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Comparative genomics of the balanced lethal system in Triturus newts

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Citation
France, J. M. (2025, April 3). *Comparative genomics of the balanced lethal system in Triturus newts*. Retrieved from https://hdl.handle.net/1887/4210100

Version:	Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/4210100

Note: To cite this publication please use the final published version (if applicable).

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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