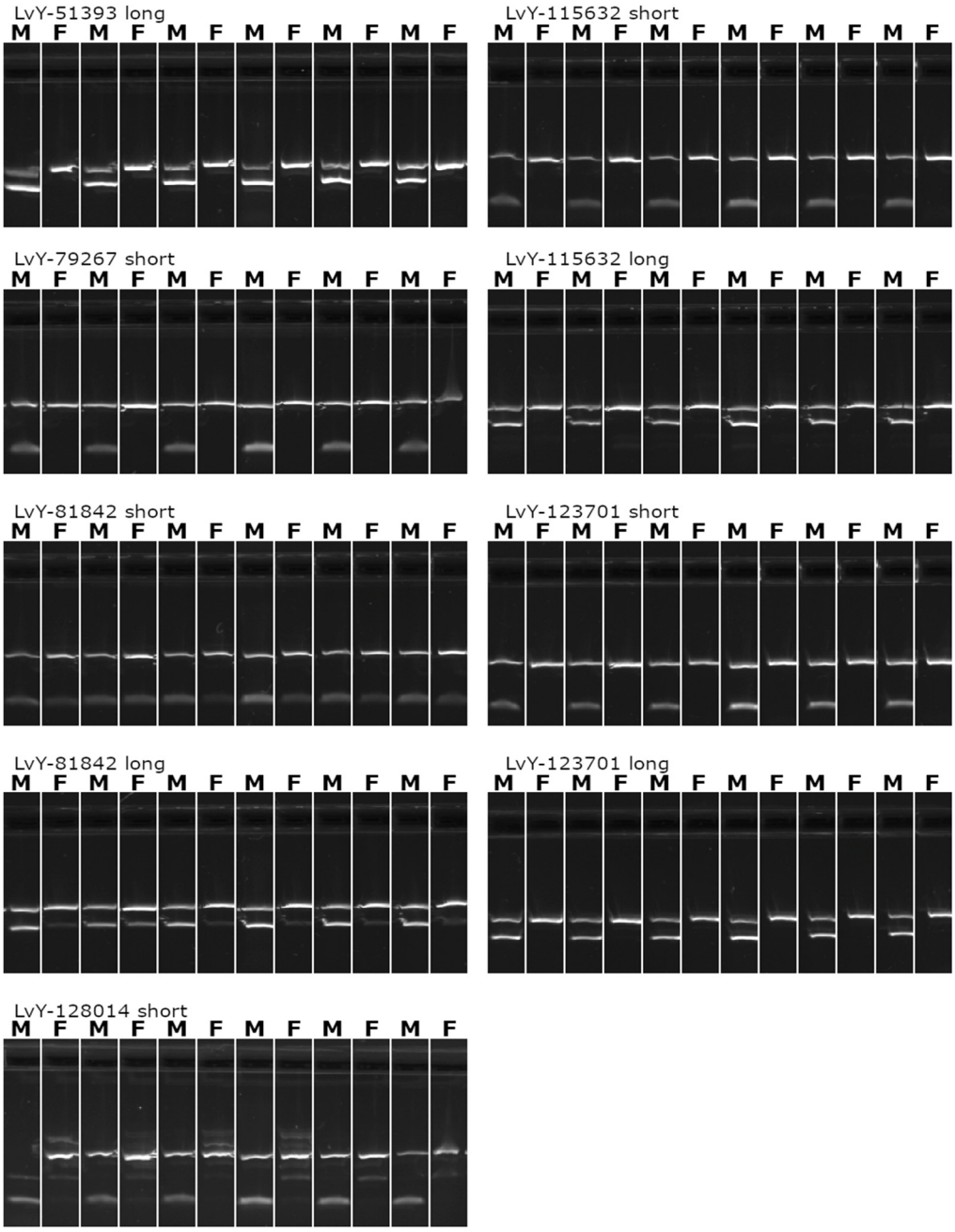

Figure S4: **PCR validation of primer pairs showing male specificity in *L. vulgaris*,** in a 12 individual panel (six male, six female, not including the two individuals previously used for screening). Primers amplifying CDK-17 were included as a control, and the resultant product appears above the test bands in all cases due to greater length (517 bp compared to 100-250 bp). Both primer pairs designed for LvY-81842 appear to amplify in their product in females and thus fail validation. All other primer pairs are validated as male specific.

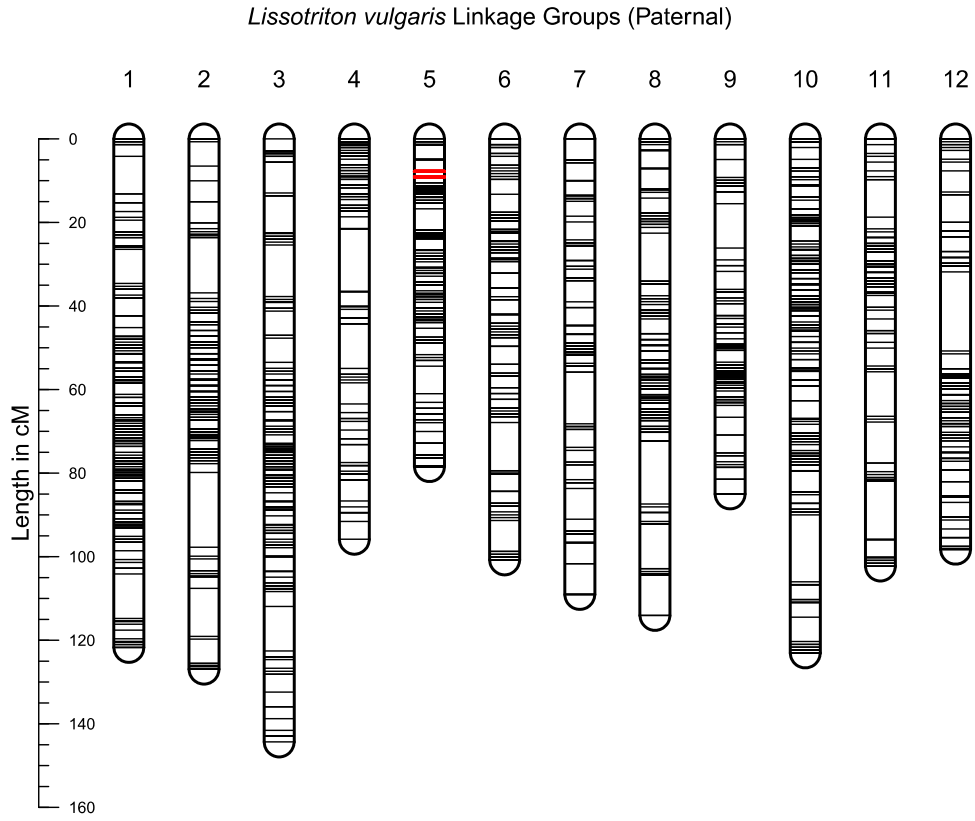

Figure S5: The paternal *L. vulgaris* linkage map, displaying 7,484 RAD markers across 12 linkage groups, including 32 Y-linked markers highlighted in red on linkage group 5. Groups are ordered according to the length of the corresponding group in the sex averaged map.

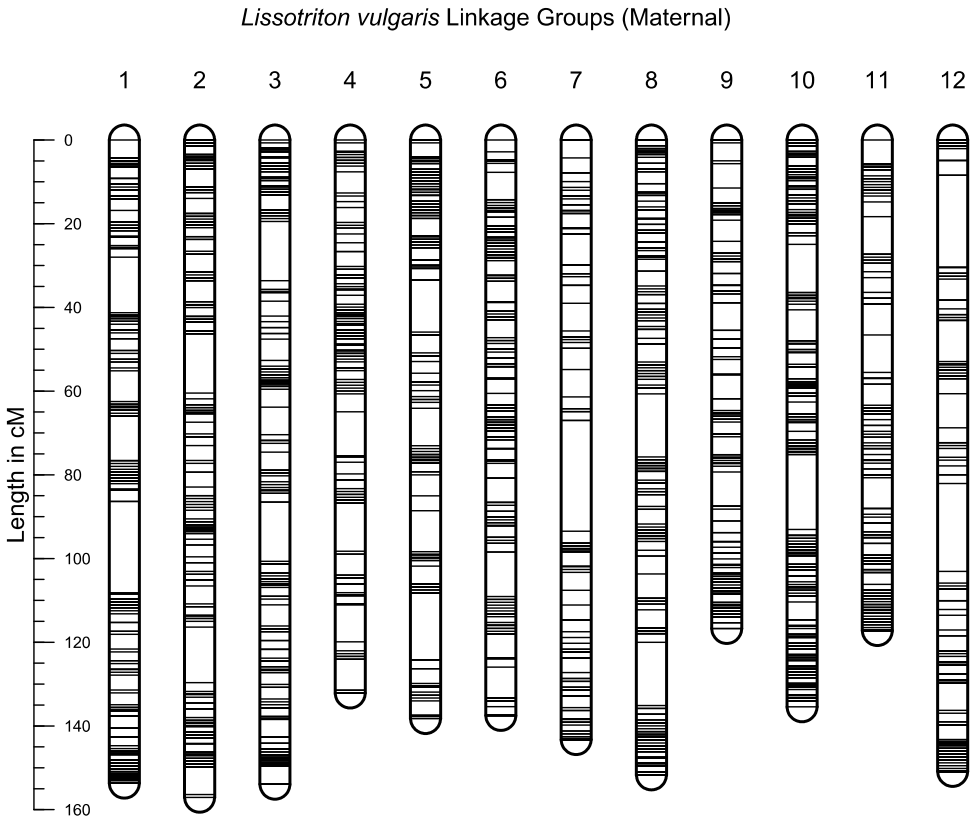

Figure S6: The maternal *L. vulgaris* linkage map, displaying 7,452 RAD markers across 12 linkage groups. Groups are ordered according to the length of the corresponding group in the sex averaged map.

Group	Sex-averaged		Paternal		Maternal	
	N markers	Length (cM)	N markers	Length (cM)	N markers	Length (cM)
1	1205	127.0	820	121.7	863	153.7
2	1317	125.4	924	126.9	916	157.0
3	1030	123.4	718	144.4	749	153.9
4	560	118.9	418	95.8	343	132.1
5	877	118.4	666	78.5	531	138.2
6	769	118.0	522	100.8	535	137.5
7	387	112.7	250	109.1	287	143.3
8	1164	111.9	831	114.1	789	151.7
9	918	108.5	626	85.0	648	116.7
10	1354	101.1	897	123.1	972	135.4
11	584	101.1	392	102.3	411	117.2
12	565	99.6	387	98.2	408	150.9

Table S1: Characteristics of the sex-averaged, paternal and maternal *Lissotriton vulgaris* linkage maps, including the number of markers and the length (in centimorgans) of each group.

Table S2: Sequences of all primers used in this study, CDK-17 is an autosomal marker used as a control, all others are candidate Y-linked markers developed for *Lissotriton vulgaris*.

Primer Pair	Forward Primer Sequence	Reverse Primer Sequence	Product (bp)
CDK-17	GGCATGGGAAGAACAGAAGA	CCATCTGCTTGGACTGTTGA	537
lvY-11521-long	GCATTTGGGCAGCTTCATTC	CAATTCAGGCACACACCAGC	>200
lvY-28978-long	TCATGCATAGCCAAAGAGTTTGTC	CCCTGATGACACTTGATCGC	>200
lvY-102891-short	CTAGATGCGCATCCACTGGG	CTGACATTAAGCAAGCCGCC	87
lvY-102891-long	GCGGCTTGCTTAATGTCAGG	CCCATAGTCTCCATGCCCTC	>200
lvY-99941-long	TTGCTGTGTGTACGTGCCAG	CGTTTGGATGGGATACAAGCAG	>200
lvY-138925-short	TGCCAATGACCAGCTCCTAC	TGGTAGCTACTCCTGGTGAAG	115
lvY-138925-long	TGCCAATGACCAGCTCCTAC	TCCACGAAGAACTGATAGAACTC	>200
lvY-81842-short	CTAGAATCTGCGGCGTCATG	TGAAGGTCACACTTTCCGCG	92
lvY-81842-long	TCAGTATGCCGTCTAGCTGC	ACCAGAGCCCCCGTTTATTG	>200
lvY-143365-short	TAGGGATCAGTTGGGGGAAC	CCGCAAAGCAAAAGAGACCC	106
lvY-143365-long	CCAGCATAAGGTGAGGAGGG	TACTGAAAAACCTGGCCCCC	>200
lvY-51393-short	GACCACTGTAGAGGAGGTTGG	GCTGCCTGTTTCTGGATGTC	124
lvY-51393-long	GACCACTGTAGAGGAGGTTGG	GATCCGTGGAGGTCGGTAAC	>200
lvY-128014-short	TTTTTGGGGGCTCTGCAGG	TGCTCAGTGTCTGTATCCTCTC	91
lvY-128014-long	GCGAGTAGATGGAAGGGTGG	TTGTTTGTCTTGCCCTTTGG	>200
lvY-65590-short	GCAGTGCAGTTCAGAGCATG	AGCCAGCACAAACAGATAGAG	104
lvY-65590-long	GCAGTGCAGTTCAGAGCATG	CAAAGCCTGTGTGCCAACTC	>200
lvY-36220-short	CTAGACTCACGCACACACCC	CCTCCTCCTCTCTCCCTAGC	97
lvY-36220-long	ACTGGTGCTAGGGAGAGAGG	GGCTTTCTTTCTCAGCACAGC	>200
lvY-123701-short	AGGCCTCAGTTCTTCTTGGG	GGTCCACTGTCCACATTGTG	126
lvY-123701-long	TGTTGCATTAGTCCTCTCCCC	GCAATTACGGACTCAGCGTTC	>200
lvY-115632-short	ACTCTACTGATACTTGCCATGCC	TGTCATCGAGCTTAGGCCAC	95
lvY-115632-long	TGTGGCCTAAGCTCGATGAC	ATTCCTCAGGGCTGTTGCAG	>200
lvY-79267-short	CAAGGCCAAAATGATCCCGC	ACTCTGGGAGCAGTAGTCAC	107
lvY-79267-long	CAAGGCCAAAATGATCCCGC	TGTGCATTGACCATAAAGCCC	>200

Identification of Y-chromosome Turnover in Newts Fails to Support a Sex Chromosome Origin for the *Triturus* Balanced Lethal System

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(in revision)

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Abstract

Non-recombining regions of the genome often have profound effects on the course of evolution, resulting in phenomena such as sex chromosomes and supergenes. Amongst the strangest examples are naturally occurring balanced lethal systems, which halve reproductive output. The evolution of such a deleterious trait is difficult to explain. European newts of the genus *Triturus* possess a balanced lethal system derived from the presence of unique non-functional alleles of essential genes within the non-recombining region of heteromorphic chromosome 1. In *Triturus* newts the genetic basis of sex determination currently unknown but an intriguing model proposes that the *Triturus* balanced lethal system evolved from an ancestral Y-chromosome. To test this hypothesis, we identify the Y-chromosome of *Triturus* and verify whether it, or the balanced lethal system, is homologous to the Y-chromosome of its sister genus *Lissotriton*, which does not possess the balanced lethal system. We identify a set of candidate Y-linked markers in *T. ivanbureschi* via a sex-associative approach and place them on a high-density linkage map that we construct with 7,233 RADseq markers. We validate male specificity of the markers across the genus and then place both the *Triturus* and *Lissotriton* Y-linked regions within previously constructed target capture linkage maps that include genes linked to the balanced lethal system. We observe that neither the *Triturus* balanced lethal system, nor the *Triturus* Y-chromosome is homologous to the *Lissotriton* Y-chromosome. We thus show the first molecular evidence of a transition between Y-chromosome systems within salamanders. However, unless additional sex chromosome turnover events are involved, our data does not support a sex chromosome origin of the balanced lethal system.

Introduction

In crested and marbled newts (the genus *Triturus*) the first and largest chromosome occurs in two distinct versions (termed 1A and 1B), which do not undergo recombination along most of their length (Callan et al. 1960). All adult *Triturus* newts possess one copy of each of these versions (genotype 1A/1B). However, as each offspring receives one of each chromosome pair randomly from both of its parents, half of the offspring will inherit two copies of the same version of chromosome 1 (1A/1A or 1B/1B). These offspring fail to develop normally and die before hatching (Macgregor & Horner 1980). The lethality of a balanced lethal system derives from the presence of unique non-functional alleles of essential genes within the non-recombining region of each version of the chromosome (Muller 1918). In *Triturus* our previous research has identified two private sets of genes – one present on each of chromosomes 1A and 1B (France et al. 2025). Any embryo that inherits a 1A/1A or 1B/1B genotype will completely lack functional alleles for one set of these genes, whereas embryos with the 1A/1B genotype will possess at least one functioning allele for all genes. As each version of the chromosome is required to compensate for the deficiencies of the other, neither can be selected against and both are maintained at equal frequencies.

Several other cases of balanced lethal systems have been described in widely divergent lineages, including central American *Drosophila* and plants of the genera *Isotoma* and *Oenothera* (Dobzhansky & Pavlovsky 1955; Steiner 1956; James et al. 1990). The repeated evolution of such a maladaptive trait, which results in the loss of 50% of reproductive output, is difficult to explain. However, there are several proposals which link the origin of balanced lethal system in *Triturus* newts to other phenomena characterized by suppressed recombination, such as supergenes (Wielstra 2020; Berdan et al. 2022) and sex chromosomes (Wallace 1984). Testing of these hypotheses may offer unique insight into how non-recombining regions of the genome can evolve into new roles systems which may exhibit with pronounced negative phenotypic effects.

A detailed model concerning the evolution of the *Triturus* balanced lethal system was developed by Grossen et al. (2012) (see also chapter 1: Fig. 2). Titled "A Ghost of Sex Chromosomes Past" it proposes that *Triturus*' chromosome 1 evolved from an ancestral Y-chromosome. As sex chromosomes typically do not undergo recombination, outside of any pseudoautosomal regions, the ancestral chromosome would be free to split into two distinct lineages, Y_A and Y_B, which would eventually become chromosomes 1A and 1B (Charlesworth et al. 2005). After the Y_A and Y_B lineages diverged, the model proposes that both began to accumulate lethal alleles. As long as these alleles were recessive they would not have been selected against, because their effect would have been masked by the presence of the X-chromosome. The model then enforces a climatic shift sufficient to override the masculinization factor present on the Y-chromosome. This results in some XY individuals having a female phenotype, and so

creates the potential of offspring with a YY genotype. Any offspring with the genotypes Y_AY_A or Y_BY_B would be non-viable due to possessing two copies of one of the lethal alleles. However, if there were no shared lethal alleles present on both the Y_A and Y_B chromosomes, then individuals with the genotype Y_AY_B could survive. If the effect of temperature-induced sex reversal grew strong enough, all XY and even some YY individuals would develop as female. The resulting female-biased sex ratio would result in selection against the X-chromosome, eventually driving it extinct, leaving Y_AY_B as the only viable genotype, and so creating a balanced lethal system. Finally, the model proposes that the biased sex ratio leads to the evolution of a new masculinizing factor on an autosome, creating a new Y-chromosome.

While the evolution of a balanced lethal system via the mechanism proposed by Grossen et al. (2012) is prerequisite on a very particular coincidence of multiple specific factors, none of these are completely implausible in isolation. Y-chromosomes will tend to degenerate and accumulate lethal factors as a direct consequence of their lack of recombination (Charlesworth & Charlesworth 2000). Temperature-induced sex reversal is common in many amphibians, including *Triturus* newts (Wallace & Wallace 2000). The lethally homozygote but inter-compatible Y-chromosome lineages proposed are an almost exact analogue of the situation observed in guppies (Haskins et al. 1970). Furthermore, the evolution of new sex chromosomes is frequent in salamanders, as evidenced by the multiple transitions between XY and ZW systems within the order (Sessions 2008).

A major virtue of the ''Ghosts of Sex chromosomes past" hypothesis is that it implies simple and readily testable predictions (see also chapter 1: Fig. 3). Firstly, because the proposed mechanism requires a sex chromosome turnover event, modern *Triturus* newts could not have retained the sex determination system that existed before the evolution of the balanced lethal system. Therefore, the Y-chromosome of *Triturus* should not be homologous to that of any relatives that diverged before the evolution of the balanced lethal system. Secondly, if *Triturus* chromosome 1 did previously function as a sex chromosome, then it should be homologous to the Y-chromosomes of other closely related newt genera - unless they had independently also lost the ancestral Y-chromosome.

In this study we test whether the Y-chromosome of *Triturus* is homologous to that of newts of its sister genus *Lissotriton* (Rancilhac et al. 2021), which does not possess the balanced lethal system. Cytological studies have identified largely homomorphic X and Y-chromosomes in both genera, but have not been able to determine if these, or any of the other 11 chromosome pairs, are homologous (Schmid et al. 1979; Sims et al. 1984). Molecular genomics is challenging due to the extremely large genome size of salamanders, estimated at approximately 30 Gbp in both *Triturus* and *Lissotriton* (Litvinchuk et al. 2007). We previously used RAD sequencing to identify Y-linked molecular markers for the smooth newt, *Lissotriton vulgaris* and place them within its

genome (France et al. 2024a) (see also chapter 2). Here, we apply the same strategy to the Balkan crested newt *Triturus ivanbureschi* to identify Y-linked molecular markers and position them within a RADseq based linkage map which we construct.

We then use two strategies to compare the Y-chromosomes of *Triturus* and *Lissotriton*. Firstly, we align the RADseq linkage maps for both genera with the genome assembly of the Iberian ribbed-newt *Pleurodeles waltl* (Brown et al. 2025) – we previously showed that the *Lissotriton vulgaris* Y-chromosome was homologous to chromosome 5 in *Pleurodeles waltl* (France et al. 2024a), if this is also the case for the *Triturus ivanbureschi* Y-chromosome it would indicate that the two genera both retain the sex chromosome of their common ancestor and so no sex chromosome turnover has occurred. Secondly, we utilize target capture based linkage maps that we previously constructed for both *Triturus* and *Lissotriton*, which show the position of genes linked to the balanced lethal system in *Triturus* and their homologs in *Lissotriton* (France et al. 2025) (see also chapter 4). We use PCR to screen the families used to construct these target capture linkage maps for the Y-linked RAD markers we identify in *Triturus* in this study (and previously in *Lissotriton*), allowing us to position the Y-linked RAD markers within the target capture maps. This allows us to identify any potential synteny between the Y-chromosomes of the two newt genera and the balanced lethal system.

Materials & Methods

Samples

For identification of candidate Y-linked markers, we performed RADseq on tissue samples of 60 adult, morphologically sexed *T. ivanbureschi* from Zli Dol (Pčinja district, Serbia). The RADseq linkage map was based on a family consisting of two adult T. *ivanbureschi* (one male, one female, also collected from Zli Dol) and 158 offspring (healthy and arrested embryos) of undetermined sex. The embryos were obtained in an experimental crossing under controlled laboratory conditions. For genus-wide validation of candidate markers via PCR we tested all other species within the genus recognized at time of sampling, including *T. anatolicus*, *T. carnifex, T. cristatus, T. dobrogicus, T. karelinii, T. macedonicus, T. marmoratus* and *T. pygmaeus* (Wielstra & Arntzen 2016) – the recently recognised *T. rudolfi* (Arntzen 2024) was not yet described at the time. For each of these species we used a single male-female pair, except for *T. carnifex*, where we used one pair each from both the Balkan and Italian lineages, which show high genetic divergence (Wielstra et al. 2021). A full list of samples used in this study is found in auxiliary supplemental table Sa1 in the associated Zenodo repository (France et al. 2024b).

DNA extraction, library preparation and RAD-sequencing

Genomic DNA was extracted from the samples with the Promega Wizard™ Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the salt-based extraction protocol of Sambrook and Russel (2001). The Adapterama III High-Throughput 3RAD (Bayona-Vásquez et al. 2019) protocol was used to prepare RADseq libraries from 100 ng of sample DNA, using restriction enzymes *Eco*RI, *Xba*I, and *Nhe*I. Fragments in the range of 490-600 bp were excised using a Pippin Prep system (Sage Science, Beverly, MA, USA), the libraries were pooled equimolarly and 150 bp paired-end sequencing was performed by Novogene (Cambridge, UK) on the Illumina NovaSeq 6000 (Illumina Inc., San Diego, CA, USA) platform.

RADseq data processing

The stacks package v2.54 (Catchen et al. 2013) was used for the processing of RADseq data obtained from the *T. ivanbureschi* samples. After demultiplexing and trimming via the process_radtags program the denovo_map.pl pipeline was used to assign reads to loci. For the linkage map family, the default settings were used without alteration. In particular, the parameter M, which determines the amount of divergence two reads may have while being assigned to the same putative locus, was kept at 2, maximising the number of loci recovered from the closely related sample set. For the adults of known-sex, the parameter M was set to 10, even though this reduces the number of loci recovered our experience with *Lissotriton* has shown this results in candidate Y-linked markers which are less likely to produce false positive results in female newts (France et al. 2024a).

Developing candidate Y-linked from known-sex *T. ivanbureschi*

In order to select loci which were present only in male individuals, the BAM files produced by denovo_map.pl were used to create a matrix listing the coverage of all markers in each sample by employing the depth function of SAMtools (Li et al. 2009). This matrix was then filtered with a custom R script to produce a list of candidate Y-linked markers, present in at least 90% of male samples and absent in at least 90% of females. To reduce the chance of selecting markers with autosomal paralogs, which may result in false positive results in PCR assays, the candidate Y-linked markers were BLASTed (Camacho et al. 2009) against the catalogue of all markers produced by denovo_map.pl and paralogous hits with > 80% sequence similarity with a query coverage of > 25% recorded. Candidate markers were ranked based on absence of residual reads in females, number of potential paralogs and average read depth in males.

Validation of candidate Y-linked markers via PCR

Primer 3 (Untergasser et al. 2012) was used to design primer pairs for the 12 highest ranked candidate markers, targeting an optimal primer length of 20 bp and

melting temperature of 60°C. For each marker we attempted to design two sets of primers to amplify both a long (ca. 200 bp) and short (ca. 100 bp) fragment. The short fragment sequences were derived entirely from the forward reads of the RADseq data, whereas the long sequences bridged both forward and reverse reads. As the majority of read pairs were non-overlapping, the long fragments incorporate an additional sequence of unknown length.

The primers were initially tested by PCR in a male-female pair of *T. ivanbureschi*. The 2x QIAGEN multiplex master mix (QIAGEN B.V, Venlo, Netherlands) was used with a PCR protocol consisting of a 95°C hot start for 10 minutes, followed by 35 cycles of denaturation for 30 seconds at 95°C, 60 seconds annealing at 63°C and 45 seconds extension at 72°C, with a final extension of 10 minutes at 72°C. All primers were used at a final concentration of 0.1 µM.

Any primer pairs which showed amplification only in the male *T. ivanbureschi* were then tested in male-female pairs of *T. macedonicus* and *T. cristatus*. Primer pairs also showing male-specific amplification in these taxa were then tested in the remaining *Triturus* species. Finally, the best performing markers were selected, and a multiplex PCR designed, incorporating CDK-17 (Meilink et al. 2024) which amplifies a product of 537 bp, as an autosomal control marker.

RADseq linkage map construction and analysis

A joint VCF file produced by Stacks was filtered with VCFtools (Danecek et al. 2011) to exclude indels and SNPs with greater than 5% missing data, a mean depth of less than 10, or a minor allele frequency of less than 0.2. The thin function was then used to select a single SNP per locus. Separately, a custom R script was used to determine the coverage of the candidate Y-linked markers, identified in the known-sex adults, within each sample used for the linkage map. This data was then converted into presence/absence genotype calls with a custom R script, treating presence of the marker as an artificial SNP locus of genotype AT and absence as AA.

Lep-MAP 3 (Rastas 2017) was then used to construct a linkage map. After the first stage of the pipeline (ParentCall2) the Y-linked presence/absence calls were appended to the output. Initial linkage groups were created with the SeparateChromosomes2 module, with a LOD limit of 20 (chosen as the number of linkage groups recovered rises rapidly with increasing LOD until 20 whereafter it plateaus) and distortion LOD set to 1. Unplaced markers were then added with the JoinSingles2All module with a LOD limit of 15. The markers were then ordered with the OrderMarkers2 module, using 12 merge iterations, 6 polish iterations, a minError value of 0.02, the scale setting M/N 2 and employing the sexAveraged option.

The sequences of the makers placed on the resulting linkage map were then blasted against the genome assembly of the Iberian ribbed newt (*Pleurodeles waltl*)

(Brown et al. 2025), using a word size of 11 and requiring a minimum E value of 1e-20. Following the methodology of Purcell et al. (2014) results were then filtered to include only hits that exceeded the significance of the next highest ranked hit by at least five orders of magnitude. The hits that remained after filtering were visualised with an Oxford plot to show syntenic relationships between *Triturus* linkage groups and *P. waltl* chromosomes.

Incorporation of Y-linked markers into target-capture linkage maps

In a previous study we used target capture to construct linkage maps based on ca. 7k coding genes for both *Triturus* (using an F_2 *T. ivanbureschi* x *T. macedonicus* family) and *Lissotriton* (F_2 *L. vulgaris* x *L. montandoni*), which allowed for the identification a set of genes associated with the balanced lethal system on *Triturus* chromosome 1, and their homologs in *Lissotriton* (France et al. 2025). However, as none of the target capture markers were sex-linked, the location of the Y-linked regions could not be ascertained from these maps. To determine whether the Y-linked region of the *Lissotriton* genome was homologous to the balanced lethal system of *Triturus* (as would be expected if the balanced lethal system evolved from the shared ancestral sex chromosome), we needed to incorporate the Y-linked RADseq markers discovered in *L. vulgaris* (France et al. 2024a) into the target capture linkage map. To this end we used PCR to screen all samples from the family used to construct the *Lissotriton* target capture linkage map for the RAD marker lvY-51393-short, which is Y-linked and amplifies a product only in males. We also used this method to incorporate the *Triturus* Y-linked RAD marker TiY-384959-short, identified in this study, into the *Triturus* target capture linkage map. In both PCR screenings the primers for the Y-linked markers were multiplexed with those for CDK-17, to provide an autosomal control.

Following genotyping of offspring, presence/absence of the Y-linked marker was converted into pseudo-SNP genotyping calls in a manner similar to that described above, with samples that amplified the Y-linked band given an artificial SNP locus of genotype AT and those which failed to amplify the band given the genotype AA for this locus. The target capture linkage maps constructed for *Triturus* and *Lissotriton* in (France et al. 2025) were then rebuilt to include these calls, thus allowing the location of the Y-linked region on the sequence capture map, otherwise using the same data, settings and pipeline as described in that study. The rebuilt maps were then compared to each other, and the *P. waltl* genome assembly, to highlight any homology between the *Lissotriton* Y-chromosome, and the region associated with the *Triturus* balanced lethal system (or if no sex chromosome turnover has occurred, the *Triturus* Y-chromosome).

Results

Sex association

After demultiplexing and initial filtering the known-sex adults yielded a total of 501 million read-pairs (median per sample: 7.62 M, interquartile range: 5.28-9.59 M). A total of 1,394,143 million loci were identified, of which 179,516 (12.88%) were present in at least 50% of all samples. The initial selection for candidate Y-linked markers generated yielded a total of 39 loci.

We designed 23 primer pairs (Supplementary Table S1) for the 12 highest ranked candidate markers (for one marker TiY-444315, Primer 3 was unable to find a valid primer pair for the shorter fragment). 11 primer pairs, covering 6 marker sequences, amplified products only in male in *T. ivanbureschi*. 6 pairs (for 5 markers) also were male specific in both *T. macedonicus* and *T. cristatus* (Table 1, Sup Figs. S1-3). No primer pairs were successful in all taxa, however TiY-384959-short showed male specificity in all species except *T. dobrogicus*, where no product was amplified in either sex. TiY-137941-long was the only primer pair to amplify in male *T. dobrogicus* and was also male specific in all other taxa that occur within the Balkans (but did not amplify sex-specifically in *T. karelinii*, *T. marmoratus, T. pygmaeus* or the Italian lineage of *T. carnifex*) (Fig. 1, primer sequences in Table 2).

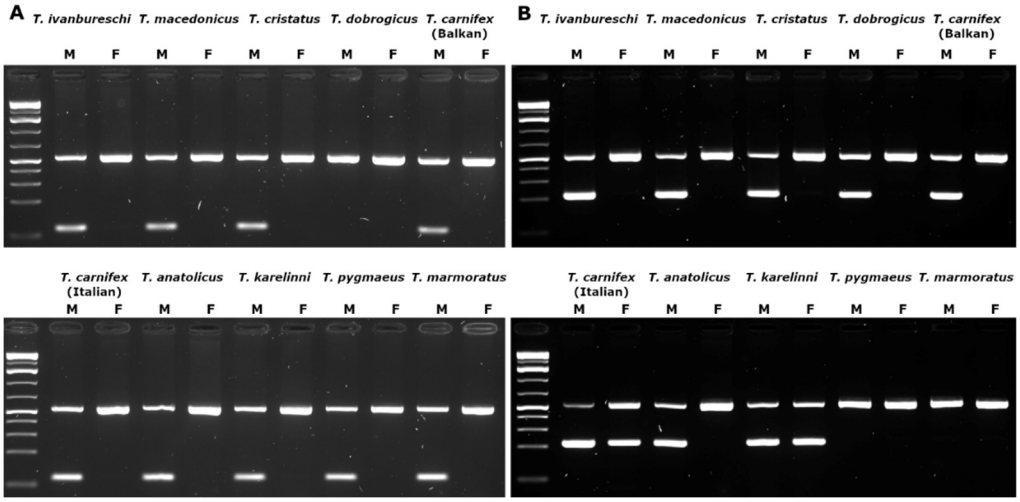

Figure 1: Gels showing amplification of Y-linked primer pairs TiY-384959-short **(A)** and TiY-137941-long **(B)**, with CDK-17 (537 bp) as control maker, in male-female pairs of various *Triturus* taxa. Male samples are labelled M and females F. TiY-384959-short (92 bp) shows male-specific amplification in all species except *T. dobrogicus*, where no product is seen. TiY-137941-long (ca. 170 bp) is male specific in *T. dobrogicus* and all other taxa which occur in the Balkans (shown on the top row of the gel) but shows no amplification in the marbled newts (*T. marmoratus and T. pygmaeus*) and non-sex specific amplification in *T. karelinii* and the Italian lineage of *T. carnifex*.

Marker	Primer pair	Male specific amplification in *Triturus* taxa									
		T. ivanbureschi	*T. macedonicus*	*T. cristatus*	*T. dobrogicus*	*T. carnifex (I)*	*T. carnifex (B)*	*T. anatolicus*	*T. karelinii*	*T. pygmaeus*	*T. marmoratus*
TiY-95401	short	○									
	long	+	+	+	×	○	○	+	○	+	+
TiY-105918	short	○									
	long	○									
TiY-106308	short	+	+	○							
	long	+	○	○							
TiY-137941	short	+	+	○							
	long	+	+	+	+	○	+	+	○	×	×
TiY-201098	short	+	+	○							
	long	○									
TiY-254147	short	○									
	long	○									
TiY-301991	short	○									
	long	○									
TiY-384959	short	+	+	+	×	+	+	+	+	+	+
	long	+	+	+	×	×	×	+	×	×	×
TiY-416318	short	○									
	long	○									
TiY-442534	short	+	+	○							
TiY-444315	short	+	+	+	×	+	×	+	○	×	×
	long	○									
TiY-817010	short	+	+	○							
	long	+	+	+	×	○	+	+	○	+	+

Table 1: Summary of results of PCR screening of primer pairs designed for candidate Y-linked markers in *Triturus* newts. Results are indicated as: + amplification only in male samples, ○ amplification in both male and female samples, × no amplification in either sex. Twenty-three primer pairs were tested in a male-female pair of *T. ivanbureschi*. Twelve primer pairs, covering eight candidate markers, show confirmed male-specific amplification in *T. ivanbureschi*. The successful primer sets were then tested in male-female pairs of *T. macedonicus* and *T. cristatus*, and the six successful in both were then tested in all available *Triturus* taxa. While candidate TiY-384959-short demonstrated broad male-specificity across the genus, it failed to amplify in either male or female *T. dobrogicus* - TiY-137941-long proved the only successful primer pair in this species.

Primer Pair	Forward Sequence	Reverse Sequence	Product (bp)
CDK-17	GGCATGGGAAGAACAGAAGA	CCATCTGCTTGGACTGTTGA	537
TiY-384959-short	TGCAGCACAGCAGTAGACTC	CCTTCTCGCATGGACCCTAC	92
TiY-137941-long	GTCACAGCAGCAAATGGTCC	CCTCTGCTCTGCCTTCACAG	ca. 170

Table 2: Primer sequences used for the sex diagnostic PCR for use within the genus *Triturus*. CDK-17 is an autosomal control marker. TiY-384959-short is male-specific in all species except *T. dobrogicus*. TiY-137941-long is male-specific in *T. dobrogicus*, as well as *T. ivanbureschi*, *T. anatolicus*, *T. macedonicus*, *T. cristatus* and the Balkan lineage of *T. carnifex*.

RADseq Linkage map

The linkage map constructed from the *T. ivanbureschi* family consists of 7,233 markers arranged into 12 linkage groups (corresponding to the 12 chromosomes of the *Triturus* genome), with a total length of 1,120 cM (Fig. 2), Supplementary Table S2). Twenty-seven Y-linked presence/absence markers were placed on the map, all located in a 3.2 cM region at the end of linkage group 8, with 24 of these markers being placed at a single point.

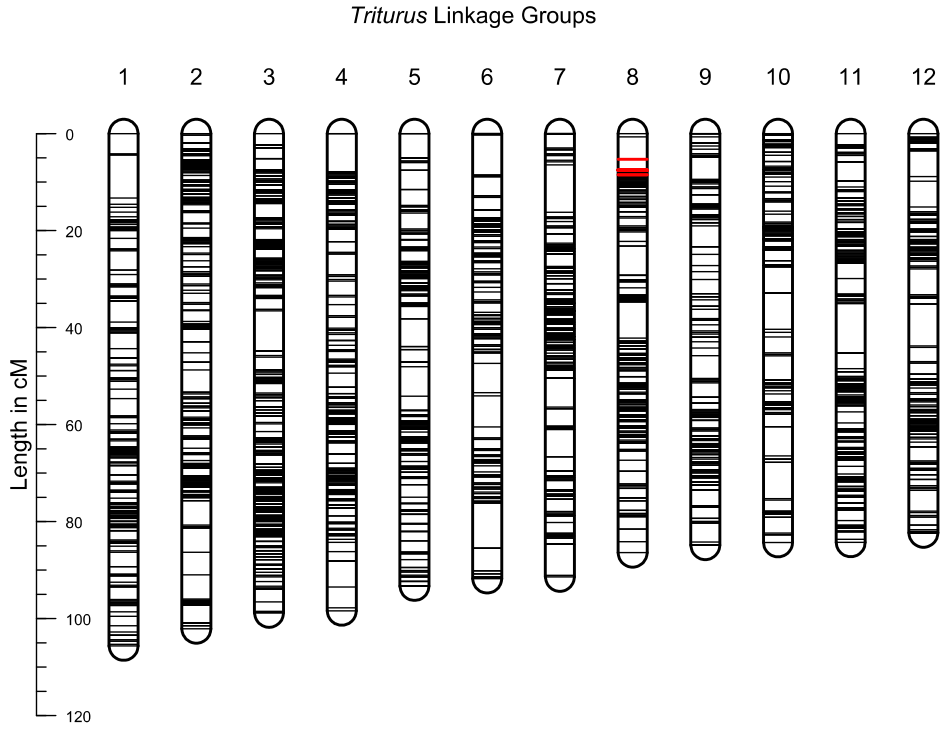

Figure 2: Linkage map for *Triturus ivanbureschi* based on 7,233 RADseq markers, arranged in 12 linkage groups. 27 male-linked presence absence markers – highlighted in red – are located at one end of linkage group 8, identifying it as the Y-chromosome.

Five hundred and twenty-four markers (7.2%) placed on the linkage map, including a single Y-linked marker, could be aligned with sequences from the *P. waltl* genome assembly. Each *T. ivanbureschi* linkage group shows a clear and reciprocal correspondence with one of the *P. waltl* chromosomes, with 374 (71%) markers mapping to their corresponding chromosome, and large-scale synteny within chromosomes. We fail to observe any clear pattern in the remaining markers, indicating that these are likely a consequence of BLAST hits against paralogous sequences, rather than a major rearrangement in the genome of either species.

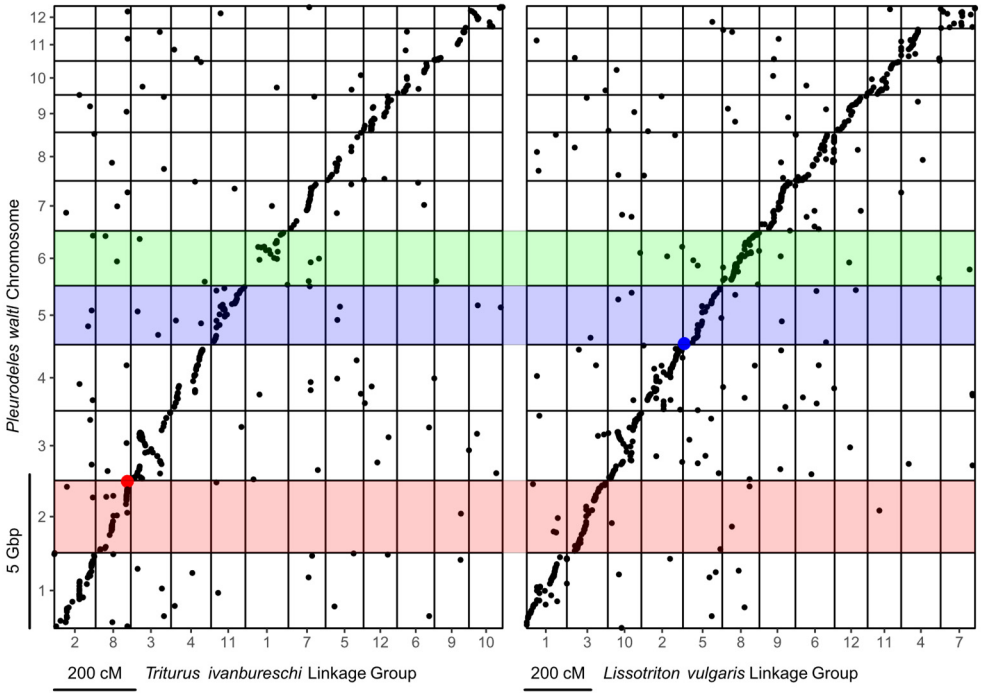

Figure 3: Oxford plots showing the *Triturus ivanbureschi* (left) and *Lissotriton vulgaris* (right) (France et al. 2024a) RADseq linkage maps against the *Pleurodeles waltl* genome assembly (Brown et al. 2025). The *T. ivanbureschi* Y-linked presence/absence markers identified in known sex-adults are highlighted in red, the *L. vulgaris* Y-linked markers in blue. The *T. ivanbureschi* Y-chromosome is shown to be homologous to *P. waltl* chromosome 2 and thus is not homologous with the *L. vulgaris* Y-chromosome (which is homologous to P. waltl chromosome 5). There is no evidence of a translocation of the sex-linked regions of either chromosome. *P. waltl* chromosome 6, highlighted in green is homologous to the *Triturus* balanced lethal system on chromosome 1

Forty-seven markers within the *T. ivanbureschi* Y-chromosome (linkage group 8) BLAST against sequences from *P. waltl* chromosome 2 (Fig. 3), including the solitary Y-linked marker. However, in the analogous RADseq linkage map previously made for *L. vulgaris*, the Y-chromosome is clearly seen as homologous to *P. waltl* chromosome 5 (France et al. 2024a). We see no evidence that this is a result of translocation of the sex-determining region, no markers from *T. ivanbureschi* linkage group 8 BLAST against sequences from *P. waltl* chromosome 5. A single marker from the *L. vulgaris* Y-chromosome (linkage group 5) is found in *P. waltl* chromosome 2, however this is located over 100 cM away from the sex-linked region.

Identification of Y-linked regions in target capture linkage maps

We incorporate Y-linked RAD markers for *Lissotriton* (France et al. 2024a) and *Triturus* (identified in this study) into the target capture-based linkage maps previously constructed for these two genera (France et al. 2025), which include genes linked to the balanced lethal system. For *Lissotriton*, 110 offspring amplified the Y-linked RAD marker lvY-51393-short, whereas 92 did not, with a single individual giving an ambiguous result (either a missing control band, or only very faint amplification of either band). For *Triturus*, 107 offspring amplified the marker TiY-384959-short, whereas 93 did not – with six giving ambiguous results. Both offspring sets are biased towards males, though this is not statistically significant – assuming an even sex ratio, two-tailed binomial p-values are 0.231 for *Lissotriton* and 0.358 for *Triturus*.

For both *Triturus* and *Lissotriton* the number of genotype calls is sufficient to confidently locate the Y-linked RAD markers within the target capture linkage maps (Fig. 4). In concordance with the RADseq linkage maps the *Triturus* and *Lissotriton* Y-chromosomes are shown not to be homologous, with the *Triturus* and *Lissotriton* Y-linked regions again located on the homologs of *P. waltl* chromosomes 2 and 5. However, the *Lissotriton* Y-chromosome also lacks homology with *Triturus* chromosome 1, where the genes associated with the balanced lethal system are located. Instead *Triturus* chromosome 1 is homologous with *P. waltl* chromosome 6.

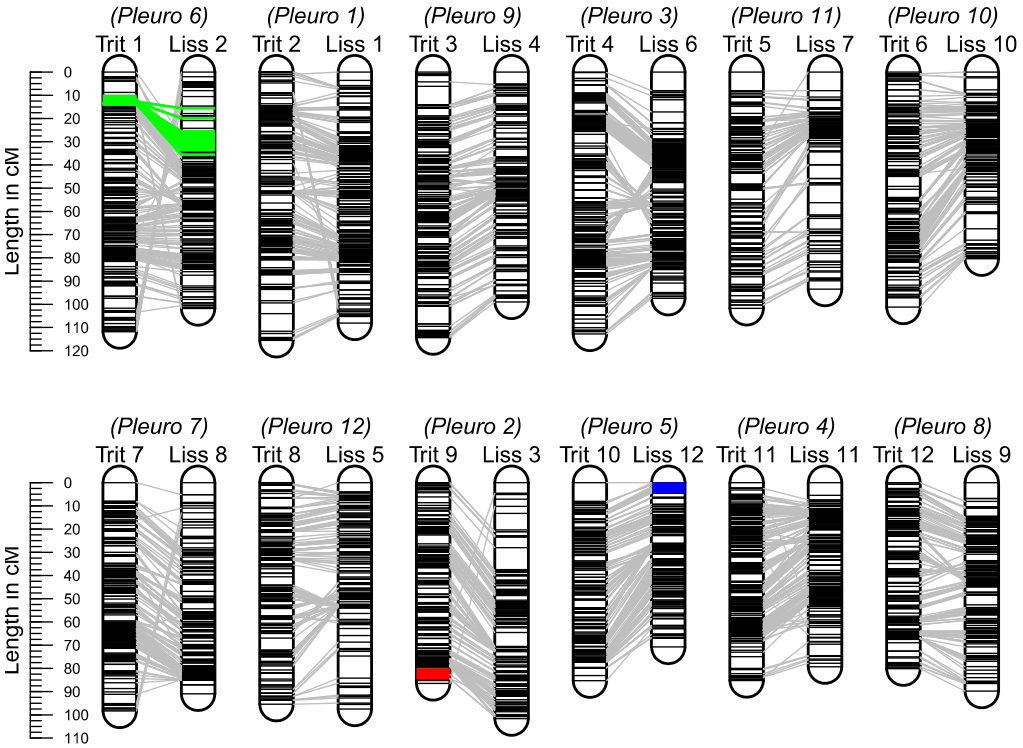

Figure 4: Target capture linkage maps (France et al. 2025) augmented with Y-linked markers (the abbreviations Trit and Liss refer to the linkage groups from *Triturus* and *Lissotriton* respectively – *Pleuro* refers to the homologous chromosome in the *Pleurodeles waltl* genome assembly). *Triturus* balanced lethal system associated markers are highlighted in green, the *Triturus* Y-linked marker in red and the *Lissotriton* Y-linked marker in blue. In accordance with the RADseq linkage maps, the *Triturus* and *Lissotriton* Y-chromosomes are shown not to be homologous. Additionally, the *Lissotriton* Y-chromosome is also not homologous with *Triturus* chromosome 1. Note that the numbering of the linkage groups differs between the RADseq and target capture linkage maps.

Discussion

No homology between the balanced lethal system and sex chromosomes

The hypothesis that the *Triturus* balanced lethal system evolved from a sex chromosome system (Grossen et al. 2012) implies a pair of predictions that we test in this study. Firstly, that sex chromosome turnover must have occurred in *Triturus* after it diverged from its sister lineage *Lissotriton* and so these taxa cannot now share a sex-determination system. Secondly, that *Triturus* chromosome 1 must be homologous to the sex chromosome of this common ancestor, and so would be homologous to the modern *Lissotriton* Y-chromosome – unless this genus has also undergone sex chromosome turnover.

We confidently identify the *Triturus* Y-chromosome by identifying a set of male-linked markers and placing them within both a newly constructed high density RADseq linkage map, and a previously constructed map based on target capture. We discover that the *Triturus* Y-chromosome is clearly not homologous to the *Lissotriton* Y-chromosome. Therefore, at least one of these lineages must have undergone a sex chromosome turnover, which is compatible with the first prediction. However, this could also be explained by sex chromosome turnover within the *Lissotriton* lineage, and as no sex-linked markers are known for any other newt taxa, we lack an outgroup to distinguish between these scenarios.

We show that, counter to the second prediction made by the sex chromosome origin hypothesis, the *L. vulgaris* Y-linked region is clearly not homologous to the balanced lethal system present on *Triturus* chromosome 1. Although we cannot rule out the possibility that sex chromosome turnover has occurred in both *Lissotriton* and *Triturus*, such that neither taxon now possesses the ancestral Y-chromosome, the most parsimonious explanation of our results is a single sex chromosome turnover after the divergence of the *Triturus* and *Lissotriton* lineages. If the *Triturus* balanced lethal system did arise from the ancestral sex chromosome, this would require at least one additional turnover. The plausibility of the sex chromosome origin hypothesis thus depends on how common sex chromosome turnover events are within newts.

Y-chromosome switching in salamanders: common or rare?

Unlike mammals and birds (Ellegren 2010; Cortez et al. 2014), sex chromosome turnover appears relatively common in amphibians (Miura 2017). However, within salamanders, the large genome size and consequent difficulty in discovering sex-linked sequences has meant that its observation has only been possible in cases where female heterogametery (ZW) has transitioned to male heterogametery (XY) or vice versa – at least three such events are known (Hime et al. 2019). Conversely, little is known about the frequency of transitions between different Y-linked (or W-linked) sex determination systems in the salamanders. There is some evidence from other amphibians that these events may be common. In the family Ranidae (true frogs), Jeffries et al. (2018) found 13 sex chromosome turnover events in 28 lineages, 11 of which were transitions between different Y-chromosomes. Additionally, the X and Y-chromosomes in *Triturus, Lissotriton,* and other related newt genera such as *Ichthyosaura* are poorly differentiated (Schmid et al. 1979; Sims et al. 1984), which may be taken as evidence that they have all evolved rather recently (Charlesworth et al. 2000).

Nonetheless, we also have evidence of sex chromosome stasis in salamanders. Despite having diverged in late Cretaceous, the giant salamanders (the family Cryptobranchidae), possess a conserved W-linked region, with the same female specific

marker shown to amplify in both the North American hellbender and the Chinese giant salamander (Hime et al. 2019). These Z and W-chromosomes also appear extremely homomorphic (Sessions et al. 1982), showing that this is not necessarily proof of a recent origin. A similar phenomenon of deceptively youthful sex chromosomes is also seen in tree frogs of the genus *Hyla* (Stöck et al. 2011). At present there is insufficient data to determine whether the Y-to-Y-chromosome turnover we observe between *Triturus* and *Lissotriton* is a common or exceptional event. The identification of sex determining regions in other salamander genera would help to answer this question.

Towards sex chromosome identification across salamanders

The sex associative RADseq methodology we employ in this study is a relatively quick and effective approach for the discovery of sex-linked sequences. These markers are not just useful for evolutionary genomics, but also invaluable for researchers interested in the ecology or population dynamics, especially in species which are difficult to morphologically sex before maturity, such as newts (Sparreboom 2014). In the case of *Triturus* we can recommend the marker TiY-384959-short in any context except when the Danube crested newt, *T. dobrogicus* may be encountered, where TiY-137941-long should be used instead.

However, while the identification of sex-linked markers is simple, determining whether they are homologous often requires locating them within a genome. Whether by linkage mapping, whole genome assembly or methods such as fluorescent in-situ hybridisation, this is often a resource intensive process. The need for such investment may be circumnavigated by aligning Y-linked RAD sequences with a genome from a related organism. In our study we show some success by employing the genome of the relatively distantly related *Pleurodeles waltl* which diverged over 60 mya (Marjanović & Laurin 2014), although only one of 27 Y-linked markers could be confidently aligned. For the investigation of newt Y-chromosomes specifically, whole genome data from a more closely related species would be extremely valuable. Given the high degree of chromosome level synteny we observe between *P. waltl*, *Triturus*, and *Lissotriton* we suggest that simply scaffolding long read data against the *P. waltl* assembly would result in a reference genome sufficient for locating sex-linked regions, similar to the strategy employed by Jeffries et al. (2018) in *Rana*. Additionally, such long read data could be used to identify sequences more conserved than those derived directly from RADseq, allowing for the development of markers that show sex-specific amplification in multiple genera.

Further investigation of Y-linked markers in newts thus promises insights into the rate of sex chromosome turnover in salamanders, as well as determining which, if either, of the *Triturus* and *Lissotriton* Y-chromosomes are ancestral – and if a sex chromosome turnover is an at all plausible explanation of the *Triturus* balanced lethal system.

Data Availability

All raw reads can be found as a part of the NCBI accession associated with Bioproject: PRJNA1173742. (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1173742) (France et al. 2024c). Information on samples and the positions and sequences of all markers in the *Triturus* RADseq linkage map can be found in a .xlsx file hosted together with scripts and bioinformatic pipelines used for analysis in the associated Zenodo repository (https://doi.org/10.5281/zenodo.14288865) (France et al. 2024b). Scripts are also available at an associated GitHub repository (Wielstra-Lab/Triturus_RADseq_Y).

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Supporting Information

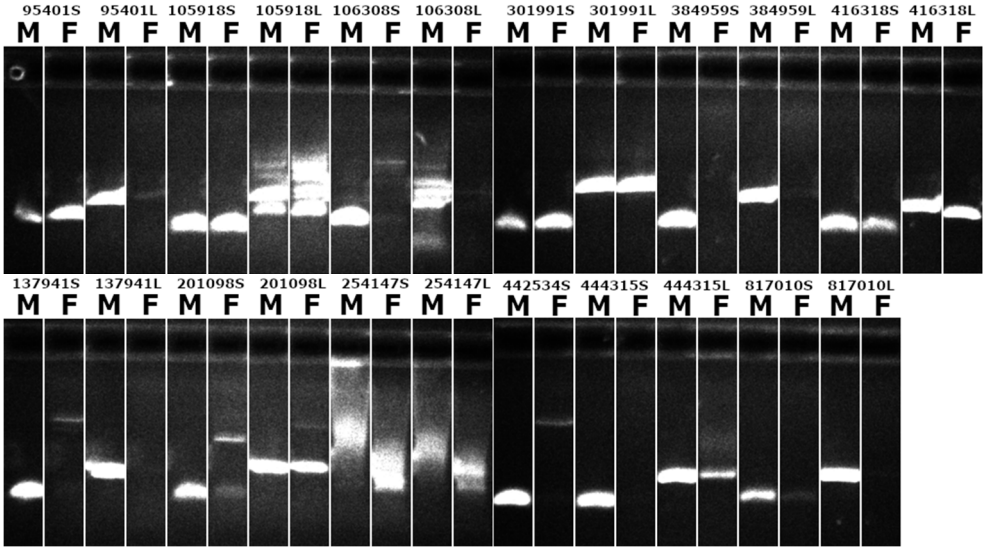

Figure S1: PCR screening of 23 primer pairs designed for candidate Y-linked markers for sex specific amplification in *Triturus ivanbureschi*. Label M indicates the male sample and label F indicates female. Markers are indicated by number followed by either S (for primer pairs designed for the short product – c.a. 100 bp) or L (for primer pairs designed for the long product – c.a. 200 bp). 12 pairs show male specific amplification.

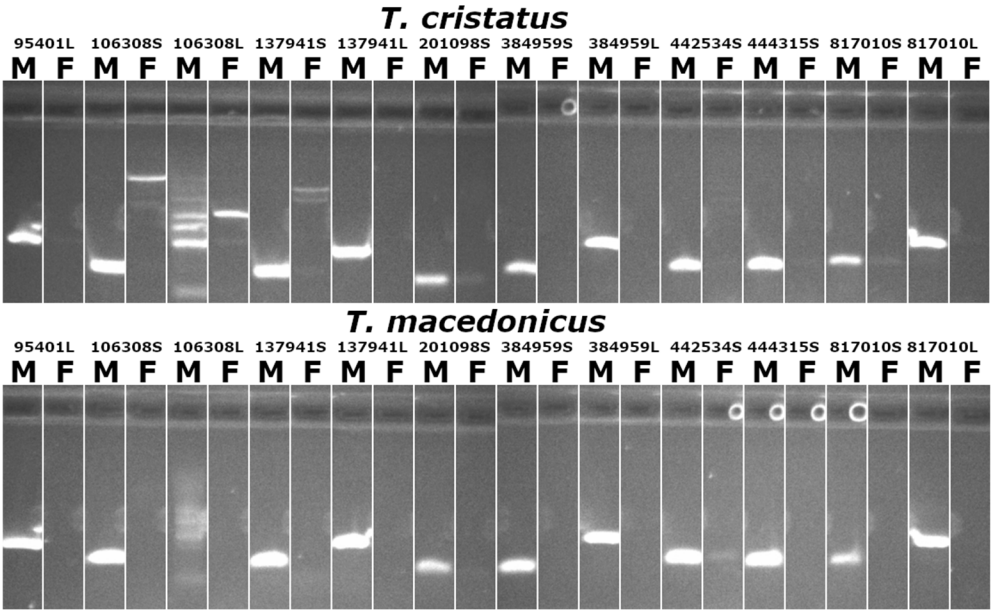

Figure S2: Further PCR screening of 12 primer pairs for Y-linked markers (that show male specific amplification in *T. ivanbureschi*) in *T. cristatus* and *T. macedonicus*. Label M indicates the male sample and label F indicates female. Markers are indicated by number followed by either S (for primer pairs designed for the short product – c.a. 100 bp) or L (for primer pairs designed for the long product – c.a. 200 bp). 6 primer pairs show strong amplification in males of both species with no product at all visible in females (several other show varying degrees of weak amplification in females).

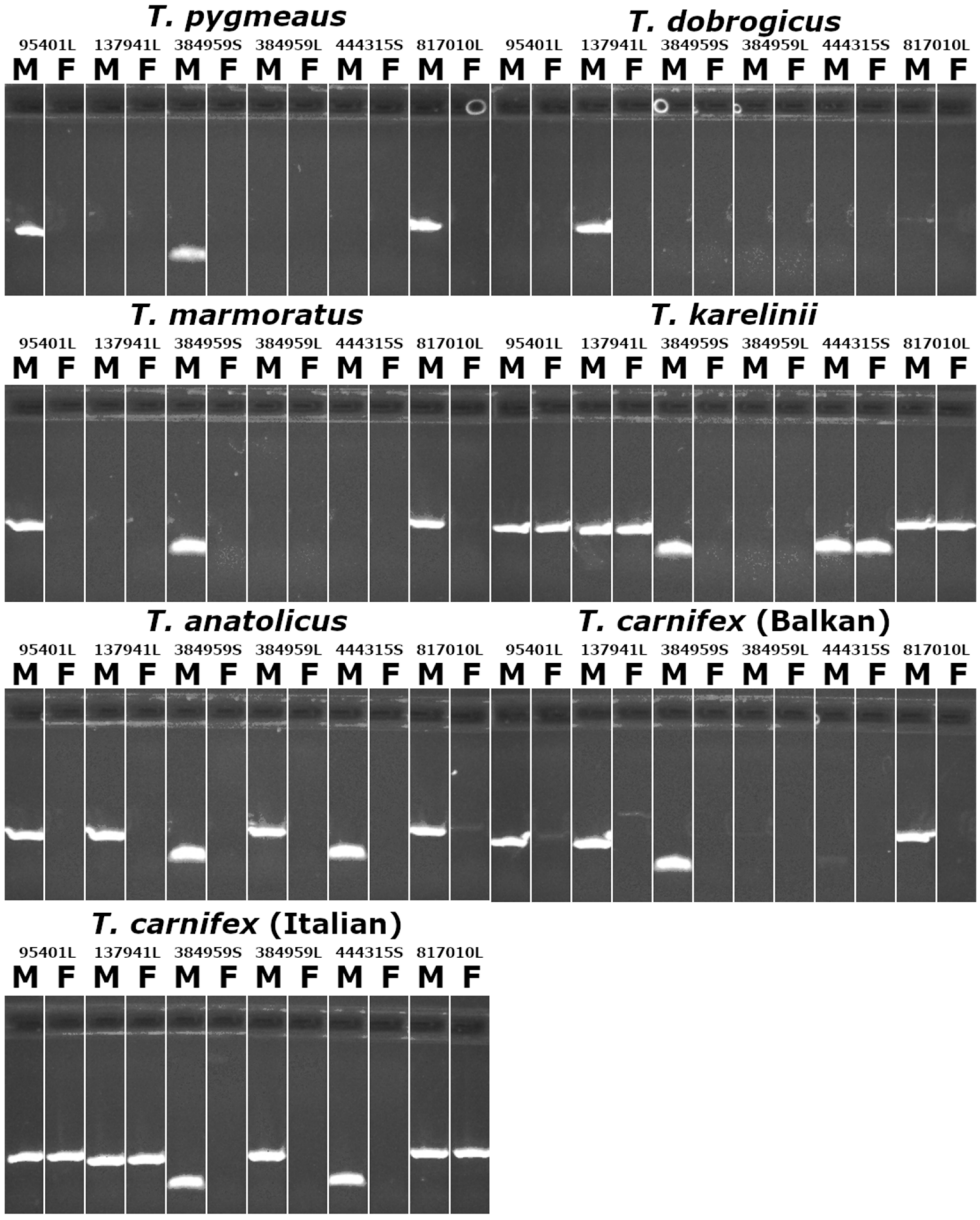

Figure S3: PCR screening of 6 primer pairs for Y-linked markers (that show confirmed male specific amplification in three *Triturus* species) in all other *Triturus* species (except for *T. rudolfi*, which was not yet described at the time of study). Label M indicates the male sample and label F indicates female. Markers are indicated by number followed by either S (for primer pairs designed for the short product – c.a. 100 bp) or L (for primer pairs designed for the long product – c.a. 200 bp). No marker shows male specific amplification in all species, however TiY-384959-short is successful in all other than *T. dobrogicus*. The only marker to show any amplification in T. dobrogicus is TiY-137941-long.

Primer Pair	Forward Primer Sequence	Reverse Primer Sequence	Product (bp)
CDK-17	GGCATGGGAAGAACAGAAGA	CCATCTGCTTGGACTGTTGA	537
TiY-444315-short	AGTTCGAGCCAGTACTTTTAGC	CAAACACACGAAAGCACAGTG	111
TiY-444315-long	CACTGTGCTTTCGTGTGTTTG	TGTACTAGAAAGGGTGGGGG	>105
TiY-137941-short	GTCACAGCAGCAAATGGTCC	CAGAAGAAGGGCATCTGGG	104
TiY-137941-long	GTCACAGCAGCAAATGGTCC	CCTCTGCTCTGCCTTCACAG	>166
TiY-95401-short	CTAGATTCCGGTGAGGCAGG	GGCCCATAGCACCAACATTC	137
TiY-95401-long	GCGTACGGAGTGATTATCCCC	ACACTGCTGCGGAACTGAAG	>199
TiY-105918-short	TGAGGATCTGGCTCAATCGC	TCTCCAAAGGTAACGCGCTG	82
TiY-105918-long	AATCTTGTCCACCAGTGTGC	AATTCAGCAGCCCACATGCC	>185
TiY-442534-short	AGGGGCATAAGTGGAGGGAC	AGGGTCTGAAAAGGGCCATC	114
TiY-416318-short	TGGGTTTCCAAGTCTCCTCAG	ACTTTCAAGAGTAAGGAGCAGAAG	89
TiY-416318-long	TGGGTTTCCAAGTCTCCTCAG	TGGAGGCCTGAAGTAATAAGCC	>166
TiY-254147-short	CCGGTCACATCTCCTTCGAG	GACTGGGCTTGAGAGTCTCG	150
TiY-254147-long	CCGGTCACATCTCCTTCGAG	TCGAAGCAGATGTGACTGGG	>163
TiY-384959-short	TGCAGCACAGCAGTAGACTC	CCTTCTCGCATGGACCCTAC	92
TiY-384959-long	GTAGGGTCCATGCGAGAAGG	AGGTGTCGTGTGCCTACTTC	>168
TiY-201098-short	TAAACCAGCAAAGCCACCAC	TGTACAATTCCTGCGTAACCG	83
TiY-201098-long	CCACCCCCAAGCACTTAAAG	TGTGTGGGTCCCAAAAGTGG	>200
TiY-817010-short	TCTGCTTTGTGTCTGAAGCTTG	TGTGTGTTCCTGTTGGGCTG	127
TiY-817010-long	TCACCTACCACCACAGTTGC	CACTCCTGACTATGGGCCTG	>186
TiY-301991-short	GGGGAGTCAGGGTTGTCATG	TCTACTAGCTCACAGGGCAC	87
TiY-301991-long	GGGGAGTCAGGGTTGTCATG	TGGGGTTTCCTACTCAGCTG	>194
TiY-106308-short	AGCAAGTTCCAGGAGCTTCC	AGAGCACATGAAGGACCAGC	125
TiY-106308-long	TCACCAGCAGAGTTTCTCCG	TGAAGGACCAGTGGATGCTG	>186

Table S1: Sequences of all primers used in this study, CDK-17 is an autosomal marker used as a control, all others are candidate Y-linked markers developed for *T. ivanbureschi*.

Group	Number of Markers	Length (cM)
1	391	105.6
2	931	102.1
3	1005	98.8
4	764	98.4
5	331	93.8
6	427	93.3
7	381	91.7
8	600	91.4
9	710	91.4
10	903	86.4
11	314	84.3
12	476	82.3
Total	**7233**	**1119.6**

Table S2: Characteristics of linkage groups within the linkage map constructed based on RADseq data from 160 *T. ivanbureschi* samples from a full-sibling family.

Genomic Evidence Suggests the Balanced Lethal System in *Triturus* Newts Originated in an Instantaneous Speciation Event

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(in review)

*This chapter is available as a preprint at **bioRxiv:** (10.1101/2024.10.29.620207)*

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.)

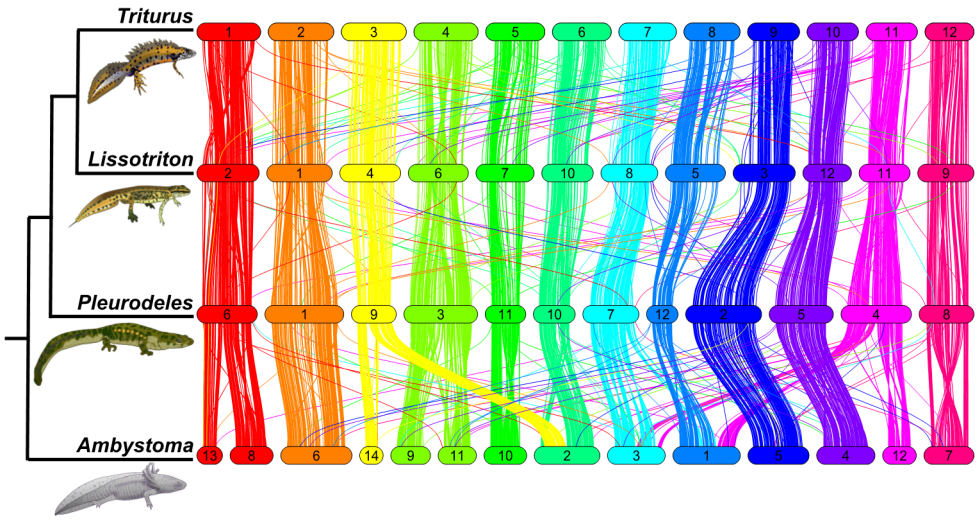

Figure 1: Comparison of the genomes of four salamander genera reveals striking conservation of synteny within newts. For *Triturus* and *Lissotriton* we construct linkage maps based on target capture data for ca. 4k coding sequences. For the more distantly related *Pleurodeles* and *Ambystoma* we incorporate 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.

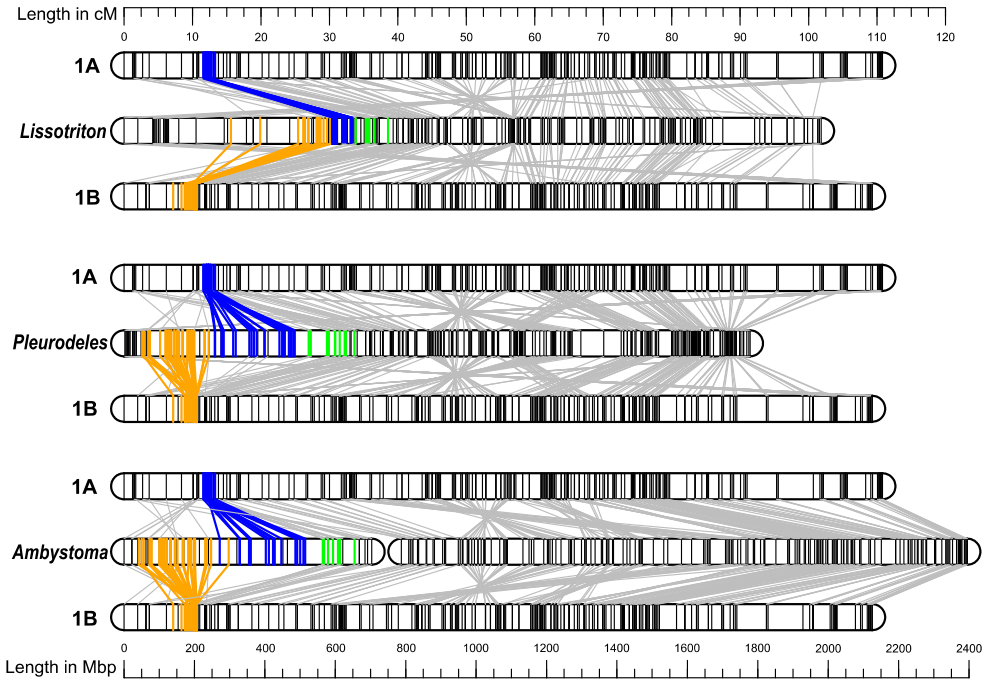

Figure 2: Groups 1A and 1B of the *Triturus* linkage map compared to the homologous group 2 of the *Lissotriton* linkage map, chromosome 6 of the *Pleurodeles waltl* genome assembly (Brown et al. 2025) and chromosomes 8 and 13 of the *Ambystoma mexicanum* genome assembly (Nowoshilow et al. 2018; Smith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

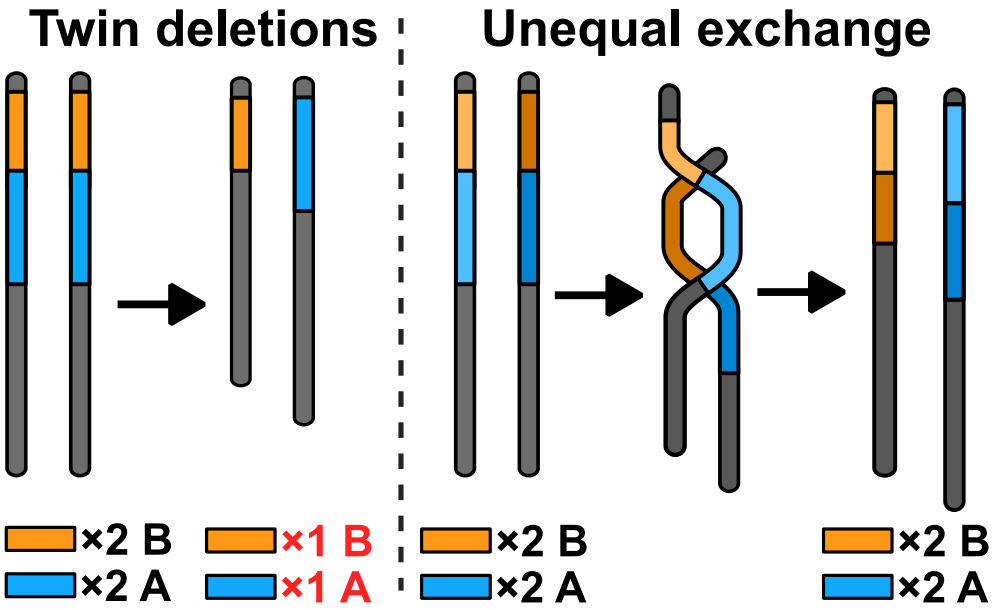

Figure 3: Contrasting scenarios of chromosome evolution in *Triturus* newts. If the absence of each set of balanced lethal system-related genes from one version of chromosome 1 is explained by simple uncompensated deletions (left) these will be left as single copy, which will reduce fitness if any of 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.

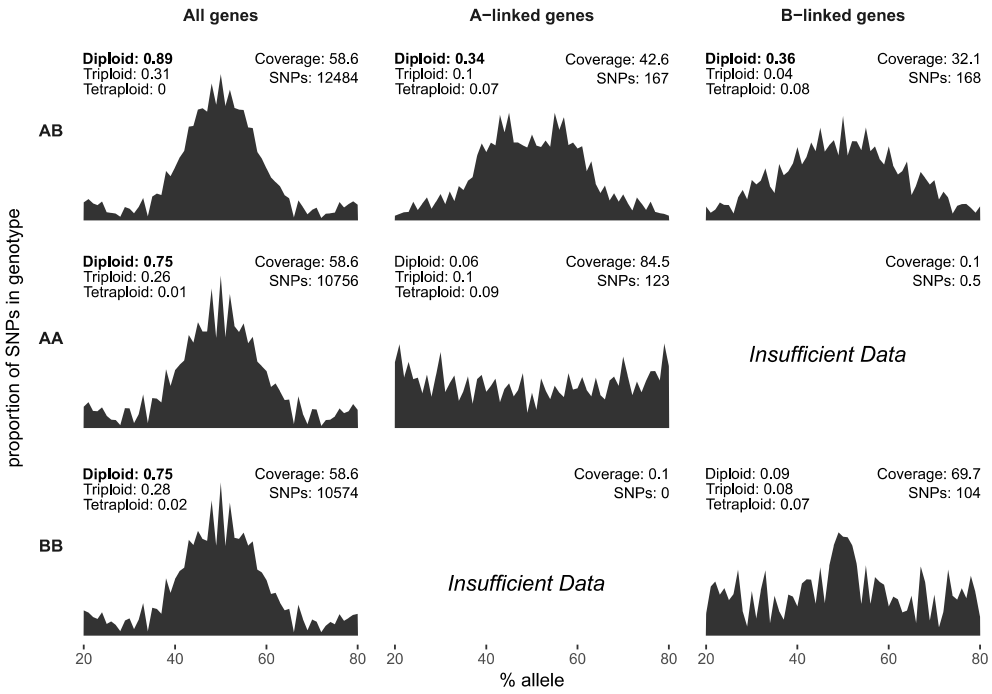

Figure 4: Profiles of allele ratios of three categories of genes (all 7,139 target sequences, 28 genes linked to chromosome 1A and 35 genes linked to chromosome 1B) based on target capture data of 30 F1 *Triturus ivanbureschi* × *T. macedonicus* F1 embryos, split evenly between three genotypes (AB, AA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

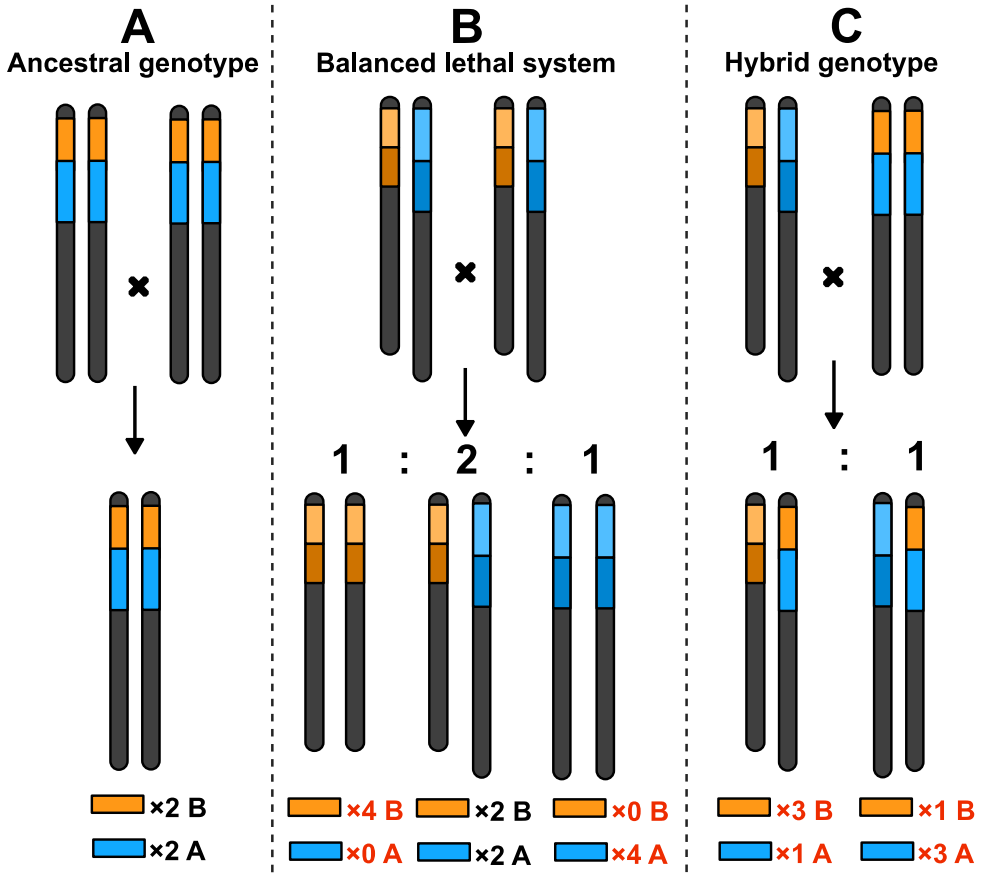

Fig. 4: Consequences of the possible combinations of rearranged and ancestral genotypes. (**A**) In the case of a cross between two individuals of the ancestral (pre-rearrangement) genotype, all offspring will inherit two copies of all genes. (**B**) If both parents are affected by the balanced lethal 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).

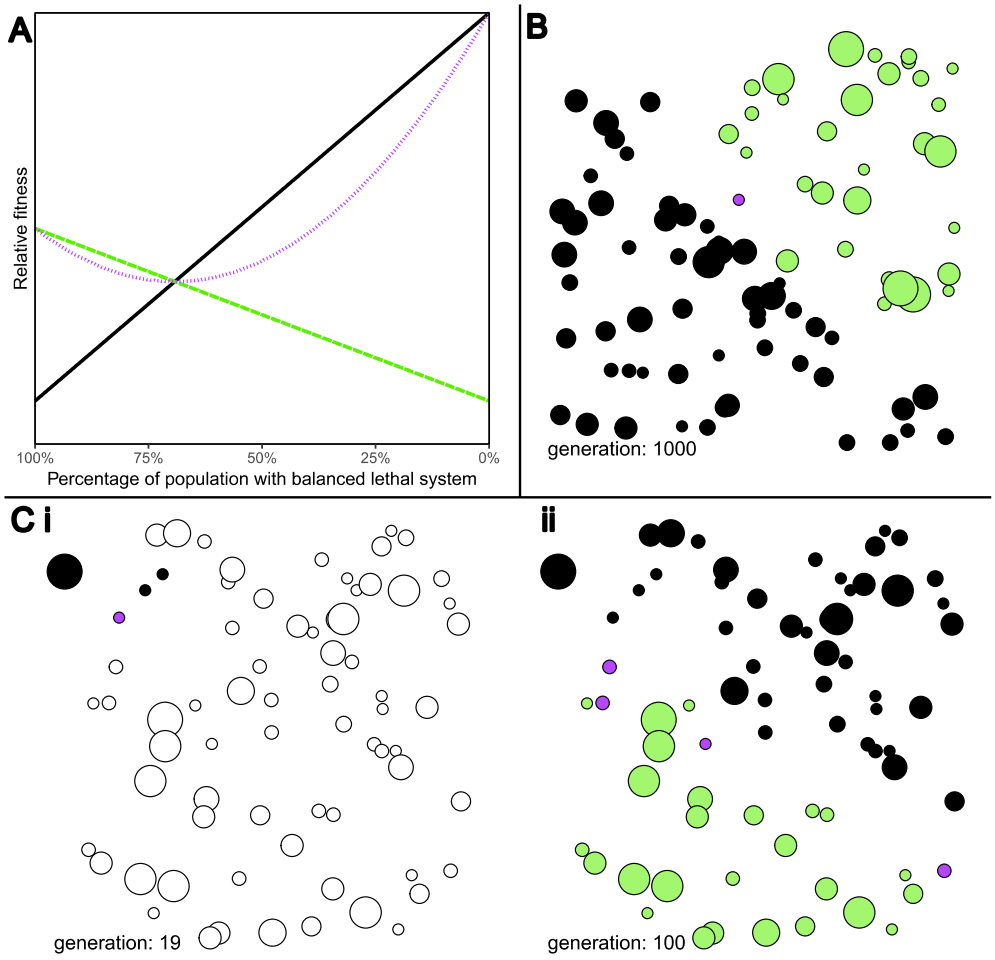

Figure 6: Simple and detailed models of outbreeding depression between the rearranged and ancestral genomes. (A) Plot showing the relative fitness of offspring from parents with the ancestral pre-rearrangement (black) and balanced lethal (green) genotypes against the proportion of the ancestral chromosome 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

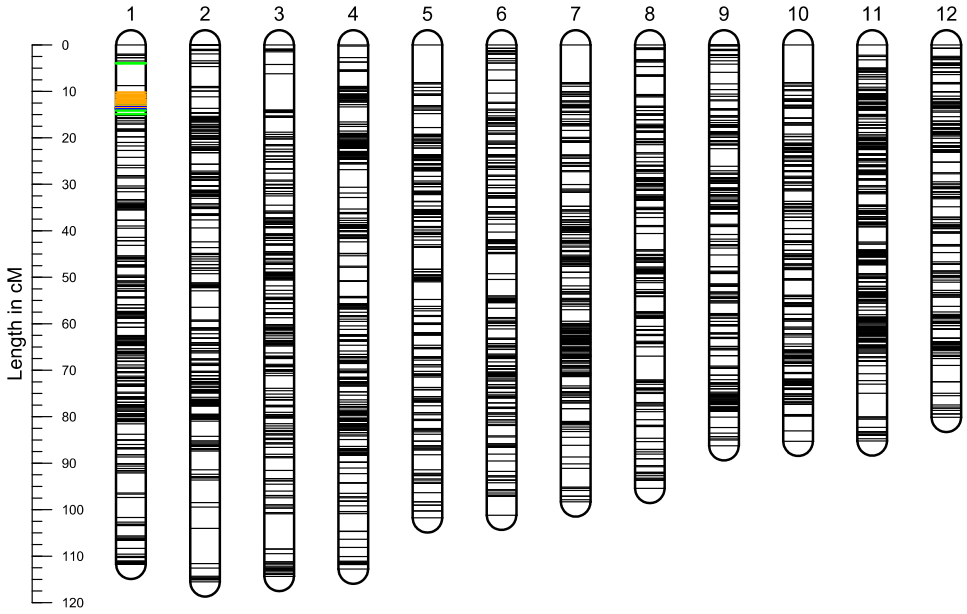

Figure S1: Linkage map based on target capture data from a full-sibling F_2 *Triturus ivanbureschi* × *macedonicus* family, consisting of four F_0 grandparents, two F_1 parents and 206 F_2 offspring. The map includes 4226 markers in 12 linkage groups spanning a total length of 1188 cM. Linkage groups 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*.

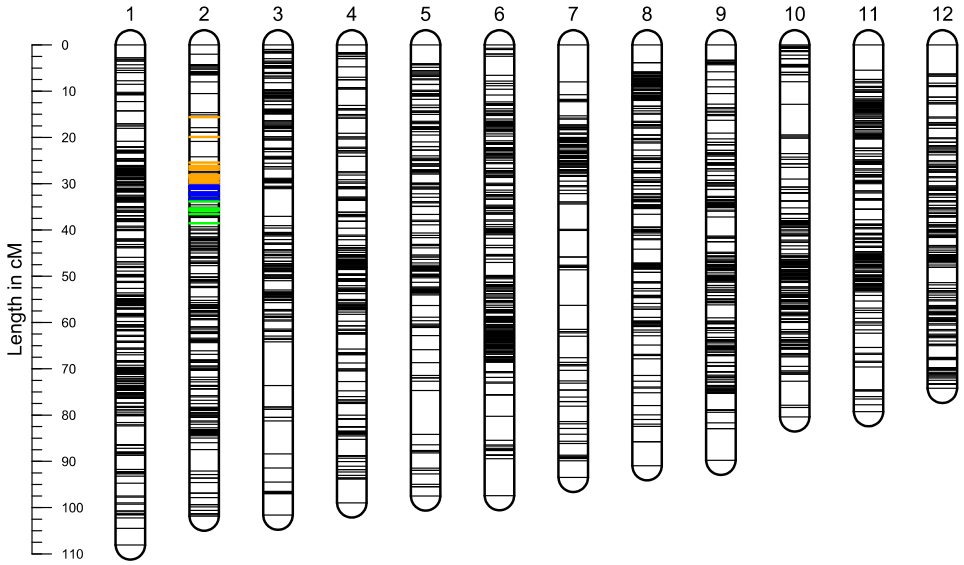

Figure S2: Linkage map based on target capture data from a full-sibling F_2 *Lissotriton vulgaris* × *montandoni* family, consisting of two F_1 parents and 203 F_2 offspring. The map includes 3693 markers in 12 linkage groups spanning a total length of 1113 cM. In *Lissotriton* the homologs of genes 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).

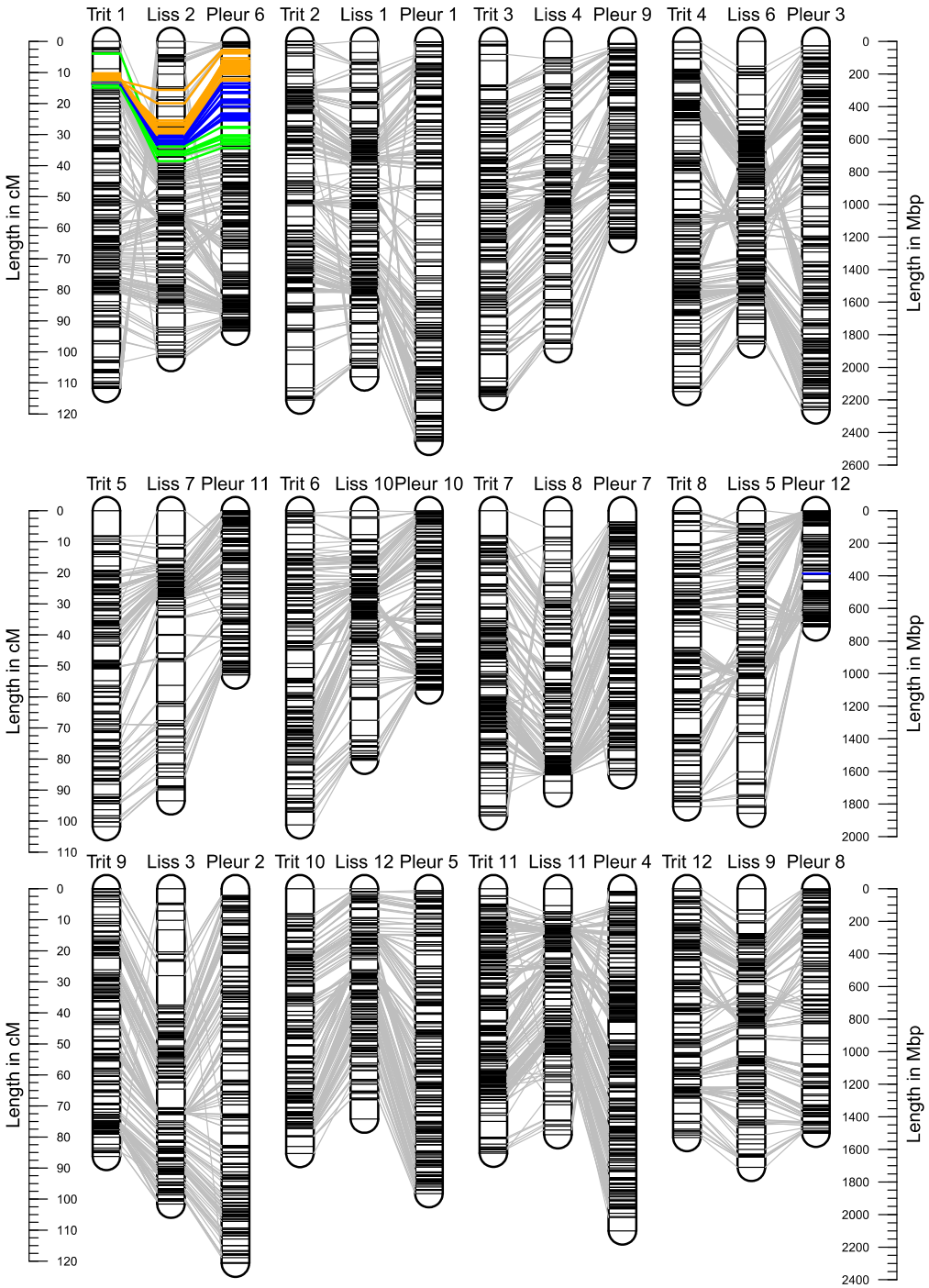

Figure S3: Homology between the *Triturus* and *Lissotriton* linkage maps and the *Pleurodeles waltl* genome assembly (Brown et al. 2025). *Triturus* chromosome 1 linked markers are highlighted: A-linked in blue, B-linked in orange and species specific in green.

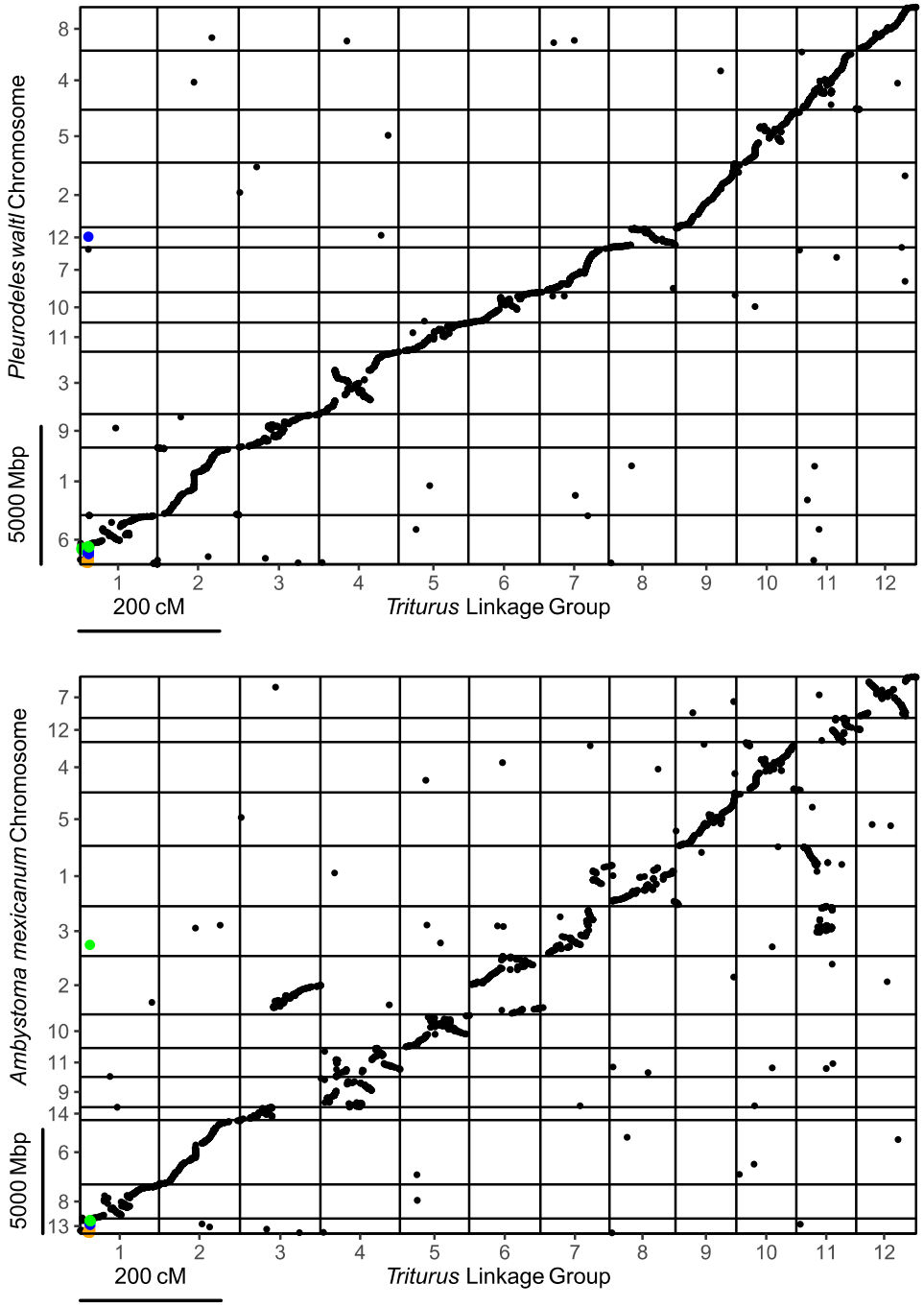

Figure S4: Oxford plots showing locations of markers within the *Triturus* linkage map and genome assemblies for *Pleurodeles waltl* (Brown et al. 2025) and *Ambystoma mexicanum* (Nowoshilow et al. 2018; Smith et al. 2019). Synteny is extremely tightly conserved between the two newt taxa, but less so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

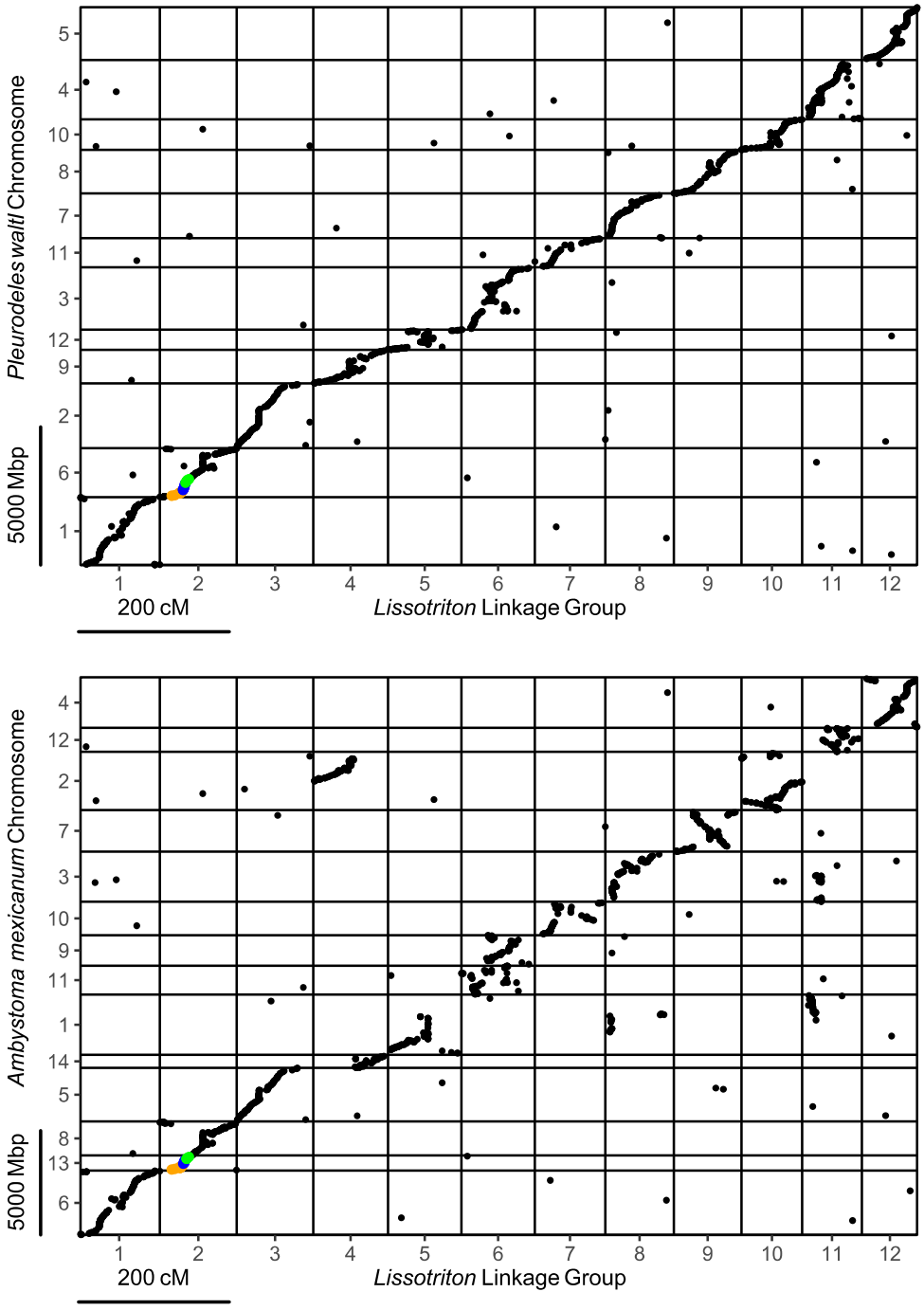

Figure S5: Oxford plots showing marker locations within the *Lissotriton* linkage maps compared to the *Pleurodeles waltl* (Brown et al. 2025) and *Ambystoma mexicanum* (Nowoshilow et al. 2018; Smith et al. 2019) genome assemblies.

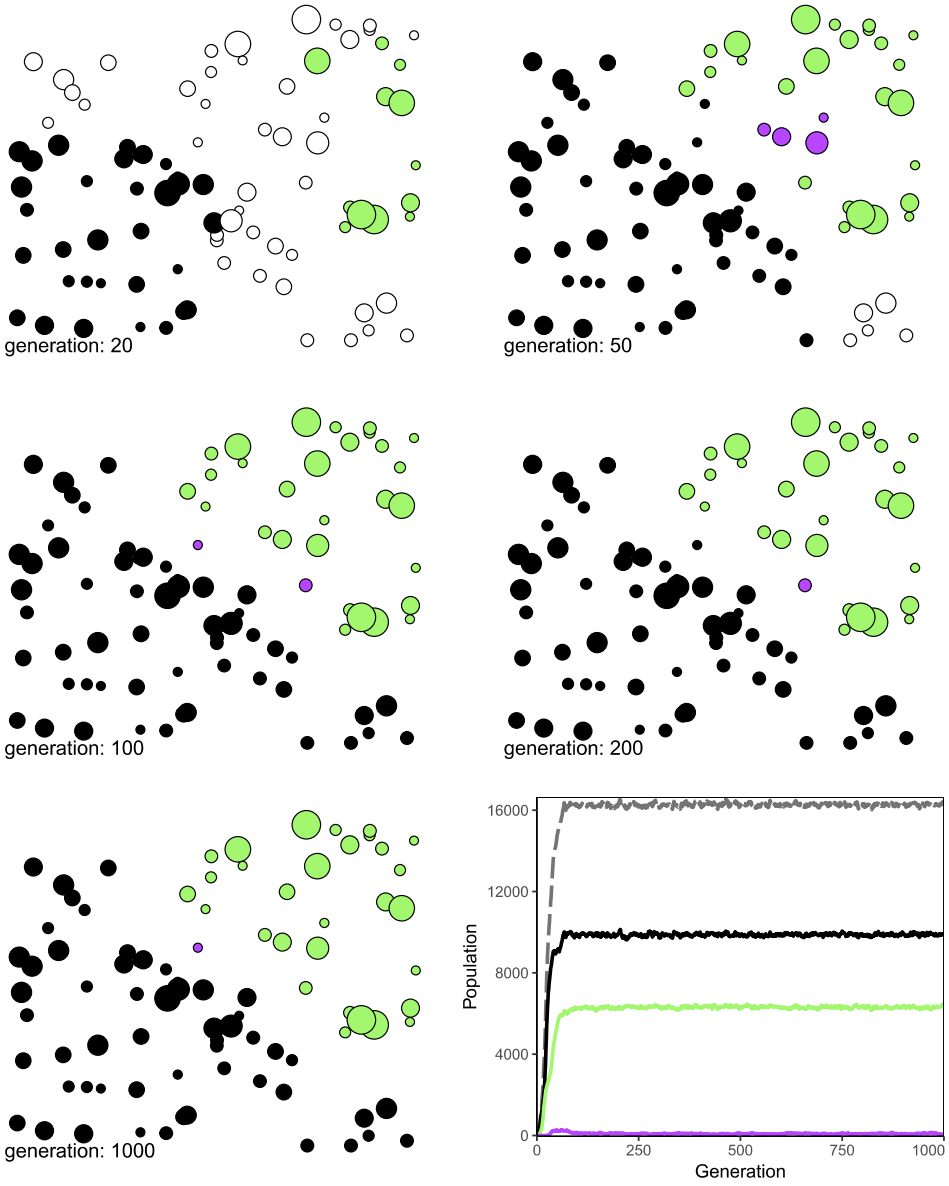

Figure S6: Simulation of secondary contact between the ancestral and balanced lethal population -shown in black and green respectively, with populations where less than 80% of individuals are of one genotype shown in purple. Despite the apparent fitness disadvantage of the balanced lethal population 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).

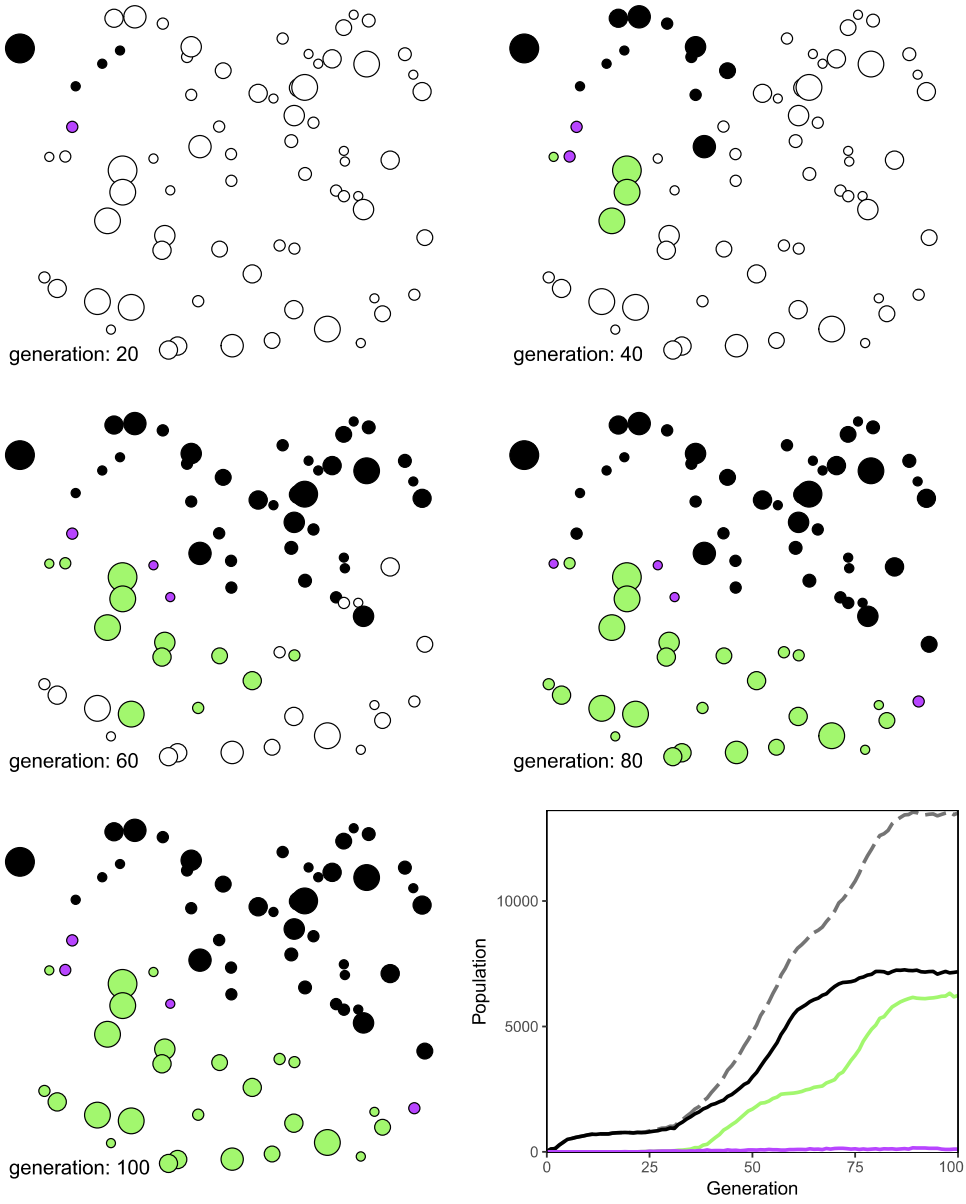

Figure S7: Simulation of evolution of a balanced lethal population from a single mutation at generation 19. By generation 100 all ponds have been colonised, and a hybrid zone has been formed between the ancestral and balanced lethal system population.

	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					
	All Markers		A-linked Markers		B-linked Markers	
Sample 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.

Synthesis

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Summary of Findings

This thesis focuses on the remarkable balanced lethal system found in crested and marbled newts of the genus *Triturus*. Our goal was to understand the genomic changes that resulted in evolution of this system. We accomplished this by mapping the genome of *Triturus*, identifying important features and structural rearrangements, and determining which, if any, of the previously proposed hypotheses regarding the evolution of the balanced lethal system are compatible with our findings.

At the time this project began the degenerate sex chromosome hypothesis proposed by Grossen et al. (2012) was the most recent and detailed model of the evolution of the *Triturus* balanced lethal system. This hypothesis makes implicit predictions concerning the Y-chromosome of *Triturus* and related genera but testing these predictions would require the development of male-linked genetic markers and the identification of the Y-linked region of the genome. As this had not yet been accomplished in any salamander, we explored methodology in *Lissotriton*, the sister genus of *Triturus*.

In **chapter 2** we identified male specific genetic markers for the common newt *Lissotriton vulgaris* by screening RADseq data generated from adults of known sex for loci associated with a male phenotype. The resulting markers facilitate genetic sex identification in all species within the *L. vulgaris* species complex, although the primers designed do not amplify sex-specifically in *Lissotriton* species outside of this lineage. Additionally, we constructed a high-density linkage map based on *L. vulgaris* hatchlings of unknown sex. The resulting map appears reliable, matching the chromosome number pairs observed in the *L. vulgaris* karyotype and showing an extremely high degree of synteny with the genome of *Pleurodeles Waltl*. Following the methodology of Brelsford et al. (2016) we attempted to locate the Y-linked chromosome purely from the information contained within this map, searching for a zone of reduced recombination, enriched in paternal-specific alleles. However, we could not definitively identify any such region, indicating that this approach cannot be relied upon on its own in species with very large genomes and small homomorphic sex-chromosomes. This was disappointing, because if the linkage map could be used in this manner, there would be no need to sequence many morphologically sexed adults. Nevertheless, by incorporating the association derived Y-linked markers into the linkage map, the Y-linked region was located and found to be homologous to *P. Waltl* chromosome 5.

With the Y-chromosome of *L. vulgaris* identified, we would apply the same methodology to identify the male-linked region in *Triturus*. If the sister genera shared a sex determination system, it would mean that no Y-chromosome turnover had occurred, and this would allow us to rule out the degenerate sex chromosome hypothesis. Therefore, in **chapter 3** we investigated the Balkan crested newt *T.*

ivanbureschi. We developed a set of Y-linked markers that amplify male-specifically across the genus and constructed a high density RADseq linkage map that also showed tight synteny with *P. Waltl*. When we localised the *Triturus* Y-linked region within this map we found it was homologous to *P. Waltl* chromosome 2, and so not homologous to the Y-chromosome of *L. vulgaris*. This indicated that at least one sex chromosome turnover event has occurred since the two genera diverged and so the degenerate Y-chromosome hypothesis could not be dismissed. We therefore examine a second implicit prediction of this model, that the ancestral Y-chromosome, presumably retained in *Lissotriton*, should be homologous to the non-recombining region in the *Triturus* balanced lethal system. By incorporating male-linked markers from both genera into target capture maps we showed that this was not the case, unless (at least) two sex chromosome turnover events had occurred. While not conclusive evidence against a Y-chromosome origin, these findings render it less plausible, especially as this scenario already relied on extremely specific conditions.

To test alternative hypotheses, it would be necessary to identify and directly locate genes involved in the balance lethal system. Therefore, in **chapter 4** we used target capture sequencing to construct linkage maps for interspecific crosses in both *Triturus* and *Lissotriton*. Surprisingly, genes associated with the balanced lethal system in *Triturus* were shown to be characterized primarily by presence/absence variation, with each gene entirely missing from either chromosome 1A or 1B. We located the homologs of these genes on the linkage map built for *Lissotriton*, as well as the published genome assemblies of both *P. Waltl* and *Ambystoma mexicanum*. This revealed that the balanced lethal system is characterised by a pair of large deletions, resulting in both chromosome 1A and 1B missing a distinct section of the chromosome that remains present on the other. This explained both the lethality of the system and the absence of recombination. A pair of deletions is one predicted consequence of an unequal exchange, a mechanism which Sessions et al. (1988) briefly speculated might explain the balanced lethal system - although at the time there was no direct evidence for this. As an unequal exchange would also result in each of the deletions being coupled with a reciprocal duplication, we also examined allele ratios for SNPs in the chromosome 1 linked genes. In heterozygous individuals, with only one copy each of chromosomes 1A and 1B, SNPs on the genes absent from either form of the chromosome 1 remained biallelic – which is strong evidence that these genes are indeed duplicated on the opposite version of the chromosome 1. As we found clear genomic evidence of both the twin deletions and reciprocal duplications that are the predicted outcome of an unequal exchange, and given the difficulty of explaining these observations via other mechanisms, we concluded that this single macromutation is the origin of the balance lethal system. We considered how such a disadvantageous mutation could spread to fixation and suggested that an answer may be found in the haploinsufficiency that would be expected in the hybrids between the balanced lethal system and the ancestral genotype. We simulated a single unequal exchange occurring within an expanding

population and found that this had the potential to produce a subpopulation with a fixed balanced lethal system that remained reproductively isolated despite being geographically connected, acting as two distinct species.

Evolution of Balanced Lethal Systems

With the exception of the unequal exchange the other hypotheses proposed for the evolution of balanced lethal systems in *Triturus* and general have posited a gradualistic model where another non-recombining region (either a sex-chromosome or inversion supergene) slowly degenerated (Grossen et al. 2012; Berdan et al. 2022). The large, contiguous deletions we observe in *Triturus* are difficult to explain in these scenarios, leading us to reject them in favour of an instantaneous origin.

Does *Triturus* represents a general case for the evolution of balanced lethal systems? The most detailed information on other naturally occurring balanced lethal systems is for plant of the genus *Oenothera*. This genus, containing around 145 species, is notorious for its complex cytogenetics, which include many other peculiarities aside from balanced lethal factors (Harte 1994). *Oenothera* exhibits complex patterns of hybridisation, connected to its genomic structure, where multiple reciprocal translocations have occurred between the seven chromosome pairs. This results in the chromosomes arranging into a complex ring during meiosis (Cleland 1962). Several *Oenothera* species act as permanent hybrids – where entire haploid genomes are transmitted as complete units (termed Renner complexes) (Rauwolf et al. 2008), and heterozygosity is required for survival. In the case of *Oenothera* the evolution of balanced lethal factors may be best explained as a consequence of maintaining advantageous heterozygosity. This is also likely to be the case with *Isotoma*, which also exhibits translocation heterozygosity, and reduced recombination except at the end of the chromosomes (James et al. 1990). In *Drosophila tropicalis* the cytology performed by Dobzhansky and Pavlovsky (1955) suggests that the balanced lethal system is a consequence of obligatory heterozygosity for an inversion. The authors also speculate that that this species remains competitive with other similar and sympatric *Drosophila* species suggests that the inversion confers a unique advantage which resembles the degenerative supergene scenarios proposed by Berdan et al. (2022). A further potential example of a balanced lethal system are polymorphic inversions in the first chromosome of the flatworm *Schmidtea mediterranea* (Charlesworth 2022), although the original authors characterise this as a proto-sex chromosome (Guo et al. 2022), illustrating the similarities of the two phenomena – and possibly being an example of a 'ghost' of a formed sex-chromosome (Grossen et al. 2012).

None of these systems closely resemble our observations in *Triturus*, suggesting that its balanced lethal system remains an anomaly among anomalies. Instead, the

examples in *Oenothera*, *Isotoma* and *D. tropicalis* all point towards a more general mechanism for the evolution of balanced lethal systems, involving the permanent fixation of a pre-existing heterozygote advantage, whether generated by hybridisation, or by a supergene complex, as modelled by Berdan et al. (2022), or by any other means. There is the possibility that this mechanism also played a role in the evolution of *Triturus* chromosome 1. If any genes within the A- or B-linked regions exhibited overdominance, then the unequal exchange could have fixed beneficial combinations of these alleles, resulting in an advantage for adults heterozygous for the two new forms of chromosome 1. Currently, this remains entirely speculative; we have no evidence of any such genes, and our simulations show that such overdominance is not necessary for establishment of the balanced lethal system.

Underdominance and Genetic Surfing

While *Triturus* chromosome 1 is likely an atypical example of a balanced lethal system, it may represent an extreme, but illustrative, case of a different phenomenon – the protection of otherwise deleterious alleles by heterozygote disadvantage, i.e. underdominance. The mechanism proposed in chapter 4 suggests that the balanced lethal system is protected against invasion by the apparently fitter ancestral genotype because the hybrids between the two would be, on average, less fit than either of parents. In this model, while the A and B versions of chromosome 1 exhibit extreme overdominance with respect to each other, they are underdominant with respect to the ancestral chromosome.

Underdominance has been proposed as a mechanism by which chromosomal rearrangements may become fixed in a population, eventually leading to speciation (White 1969, 1978; King 1993). Rearrangements such as inversions and translocations have a theoretical tendency towards underdominance, as recombination between the derived and ancestral chromosomes within the affected region will result in deletions and/or segregation defects during meiosis, which should result in non-viable gametes (Rieseberg 2001). A well-studied example in which this mechanism has been claimed to act is Australian Morabine grasshoppers, where multiple "chromosomal races" exist in parapatry (White 1974). However, this mechanism has been criticised on theoretical grounds, as it is unlikely for any strongly underdominant mutation to drift to fixation, except in a very small and inbred population. If the mutation did become established in a subpopulation, then underdominance means further spread at the expense of the ancestral genotype is difficult, even if the mutation is adaptive (when homozygous), and especially if the mutation is maladaptive, as in the balanced lethal system (Futuyma & Mayer 1980; Walsh 1982).

The dynamics change significantly if we consider a population with a rapidly expanding range. As modelled in chapter 4, mutations present at the colonising front can rapidly increase in frequency and become common over a large range as the population expands, in a process known as genetic surfing (Edmonds et al. 2004; Klopfstein et al. 2006). Importantly, because genetic surfing acts randomly upon alleles with little regard to how adaptive they might be, deleterious alleles can also achieve very high frequency via this mechanism. Indeed, because maladaptive mutations are more common than adaptive, a rapidly expanding population can be expected to suffer from reduced fitness due to 'expansion load' (Peischl & Excoffier 2015). The capacity of genetic surfing to promote deleterious mutations is well understood theoretically and several examples have been described (Peischl et al. 2013, 2018; Henn et al. 2016; Rougemont et al. 2023). Interaction between genetic surfing and underdominant mutations has received less attention, but a recent publication models the establishment of clines for loci exhibiting heterozygote disadvantage within an expanding population (Gilbert et al. 2022). The authors demonstrate that not only are clines for underdominant alleles easily established in these populations, but that these clines will tend to attract each other (cline coupling), potentially resulting in parapatric speciation. *Triturus* may be a good example of this mechanism.

The Balanced Lethal System and Speciation

In chapter 4 we describe the population in which we simulate the balanced lethal system becoming fixed as a new species, distinct from the ancestral species with the unrearranged form of chromosome 1. However, while the mechanism we propose requires significant underdominance between the ancestral and derived versions of this chromosome, which will result in a barrier to gene flow, reproductive isolation between these populations cannot be complete. This is because chromosomes 1A and 1B must pass through at least one 'hybrid' generation after the initial mutation. Consequently, the applicability of the term 'species' may be considered debatable.

'Species' is famously not a term with a single unambiguous definition (Zachos 2016). While a full description of the 'Species Problem' is far beyond the scope of this discussion, there exist multiple competing species concepts, many of which are themselves somewhat subjective (Mayr 1996; Hey 2001; Stankowski & Ravinet 2021). The categorisation of the new population in our model as a species can be defended on several grounds. Firstly, while reproductive isolation is incomplete, it is real and significant. Secondly, the new population is distinguished by a distinct phenotype, the premature death of half of its offspring, which is directly linked to the locus driving reproductive isolation. Thirdly, the new population will experience different selective pressures as a direct result of its phenotypic divergence. For example, Sessions et al.

(1988) speculate that the decrease in embryonic survival would favour an increase in clutch volume, explaining the larger body size of *Triturus* compared to its close relatives - as in salamanders clutch volume is correlated with body size (Kaplan & Salthe 1979).

Stable Genomes Going Rogue?

The target capture (chapter 4) and RADseq (chapters 2 and 3) linkage maps constructed for *Triturus* and *Lissotriton* show highly conserved synteny at the genome level. When compared with the genome assembly of *P. waltl*, no chromosomal fusions, fissions or translocations are observed. If these lineages are representative, the genomic structure of newts has changed very little since their last common ancestor lived an estimated 60 mya (Marjanović & Laurin 2014). Across the family Salamandridae the only known deviation from the ancestral chromosome number of 2n=24 is in north American newts (the genera *Taricha* and *Notophthalmus*), which form a monophyletic lineage with 11 chromosome pairs, likely due to a single fusion event (Sessions 2008). This genomic stability is also seen in some other salamander families, for instance all the Ambystomatidae possess 14 chromosome pairs, but it is much less dynamic than many vertebrate taxa of comparable age and species richness (Wienberg & Stanyon 1998; Sessions 2008; Degrandi et al. 2020).

Notwithstanding this apparent stability, newts seem to have a talent for surprising and consequential genomic alterations. Only two Y-chromosomes have been molecularly identified in salamanders, those described in chapters 2 and 3. Although these are from sister genera, they are revealed to be completely non-homologous. As *P. waltl* possesses Z-and W-chromosomes, we now know of three distinct sex determination systems within newts, implying at least two turnover events, despite only having investigated two species at the genomic level. Of course, while frequent sex-chromosome turnovers may be considered unusual, they are prosaic compared to the extraordinary rearrangements involved in the evolution of the *Triturus* balanced lethal system. Although it may be entirely coincidental that the same taxa exhibit gigantic genomes, almost perfect conservation of inter-chromosome synteny, frequent sex-chromosome turnover and a naturally occurring balanced lethal system, it would be interesting to known if there is any causal relationship between these phenomena.

Outlook for Future Research

The research described in this thesis relies heavily upon linkage maps, based on both target capture and RADseq data. Constructing these maps involves placing markers according to the frequency of recombination between them. It is therefore somewhat ironic that the primary areas of interest, sex-chromosomes and the balanced lethal system, are characterised by the absence of recombination. Although the linkage maps can reveal which loci are associated with these regions, they cannot resolve the order of these genes. At present we would be unable to detect, for example, an inversion within the Y-linked region of *Triturus*.

The obvious alternative is whole genome sequencing and assembly, which would be extremely useful for answering may of the questions addressed in this thesis. Unfortunately, the size of the *Triturus* genome made whole genome assembly appear wildly ambitious, at least at the time when this project was initiated. In the intervening period, however, several similarly large genomes have been assembled, including that of *P. Waltl* (Brown et al. 2025). A *Triturus* genome assembly would enable the identification of many more genes within both the balanced lethal system and the Y-linked region, potentially revealing the primary sex-determining gene. A whole genome assembly would also facilitate testing of whether that the A- and B-linked regions have been duplicated as complete consecutive blocks, as would be predicted in an unequal exchange. Additionally, with an assembly it would become much easier to disentangle the two paralogs of each gene that should have arisen from the duplication and group these together as the two blocks we expect to observe.

Looking beyond *Triturus* and the balanced lethal system, there are many curious genomic phenomena that were the subject of much study in the twentieth century, but received less attention as researchers ran up against the limitations of the techniques of the time. With technology such as target capture and whole genome sequencing now well established and capable of being easily and effectively applied to a very large variety of non-model organisms, we have the opportunity to revisit many old evolutionary puzzles.

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Summary

The balanced lethal system found in the crested and marbled newts that make up the genus *Triturus* is an especially puzzling phenomenon. In these newts half of all fertilised eggs undergo developmental arrest and die before hatching. This phenomenon results in a catastrophic loss of reproductive output and is not seen in any other species of newt or salamander. While we understand evolution to be a stochastic process with no ability to recognise or progress towards any 'objective', it is still surprising that a lethal mutation has become fixed in an entire genus. This thesis aims to reveal the nature of this mutation and the evolutionary mechanisms that produced it.

In Chapter 1 I give a broad overview of the research background. I introduce the organisms of interest (*Triturus* newts) and place them in a phylogenetic context, including their sister genus *Lissotriton* and more distant relatives inside and outside of the family Salamandridae. I explain the general mechanics of balanced lethal systems and discuss the literature regarding the system found in *Triturus*, which comprises the two distinct, non-recombining forms of chromosome 1. I describe several possible hypotheses which seek to explain the evolution of the balanced lethal system, including evolution from sex chromosomes, and degeneration of a supergene system. I then set out the objectives of the PhD project: to examine these hypotheses by mapping the genomes of *Triturus* and its relatives, and identifying structural changes associated with the evolution of the balanced lethal system.

In Chapter 2 we begin the process of testing the sex chromosome origin hypothesis by identifying the Y-chromosome of *Lissotriton*, which in this hypothesis would be expected to be homologous to the non-recombining region of *Triturus* chromosome 1. This is challenging because newts have extremely large genomes (20-30 Gbp) and small, homomorphic, sex-linked regions. Two RADseq based approaches are attempted, identifying male specific markers in adults of known sex, and identifying a paternal-linked, non-recombing region via linkage mapping. The sex association approach proves to be effective, identifying several Y-linked markers which are validated in multiple *Lissotriton* species. However, despite the construction of a high-density linkage map, a Y-linked non-recombing region is not immediately apparent until the markers discovered in the adult males are highlighted.

In Chapter 3 we apply the techniques used in Chapter 2 to *Triturus* itself. A high-density RADseq linkage map is constructed, and Y-linked markers identified via sex association in adult males. The Y-linked markers are then incorporated into the linkage map to highlight the Y-chromosome. By comparing the *Triturus* and *Lissotriton* linkage

maps, we observe that the two genera have two different Y-chromosomes. This indicates that at least one of the lineages evolved a new Y-chromosome after they split from their last common ancestor. This is important because the sex chromosome origin model requires this kind of Y-chromosome turnover event to occur in this time frame. However, we also observe that the *Lissotriton* Y-chromosome is not homologous to *Triturus* chromosome 1. Consequently, we conclude that, while there is clear evidence of recent Y-chromosome turnover within newts, this is likely incidental to the evolution of the *Triturus* balanced lethal system.

In Chapter 4 we seek to explore other models of the evolution of the balance lethal system by directly identifying any genomic rearrangements that have occurred since *Triturus* and *Lissotriton* diverged from their last common ancestor. We use target capture sequencing to construct linkage maps for both genera, each including the position of over 3,500 coding genes. We also compare these maps to the genome assemblies of the Iberian Ribbed Newt (*Pleurodeles waltl*) and Axolotl (*Ambystoma mexicanum*). Surprisingly, at the whole genome level, there is very little structural variation between the three newt genera, even though their last common ancestor lived over 60 million years ago. However, in *Triturus* chromosome 1 specifically, there are rearrangements associated with the balanced lethal system. We observe that each of the two forms of chromosome 1 is missing a single large section of DNA, approximately 200 Mbp in length. This explains the lethality of the system: the 50% of embryos unlucky enough to inherit two copies of the same version of chromosome 1 are completely missing a significant portion of their genome. Curiously, it also appears that each deletion is compensated for by duplication of the homologous region of the opposite version of the chromosome, so the 50% of embryos that inherit one copy of each version of chromosome 1 still have two copies of all genes. This pattern of deletion and duplication is the predicted result of an unequal exchange between sister chromosomes, which is a mechanism that has been hypothesised to create a balanced lethal system in a single step, in a single individual. We model this scenario and find that, counterintuitively, the balanced lethal system can become fixed in a sub-population that then becomes resistant to invasion by the ancestral, pre-rearrangement form of chromosome 1. This resistance is due to the fact the duplications in the balanced lethal system fully compensate for the deletions, which is not the case for 'hybrids' between the balanced lethal system and ancestral chromosome.

In Chapter 5 I synthesise the findings of the preceding three chapters and provide directions for future research. The identification of two completely different Y-chromosomes in two sister genera is somewhat surprising and suggests a need for further investigation of sex determination systems across newts, which I suggest could be accomplished using the same methodology as I describe in Chapters 2 and 3. A consistent finding across the chapters is a tight conservation of genome scale synteny within newts, and it would be interesting to know whether this is restricted to *Triturus*,

Lissotriton and *Pleurodeles*, or is characteristic of the entire family Salamandridae. I also discuss the implications of the reproductive isolation modelled in Chapter 4 and the unexpected link this suggests between the balanced lethal system and speciation. I conclude that the *Triturus* balanced lethal system offers an excellent example of how applying modern genomic tools to previously intractable problems can offer fascinating and surprising new insights

Samenvatting

Het zogenaamde 'balanced lethal system' (gebalanceerd dodelijk systeem), dat voorkomt bij de kam- en marmersalamanders die samen het geslacht *Triturus* vormen, is een bijzonder raadselachtig fenomeen. Bij deze salamanders komt de helft van alle bevruchte eitjes niet uit; in plaats daarvan stopt de ontwikkeling en sterven de embryo's uiteindelijk. Dit fenomeen leidt tot een catastrofale afname van het reproductieve succes, wat bij geen enkele andere (water-)salamandersoort wordt waargenomen. Ondanks dat we evolutie begrijpen als een stochastisch proces zonder doelgerichtheid, blijft het uiterst verrassend dat zo een overweldigend nadelige mutatie zich heeft kunnen vestigen in een volledig geslacht. Mijn proefschrift richt zich op het blootleggen van de aard van deze mutatie en de evolutionaire krachten die dit systeem mogelijk voortgebracht hebben.

In hoofdstuk 1 beschrijf ik de achtergrond van het onderzoek. Ik introduceer de onderzochte organismen (*Triturus*-salamanders) en plaats ze in een fylogenetische context, inclusief hun zuster-geslacht *Lissotriton* en minder nauw verwante soorten binnen en buiten de familie Salamandridae. Vervolgens leg ik de algemene mechanismen van balanced lethal systems uit en bespreek ik de literatuur over het systeem in *Triturus*, dat bestaat uit twee verschillende, niet recombinerende vormen van chromosoom 1. Ik bespreek verschillende hypothesen die het ontstaan van het balanced lethal system proberen te verklaren, waaronder de evolutie vanuit geslachtschromosomen en de degeneratie van een supergen-systeem. Tot slot presenteer ik de doelstellingen van mijn promotieonderzoek: het testen van deze hypothesen door de genomen van *Triturus* en verwante soorten in kaart te brengen en structurele veranderingen te identificeren die gerelateerd zijn aan de evolutie van het balanced lethal system.

In hoofdstuk 2 beginnen we met het testen van de hypothese dat het systeem zijn oorsprong vindt in geslachtschromosomen. Dit doen we door het Y-chromosoom van *Lissotriton* te identificeren, dat in dit model homoloog zou moeten zijn aan het niet-recombinerende gebied van *Triturus* chromosoom 1. Dit onderzoek is ingewikkeld omdat salamanders zeer grote genomen hebben (20-30 Gbp) en kleine, homomorfe geslachtschromosomen. We proberen twee RADseq-gebaseerde methodes: het identificeren van genetische merkers bij volwassen individuen met een bekende sekse die uniek zijn aan de mannelijke salamanders en het in kaart brengen van een paternaal overgeërfd, niet-recombinant gebied via een genkoppelingskaart (linkage map). De geslachtsgebonden benadering blijkt effectief; er worden meerdere Y-gebonden merkers geïdentificeerd die in verschillende *Lissotriton* soorten gevalideerd worden.

Echter, ondanks het samenstellen van een genkoppelingskaart met hoge dichtheid, wordt een Y-gebonden, niet recombinerende regio niet onmiddellijk zichtbaar, zonder de merkers die eerder bij volwassen mannetjes gevonden zijn eruit te lichten.

In hoofdstuk 3 passen we de technieken uit hoofdstuk 2 toe op *Triturus* zelf. We stellen een gedetailleerde RADseq genkoppelingskaart samen en identificeren Y-gebonden merkers via geslachts associate in een populatie volwassen mannetjes. Vervolgens worden deze Y-merkers geïntegreerd in de genkoppelingskaart om het Y-chromosoom te lokaliseren. Door de genkoppelingskaarten van *Triturus* en *Lissotriton* met elkaar te vergelijken, ontdekken we dat beide geslachten verschillende Y-chromosomen hebben. Dit betekent dat ten minste één van de twee geslachten een nieuw Y-chromosoom heeft ontwikkeld sinds hun laatste gemeenschappelijke voorouder. Dit is belangrijk, omdat het model van geslachtschromosoom-oorsprong een dergelijke Y-chromosoom vervanging vereist binnen deze tijdsperiode. We constateren echter ook dat het *Lissotriton* Y-chromosoom niet homoloog is aan *Triturus* chromosoom 1. Daarom concluderen we dat, hoewel er duidelijk bewijs is voor recente Y-chromosoomvervanging binnen salamanders, dit waarschijnlijk geen rol heeft gespeeld in de evolutie van het balanced lethal system van *Triturus*.

In hoofdstuk 4 onderzoeken we alternatieve modellen voor de evolutie van het balanced lethal system door direct chromosomale mutaties te identificeren die hebben plaatsgevonden sinds *Triturus* en *Lissotriton* zich van hun laatste gemeenschappelijke voorouder hebben afgesplitst. We gebruiken target-capture-sequencing om genkoppelingskaarten te construeren voor beide geslachten, elk met de locatie van meer dan 3.500 coderende genen. Daarnaast vergelijken we deze kaarten met de genoomassemblages van de ribbensalamander (*Pleurodeles waltl*) en de axolotl (*Ambystoma mexicanum*). Opvallend genoeg vinden we op het niveau van het gehele genoom zeer weinig structurele variatie tussen de drie watersalamandergeslachten, ondanks het feit dat hun laatste gemeenschappelijke voorouder meer dan 60 miljoen jaar geleden leefde. Specifiek in *Triturus* chromosoom 1 zijn er echter wel herschikkingen die verband houden met het balanced lethal system. We constateren dat beide varianten van chromosoom 1 een grote deletie van ongeveer 200 Mbp hebben. Dit verklaart de dodelijke aard van het systeem: de 50% van de embryo's die de pech hebben twee kopieën van dezelfde variant te erven, missen een aanzienlijk deel van hun genoom. Verrassend genoeg lijkt elke deletie gecompenseerd te worden door een duplicatie van hetzelfde DNA-segment op de andere variant van chromosoom 1, waardoor de 50% van de embryos die beide varianten erven alsnog over een enkel exemplaar van elk gen beschikken. Dit patroon van deletie en duplicatie is het voorspelde resultaat van een ongelijkmatige uitwisseling tussen zusterchromosomen, een mechanisme dat in theorie een balanced lethal system in één enkele stap zou kunnen creëren. We modelleren dit scenario en ontdekken dat het balanced lethal system zich contra-intuïtief kan vestigen in een subpopulatie en vervolgens bestand kan

worden tegen invasie door de ancestrale chromosoomvariant. Dit komt doordat de duplicaties binnen het systeem de deleties volledig compenseren, terwijl dit niet het geval is voor 'hybride' combinaties van het balanced lethal system en het voorouderlijke chromosoom.

In hoofdstuk 5 vat ik de bevindingen uit de voorgaande hoofdstukken samen en bepaal ik richtingen voor toekomstig onderzoek. De identificatie van twee totaal verschillende Y-chromosomen in twee nauw verwante geslachten is verrassend en suggereert dat het geslachtsbepalingssysteem bij salamanders verder onderzocht moet worden. Ik stel voor om hiervoor dezelfde methodologie te gebruiken als in hoofdstuk 2 en 3. Een terugkerende bevinding is het sterke behoud van genomische syntenie binnen salamanders. Het zou interessant zijn om te onderzoeken of dit beperkt is tot *Triturus*, *Lissotriton* en *Pleurodeles*, of een algemeen kenmerk is van de gehele familie Salamandridae. Daarnaast bespreek ik de implicaties van de in hoofdstuk 4 gemodelleerde reproductieve isolatie en de hierdoor gesuggereerde onverwachte verbinding tussen het balanced lethal system en soortvorming. Ik concludeer dat het balanced lethal system in *Triturus* een uitstekend voorbeeld is van hoe het toepassen van moderne genomische technieken op voorheen onoplosbare evolutionaire vraagstukken nieuwe en verrassende inzichten kan bieden.

Curriculum Vitae

James France was born on 7th April 1990 in Bolton, Lancashire, United Kingdom. After completing his secondary education at Queen Elizabeth's Grammar School, Blackburn, he pursued an integrated master's degree at the University of Durham (2008–2013), graduating with joint honours in chemistry and biology. During his undergraduate studies he completed a research internship at the University of Washington, Seattle, USA, characterizing lateral line regeneration in zebrafish. For his master's thesis, *Characterisation of Nucleotide Binding Dynamics in the IRE1 Kinase Domain*, James investigated the activation mechanism of the enzyme IRE1, which regulates the response to misfolded proteins.

After earning his degree James became a research assistant at the University of Central Lancashire's School of Medicine and Dentistry. His research focussed on developing cell culture models of Alzheimer's disease to investigate the biochemical mechanisms that make oral bacteraemia a risk factor for dementia. Additionally, James taught as part of master's programmes, including research methods, literature appraisal and biochemistry.

James grew fascinated with the processes that might drive the evolution of apparently harmful traits. In 2019 he was given the opportunity to begin his PhD studies in the laboratory of Dr. Ben Wielstra at the Institute of Biology Leiden, Leiden University and Naturalis Biodiversity Center. These studies focus on the genomics and evolution of the mysterious balanced lethal system found in newts of the genus *Triturus*. His research has involved optimising a target capture sequencing protocol to obtain large scale genomic data from hundreds of samples of *Triturus* and other newt species. He analysed these data to construct high-density genetic maps which revealed the rearrangements that created the balanced lethal system, as well as allowing for the identification of a recent sex chromosome turnover within European newts. He then developed simulations to model the spread and paradoxical survival of the balanced lethal system. Alongside research, James was also involved in lecturing on sequencing technology and genomic architecture as part of bachelor's and master's programs, developing teaching lab protocols that explore genetic linkage, and supervising a total of twenty bachelor's and master's internship students.

After completing his PhD thesis, James hopes to continue his research into evolutionary mysteries with the genome of another European amphibian, the Edible Frog (*Pelophylax kl. Esculentus*), and its bizarre mode of reproduction, known as hybridogenesis.

List of Publications

Published

J. Mars, S. Koster, W. Babik, **J. France**, K. Kalaentzis, C. Kazilas, I. Martínez-Solano, M. C. de Visser, B. Wielstra, Phylogenomics yields new systematic and taxonomical insights for *Lissotriton* newts, a genus with a strong legacy of introgressive hybridization. *Molecular Phylogenetics and Evolution* **204**, 108282 (2025).

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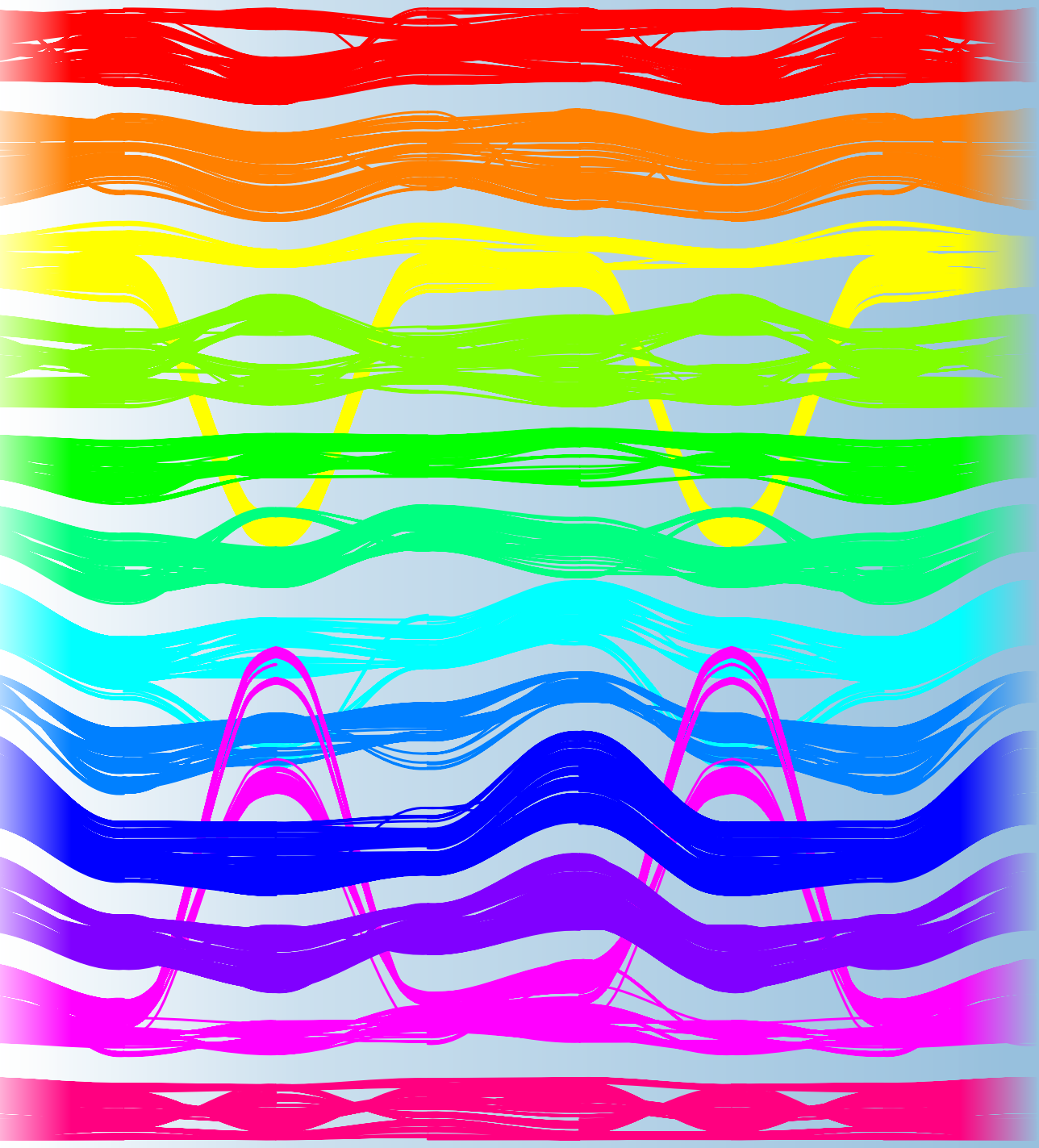

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