Hybrid zone dynamics in amphibians

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Discussion

This thesis focusses on hybrid zone dynamics. I will highlight important results that apply specifically to the genera studied. Using the effective selection against hybrids to compare the barrier effect between taxa is a promising new lead. The shape of a peak of D’ may provide a further avenue to investigate the underlying mechanisms of asymmetric introgression in dynamic hybrid zones. Future perspectives of research on dynamic hybrid zones are discussed.

Specific findings

In Chapter 1 I show that the banded newts (*Ommatotriton*) represents three distinct species, rather than two as in previous treatments. A phylogenetic tree based on mitochondrial DNA reveals high divergence between the species and they do not share nuclear DNA genotypes. As the morphology of *O. nesterovi* and *O. ophryticus* is highly similar, they were considered cryptic species. However, building on the information from Chapter 1, a recent publication supports the species status of *O. nesterovi* and *O. ophryticus* by subtle divergence in phenotype (Uzum et al., 2019).

In Chapter 2, hybrid offspring of *O. nesterovi* and *O. ophryticus* were found in Spain. All Spanish individuals possessed mitochondrial haplotypes of *O. ophryticus*, which was in line with the identification of one introduced Spanish individual as *O. ophryticus* based on the number of rib-bearing vertebrae (Fontelles, Guixé, Martínez--Silvestre, Soler, & Villero, 2011). However, the nuclear markers showed all individuals contain both *O. nesterovi* and *O. ophryticus* haplotypes. Hybrid offspring is able to successfully reproduce as eleven out of twelve individuals from Spain were not first generation offspring. The geographic origin of the *O. ophryticus* mitochondrial haplotypes, which occur 400 km eastward from the species boundary. This implies that the hybridisation has not happened in a natural population, but must have taken place during or after captivity.

Finding hybrid offspring was surprising, because we found no admixture in the natural range of *Ommatotriton* (Chapter 1). We could narrow down the area of an expected hybrid zone to a 60 km gap (Fig. 6.1a). The possibility remains that low levels of introgression near this gap remained undetected, as only two nuclear markers were used. Two other newt genera with distributions similar to that of *Ommatotriton,* however, have shown considerable movement of up to 600 km (Nadachowska & Babik, 2009; Wielstra et al., 2017). If there is a hybrid zone present between *O. nesterovi* and *O. ophryticus*, selection against hybrids may be very high, or the hybrid zone may be trapped in an area with low population density (Endler, 1977; Barton & Hewitt, 1985). Therefore I developed a set of ~ 200 nuclear markers based on transcriptome data (data unpublished).

In Chapter 3 I address the hypothesis of movement in a common toad hybrid zone by employing 31 nuclear gene based markers. Introgression from the southern *B. spinosus* into *B. bufo* is not significantly asymmetric, and the peak of admixture linkage disequilibrium appears to coincide with the larger portion of introgression. If this pattern was significant, it would rather point to asymmetric reproductive isolation, with hybridisation more successful north of the hybrid zone than south of the hybrid

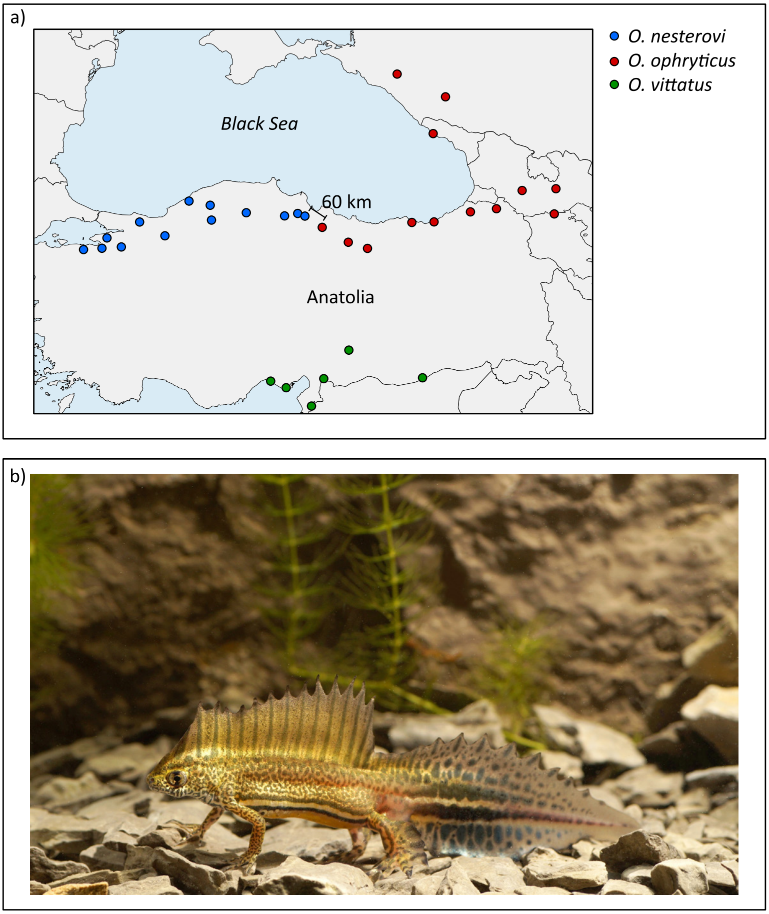

Figure 6.1: Map (a) showing northern part of the *Ommatotriton* distribution, with the 60 km gap between *O. nesterovi* and *O. ophryticus.* (b) Photograph of an *O. ophryticus* male in breeding season from Trabzon (Turkey). Photograph kindly provided by Michael Fahrbach.

zone. In Chapter 3 I propose an alternative explanation of low population sizes and slow dispersal, which would have increased genetic drift and obscured patterns of asymmetry.

The data for Chapter 3 is gene-coding, which may be more prone to selective pressures. In addition, the length of the *Bufo* hybrid zone makes it ideal to draw multiple transects. In Chapter 4 a replicate transect was thus included to test consistency of barrier genes (genes restricting introgression) and patterns of introgression and admixture linkage disequilibrium using a RAD dataset. I show asymmetric introgression in northwest France, with higher levels of introgression and admixture linkage disequilibrium coincident at the northern side of the hybrid zone, following predictions of asymmetric reproductive isolation. This strengthens the non-significant patterns found in Chapter 3. Introgression is symmetric in southeast France, and a peak of admixture linkage disequilibrium is found in the centre of the hybrid zone, following the prediction of a stable hybrid zone. The number of genomic outliers suggestive of heterozygote advantage and disadvantage is about half in

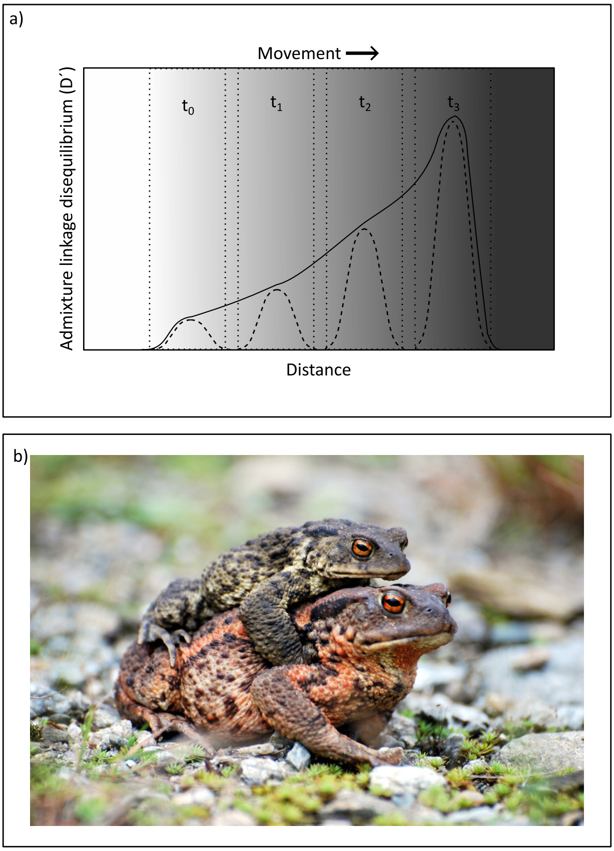

Figure 6.2: Hypothesis (a) for the shape of a peak of admixture linkage disequilibrium (D'; y-axis) under hybrid zone movement, with the position of the hybrid zone centre at four different time points (starting at t0 and ending at t3) indicated between dotted lines, distance on the x-axis. The peaks of D' under a stable situation, are expected to quickly disappear when migration no longer adds unadmixed individuals to the location. These peaks are drawn as dashed lines. The peak of D' expected during movement at time point t3 is drawn as a solid line. (b) Photograph of a *B. bufo* pair in amplexus with the male on top, from Cerna Sat (Romania). Photograph is kindly provided by Ioan Ghira.

northwest France compared to southeast France, and is in support of a difference in the number or strength of barrier genes between both transects. However, more markers are behaving as barrier markers in both transects than would be expected from random resampling. This suggests at least part of the barrier effect is shared between both transects.

Any explanation of these results should unify asymmetry and symmetry in different transects, explain the difference in the number of barrier markers in both transects, and explain the overlap of outlier markers in both transects. The involvement of divergent genetic groups involved in the hybrid zone may be underlying the differences between the transects. Only one mitochondrial lineage, originating from a refugium from the last glacial maximum in the Balkans, is involved on the *B. bufo* side of the zone in transect one, whilst three different mitochondrial lineages, originating from refugia in Italy, the northern Balkans and the Balkans, are involved in transect two (Fig. 6.2a; Garcia-Porta et al., 2012; Recuero et al., 2012; Arntzen et al., 2017). The lineage of *B. spinosus* involved in the hybrid zone originated from the same Iberian refugium (Garcia-Porta et al., 2012; Recuero et al., 2012; Arntzen et al., 2017). This indicates that a deeper genetic structure can be found on the *B. bufo* side of the hybrid zone.

To explain our results, however, an underlying mechanism is needed. This mechanism might be found in a change in chromosome morphology related to the intraspecific variation in *B. bufo*. In northwest France, *B. bufo* with presumably homomorphic sex chromosomes are meeting with *B. spinosus* with a heteromorphic sex chromosome in females (Birstein & Mazin, 1982; Pisanets et al., 2009; Skorinov et al., 2018). In southeast France, *B. bufo* with presumably heteromorphic sex chromosome in males are meeting *B. spinosus* with a heteromorphic sex chromosome in females (Morescalchi, 1964; Skorinov et al., 2018). These different sex chromosome systems explain the different introgression patterns in the two transects, and a lower amount of barrier markers found in northwest France than in southeast France. Because the same chromosome is involved in all interactions, this would also explain the overlap between the barrier markers found in both transects.

The spatial variation in introgression, potentially related to intraspecific variation in sex-determination system, shows that the common toad hybrid zone is more complex than previously appreciated.

The role of sex chromosomes in Anuran speciation

A pair of chromosomes with a difference in morphology, are usually the sex chromosomes. Homomorphy of sex chromosomes and changes of sex chromosome systems (when the heterozygosity of sex chromosomes switches from male to female or vice versa), are well studied in amphibians. In XY systems where males are the heterogeneous sex, the genes causing ‘maleness’ are situated on the Y chromosome. Recombination between Y and X chromosomes are predicted to become suppressed. Genes on the Y chromosome that are not related to male development will be lost (Mueller’s ratchet), and the Y chromosome will degenerate, making its’ morphology visibly different from the X chromosome. Homomorphic sex chromosomes, such as is the case in *B. bufo* in northwest France, are thought to be maintained by recombination in phenotypic females with a male sex chromosome combination (phenotypic female with both X and Y chromosomes) which generates new Y haplotypes and thus prevents evolutionary decay of the Y chromosome (Perrin, 2009).

Changes in sex chromosome system occur exceptionally often in Anurans, even within genera (Hillis & Green, 1990; Uno et al., 2008; Stöck et al., 2011; Jeffries et al., 2018; Skorinov et al., 2018). In *Rana temporaria*, it has been shown that, indeed, recombination between sex chromosomes is not dependent on the genetic sex of an individual, but on the phenotypic sex (Rodrigues, Studer, Dufresnes, & Perrin, 2018). In the *Bufo viridis* subgroup recombination between sex chromosomes is also linked to sex chromosome homomorphy (Stöck et al., 2013). In the Eurasian Hyla radiation, homomorphy of the sex chromosomes was driven by both recombination between the sex chromosomes and by sex chromosome turnover (Dufresnes et al., 2015).

The possibility of a chromosome turnover still needs to be verified for the *Bufo* hybrid zone. Having both homomorphic and heteromorphic sex chromosome systems within the same species complex may make *Bufo* a great system to study the role of sex chromosomes in Anuran speciation.

Effective selection against heterozygotes

The average effective selection against hybrids on a locus (s*) can be calculated using a few markers. However, since the introduction of s* in 1993 by Barton and Gale, the amount of genetic data available greatly increased, which would make the estimate more precise. I developed a script to calculate s* for many markers in a reproducible manner. Chapter 3 compares s* from hybrid zones in various taxa (Table 6.1). When s* is lower, it appears individual clines are more often displaced. This makes sense as with the alleviation of selection against hybrids, clines are free to move away from the hybrid zone centre. In hybrid zones where drift causes variation in the position of the individual (narrow) clines by locally driving alleles to fixation, such as I suggest may be the case in the *Bufo* hybrid zone, the cline of the expected frequency becomes wider (Polechová & Barton, 2011). This means that s* will, in general, be underestimated in hybrid zones where the effect of drift is high.

On the other hand, Table 6.1 shows that when the amount of markers used for the calculations increases, s* appears to decrease. This may be because using more markers makes the estimate more precise. It was possible to compare the effective selection against heterozygotes estimated using both the 32 nuclear marker dataset from Chapter 3 and the 1,189 nuclear marker dataset from Chapter 4 for the transect in northwest France. We show that the estimated s* is similar when expanding the dataset from 31 loci to 1,189 loci. For the *Bufo* system, apparently, tens of loci already capture the variability well enough to estimate s*. However, the confidence interval is narrower with 1,189 loci than with 31 loci.

To test if increasing amounts of markers decreases s*, larger datasets can be generated for earlier published hybrid zones with high estimates of s*. Such datasets probably do not need to contain more than a hundred markers.

Table 6.1: Estimated effective selection against heterozygotes (confidence intervals for s*). The table is ordered from low to high s*, and a line is drawn between low estimates of s* in studies which also report displaced clines, and high estimates of s* reporting only coincident clines.

Comon name	Estimated s*	Displaced	No. loci	Citation
Bufo bufo and *B. spinosus*	0.0001-0.004	Yes	31	van Riemsdijk, et al. (2019)
Bufo bufo and *B. spinosus*	0.001-0.003	Yes	1,189	van Riemsdijk, et al. in prep.
Malurus melanocephalus subsp.	0.002-0.03	Yes	103	Baldassarre, et al. (2014)
Two ecotypes of *Littorina saxatilis*	0.005-0.32	Yes	57	Hollander, et al. (2015)
Triturus anatolicus & *T. ivanbureschi*	0.004-0.019	Yes	49	Wielstra, et al. (2017)
Vandiemenella viatica sp.	0.058-0.405	No	11	Kawakami, et al. (2009)
Two lineages of *Carlia rubrigularis*	0.22-0.49	No	8	Phillips, et al. (2001)
Heliconius melpomene & *H. erato*	0.23	No	4	Mallet, et al. (1990)
Mus m. musculus & *M. m. domesticus*	0.28-0.48	No	7	Macholán, et al. (2007)
Bombina bombina & *B. variegata*	0.15-0.58	No	6	Szymura & Barton (1991)
Pontia daplidice & *P. edusa*	0.47-0.64	No	17	Porter, et al. (1997)
Oryctolagus c. cuniculus & *O. c. algirus*	0.5-0.64	No	17	Carneiro, et al. (2013)

Asymmetric introgression & admixture linkage disequilibrium

Alleles originating from the same parent species are found together within the genomes of hybrid offspring, causing admixture linkage disequilibrium. The benefit of plotting the admixture linkage disequilibrium (D') is that it shows where most recently fresh genetic material from one of the parent species, which has not had the chance to recombine during backcrossing, has come into the hybrid zone. This peak is hypothesised to be shifted ahead of the hybrid zone movement, to the opposite side of the trail of introgression (Gay, Crochet, Bell, & Lenormand, 2008; Wang et al., 2011). During asymmetric reproductive isolation the peak is hypothesised to be more coincident with the tail of introgression (Devitt, Baird, & Moritz, 2011). However, the distance a peak of D' can move within the hybrid zone centre is per definition restrained to the area in which introgression occurs. If the hybrid zone is a stepped cline, this distance becomes rather small, and the highest D' may be coincident with the place where the expected frequency cline is most steep, regardless of what process occurs.

In a neutral situation, the peak of D' is expected to follow a Gaussian curve. What shape would be expected during movement or asymmetric reproductive isolation is not clear yet. In a hybrid zone between two gull species, it appears however that the peak of D' is not following a Gaussian curve, but rather a Gaussian curve with a tail, a type of β distribution (Gay et al., 2008; Fig. 6.2). The further the hybrid zone has moved away from its original position, the longer recombination has taken place to decrease the effect of D', and the lower the local D' will be. In both transects from Chapter 4, the shape of the peak of D' is not very well described by a Gaussian curve, either. I would argue the shape of the peak under asymmetric reproductive isolation could be highly dependent on where the clines of the barrier genes are located. As barrier genes are causing the incompatibility between the species, the place where the cline centres of the barrier genes coincide is the place where most likely highest D' will be measured. What will happen to the shape of the peak of D' during hybrid zone movement caused by asymmetric reproductive isolation, is not clear yet either.

The concept of a peak of D' was more nuanced in the paper by Gay et al. (2008), as they described an excess of intermediates in the tail of the hybrid zone movement and a deficit of intermediates ahead of the hybrid zone movement. The two gull species have different morphologies and intermediates were defined by the morphology in the hybrid populations. The observation supporting this concept was (1) that an excess of individuals with intermediate morphology were found in the tail of the movement, and (2) a deficit of intermediates was found at the leading edge of the hybrid zone where only individuals with a morphology of either species (and no individuals of hybrid morphology were present) were found. In absence of morphological differences, other measures are available to assess both excess and deficit of intermediates, besides the measure of D' we used for the *Bufo* hybrid zone. For example, the squared correlation of alleles at two sites (r^2) could be used, which shows less inflation than D' in small samples (Weir, 1996; Ardlie, Kruglyak, & Seielstad, 2002; Weiss & Clark, 2002; Wang et al., 2011).

The shape of the peak of D' may be indicative of different processes, but to hypothesise without further evidence would be pure conjecture. Extensive simulations may provide a first step on the way to be able to clearly outline the expectations of a peak of D’ under different scenarios.

Future perspectives

This thesis contributed to our knowledge of the *Ommatotriton* and *Bufo* systems. Many new questions have arisen for both these systems, and for the study of dynamic hybrid zones in general.

For *Ommatotriton*, the question remains if there is introgression between *O. nesterovi* and *O. ophryticus*. More populations may be sampled in the 60 km gap between the two species distributions, and I developed a set of ~ 200 nuclear markers based on transcriptome data to asses introgression (data unpublished).

The existence of intraspecific *B. bufo* groups can be studied using many nuclear markers. A possible setup would be to use sequence capture or RAD sequencing in samples from different sections of the hybrid zone and include samples covering the wider distribution of *B. bufo* to study nuclear phylogeographic patterns. This allows to determine the genetic groups involved in the hybrid zone first, and then determine diagnostic markers based on reference populations. After establishing which genetic groups are involved in the hybrid zone, one can also make an informed decision about the setup of a chromosome study by karyotype analyses, linkage map, or genome sequencing. Crossings within and between *B. bufo* groups and *B. spinosus* can be used to investigate mechanisms involved in genetic sex determination and patterns of reproductive isolation.

The expectations about the shape of a peak of D’ in a dynamic hybrid zone remain speculative. Two lines of investigation can be set out to determine the shape of this peak in more detail. The first is to simulate different scenarios of hybrid zone movement and asymmetric reproductive isolation and test for different variables, such as selection against hybrids, dispersal, and recombination rate. The other is to gather some published datasets, which show asymmetric introgression and for which good additional evidence for the cause of the asymmetry is available. Both simulations and published datasets can further inform expectations of the shape of the peak of D’, or expectations of other measures such as r^2.

Instead of estimating measures of linkage disequilibrium it is possible to directly infer linkage of markers if reliable genome resources such as dense linkage maps or genome assemblies are available. Chromosome or ancestry blocks are stretches of chromosomes which can be assigned to the parent species and reflect inheritance patterns (Fisher, 1954; Baird, Barton, & Etheridge, 2003; Luikart, England, Tallmon, Jordan, & Taberlet, 2003; Wall & Pritchard, 2003; Gravel, 2012; Harris & Nielsen, 2013; Hellenthal et al., 2014; Sedghifar, Brandvain, Ralph, & Coop, 2015; Sedghifar, Brandvain, & Ralph, 2016). A junction, the border of two chromosome blocks, is a break in a chromosome caused by a recombination event, and is inherited as a point mutation (Fisher, 1954; Baird, 2006). The more generations of backcrossing occur, the more recombination has taken place, the more junctions will be present, and the smaller the genome blocks will become. The size of chromosome blocks can thus be used to investigate the amount of time passed since hybridisation first occurred (Baird, 1995; Chapman & Thompson, 2002; Pool & Nielsen, 2009).

Where chromosomes contain barrier genes, such as heterozygote disadvantage or Dobzhansky-Muller incompatibilities, chromosome blocks are predicted to be long and junctions few (Barton, 1983; Sedghifar et al., 2016; Hvala, Frayer, & Payseur, 2018). For example, a linkage map based on a single cross of two divergent lineages of *Rana temporaria* showed low recombination rate linked to genetic incompatibilities between the parental populations (Palomar et al., 2016). When a hybrid zone has moved, longer chromosome blocks can be found at the leading edge (Wang et al., 2011; Seixas, Boursot, & Melo-Ferreira, 2018).

These chromosome block studies all have one thing in common; the availability of genomic resources. During the last years, sequencing methods have become cheaper, more advanced, and more reliable. It is now possible to complete a full-scale genomic analysis within a PhD project for many non-model organisms (Matz, 2017). Amphibian genomes vary widely in size and contain many repetitive regions, which has made it more difficult to accumulate genomic resources for amphibians (Liedtke, Gower, Wilkinson, & Gomez-Mestre, 2018; Nowoshilow et al., 2018). Long reads (> 10,000 base pairs [bp]) can be used to bridge repetitive regions, and reliably assemble such large genomes.

Two well-known methods are available for sequencing long reads; Pacific Biosciences (PacBio), and Oxford Nanopore Technologies (ONT) platforms (Matz, 2017). The accuracy for PacBio has improved from 82% to 87%, and maximum read length is 50,000 bp (Koren and Phillippy, 2015, and references therein; Watson and Warr, 2019). The accuracy of ONT has improved from 70-80% to over 85%, and maximum read length is up to 800,000 bp (Quick, Quinlan, & Loman, 2014; Risse et al., 2015; Michael et al., 2018; Watson & Warr, 2019). For chromosome block analysis it is not necessary to have a highly accurate reference genome. As polymorphism in non-model populations is usually higher than 1%, the reference based on a single individual is inaccurate with respect to the genomes of the rest of the population, no matter how accurate the reference genome is (Matz, 2017; Koren, Phillippy, Simpson, Loman, & Loose, 2019).

At the start of 2019, five genome assemblies of considerable quality have been published for amphibians (Table 6.2). The *Xenopus tropicalis* genome is the most complete assembly available for amphibians. The genome was originally sequenced

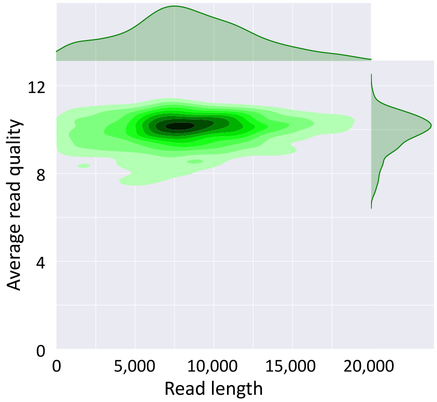

Figure 6.3: Plot for average read quality (phred) and read length (bp) of the raw reads obtained from a single MinION flow cell run for liver tissue of *B. bufo*.

Table 6.2: Amphibian genome assemblies available in February 2019, ordered by scaffold N50. The N50 is a measure of assembly quality, and represents the minimum contig length of the contigs covering half the total genome assembly length.

Species (Genome size)	**Scaffold N50 (kbp)**	**Reference**
Xenopus tropicalis (1.4 Gbp)	135,000	Hellsten et al. (2010)
Ambystoma mexicanum (32 Gbp)	3,000	Nowoshilow et al. (2018)
Nanorana parkeri (2.3 Gbp)	1,050	Sun et al. (2015)
Rhinella marina (2.5 Gbp)	168	Edwards et al. (2018)
Rana catesbeiana (6 Gbp)	69	Hammond et al. (2017)

with short read sequences and transcriptome data, and has been updated and improved on since publication (Table 6.2: Hellsten et al., 2010; Vize and Zorn, 2016). The *Nanorana parkeri* and *Rana catesbeiana* genomes have, too, been sequenced with short reads and transcriptome data but are much less complete than the *Xenopus assembly* (Sun et al., 2015; Hammond et al., 2017). For the *Ambystoma mexicanum* and *Rhinella marina* genome assemblies, PacBio long read sequencing and short read sequencing or transcriptome data were used. This resulted in more complete assemblies than for *N. parkeri* and *R. catesbeiana*, but the assemblies are still nowhere near the quality of the *Xenopus* assembly (Edwards et al., 2018; Nowoshilow et al., 2018).

The use of ONT sequencing for amphibian genomes has not yet been explored, whilst its reads are of similar quality and may be much longer than PacBio reads. I tested whether ONT could be applied to assemble the *B. bufo* genome, and successfully sequenced high quality reads (unpublished data; Fig. 6.3). The application of long read sequencing to amphibian study systems has great potential for future evolutionary research.

Conclusion

The three species in the genus of *Ommatotriton* have split a long time ago in a relatively short time span. If introgression occurs between *O. nesterovi* and *O. ophryticus* in their natural range, it is geographically limited. Hybridisation between *O. nesterovi* and *O. ophryticus* can occur, and I narrowed down an area in the natural distribution where introgression between the two species could take place. I have developed genetic tools for detailed analysis of genetic introgression.

Previously identified asymmetries in introgression along the transect of *Bufo bufo* and *B. spinosus* were confirmed for northwest France, but in southeast France the zone appears to be symmetric. There appears to be asymmetric reproductive isolation between the two *Bufo* species, and a difference in reproductive isolation with *B. spinosus* for different genetic groups within *B. bufo*. It appears the variation in introgression is linked to differences in chromosome morphology.

The availability of the two datasets for the *B. bufo* hybrid zone allowed to compare the estimated effective selection against hybrids (s*) based on 31 and 1,189 markers. From this, it appears datasets of no more than a hundred markers may already enable stable estimations of s*. With the availability of a script to estimate s* for larger datasets, it is possible to reproducibly estimate s* and compare the barrier effect within or between different taxonomic groups.

The position of a peak of admixture linkage disequilibrium (D') has been useful in studying hybrid zones, but many questions remain about what is expected for the shape and position under different scenarios of hybrid zone movement and asymmetric reproductive isolation. The shape of the peak under hybrid zone movement may be more like a peak with a tail than a Gaussian curve. Where possible, a more nuanced hypothesis of a heterozygote excess and deficit may be more informative for hybrid zones involving movement. By using simulations and previous datasets, expectations of the shape of the peak and behaviour of different statistics needs to further be tested.

With the increasing availability of genome resources, a chromosome block approach will be applicable to many more non-model systems, and will allow detection of more detailed patterns of differential introgression along the genome. This will aid in the identification of barrier genes and the causes underlying asymmetries in hybrid zones. I anticipate that researchers will be able to answer many of the open questions about amphibian evolution with the aid of long read sequencing.

We have surpassed the point where hybrid zones are thought to be in a stable situation by default.

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