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On mutations, recombination, and sexual antagonism in songbirds

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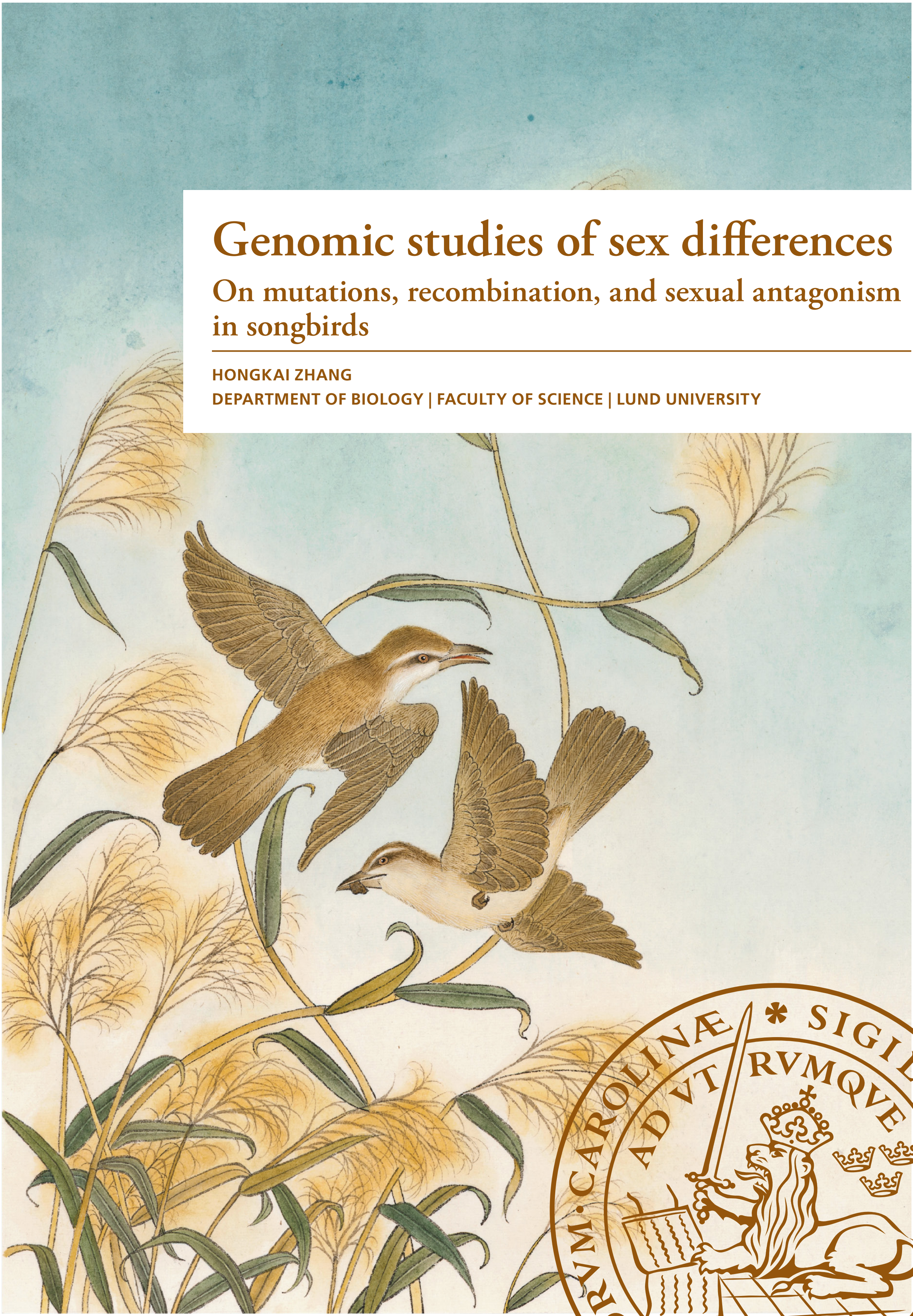
DEPARTMENT OF BIOLOGY | FACULTY OF SCIENCE | LUND UNIVERSITY



Genomic studies of sex differences
On mutations, recombination, and sexual antagonism in songbirds

Hongkai Zhang

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Science at Lund University to be publicly defended on 24th of February at 09.30 in Blue Hall, Ecology Building, Sölvegatan 37, Lund, Sweden

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In this thesis, I used genomic approaches to study sex differences in a songbird, the great reed warbler (*Acrocephalus arundinaceus*), in which male and females are monochromatic and genetically determined by a pair of sex chromosomes. The thesis starts with a study presenting and evaluating two alternative phylogenetic approaches (the expected likelihood weight (ELW) and the BEAST approach) to determine when different parts of the sex chromosomes stop recombining. My findings highlight the benefits of using fixed topologies to estimate the timing of recombination cessation as done by these approaches. Thereafter, I focus on molecular sex differences using genomic and bioinformatic methods to specifically investigate sex biases in *de novo* mutations and in recombination patterns, and search for sexually antagonistic loci in the genome.

By using whole genome sequencing data from a three-generation pedigree of the great reed warbler, I found a strong sex bias in the numbers of *de novo* mutations, with males having three times as many mutations as females. Regarding recombination, I found no statistical support for sex-specific recombination rates, but the recombination landscape differed between sexes, with males having more crossovers towards the chromosome ends compared to females. Besides, I developed an interactive R application, *RecView* ShinyApp, to implement the methodology of locating recombination for future studies within similar topic.

Finally, I used statistical approaches based on allele frequency differences and associations with sex *per se* to search for sexually antagonistic loci with whole genome sequencing data from 100 old great reed warblers that aged between 3 and 5 years. By comparing the top 100 SNPs with the strongest allelic differentiation between the sexes, and the most significant associations with sex, I discovered 50 overlapping SNPs that constitute candidates for future studies of sexual antagonistic selection.

To conclude, this thesis has improved the methodology for studying the timing of recombination cessation on sex chromosomes as well as to study recombination *per se*, identified sex-specific *de novo* mutation rates and sexually dimorphic recombination landscapes, and obtained candidate loci for sexual antagonism.

Key words: sex differences, mutation, recombination, sexual conflict, sexual antagonism, sexually antagonistic selection, Great reed warbler, *Acrocephalus arundinaceus*

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献给我敬爱的爷爷奶奶，外公外婆

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I. **Hongkai Zhang,** Hanna Sigeman, Bengt Hansson. 2022. Assessment of phylogenetic approaches to study the timing of recombination cessation on sex chromosomes. *Journal of Evolutionary Biology,* 35, pp.1721-1733, doi: 10.1111/jeb.14068.

II. **Hongkai Zhang,** Max Lundberg, Maja Tarka, Dennis Hasselquist, Bengt Hansson. 2022. Male-driven mutations: a pedigree-based study in the great reed warbler. Manuscript.

III. **Hongkai Zhang**, Bengt Hansson. 2022. RecView: an interactive R application for viewing and locating recombination positions using pedigree data. *bioRxiv*, doi: 10.1101/2022.12.21.521365.

IV. **Hongkai Zhang,** Max Lundberg, Dennis Hasselquist, Bengt Hansson. 2022. A genomic analysis of a great reed warbler pedigree reveals sex-specific recombination landscapes. Manuscript.

V. **Hongkai Zhang**, Max Lundberg, Maja Tarka, Dennis Hasselquist, Bengt Hansson. 2022. The genetics of sex differences: searching for signs of sexual antagonism in the genome of the great reed warbler. Manuscript.

In addition, I am co-author for the following papers published during my PhD. These papers are **NOT** included in this thesis.

VI. Sigeman, H., Ponnikas, S., Videvall, E., **Zhang, H.**, Chauhan, P., Naurin, S. and Hansson, B., 2018. Insights into avian incomplete dosage compensation: sex-biased gene expression coevolves with sex chromosome degeneration in the common whitethroat. *Genes*, 9(8), p.373.

VII. Sigeman, H., Strandh, M., Proux-Wéra, E., Kutschera, V.E., Ponnikas, S., **Zhang, H.**, Lundberg, M., Soler, L., Bunikis, I., Tarka, M., Hasselquist, D., Nystedt, B., Westerdahl, H. and Hansson, B., 2021. Avian neo-sex chromosomes reveal dynamics of recombination suppression and W degeneration. *Molecular Biology and Evolution*, 38(12), pp.5275-5291, doi: 10.1093/molbev/msab277.

VIII. Sigeman, H., **Zhang, H.**, Ali Abed, S. and Hansson, B., 2022. A novel neo-sex chromosome in *Sylvietta brachyura* (Macrosphenidae) adds to the extraordinary avian sex chromosome diversity among Sylvioidea songbirds. *Journal of Evolutionary Biology*, 35, pp.1797-1805, doi: 10.1111/jeb.14096.

Author contributions

I. H.Z. and B.H. conceptualized the study. H.Z. conducted the analyses with input from B.H. H.S. generated the dataset. H.Z. and B.H. wrote the manuscript with input from H.S. All authors reviewed and approved the final manuscript.

II. H.Z., D.H. and B.H. designed and planned the study. H.Z. performed the laboratory work of preparing genomic DNA. H.Z. performed the bioinformatic analyses with input from M.L and B.H. H.Z. and B.H. wrote the manuscript with input from M.L., M.T. and D.H. All authors reviewed and approved the final manuscript.

III. H.Z. and B.H. conceptualized the study. H.Z. developed the software with input from B.H. H.Z. performed the bioinformatic analyses. H.Z. and B.H. wrote the manuscript.

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Authors:

Hongkai Zhang (H.Z.), Bengt Hansson (B.H.), Dennis Hasselquist (D.H.), Hanna Sigeman (H.S.), Max Lundberg (M.L.), Maja Tarka (M.T.).

Abstract

Many organisms have separate sexes, *i.e.*, males and females. The presence of separate sexes causes sex-specific selection regimes and sexual antagonism, which can lead to sex differences in morphology, physiology, and behaviours. Sex and sex differences can be genetically governed and regulated by a pair of sex chromosomes (*e.g.*, X and Y, or Z and W), on which there often are regions without recombination.

In this thesis, I used genomic approaches to study sex differences in a songbird, the great reed warbler (*Acrocephalus arundinaceus*), in which male and females are monochromatic and genetically determined by a pair of sex chromosomes. The thesis starts with a study presenting and evaluating two alternative phylogenetic approaches (the expected likelihood weight (ELW) and the BEAST approach) to determine when different parts of the sex chromosomes stop recombining. My findings highlight the benefits of using fixed topologies to estimate the timing of recombination cessation as done by these approaches. Thereafter, I focus on molecular sex differences using genomic and bioinformatic methods to specifically investigate sex biases in *de novo* mutations and in recombination patterns, and search for sexually antagonistic loci in the genome.

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Popular summary

Have you ever noticed that some peacock individuals have exaggerated long and decorated tail feathers while some other individuals don't? Have you ever noticed some lions, usually one in a pride, have conspicuous mane around their necks while others don't? If you find these cases easy, have you ever noticed if there are differences between the individuals of hares, or zebra finches? These examples show that the differences we observe between males and females vary among different species of animals, and we use the term sexual dimorphism, which literally specifies that males and females have two morphs, to quantify these sex differences. However, while it sometimes is very easy to define two subgroups of individuals as males and females based on their morphology, it is sometimes very difficult. In such cryptic situations, it is instead often possible to define males and females based on how they behave during reproduction or on the gametes they produce, for example, sperms and eggs.

We can also notice that the physical differences between males and females are quite consistently present in the next generation and even the next generation after the next, because these features are encoded in the DNA, the genes. And these genes can affect the males and females along their development, so males and females start as two similar embryos but gradually they develop and mature to become different. Studying the differences between males and females are not only useful for knowing and understanding the life in nature, but also useful for ourselves humans. For example, investigating the sex differences in susceptibility and response in diseases may help us perform different treatments suitable for each sex.

The twisted-ladder structure of DNA, the double-helix, was described in 1953. This discovery unfolded a new chapter of molecular biology, the use of DNA as studying material. The technology of getting DNA sequences took off with the Sanger sequencing method in 1977, but at that time obtaining even a few hundred base pairs of DNA was expensive and difficult to automate. Until now, next-generation sequencing technology has replaced Sanger sequencing method, because it increases the automation of sequencing procedure, it is able to generate DNA sequences that altogether cover the whole genome, and more importantly, the cost is much reduced.

The world has a vast diversity of organisms, thanks to two fundamental processes, mutation and recombination. Mutation describes the change of DNA sequences and if such changes happen inside genes, they may cause physical changes to the individual. Recombination describes the swap of DNA between one chromosome inherited from the father and the other one inherited from the mother. Therefore, the DNA in the offspring is mosaic with some gene copies from father while some other from mother. This new combination of gene copies may cause physical changes to the offspring in a way that the offspring does not look exactly the same as its father or mother. With the presence of separate sexes, these changes, for example in father, can be passed down to his daughter and next to his grandson. Then, some questions can be raised: Will males and females have different patterns in mutation and recombination? What are the outcomes for these DNA changes after they experience differentiated selection in males and females? How can we study these outcomes?

To answer these questions, I used the great reed warbler as a study species. This species is relatively common in parts of Sweden, for example, at Lake Kvismaren close to the city Örebro. Every year during the last 40 years, this population has been studied daily during the breeding season and thousands of blood samples and biological data have been collected, which provides a comprehensive database for my thesis. I used next-generation sequencing technology to obtain the DNA sequences data for the bird's genome, and I analysed the mutation rate, and the recombination positions and rates, within a great reed warbler family consisting of grandparents, parents, and the offspring born in 1998. I found that males had more mutations compared to females, which can be explained by sperms going through more cell divisions during their development than eggs (more cell divisions mean more chance for mutations to happen). I did not find that male and female great reed warblers differed in recombination rate, but there were more recombination events close to the ends of the chromosomes in males. Moreover, by using sequencing data from another 100 great reed warblers, I found traces in the genome indicating that males and females had been affected differently by selection.

Taken together, I learned that even within a single species, the great reed warbler, the sexes can differ in subtle ways like how often mutations happen and in the pattern of recombination. These are both very important processes that generate genetic variation that selection can act upon. What about other species? I'll leave you to find out!

1 Background

1.1 How is sex evolved?

As described by Bell (1982), “Sex is the queen of problems in evolutionary biology. Perhaps no other natural phenomenon has aroused so much interest among evolutionary biologists; certainly, none has sowed as much confusion.” Defining sex is difficult, especially for a vast diversity of organisms. For mammals (including humans) it may seem natural to use a phenotypic sex definition, where the distinction between the males and females is characterised by their phenotypic differences in internal and external reproductive organs (the traits that are identifiable at birth, the so-called primary sex characteristics), secondary sex characteristics (the traits that are not identifiable at birth, but develop over time as the individuals mature), and behaviours (Purves *et al.*, 2001). However, some ancient lineages of eukaryotes, such as protists, do not have reproductive organs, but still undergo sexual reproduction involving individuals with differentiated roles and gametes (Schärer *et al.*, 2012). Sex can be more clearly defined by anisogamy, a form of sexual reproduction where males produce the small and often motile gametes, and females produce the large and sedentary gametes. Sexual reproduction has not been observed in many taxa on the Tree of Life (Schurko and Logsdon, 2008), but always involves a pivotal step, meiosis, during which germline cells undergo a second round of cell division resulting in sperm or egg cells that are haploid (containing only a single copy of the genome). Therefore, the presence of genes involved in meiosis can be used to verify the presence of sex (Schurko and Logsdon, 2008; Speijer *et al.*, 2015). If we use the presence of meiosis as the defining feature of sex, it is estimated that sex originated 1,500 M.y.a. (Stearns, 2013).

Why and how sex evolves and why it has persisted within so many diverse lineages is paradoxical. Some suggestions for benefits of sexual reproduction have been proposed, *e.g.*, that sexual reproduction increases the ability to adapt in a changing environment due to the positive effects of meiotic recombination, because it reshuffles alleles and generates the genetic variation for selection to act on (Barton and Charlesworth, 1998; Felsenstein, 1974; Otto, 2021). However, there are also costs related to sexual reproduction. For example, sex comes with an unavoidable cost originating from producing males that only share a part of the genome with their mothers, compared to asexual offspring which inherit the whole genome from their mothers. Moreover, there are other costs such as development of specialised reproductive organs, investment in mate searching, risk of higher exposure to predation, and disease transmission during mating (Smith, 1978; Stearns, 2013).

1.2 Anatomical and physiological sex differences

The primary phase of sexual development results in reproductive systems of very distinct morphologies and differentiated functions between males and females. Male and female reproductive systems can exist in the same individual, as exemplified by most plant species, for which several terms were coined, from monoecy describing male and female reproductive organs on different flowers (unisexual flowers) in the same individual, to hermaphrodite describing male and female reproductive organs on the same flower on the same individual. A small proportion (*ca.* 6%) of angiosperm plant species are dioecious, so male and female reproductive organs grow on separate individuals (Renner and Ricklefs, 1995), for example, willow (*Salix* spp.), cannabis (*Cannabis* spp.) and ginkgo (*Ginkgo* spp.). Hermaphroditism in animals has been reported in about one-third of species, when insects are excluded (Jarne and Auld, 2006). In vertebrates, almost all species have individual males and females with a few exceptions, *e.g.*, the mangrove killifish (*Rivulus marmoratus*) where hermaphrodites and males exist (Mackiewicz *et al.*, 2006), and female bluehead wrasse (*Thalassoma bifasciatum*) which can be sequential hermaphrodites (Warner and Swearer, 1991).

The next phase of sexual development involves the development of secondary sex characteristics through the influence of sex hormones which are secreted by the gonads, *e.g.*, oestrogen in females and testosterone in males. Sex hormones are also involved in a variety of trade-offs between immunity and sexual behaviours, and thus they can be subject to selection. Compared to mammals, birds and insects seem to have evolved a different and cell-specific mechanism (*i.e.*, cell-autonomous sex identity). For example, studies of chicken and *Drosophila* indicate that the development of secondary sex characteristics is less influenced by the hormonal environment (Bear and Monteiro, 2013; Ioannidis *et al.*, 2021; Janzer and Steinmann-Zwicky, 2001; Zhao *et al.*, 2020).

Sexual development can lead to differentiated morphological traits between males and females. These morphological differences between the sexes are collectively described as sexual dimorphism. Sexual dimorphism can be shaped by sex-specific selection regimes, different mating systems, cryptic female choice, parental care, and plant-pollinator interaction (Okamoto *et al.*, 2013; Owens *et al.*, 1998; Post *et al.*, 1999). Some sexually dimorphic traits are visually obvious, like plumage colouration in some bird species where males often sport colourful plumage and occasionally also exaggerated ornaments, such as the peacock’s train, and elongated feathers in birds-of-paradise. In fact, there are some exceptions to the common pattern of sexual dimorphism among species, such as female phalaropes exhibiting more colourful plumage than the males, and the smaller body size of the males than the females of birds of prey (Falconiformes) and owls (Strigiformes) (Wheeler and Greenwood, 1983).

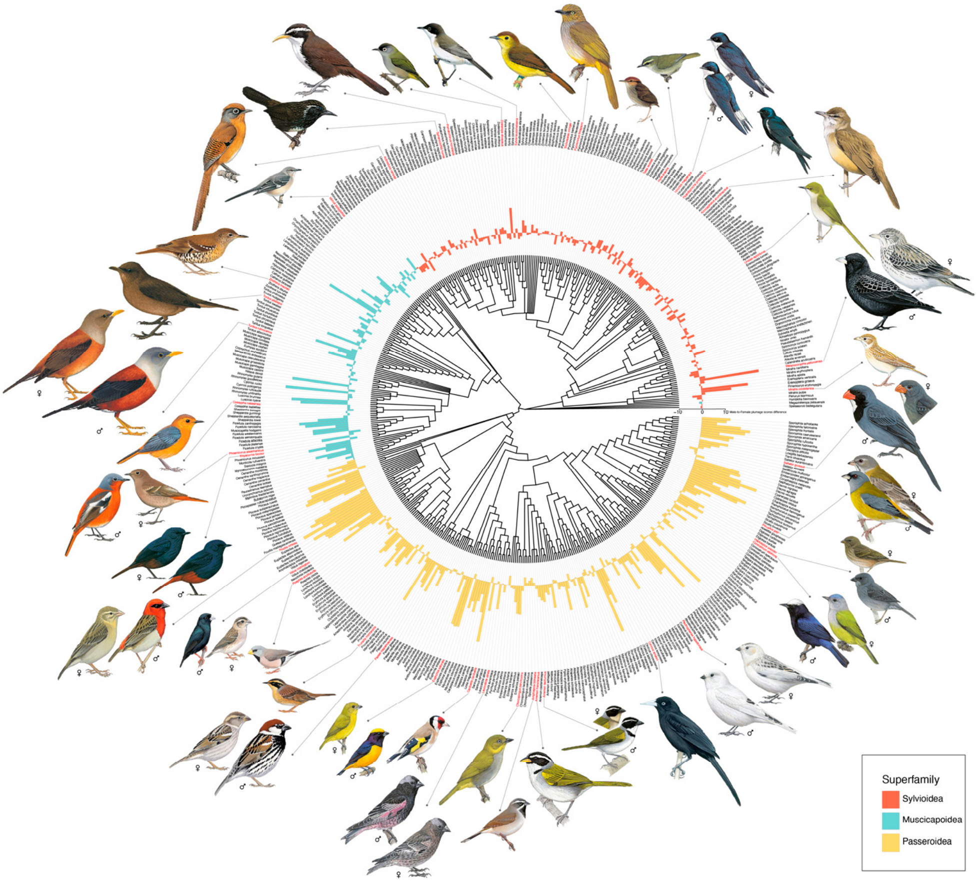


Figure 1. Sexual dimorphism quantified as the male-to-female difference in plumage colouration scores among selected species in Sylvioidea, Muscicapoidea, and Passeroidea superfamilies. Illustrations show monochromatic (*e.g.*, great reed warbler, *A. arundinaceus*), and dichromatic (*e.g.*, Spanish sparrow, *P. hispaniolensis*), males and females of some species. Birds illustrations were from the Handbook of the Birds of the World (del Hoyo and Elliott, 2006).

Sexual dimorphism in plumage colouration becomes more interesting when compared across different avian lineages (**Figure 1**). The Sylvioidea and Passeroidea superfamilies diverged approximately 37 M.y.a. (Oliveros *et al.*, 2019), and the evolution of plumage colouration in the two has had quite different outcomes, with most monochromatic members belonging to the Sylvioidea, such as great reed warbler (*Acrocephalus arundinaceus*), common reed warbler (*A. scirpaceus*), and sedge warbler (*A. schoenobaenus*), while most dichromatic members belong to the Passeroidea, such as Spanish sparrow (*Passer hispaniolensis*), black rosy-finch (*Leucosticte atrata*), and Scarlet Tanager (*Piranga olivacea*). However, monochromatic species could have evolved other sexually dimorphic traits besides plumage colouration, such as wing length, songs, and vocal learning (Hasselquist *et al.*, 1996; Tarka *et al.*, 2014; Yamaguchi, 2001).

1.3 Molecular sex differences

1.3.1 Sex determination

Sex determination refers to the process of differentiation into males or females in sexually reproductive organisms. Traditionally, the sex determination systems are easily categorised into genetic sex determination (GSD; *e.g.*, Y or W chromosomes), and environmental sex determination (ESD; *e.g.*, temperature) (Charlesworth and Mank, 2010). However, GSD and ESD are not mutually exclusive, and can interactively affect a complex network of genes involved in sexual development. Hence, sex determination systems exhibit a continuum of genetic and environmental mechanisms (Gamble and Zarkwer, 2012), including temperature-dependent sex determination (TSD), and susceptibility to hormonal influence (Capel, 2017; Sarre *et al.*, 2004). For example, the Australian bearded dragon (*Pogona vitticeps*) has a ZZ/ZW sex chromosome system where ZW genetically determines the female development, but extreme incubation temperature can override the genetic sex determination and result in sex reversal that ZW individuals develop into males and ZZ individuals develop into females (Holleley *et al.*, 2015). In addition, some intrinsic signals (*e.g.*, depletion of oocytes) can cause sex reversals in some fishes (*e.g.*, zebra fish; *Danio rerio*; Kossack and Draper, 2019).

Although there are various modes of sex determination (Bachtrog *et al.*, 2014), some genes are conserved in regulating the pathways to develop into a male or female. This conservation is exemplified by the members of the Doublesex-Mab-3 Related Transcription factors (*DMRT*) gene family, which are expressed in the gonads of almost all vertebrates and invertebrates (Bopp *et al.*, 2014; Cho *et al.*, 2007; Chue and Smith, 2011; Raymond *et al.*, 2000; Shoemaker *et al.*, 2007; Wexler *et al.*, 2014).

1.3.2 The evolution of sex chromosomes

GSD is governed by the sex-determining genes, which are located on a pair of chromosomes and distinguish them as sex chromosomes from autosomes. In most mammals, males are the heterogametic sex with heteromorphic X and Y chromosomes, while females are the homogametic sex with two homomorphic X chromosomes, referred to as the XY system. In contrast, females in birds are the heterogametic sex with heteromorphic Z and W chromosomes, referred to as the ZW system. For reptiles and fishes, both XY and ZW systems have been reported. Besides, there are other sex chromosome systems, such as the UV system in some algae and bryophytes (Coelho *et al.*, 2018).

At present, the most widely accepted theory states that sex chromosomes originate from autosomes that gain two or more sex-determining genes in the hermaphrodite population (Abbott *et al.*, 2017; Charlesworth and Charlesworth, 1978). The two-mutations model of evolutionary transitions from hermaphroditism to separate sexes proposes that genetic sex determination requires at least two unisexual sterility mutations causing female and male sterility, respectively. First, the evolutionary invasion of a male sterility mutation results in a mixed population of females and hermaphrodites. The presence of unisexual females can then allow selection for the remaining hermaphrodites to become increasingly masculinised and eventually males, by the fixation of one or multiple female suppressor mutations (Charlesworth and Charlesworth, 1978).

Following the establishment of a sex-determining region (SDR) where recombination is selectively suppressed, the non-recombining region may continue to expand along the sex chromosome. Compared to the non-recombining region, the recombining region, called the pseudoautosomal region (PAR), maintains correct homologous pairing during meiosis (Rappold, 1993). In the non-recombining region on the sex-limited chromosomes (*e.g.*, Y or W), deleterious mutations start to accumulate by the Muller's ratchet effect or other processes (Bachtrog, 2006). Influenced by the combination of recombination cessation, genetic drift, and selection, DNA sequences on the X and Y (or Z and W) chromosomes diverge and the Y-linked (W-linked) gene copies lose their functions (a feature called degeneration). This could eventually lead to genetically and morphologically different X and Y (or Z and W) chromosomes. The degeneration can result in loss of the gene copy on Y (or W) chromosomes. The loss of functions of the degenerated genes can result in inadequate dosage for those gene products. To restore ancestral gene expression levels, some mechanisms of dosage compensation have to evolve. Ultimately, Y and W chromosomes are predicted to disappear. However, the progression can be perturbed by sex chromosome turnover, for example, another chromosome gains new sex-determining factors, or there are fusions between (a part of) autosomes and sex chromosomes.

1.3.3 Dosage compensation and epigenetic sex differences

There are several molecular mechanisms for dosage compensation (Marín *et al.*, 2000), including X chromosome inactivation in the females of mammals (Brockdorff and Turner, 2015), doubled transcription of X chromosomes in males of *Drosophila* (Lucchesi and Kuroda, 2015), or decreased transcription of both X chromosomes in males of *Caenorhabditis elegans* (Meyer, 2000). Noticeably, a theory about the regulatory evolution was proposed that after recombination cessation, dosage compensation can evolve if the autosomal *trans*-regulatory sequences can compensate the reduced gene expression on Y (or W) chromosome caused by the *cis*-regulatory divergence on sex chromosomes (Lenormand *et al.*, 2020).

Interestingly, complete dosage compensation seems generally absent in birds (Itoh *et al.*, 2007), and there are indications of sex-specific profiles of incomplete dosage compensation (Itoh *et al.*, 2010). Furthermore, female-to-male expression ratios appear to correlate negatively with non-synonymous substitutions between the Z and W gametologs in common whitethroat (*Sylvia communis*), suggesting that incomplete dosage compensation has evolved in some avian lineages (Sigeman *et al.*, 2018).

Males and females share most of the genome, except for the sex chromosomes. Yet, the phenotypic sex differences, *i.e.*, sexual dimorphism, are so ubiquitous that some other downstream processes are likely involved. For example, the development of the human brain, which is anatomically sexually dimorphic (Goldstein *et al.*, 2001; Lenroot *et al.*, 2007), is epigenetically influenced by the steroid hormones via sex-specific methylation patterns of the oestrogen and progesterone receptor genes (McCarthy *et al.*, 2009). Notably, epigenetic sex differences can occur in the absence of hormones, as exemplified by the evidence showing that sex-specific expression of autosomal genes can be epigenetically induced by the expression of other genes located on the sex chromosomes (Wijchers and Festenstein, 2011).

1.4 Evolutionary implications with the presence of separate sexes

1.4.1 Sexual antagonism

The presence of separate sexes can lead to several important evolutionary processes, including sex-specific selection regimes. The divergent evolutionary interests of males and females, and sex-specific selection regimes, give rise to what is called ‘sexual conflict’. Sexual conflict can be categorized as antagonistic interactions between different loci within the genome (inter-locus conflict) or between alleles at the same locus (intra-locus sexual conflict) (Rowe *et al.*, 2018).

Inter-locus sexual conflict is manifested by the antagonistic interaction between different traits in males and females (Rowe *et al.*, 2018), with the assumption that these traits are governed by different genes. A typical example of inter-locus sexual conflict is the copulatory wounding during reproduction in cowpea seed beetles (*Callosobruchus maculatus*). The male *C. maculatus* bears strongly sclerotised spines on the tip of the genitalia (Crudgington and Siva-Jothy, 2000), which is used to prolong copulation times and causes damage to females, discouraging them from remating and also increases their risk of infection (Edvardsson and Tregenza, 2005). However, female *C. maculatus* can evolve resistance adaptations, such as increasing tract volume, and immunity (Dougherty *et al.*, 2017). Ultimately, this conflict between male and female *C. maculatus* results in a coevolutionary arms race between male-coercive mating and female resistance traits (Dougherty *et al.*, 2017; McNamara *et al.*, 2020).

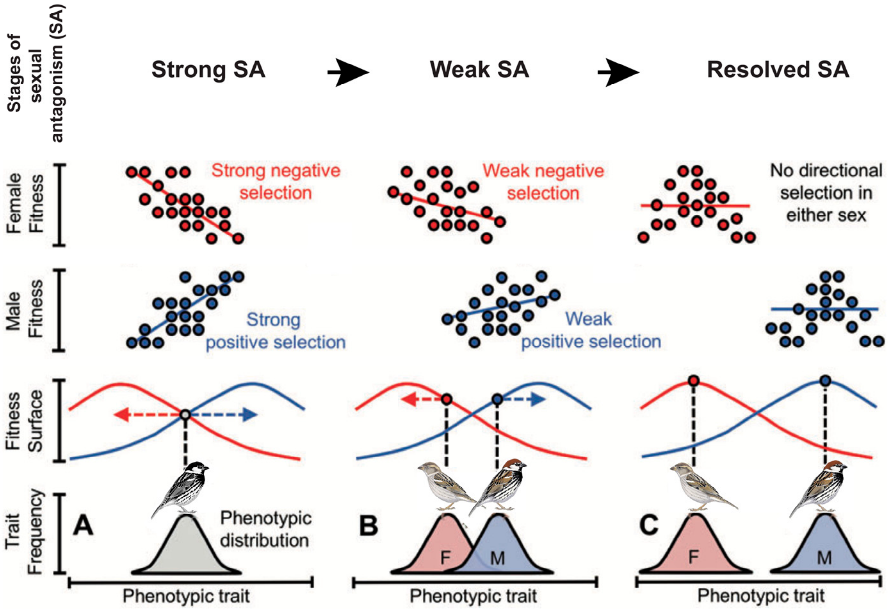


Figure 2. Illustration of different stages in the evolution of sexual antagonism. (A) A sexually monomorphic trait with different fitness optima in males and females, resulting in sexually antagonistic selection that is quantified by the opposing slopes of the relationships between relative fitness and phenotype across individual females and males. (B) Sexual dimorphism evolves as male and female phenotypic distributions move toward their respective fitness optima in response to sexually antagonistic selection. (C) A hypothetical endpoint in which the phenotypic distributions of males and females match their fitness optima, and sexual antagonism is resolved at the loci for this trait. This figure was adaptated from Cox (2017).

In contrast, intra-locus sexual conflict (also called sexual antagonism) is characterized by the shared traits with a shared genetic basis under sexually antagonistic selection between males and females (Rowe and Day, 2006; **Figure 2**). At the sexually antagonistic locus, the expression of an allele is beneficial for one sex but detrimental for the other sex, which can be reflected by the negative genetic correlation between the sexes (Bonduriansky and Chenoweth, 2009). An example of sexual antagonism is the study of red deer (*Cervus elaphus*) where male red deer with relatively high fitness often had daughters with relatively low fitness (Foerster *et al.*, 2007). Sexual antagonism could be ubiquitous, because sexual antagonistic selection is commonly detected, at least among the animal studies that were examined by Cox and Calsbeek (2009). Also, evolutionary theory suggests that sexually antagonistic selection is an inevitable consequence of the evolution of sex (Connallon and Clark, 2014; Fisher, 1930),

The presence and intensity of sexual conflict is dynamic over the developmental process (Ingleby *et al.*, 2015), because gene expression varies between the sexes during development. Sex-biased gene expression can cause differentiated phenotypes between the sexes and therefore is thought to underlie sexual dimorphism. Sex-biased gene expression is often associated with sexual conflict, because the decoupling of gene expression between the sexes allows them to approach their respective fitness optima resulting in an alleviated sexual conflict. As morphological and behavioural differentiation between the sexes continues to increase during development (Badyaev, 2002), perhaps the signs of sexual conflict become more identifiable when the distinct sexual dimorphic traits are fully developed.

1.4.2 Sex differences in mutation rate and recombination

Different selection regimes in males and females can have various consequences, such as divergent patterns of mutations and recombination events between the sexes.

Mutations are the raw material for evolutionary change, because they endlessly fuel the genetic variation that selection can act upon to drive adaptation (Loewe and Hill 2010; Lynch *et al.*, 2016). Sex differences in mutation rates, with most often a higher mutation rate in males than females, have been reported in mainly primates as well as in one bird species (Campbell *et al.*, 2021; Gao *et al.*, 2019; Smeds *et al.*, 2016; Thomas *et al.*, 2018; Venn *et al.*, 2014). One possible explanation for higher mutation rates in males is that the continuous division of germ-line cells during spermatogenesis creates increased opportunities for mutations to occur compared to the fewer cell divisions during oogenesis in females (Zhang *et al.*, 2022). In addition, the increasing trend in mutation rate over the developmental process has been found to be more prominent in males than in females in humans (Wong *et al.*, 2016; **Figure 3**).

Recombination during meiosis is also an important process to generate genetic variation, in addition to the mechanistic function for ensuring connection and proper segregation between homologous chromosomes during meiosis (Petronczki *et al.*, 2003). However, suppression of recombination is a pivotal process for the formation of the differentiated, heteromorphic sex chromosomes (Bachtrog *et al.*, 2011; Bergero and Charlesworth, 2009; Ponnikas *et al.*, 2018). Moreover, suppression of recombination between the sex chromosomes in the heterogametic sex is likely to facilitate alleviating the sexual antagonism via accumulation of, for example, male-beneficial alleles on the Y chromosome.

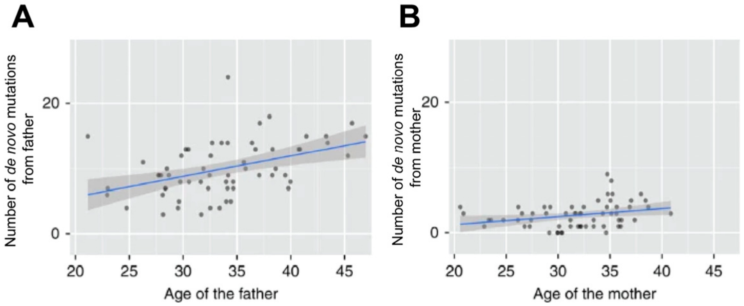


Figure 3. (A) The number of DNMs of paternal origin is plotted against the father's age (in years). The blue line shows the linear fit (estimate of the slope=0.31, p=5.15 × 10^{-4}) and the grey band represents the 95% confidence interval. (B) The number of DNMs of maternal origin is plotted against the mother's age (in years), the blue line shows the linear fit (estimate of slope=0.12, p=0.02), and the grey band represents the 95% confidence interval. The figure was adapted from Wong *et al.* (2016).

Recombination rates may vary between the sexes, a phenomenon known as heterochiasmy. The degree of heterochiasmy can be so extreme that the recombination is absent during meiosis in one sex (usually the heterogametic sex; Haldane, 1922; Huxley, 1928; John *et al.*, 2016). Less extreme heterochiasmy is observed across the vertebrate lineages (Bergero *et al.*, 2019; Berset-Brändli *et al.*, 2008; Broman *et al.*, 1998; Maddox *et al.*, 2001; Malinovskaya *et al.*, 2020). In addition, there is significant variation in the recombination landscape, *i.e.*, the variation of recombination rate along each chromosome, with higher recombination rates generally observed closer to the chromosome ends in males, in contrast to a flatter recombination landscape in females (Backström *et al.*, 2010; Ponnikas *et al.*, 2022; Sardell and Kirkpatrick, 2020; **Figure 4**).

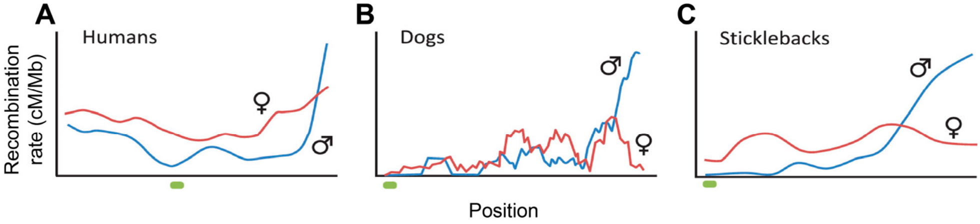


Figure 4. Examples of sex differences in recombination landscapes. Local recombination rates are greater in males at chromosome ends, while local rates are often greater in females near the centromere (shown by the green bars). (A) Recombination along human chromosome 7 (Broman *et al.*, 1998). (B) Recombination along domestic dog chromosome 19 (Wong *et al.*, 2010). (C) Distribution of crossovers as function of relative distance from centromere across long arms of all Gasterosteus stickleback chromosomes (Sardell *et al.*, 2018). The figure was adapted from Sardell and Kirkpatrick (2020).

2 Aims

My thesis centres around genomic studies of sex differences in a songbird, the great reed warbler. Male and female great reed warblers are monochromatic and genetically determined by a pair of sex chromosomes. I took advantage of the available blood samples from the long-term study of great reed warblers from lake Kvismaren that has been going on since the mid 1980s (Bensch *et al.*, 1998; Hansson *et al.*, 2000; Hasselquist 1998).

The aims of each chapter are listed in **Table 1**. Sex chromosomes play a pivotal role in GSD. The expansion of non-recombining regions on the sex chromosome could be in a gradual rather than stepwise fashion. To understand how the non-recombining regions expand necessitates studying the timing of recombination cessation (t_{RC}) along the sex chromosomes. However, two presently popular approaches, *i.e.*, synonymous substitution rate (dS) and maximum likelihood (ML) tree approaches, have their own problems that pose challenges for interpreting the t_{RC} on sex chromosomes. Therefore, **Paper I** mainly tackled this methodological problem assessing two alternative phylogenetic approaches that I developed for studying t_{RC}.

The presence of separate sexes can cause sex-specific selection regimes, which possibly leads to phenotypic and genetic differences between the sexes. As being the fundamental parameters to many population genetics and evolutionary studies, it is important to investigate to what extent mutations and recombination events could exhibit sex-specific patterns. With a three-generation pedigree of great reed warblers, **Paper II** estimated the *de novo* mutation rates and **Paper IV** located crossovers (one of the outcomes from recombination) on the chromosomes. These two chapters further investigated the patterns of these estimates between males and females. Moreover, the approaches and strategies to locate recombination positions using sequencing data from a pedigree were implemented in the *RecView* ShinyApp, an interactive R application. The design of the application and two algorithms to locate the recombination positions were documented in **Paper III**.

Sexual antagonism specifies that the adaption to different sex-specific fitness optima is constrained by traits with a shared genetic basis between the sexes. Considering that males and females share most of the genome except for the sex chromosomes, sexually antagonistic genes could be located anywhere in the genome, instead of being only on the sex chromosomes. In **Paper V**, I aimed to use the whole-genome sequencing data from 100 great reed warbler individuals to search for signs of sexual antagonism in the genome.

Table 1. Aims in each chapter

Chapters	Aims
Paper I	Improving methodology to study the timing of recombination cessation on the sex chromosome.
Paper II	Estimating the *de novo* mutation rate; investigating sex differences in mutation rates.
Paper III	Developing *RecView* ShinyApp to facilitate viewing and locating recombination positions using sequencing data from a pedigree.
Paper IV	Locating crossover positions on chromosomes; investigating sex differences in recombination rates and positions.
Paper V	Searching for sexually antagonistic genes in the genome, using F_{ST} -based and GWAS approaches.

3 Methodology

3.1 Study species

The great reed warbler (*Acrocephalus arundinaceus*) is passerine bird which has a present breeding range across Eurasia, from Spain in the west to Mongolia in the east (**Figure 5**). Great reed warblers have been breeding at the marshes of Lake Kvismaren, Örebro, Sweden, since 1978 (Bensch *et al.*, 1987), and a long-term study of great reed warbler has been set up for surveying this population since 1983 (Hasselquist, 1994; Hasselquist *et al.*, 1995). The great reed warbler population breeding at Lake Kvismaren is at the north-western edge of the species' breeding range, and it has gradually increased until 1988, and then stabilised at 40-60 individuals over the last 35 years (Bensch, and Hasselquist, 1991; Hansson *et al.*, 2000). For this population, phenotypic measurements, blood samples, and pedigree records, have been collected for almost 40 years, providing an adequate database for answering many scientific questions, such as physiological and immunological studies (Asghar *et al.*, 2015; Roved *et al.*, 2018; Westerdahl *et al.*, 2005), reconstructing a reference genome (Sigeman *et al.*, 2021), studying recombination (Hansson *et al.*, 2005), and searching for signs of sexual antagonism in the genome (Tarka *et al.*, 2014).

Great reed warbler is classified as a member of Sylvioidea superfamily that is under the order of Passeriformes and infraorder Passerida, according to the Sibley–Ahlquist taxonomy of birds (Alström *et al.*, 2013; Sibley and Ahlquist, 1990). The Sylvioidea superfamily consists of approximately 1,300 species, encompassing approximately 10% of the bird species that have been described so far (Gill and Donsker, 2021). The sex chromosome of birds originated from an autosome more than 100 M.y.a. (Zhou *et al.*, 2014). Recent works of identifying sex-linked regions discovered autosome-to-sex chromosome fusion(s) in many Sylvioidea species, forming a so-called neo-sex chromosome (Pala *et al.*, 2012a, 2012b; Sigeman *et al.*, 2019, 2020, 2022). The neo-sex chromosomes (or more specifically the neo-Z chromosome) of the great reed warbler comprise the ~78-Mb ancestral part on which recombination has been suppressed (except for a 1-Mb pseudoautosomal region, PAR), and recombination was not detected on the added part (Sigeman *et al.*, 2021). However, a phylogenetic analysis revealed that the ~10-Mb added part could have maintained recombination several million years after the fusion, serving as another PAR (Sigeman *et al.*, 2021). In comparison, some other Sylvioidea members have even more complex sex chromosome compositions with multiple added regions, *e.g.*, the added part is formed by regions of autosome 3, 4A, and 5 in the Eurasian skylark (*Alauda arvensis;* Sigeman *et al.*, 2019). The degree of recombination cessation on the added part also varies, as exemplified by the added part of the neo-sex chromosome in northern crombec (*Sylvietta brachyura*) which still recombines over parts of its length (Sigeman *et al.*, 2022).

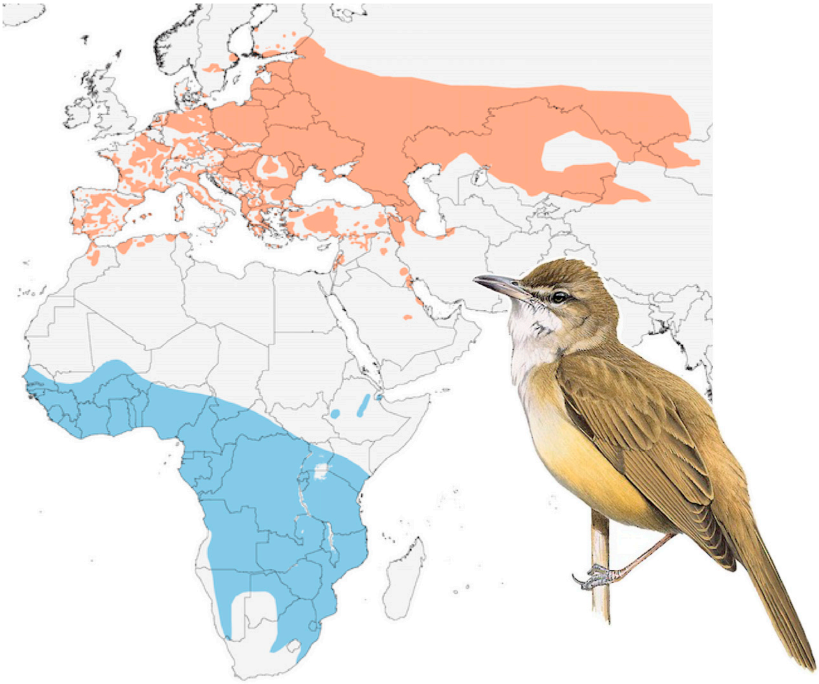


Figure 5. Distribution range of great reed warbler in summer (orange) and winter (blue). The map and bird illustration were from the Handbook of the Birds of the World (del Hoyo and Elliott, 2006).

Male and female great reed warblers are monochromatic. During the breeding season males often sing at the top of reeds using a complex song to mark the dominance in its territory and to attract females. As a migratory bird wintering in sub-Saharan Africa, the arrival of males at Lake Kvismaren is as early as the very beginning of May (Lemke *et al.*, 2013), followed by the females *ca.* 10 days later (Tarka *et al.*, 2015). The great reed warbler has a facultative polygynous social mating system, with approximately 40% of the males having more than 1 female, and approximately 20% of the males do not manage to mate with any female in a season (Hasselquist, 1998). Higher reproductive success of males is associated with an attractive territory, early spring arrival and a high song repertoire size (Hasselquist, 1998). The probability of occupying an attractive territory appear to be proportional to the spring arrival date, and spring arrival date (possibly reflecting migration speed) is negatively associated with wing length (Tarka *et al.*, 2014). Compared to males, it was hypothesised that growing a long wing is less important for females, because shorter wings increase their flying manoeuvrability during foraging among the reeds (Tarka *et al.*, 2014). Therefore, this discrepancy of sex-specific fitness optima potentially results in the sexually antagonistic selection on wing length (Tarka *et al.*, 2014).

3.2 Reference genome assembly

Reference genome assembly plays a pivotal role in my analyses, as it provides reference sequences for alignment and coordinates of scaffolds or chromosomes for any allele variants detected in the sequencing data of the actual samples. For all the analyses in this study, I used a good-quality and highly complete reference genome assembly of a great reed warbler from the Lake Kvismaren study population, named as acrAru1, which is publicly accessible on NCBI (BioProject ID: PRJNA578893).

With a size of approximately 1.2 Gb, this reference genome assembly consists of 3,013 scaffolds and has a N50 of 21.4 Mb (Sigeman *et al.*, 2021). The reference genome assembly has 93.1% complete genes, and the annotation covers 22,524 genes. Moreover, the scaffolds belonging to the non-recombining part of the Z and W chromosomes are likely to be reliable, as they were determined with additional analyses on another 5 male and 5 female individuals (Sigeman *et al.*, 2021).

3.3 Datasets

There were three datasets used in the thesis. For concise description, these three datasets are named as *Sylvioidea sequences dataset*, *Pedigree dataset*, *GRW100 dataset* (**Table 2**).

Table 2. The used dataset in each chapter

Dataset	Used in Chapter	Description
Sylvioidea sequences dataset	I	Z and W gametologous sequences of 51 genes from 6 Sylvioidea species (including great reed warbler) and 7 outgroup species.
Pedigree dataset	II, III, IV	Illumina sequence data of 12 individuals from a three-generation pedigree of great reed warbler, consisting of 150 bp paired-end reads at ~50X coverage.
GRW100 dataset	V	Illumina sequence data of 49 males and 51 females of great reed warbler aged between 3 and 5 years old, consisting of 150 bp paired-end reads at ~30X coverage.

The *Sylvioidea sequences dataset* was used in **Paper I**, which comprises Z and W gametologous sequences of 51 genes from 13 species, including 6 Sylvioidea species – great reed warbler (*Acrocephalus arundinaceus*), clamorous reed warbler (*A. stentoreus*), marsh warbler (*A. palustris*), western olivaceous warbler (*Iduna opaca*), Savi's warbler (*Locustella luscinioides*), bearded reedling (*Panurus biarmicus*); and 7 outgroup species – great tit (*Parus major*), zebra finch (*Taeniopygia guttata*), blue-crowned manakin (*Lepidothrix coronata*), budgerigar (*Melopsittacus undulatus*), chicken (*Gallus gallus*), emu (*Dromaius novaehollandiae*), and green anole (*Anolis carolinensis*). The selection of these 13 species takes into consideration their speciation times and phylogenetic relationships (**Figure 6**).

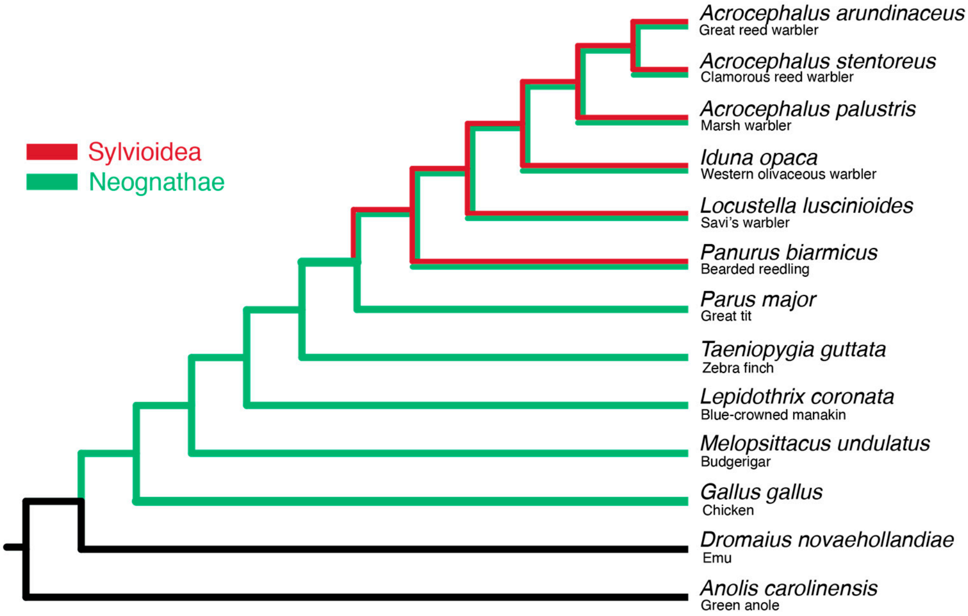


Figure 6. Phylogeny of the 13 selected species.

The *Pedigree dataset* was used in **Paper II**, **Paper III**, and **Paper IV**. A three-generation pedigree was chosen. This pedigree consists of 12 individuals, including 4 grandparents, 2 parents and 6 offspring (**Figure 7**). The genomic DNA was extracted from blood samples and sent to SciLifeLab to perform library preparation and sequencing. I got Illumina sequence data of 150 bp paired-end reads at ~50X coverage.

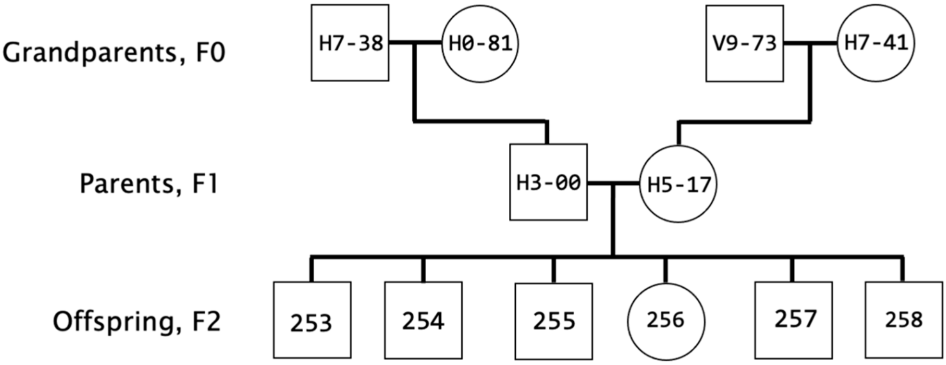


Figure 7. The three-generation pedigree of great reed warblers (*Acrocephalus arundinaceus*). Shown are generation (F0, F1, and F2), individual code (*e.g.*, H7-38) and sex (square: male; circle: female).

The *GRW100 dataset* was used in **Paper V**. I selected 49 males and 51 females aged between 3 and 5 years from the long-term study of great reed warbler at Lake Kvismaren, Sweden. The genomic DNA was extracted from blood samples and sent to SciLifeLab to perform library preparation and sequencing, using a paired-end (2 × 150 bp) setup and a targeted ~30X coverage.

3.4 Analyses summary

The analyses of the three datasets involve various considerations and designs, so there will be differences in the analysing approaches between papers. Given that the details are already described in each paper, I will here highlight some key points for each paper, but avoid intricate technical and bioinformatic details.

3.4.1 Paper I

Paper I highlights the evaluation of two alternative phylogenetic approaches that I developed for studying the timing of recombination cessation (t_{RC}). The evolution of sex chromosomes can be punctuated by several recombination cessation events, thus forming the so-called evolutionary strata along the sex chromosomes. However, the evolutionary strata hypothesis has been challenged by studies sampling more genes along the sex chromosomes, and a gradual expansion of the non-recombining region was instead suggested (Natri *et al.*, 2013; Sigeman *et al.*, 2021). With respect to studying t_{RC}, one approach is to analyse the synonymous substitution rate (dS; Lahn and Page, 1999; Nam and Ellegren, 2008; Peichel *et al.*, 2020), assuming dS is proportional to when recombination was suppressed in the past. Another approach to study the clustering pattern of gametologous sequences of several species in phylogenetic trees, *e.g.*, maximum likelihood (ML) trees. The latter method assigns the t_{RC} as occurring before the oldest speciation event in the topology when the gametologs of a set of species cluster exclusively according to chromosome type rather than to species (Charlesworth *et al.*, 2005). With dense sampling of genes, the results of these two approaches often deviate from the expectation. For example, dS values along the sex chromosome often show gene-specific patterns (Nam and Ellegren, 2008; Peichel *et al.*, 2020; Sandstedt and Tucker, 2004; Sigeman *et al.*, 2021; Wright *et al.*, 2014), which is contradictory to either the stepwise or gradual expansion of non-recombining part. Also, the ML tree approach suffers from unresolved topologies, making it difficult to understand whether the gametologs are clustered according to chromosome type rather than to species. These problems compromise the interpretation of the results for t_{RC}. Also, based on such results, the demarcation of evolutionary strata would become somewhat subjective.

In **Paper I**, I developed two alternative phylogenetic approaches, the ELW (expected likelihood weight) and the BEAST approaches, to circumvent these problems and evaluated their performances alongside the two frequently used dS and ML_{CT} approaches. ML_{CT} stands for maximum likelihood tree collapsed for the unreliable branches (bootstrapping value < 0.7), which resolves the topology by allowing polytomies. Thereafter, the t_{RC} inferred from these topologies can be compared with the results of other methods. The ELW approach finds the most likely topology among a set of hypothetical topologies representing different t_{RC}. In comparison, the BEAST approach estimates t_{RC} through estimating the posterior probability distribution for the divergence node between the Z and W gametologs of a focal species in a reference species topology (*e.g.*, **Figure 6**) with specified calibration priors on the other nodes.

These two alternative phylogenetic approaches, *i.e.*, ELW and BEAST, highlight the usage of fixed topologies. Since the likelihood of a model represents the probability of the data given the model, using fixed topologies allows us to evaluate which model (topology) is most likely to generate the data. The ML topology is not necessarily but closest to the true topology, which can be complemented with some *post hoc* statistics, *e.g.*, bootstrapping. Notably, other tested models (topologies) are less likely, but they can be used to form a distribution of relative likelihoods across the models (topologies) and thereby reflect the uncertainty around the maximum likelihood topology. On the other hand, using fixed topology in BEAST approach controls the certainty of the tree topology for Markov chain Monte Carlo algorithm to instead estimate the posterior probability distribution for t_{RC}. Essentially, these two alternative phylogenetic approaches transform the problem of unresolved topology to the estimation of the uncertainty for t_{RC}.

The evaluation of each approach was done by comparing the clustering results using the t_{RC} estimates and the gene locations, *i.e.*, on ancestral and added parts of the neo-sex chromosomes in great reed warbler.

3.4.2 Paper II

In **Paper II**, I identified germline mutations (called *de novo* mutations or DNMs) using the pedigree dataset and compare the number of DNMs between males and females. The sequencing data were mapped to the reference genome assembly of the great reed warbler, and next the variants were called with bioinformatic programme. Through the analysis at each bi-allelic SNP, a DNM will be shown as a singleton, that is, the rare allele occurs in only one of the examined individuals.

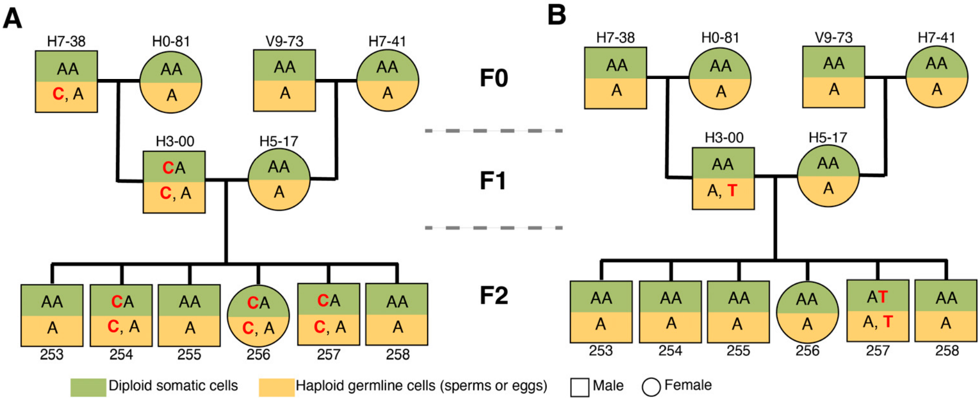


Figure 8. Two scenarios of the occurrence and fate of germline mutations in a three-generation pedigree. (A) The first scenario involves germline mutations occurring in one individual (*e.g.*, H7-38) of F0 generation, detected in one individual (*e.g.*, H3-00) of F1 generation, and then present in several individuals (*e.g.*, 254, 256, 257) of F2 generations. (B) The second scenario involves the germline mutations occurring in one individual (*e.g.*, H3-00) of F1 generation and detected in one individual (*e.g.*, 257) of F2 generation.

There are two scenarios where a DNM occurs and can be detected within the pedigree (**Figure 8**). The first scenario involves a DNM occurring in one individual of the F0 generation (**Figure 8A**). This DNM can be inherited by an individual of the F1 generation, and then this mutation will be present in both the somatic and germline cells in this individual of the F1 generation (**Figure 8A**). By using sequencing data from blood samples, which is a somatic tissue, the DNM can be detected by singleton analysis including the individuals of both the F0 and F1 generations. Furthermore, this DNM can be passed down so that it will also be present in one or several individuals of the F2 generation. Considering 50% chance of inheritance for an allele, we can expect a normal distribution of transmission to individuals of the F2 generation for all DNMs in this first scenario. Importantly, deviations from a normal distribution would have indicated the presence of false positive DNMs. For example, skewed distribution towards zero individuals of the F2 generation would have indicated somatic mutations rather than DNMs. In contrast, the second scenario involves a DNM occurring in an individual's germline cells in the F1 generation (**Figure 8B**). Such a DNM can only be passed down to one individual of the F2 generation. With sequencing data from blood samples, the DNM can be detected in an offspring of the F2 generation by singleton analysis.

To analyse whether the number of DNMs differs between males and females, I needed to investigate the parent-of-origin for each DNM showing whether it was passed down from the father or the mother. This can be facilitated by examining the genotypes of the individuals within the pedigree at an additional SNP. This additional SNP is only informative when it satisfies the following criteria: (i) this SNP is located on the same read or read-pair as the DNM, (ii) the individual with the DNM is heterozygous at this SNP, and (iii) one of the parents is homozygous for the reference allele at this SNP.

3.4.3 Paper III

In **Paper III**, I developed *RecView* ShinyApp, an interactive R application to facilitate viewing and locating recombination positions using sequencing data from a pedigree. I further demonstrated its applicability with the example of locating recombination positions on chromosome 1 in one of the offspring in the great reed warbler pedigree dataset (ID-256). The *RecView* ShinyApp is wrapped into a R package as a workaround for accessibility.

The analysis of locating recombination positions is conducted separately for each offspring (*i.e.*, the individual of the F2 generation). *RecView* takes two input files, a genotype file of individuals from a three-generation pedigree and a scaffold file specifying the order and orientation of the scaffolds on each chromosome. As indicated by the basic workflow (**Figure 9**), the input genotype file can be prepared by using *VCFtools* to extract individual genotypes from the VCF file and then running a built-in function *make_012gt()*. Next, *RecView* infers the grandparent-of-origin (GoO) for the alleles at each SNP by comparing the genotype strings to the "dictionary of GoO". More specifically, the genotype string is constructed from the genotypes of paternal grandfather, paternal grandmother, maternal grandfather, maternal grandmother, father, mother, and the offspring of interest. The dictionary and the inference are described in **Paper III**. Alleles with a specific GoO are informative, and as one of the results, *RecView* visualizes the inferred GoO at each informative allele along the specified chromosome. Also, the local density of informative alleles is calculated.

Moreover, *RecView* includes the option to infer putative recombination positions with either proportional difference algorithm or cumulative continuity score algorithm (**Figure 9**). The details of these two algorithms are described in **Paper III**. The results can be previewed in the result panel of the graphic user interface. *RecView* outputs the result figures from the chosen algorithm, a table with the putative positions and the estimated errors for the recombination on the chromosome, and a figure showing the local density of the informative SNPs along the chromosome. Furthermore, *RecView* provides options to save these results to the local directory.

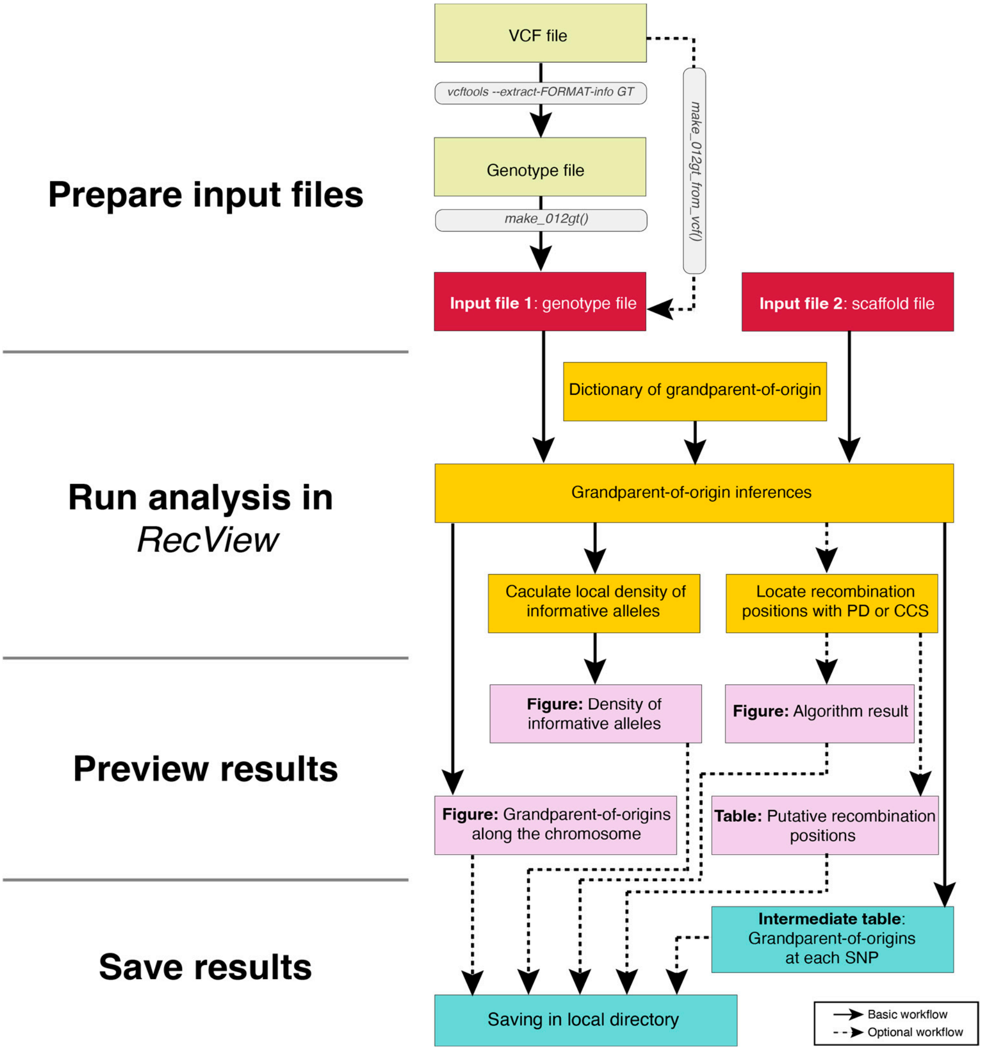


Figure 9. The workflow of using *RecView*. Solid lines indicate the basic workflow while dashed lines indicate the optional workflow. *RecView* requires an input genotype file which can be generated by using *make_012gt()* on the output file from *VCFtools*, or using *make_012gt_from_vcf()* on the VCF file. *RecView* further requires an input scaffold file containing the order and orientation of the scaffolds. These two input files are used together with the built-in dictionary of grandparent-of-origin (GoO) to produce the GoO figure showing the GoO inferences of alleles along the scaffolds, and a figure showing the informative alleles density. *RecView* can further locate putative recombination positions with the proportional difference or cumulative continuity score algorithms and output result figures and tables. The result figures and tables can be saved, including an intermediate table containing the GoO inferences at each SNP.

3.4.4 Paper IV

In **Paper IV**, I used *RecView* to locate the recombination positions on the chromosomes of each offspring from the three-generation pedigree (**Figure 7**). Given the low nucleotide diversity of the great reed warbler, I specifically focused my analyses on crossovers (CO), which is one of the two outcomes from resolving the Holliday junctions during recombination. Alternatively, recombination can result in non-crossovers (gene conversions), however, because it only covers a dozen to several hundred base pairs it is unlikely to be distinguishable from the mapping errors in the case of the great reed warbler.

The principle of locating CO is to infer GoOs for different chromosomal regions in the individuals of the F2 generation, and to locate the positions with switching GoOs along a chromosome or scaffold (**Figure 10**). In the F1 generation, the GoOs for the homologous chromosomes are certain, *i.e.*, the paternal chromosome is inherited from the father (that belongs to the F0 generation), and the maternal chromosome is inherited from the mother (belonging to the F0 generation) (**Figure 10**). In the F1 generation, recombination events occur between the two homologous chromosomes. A minimum of one recombination per chromosome arm will be resolved as CO to ensure the proper segregation of homologous chromosomes, a pattern called “obligate CO requirement”. Moreover, each CO involves two non-sister chromatids. Therefore, it has a 50% probability that the recombined chromatids will be inherited by the individuals of the F2 generation (**Figure 10**).

Using *RecView*, I obtained CO positions on the chromosomes, and I further translated these positions to the positions on chromosome arms, assuming recombination events are independent between the chromosome arms. Note that some chromosomes, especially the microchromosomes, are telecentric and thus have a single arm. Next, I calculated the recombination rates for the paternal and maternal chromosome arms, respectively. Because the comparison of chromosome arms between paternity and maternity is equivalent to the comparison of chromosome arms between the sexes, I investigated whether recombination rates differed between the sexes by statistically test for differences of recombination rates between the paternal and maternal chromosome arms.

The chromosome arms were aligned from the chromosome end (*i.e.*, the telomere) to the centromere. Therefore, the arm positions of COs correspond to the distance to the chromosome ends. Afterwards, I tested whether these CO positions differed between males and females, and followed the general pattern of more COs close to the chromosome ends in males than in females (**Figure 4**).

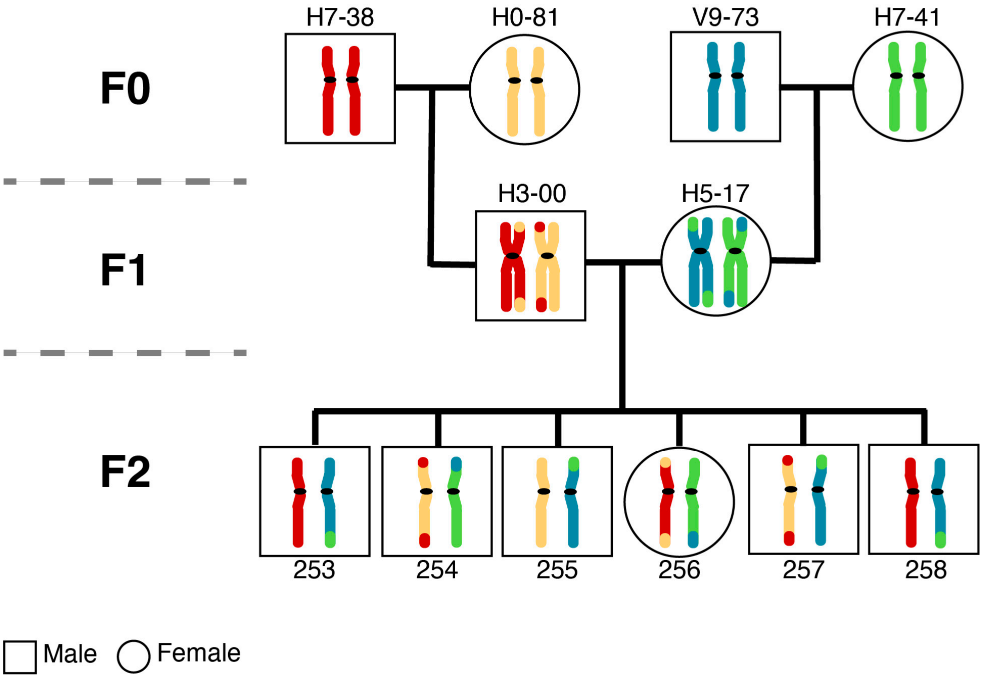


Figure 10. Examples of the occurrence and inheritance of crossover in individuals of F1 generation. The locations of recombination are visualised as the boundaries of two chromosome regions with different colours in individuals of F2 generation. Different colours indicate different grandparent-of-origins (*i.e.*, individuals of F0 generation).

3.4.5 Paper V

Sexually antagonistic selection can result in the evolution of sexual dimorphism (Cox and Calsbeek, 2009), which can be mechanistically modulated by sexually differential gene expression. However, sexually antagonistic selection can also differentiate the allele frequencies at numerous loci in males and females, through selection acting at (probably) many different loci coding for shared traits. In **Paper V**, I tried to search for sexually antagonistic loci with whole-genome sequencing data of 49 males and 51 females that were aged between 3 and 5 years. Considering that only approximately 30% of nestlings survive their first year, and that the adult annual survival rate is approximately 45% (Bensch *et al.*, 1998; Hansson *et al.*, 2002), these 3–5-year-old survivors represent less than 6% of the total new-born population, suggesting that they have been exposed to strong selection for survival.

The sequencing data of these 100 individuals were mapped to the reference genome assembly of the great reed warbler and the variants were called. In addition to some technical filtering (details were described in **Paper V**), I kept the SNPs with two alleles for the following analyses. Next, I performed the analyses with both F_{ST} -based and GWAS approaches.

F_{ST}, which represents a fixation index, is the proportion of genetic variance within subpopulations (s subscript) in relation to the total genetic variance (t subscript). In brief, F_{ST} quantifies the differentiation between subpopulations, so higher values indicate that two subpopulations are more genetically different. In practice, the F_{ST} -based approach aims to locate markers, and eventually genes, showing differences in allele frequencies between subpopulations, which are males and females in the case of **Paper V**. It premises that different selection regimes between the sexes will result in detectable differences in allele frequencies at the genes under selection and their linked markers (Vitti *et al.*, 2013). In comparison, GWAS is the acronym for genome-wide association study. The GWAS (Ozaki *et al.*, 2002) approach aims to locate the loci at which genetic variation is statistically associated with the phenotypic variation, such as the sex *per se*. GWAS could be less powerful when the alleles of interest are rare (Bush and Moore, 2012).

Before the analyses, I investigated the pairwise linkage disequilibrium (LD) between SNPs in the genome, which helps understand how SNPs are linked and allows for a rough estimation of the minimal number of SNPs required to cover the whole genome. Next, for the F_{ST} -based approach, I used *vcftools* to calculate the F_{ST} estimates. In addition, weighted mean F_{ST} in sliding windows (the window size was approximated from the LD analysis) was also calculated along the chromosomes. For the results from the F_{ST} -based analysis, I visualised the locations of the top 10 and the top 100 SNPs with the highest F_{ST} values, and likewise the locations of the top 10 and top 100 windows with the highest weighted mean F_{ST} values.

For the GWAS analysis, a linear model was performed to study the association between the sex differences in allele frequencies and the sex as a binary trait (*i.e.*, male or female) of 100 individuals at each separate SNP. The relatedness among the 100 individuals were also accounted for. Wald test was used to compute the significance p value, and Bonferroni correction was performed to account for multiple tests. Aa an alternative, I also calculated the false discovery rate (FDR, also known as the q value) based on the p values for each SNP. For the results of the GWAS analysis, I visualised the locations of the top 10 and the top 100 SNPs with the lowest p values.

Next, I compared the results to search for the overlapping SNPs from the respective top 100 SNPs reported by the F_{ST} -based and GWAS analyses, respectively, as this should give a subset of candidate SNPs to verify the presence of sexually antagonistic selection in the future.

4 Results and Discussions

4.1 Paper I

I evaluated two alternative phylogenetic approaches, ELW and BEAST, together with the frequently used dS and ML_{CT} approaches, for studying the timing of recombination cessation (t_{RC}) on the sex chromosome. The evaluation was done on a dataset of 22 genes on the ancestral part, and 29 genes on the added part, of the Sylvioidea neo-sex chromosome. Considering that the fusion occurred approximately 75 M.y. after the origin of the ancestral avian sex chromosomes, I expected that the ancestral part had fully ceased to recombine long before the added part, and therefore, the timings of recombination cessation of the sampled genes should distinctly cluster according to the different parts of the neo-sex chromosome.

The results from dS approach show different patterns between the ancestral and added parts of neo-sex chromosome. However, the clustering result was not consistent with the demarcation between the added part, because many genes on the ancestral part exhibit low dS values and they were clustered with the genes on the added part (**Figure 11A**).

The ML_{CT} approach is based on the phylogenetic clustering pattern of the species. To resolve the topology, I collapsed tree branches with bootstrap value < 0.7, and the collapsed trees were drawn in cladograms. The collapsed trees were compared to the reference species topology indexed on the biologically meaningful phylogenetic positions for recombination cessation. The assigned tree position range represents where the great reed warbler W gametolog branched off from the collapsed tree. The results of the ML_{CT} analysis showed imprecise tree positions in many genes, though, it still improves the clustering of the genes according to the ancestral and added parts of neo-sex chromosome (**Figure 11B**).

Notably, three genes of the ancestral part (T02835, T01777, T03277) showed a tree position range between 4 and 12 (**Figure 11B**), implying that continued recombination after the formation of Sylvioidea could not be excluded. The other wrongly classified gene was located on the added part (T02964) and showed a narrow tree position range, *i.e.*, 4, which in turn accounted for its unexpected clustering with the majority of genes on the ancestral part of which some also have narrow tree position ranges (**Figure 11B**). I believe that this is caused by the clustering analysis using Euclidean distances which are computed as single values from multi-dimensional vectors. Such scaling of a high dimensional space is always likely to lead to lost information to some extent, in this case giving similar distance values to some genes with narrow tree position range despite differing in tree position. On the added part, no fewer than 27 genes had a tree position range exceeding 6.5 (**Figure 11B**), indicating the erroneous possibility of recombination cessation even before the chromosome fusion took place.

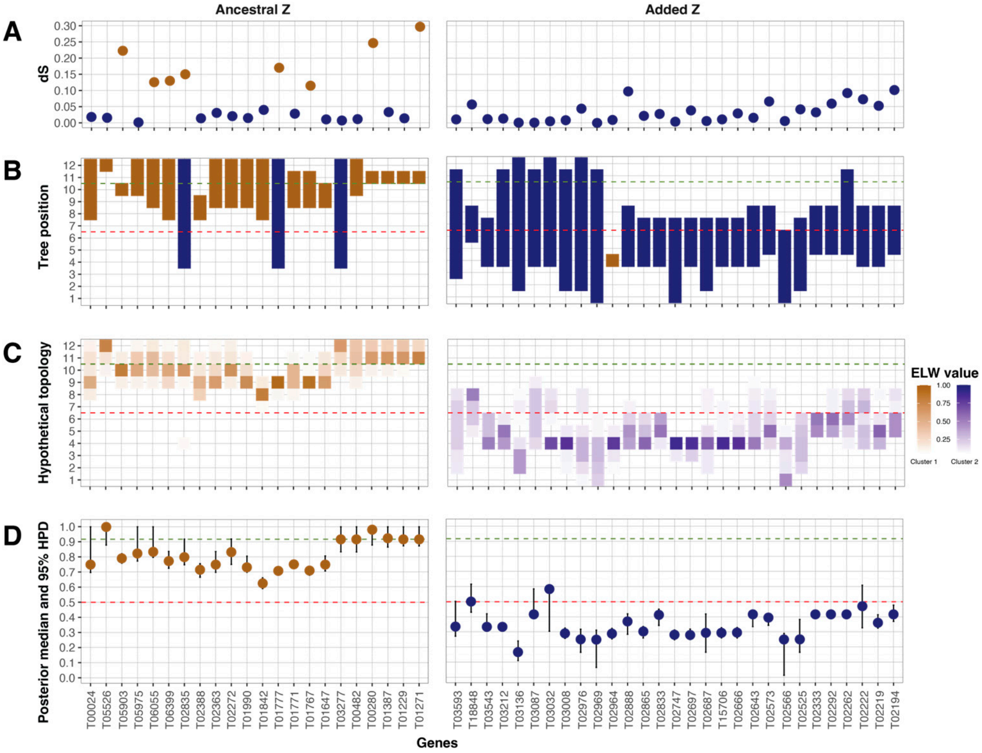


Figure 11. Estimates of t_{RC} across 51 neo-sex chromosome genes from (A) the dS approach, (B) the ML_{CT} approach, (C) the ELW approach, and (D) the BEAST approach. Genes were ordered according to the physical positions on great reed warbler's neo-Z, and included 22 genes from the ancestral part and 29 genes on the added part (Sigeman *et al.*, 2021). Colours correspond to clusters of a K-means clustering analysis (k = 2), with brown indicating Cluster 1 and blue Cluster 2. The colour gradient (heatmap) in the ELW approach indicates the ELW values for each hypothetical topology. The two horizontal lines mark the origin of Neognathae (green) and Sylvioidea (red).

In contrast to the dS and ML_{CT} approaches, the ELW and BEAST approaches separated clearly between genes of the ancestral and added part of the neo-sex chromosome by grouping them in Cluster 1 and 2, respectively (**Figure 11C, D**). Most importantly, both approaches gave some uncertainty in the t_{RC} estimates (**Figure 11C, D**), underlining the point I previously made, *i.e.*, that the advantage of these latter two approaches is that they transform the problem of unresolved topology into the uncertainty of t_{RC}.

Notably, the ELW and the BEAST approaches resulted in varying degrees of uncertainty of the t_{RC}, with the ELW approach having more genes with a larger degree of uncertainty. This may be explained by the different set-ups of the two approaches. For the ELW approach, the estimation is based on a finite set of hypothetical topologies, so that possible t_{RC} estimates are finite and discrete, and dependent on the number of species. In contrast, the BEAST approach provides continuous t_{RC} estimates between 0 and 1 and should therefore in theory have better resolution. In general, the erroneous calling of W variants will affect the t_{RC} estimates. For example, interpreting Z polymorphisms as W-specific sites will overestimate t_{RC} to be more ancient than it should be. We called W variants based on a single individual of each sex, but we believe that this did not inflate our t_{RC} estimates as we have found almost identical W variants when using two individuals of each sex (Sigeman *et al.*, 2019, 2021; H. Sigeman and B. Hansson unpublished).

4.2 Paper II

With the pedigree dataset, I found 78 DNMs on autosomes, and 4 DNMs on the Z chromosome. I managed to infer the parent-of-origin for 43 of the 78 DNMs on autosomes to F0 or F1 individuals (**Table 2**). Statistics showed a significant sex-bias among these DNMs with 32 DNMs inherited from the male parent, and only 11 DNMs from the female parent ($\chi^2 = 10.26$, df = 1, p = 0.001, **Figure 12**). Therefore, the number of DNMs in males was approximately three times higher than in females.

It is thought that the mutation rate is associated with the number of cell divisions. The presence of separate sexes can result in sex-specific mutation rates probably due to different numbers of postnatal cell divisions between the male and female germlines, caused by continuous cell division in spermatogenesis but a limited number of cell divisions in oogenesis (Ellegren and Fridolfsson, 1997; Makova and Li, 2002). Pronounced male-to-female-bias in number of germline mutations has previously been detected in mammals as well as in one bird species. However, there seem to be a considerable range in the strength of the male-biased germline mutation rate, with chimpanzees (*Pan troglodytes*) showing very strong (male-to-female mutation rate ratio is 5 – 6; Venn *et al.*, 2014) and collared flycatchers (*Ficedula albicollis*) only a modest male bias (male-to-female mutation rate ratio is 1.2; Smeds *et al.*, 2016).

The variation in the magnitude of the male-biased effect between different studies can be caused by a confounding effect of age of the sampled individuals, because the number of germline mutations can increase with age, and in particular so in males (Kong *et al.*, 2012; Wong *et al.*, 2016). Although I may have a rather limited dataset to analyse to what extent the age effect is responsible for the different numbers of DNMs between the sexes, this three-generation pedigree contains three pairs of males and females of various ages where I might be able to obtain an indication of the male-biased effect in relation to age at reproduction, including two pairs in F0 generation: paternal grandfather and paternal grandmother, maternal grandfather and maternal grandmother, and one pair in F1 generation: father and mother (**Table 3**).

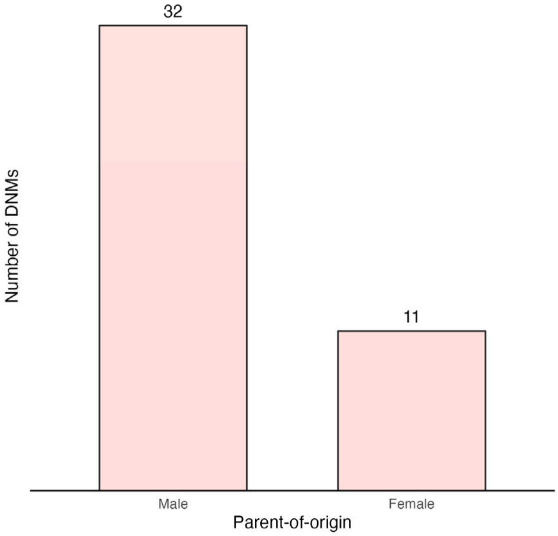


Figure 12. Parent-of-origin for the DNMs.

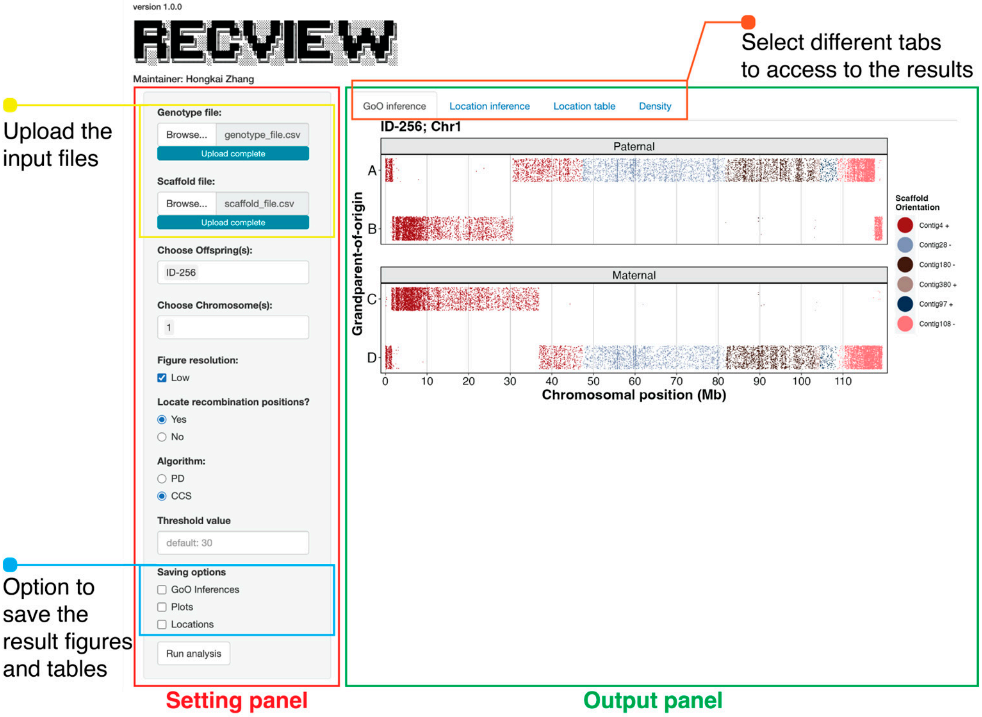
Table 3. Number of traceable DNMs to F0 and F1 individuals.

Generation	Individual	Sex	Age (yr)	Traceable DNMs
Paternal F0	H7-38	Male	>2	6
	H0-81	Female	>2	2
Maternal F0	V9-73	Male	2	3
	H7-41	Female	>6	0
F1	H3-00	Male	4	23
	H5-17	Female	2	9

Within all these three pairs, there were more DNMs in the male than the female. In the pair of paternal grandfather and paternal grandmother, and the pair of father and mother, the difference in numbers of DNMs could possibly be explained by both sex and age effects. Nonetheless, the remaining pair, the maternal grandfather and maternal grandmother, is highly informative, because the male that is much younger (2 yrs) than the female (>6 yrs) still shows more mutations (n=3) than the female (n=0; **Table 3**). Therefore, these results collectively suggest that there is a male-biased effect on DNMs, and thus provide support for the general view that males have more germline mutations than females. However, to statistically tease apart the age effect from the sex effect, it probably needs to include more pedigrees.

4.3 Paper III

RecView has an easy-to-use GUI to view and locate the recombination positions with pedigree data (**Figure 13**). One of the outputs visualises the GoO for the informative alleles at each SNP along the chromosome. Taking the example of chromosome 1 in offspring ID-256 (**Figure 14**), an approximately 4-Mb starting region of the paternal chromosome has informative alleles showing the inferred origin from grandparent A, followed by another approximately 27-Mb region with informative alleles showing the inferred origin from grandparent B. In between these two regions a switch between different GoOs locates a CO event. Likewise, there are visually three CO events on the paternal chromosome (**Figure 14** upper panel), and two CO events on the maternal chromosome (**Figure 14** lower panel).



version 1.0.0

RECVIEW

Maintainer: Hongkai Zhang

Genotype file:
Browse... genotype_file.csv
Upload complete

Scaffold file:
Browse... scaffold_file.csv
Upload complete

Choose Offspring(s):
ID-256

Choose Chromosome(s):
1

Figure resolution:
☑ Low

Locate recombination positions?
● Yes
○ No

Algorithm:
○ PD
● CCS

Threshold value
default: 30

Saving options
☐ GoO Inferences
☐ Plots
☐ Locations

Run analysis

Upload the input files

Select different tabs to access to the results

Option to save the result figures and tables

Setting panel

Output panel

Figure 13. The GUI of *RecView* with the setting panel (red square) for uploading input files (yellow square) and setting options and the output panel (green square) where results can be accessed by selecting different tabs (orange square).

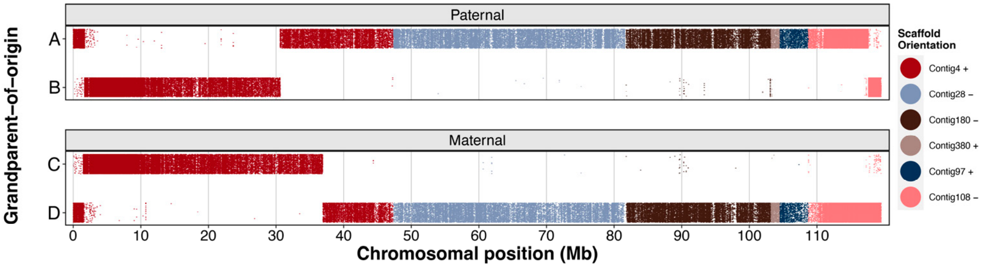


Figure 14. The grandparental-of-origin of informative alleles at all SNPs along chromosome 1 in great reed warbler offspring ID-256 for. Dots are plotted with noise on the y-axis to alleviate the degree of overlap. Colouration indicates different scaffolds on chromosome 1 in the great reed warbler genome assembly (Sigeman *et al.*, 2021).

4.4 Paper IV

In total, I located 224 COs on 408 autosomal arms in 6 offspring, including 113 COs on the paternal chromosome arm and 111 COs on the maternal chromosome arm in the great reed warbler three-generation pedigree. For each autosomal arm in each sex, I obtained the recombination distance by dividing the number of COs by the total number of meiosis in 6 offspring, *i.e.*, 12 meiosis. The calculated male recombination distances varied between 0 – 116.7 cM and the females' varied between 0 – 100 cM. Using the recombination distances, I calculated the recombination rates with the division by the size of the autosomal arms, respectively. I obtained the male recombination rates which varied between 0 – 21.2 cM/Mb (median: 2.55 cM/Mb) and female recombination rates which varied between 0 – 14.1 cM/Mb (median: 2.41 cM/Mb). Statistical tests suggested recombination distances were similar between the sexes (paired T-test, t = -0.210, d.f = 33, p = 0.835), and the same for the recombination rates (paired T-test; t = -0.578, d.f = 33, p = 0.567; **Figure 15A**).

Notably, the CO positions differed between males and females, irrespective of analysing the data as physical positions (Mann-Whitney U test: U = 7732, p = 0.00261; **Figure 15B**), or as proportional positions (Mann-Whitney U test: U = 7254, p = 0.0429; **Figure 15C**). Males had an increasing number of COs towards the telomere, contrasting to the females that had more COs off the telomere (Median $\text{position}_{\text{male}}$ = 1.57 Mb or 8.85%; Median $\text{position}_{\text{female}}$ = 2.10 Mb or 13.1%; **Figure 15B, C**).

The mechanism accounting for the sex-specific recombination landscapes is thought to be related to the combined effects of the sex-specific centromere effect and the non-sex-specific telomeric effect. The sex-specific centromere effect, proposed by several studies, results in reduced recombination rate in males and/or increased recombination rate in females. Together with the obligate CO requirement, it can be expected that COs will occur more frequently towards the telomeres in males. Also, the telomere-guided initiation of recombination could cluster COs in sub-telomeric regions (Haenel *et al.*, 2018; Higgins *et al.*, 2012; Otto and Payseur, 2019). Furthermore, the sex-specific recombination landscapes imply sex differentiation in local recombination rates which can be attributed to some genes with sexually antagonistic effect in recombination rate. There have been some studies reporting such genes, for example, *RNF212* gene in human and Soay sheep (Johnston *et al.*, 2016; Kong *et al.*, 2008).

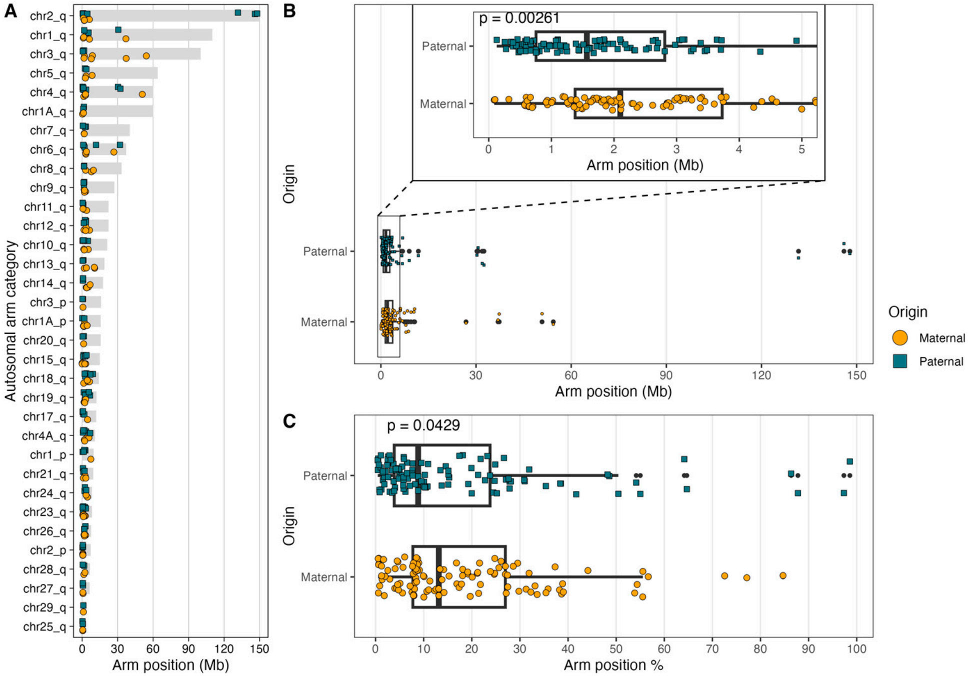


Figure 15. CO rates and locations between sexes. (A) Recombination rates were not significantly different between males and females. (B) Physical distance and (C) proportional distance of COs from the telomeric end of chromosome arms were significantly different, with more COs near the telomeric end in males than females.

4.5 Paper V

I searched for signs of sexually antagonistic selection in the genome by investigating the difference in allele frequencies at SNPs between 49 male and 51 female great reed warblers aged between 3 and 5 years. Among 15,966,921 SNPs, F_{ST} values ranged between 0–0.232. The F_{ST} values of the top 10 and the top 100 SNPs were above 0.190 and 0.147, respectively (**Figure 17A**). The top 10 SNPs with highest F_{ST} values were located on chromosome 4, 7, 17, and on the Contig 146, 151, 3637 that were unable to be assigned to any chromosome. Furthermore, among the top 100 SNPs with the highest F_{ST}, there was *not* any on chromosome 10, 13, 19, 20, 23, 25, 27, and 30.

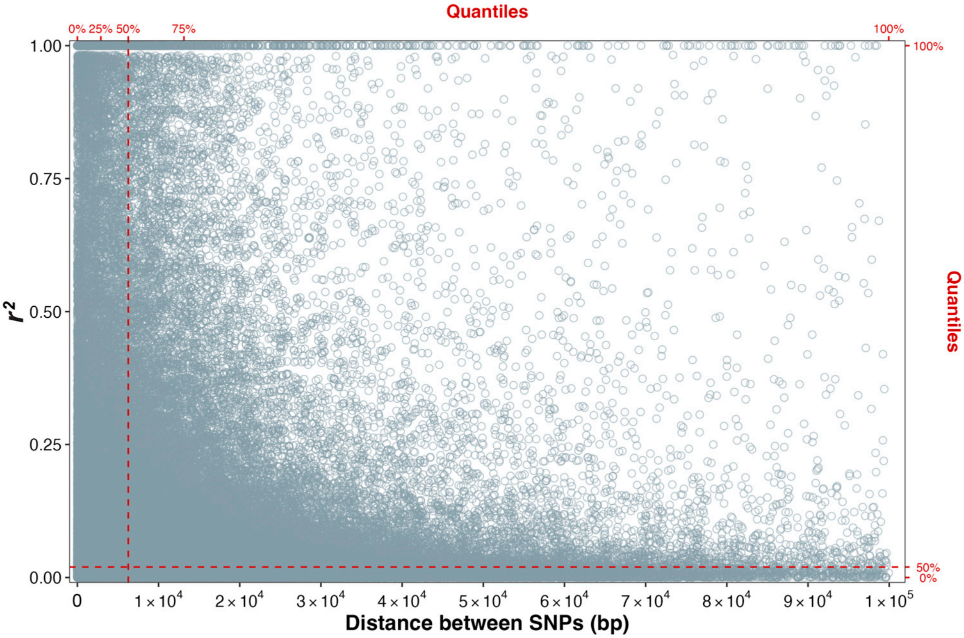


Figure 16. Pairwise linkage disequilibrium squared correlations (r^2) between the SNPs within 100 kb. The scale at the bottom (black) indicates the physical distance (bp) between the SNPs. The scale at the top (red) indicates the distribution of the data points within the 100 kb. The scale on the right (red) indicates the distribution of the data points between 0.00 – 1.00 of r^2.

LD between the SNPs within 100-kb windows were analysed, showing a non-linear reduction of LD correlations (r^2) with increasing distance between the pairwise SNPs (**Figure 16**). The median distance between the pairwise SNPs was 6,267 bp, and the median r^2 was 0.02, which suggests that r^2 quickly dropped within 10 kb (**Figure 16**). With a setting of 10-kb non-overlapping windows, the weighted mean F_{ST} values were calculated within each window, and the top 10 and 100 windows were above 0.057 and 0.039 (**Figure 17B**). The top 10 windows with highest weighted mean F_{ST} values were located on chromosome 2, 3, 4, 5, 11, and scaffold 54 that was not assigned to any chromosomes. In addition to the latter chromosomes, the top 100 windows with the highest weighted mean F_{ST} values were also located on chromosome 1, 1A, 4A, 7, 9, 10, 15, and 20.

Comparing the results between the F_{ST} analyses on the basis of single SNPs and 10-kb sliding windows, chromosome 4 harbours two of the top 10 SNPs with highest F_{ST} values and two of the top 10 windows with highest weighted mean F_{ST} values. However, their locations did not overlap.

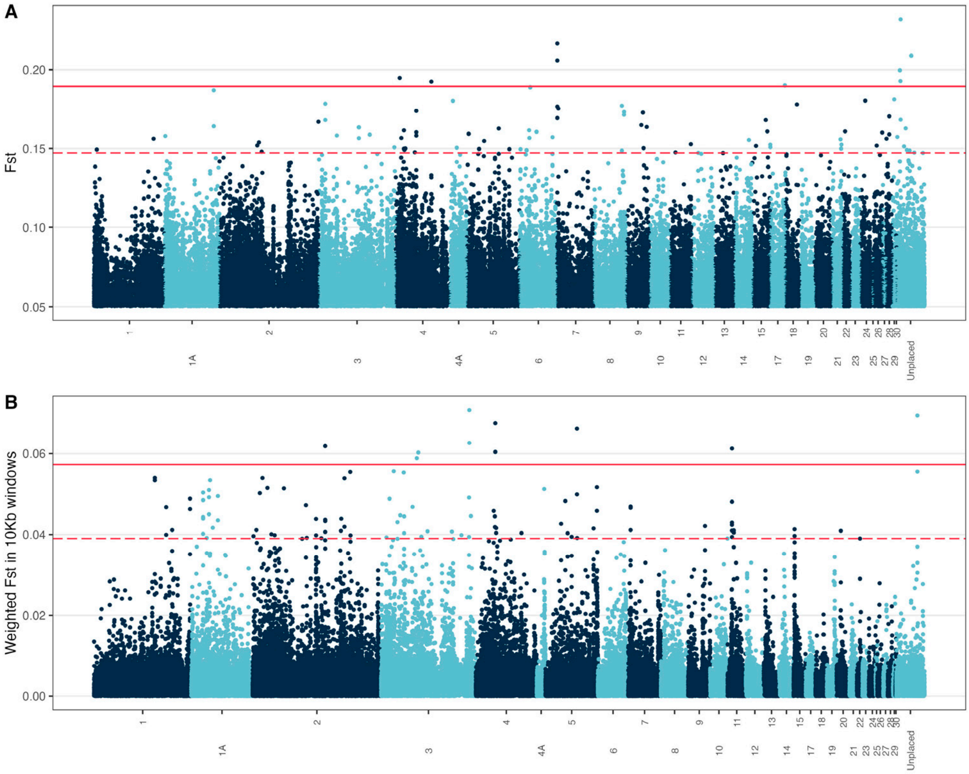


Figure 17. F_{ST} values (A) and weighted mean F_{ST} values in a 10-kb window (B) on different chromosomes. Solid red line delimits the top 10 and dashed red line delimits the top 100 SNPs or windows with highest mean F_{ST}. "Unplaced" groups the data points on the scaffolds that were not assigned to chromosomes.

In comparison, the GWAS approach was used to investigate the association between sex differences in allele frequencies and the sex of the 100 individuals at each SNP individually. The results showed that none of the SNPs were significant. I visualised the locations for the top 10 and top 100 SNPs with lowest p values after Bonferroni correction for multiple tests (**Figure 18**). The top 10 SNPs with lowest p values were located on chromosome 3, 4, 7, 18, and scaffolds (98, 146, 3637) that were not assigned to any chromosomes (**Figure 18**). The top 100 SNPs with the lowest p values were located on many chromosomes *except* for chromosome 9, 20, 23, 25, 27 (**Figure 18**).

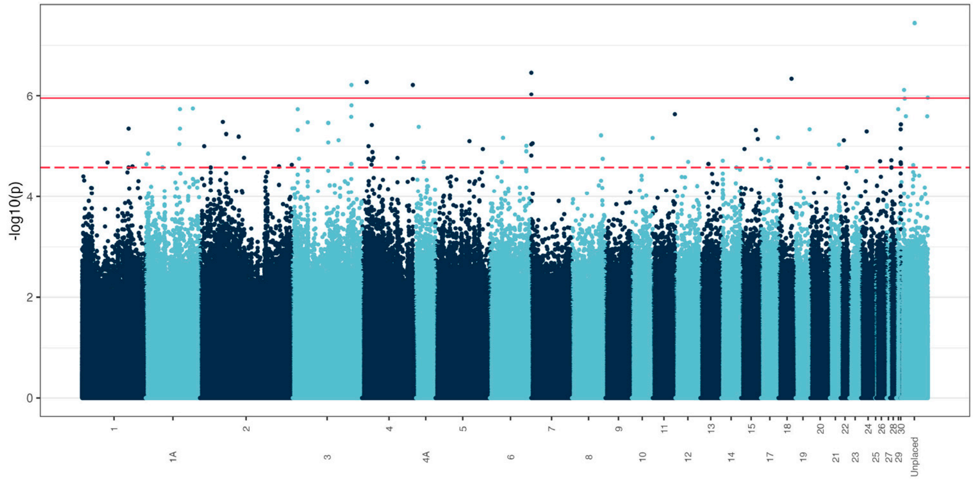


Figure 18. Significance values of single SNPs along the different chromosomes from a GWAS analysis with sex. Solid red line delimits the top 10 most significant SNPs and dashed red line delimits the top 100 most significant SNPs. “Unplaced” groups the data points on the scaffolds that were not assigned to chromosomes.

Since the GWAS analysis performs a statistical test at each SNP individually, p values must be corrected for multiple testing to lower the risk of identifying false positives. One way is to use Bonferroni correction. However, considering that adjacent SNPs are correlated to some extent, which is indicated by LD values (**Figure 16**), the assumption that all tests are independent in Bonferroni correction is violated. Therefore, Bonferroni correction may be too conservative, leading to over-correction of p values for GWAS (Johnson *et al.*, 2010). In contrast, the false discovery rate (FDR), which specifies the expected proportion of false positives, is suggested as an alternative. However, I did not detect any SNPs with FDR lower than 0.05. Furthermore, the most significant SNP was estimated to have 58% probability as a false positive.

Comparing the F_{ST} values and the significance of association with sex from GWAS for the top 100 SNPs in respective analyses, I identified 50 overlapping SNPs that had relatively high F_{ST} values and low p values than the non-overlapping SNPs (**Figure 19**). The results suggested that these 50 overlapping SNPs are candidates for studying sexually antagonistic selection.

While a F_{ST} -based approach quantifies the difference in allele frequencies between males and females at each locus, the GWAS approach further tests whether this difference in allele frequencies is associated with phenotypic variation. However, identifying signs of sexually antagonistic selection is challenging with GWAS, given the potential technical artefacts, genotyping errors, and high sampling variance when using small sample sizes. In addition, sexually antagonistic variants can rapidly reach fixation (Kasimatis *et al.*, 2019; Rowe *et al.*, 2018), and such historic variants are therefore less likely to be discovered.

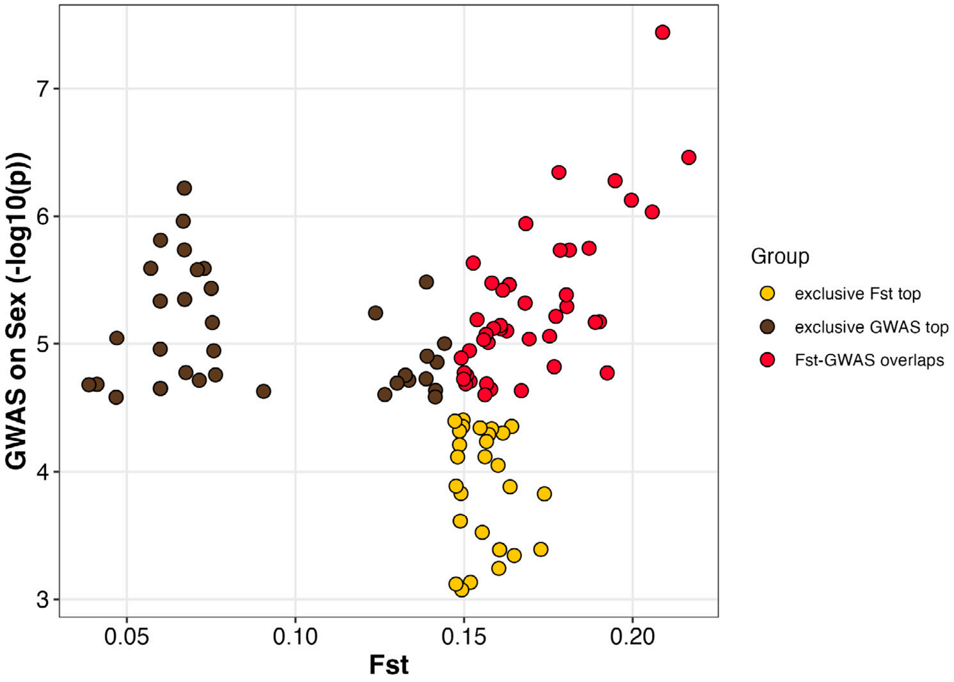


Figure 19. Comparing F_{ST} and significance of association with sex from GWAS for the top 100 SNPs in respective analyses, with the SNPs exclusively identified in F_{ST} -based approach (yellow), exclusively identified in GWAS approach (brown), the SNPs identified in both F_{ST} -based and GWAS approaches (red).

5 Conclusions and Perspectives

Sex can be genetically determined by sex chromosomes. To study the timing of recombination cessation (t_{RC}) between sex chromosomes, I presented two alternative phylogenetic approaches (ELW and BEAST) to circumvent problems present when using two popular methods (dS and ML). Moreover, I investigated whether and how much males and females have differentiated in two fundamental mechanisms generating genetic variation: germline mutations and recombination. For the DNMs, I found a strong male bias on the number of DNMs with approximately three times as many mutations in males than in females. The recombination rates were similar between the sexes. However, I found distinct sex-specific recombination landscapes near the telomeres. Compared to females, the pattern of higher recombination rates in sub-telomeric regions and lower recombination rates in centromeric regions in males could be caused by the obligate CO requirement, the general telomeric effect, and the sex-specific centromeric effect in the CO positioning. I implemented the methodology of analysing recombination positions in *RecView*, an interactive R application to facilitate future studies aiming to localise recombination. In addition, I used F_{ST} -based and GWAS approaches to search for the sexually antagonistic loci in the genome of the great reed warbler, and identified 50 overlapping SNPs which could be the candidate loci for future investigations of sexually antagonistic selection in this species.

There are several possible directions for future studies.

5.1 The t_{RC} on added part of the neo-sex chromosome among Sylvioidea songbirds

The alternative phylogenetic approaches proved to be effective using the Sylvioidea sequences dataset with focus studying the timing of recombination cessation between Z and W gametologs of great reed warblers. It was therefore feasible to ask how the non-recombining region of the neo-sex chromosomes among Sylvioidea songbirds has expanded over time. With the Sylvioidea sequences dataset used in this study, the t_{RC} on the added part of the neo-sex chromosome had two important implications: i) the added part of the neo-sex chromosomes functioned as a PAR after the fusion, and ii) another chromosomal rearrangement(s) occurred within the added part after the fusion. So far, it is established that there is variation in the composition of the enlarged neo-sex chromosomes among Sylvioidea species. For example, the added part involves parts of chromosome 3, 4A, and 5 in skylark (*Alauda arvensis*), parts of chromosome 4 and 4A in zitting cisticola (*Cisticola juncidis*), and parts of chromosome 4A and 8 in northern crombec (*Sylvietta brachyura*) (Sigeman *et al.*, 2019, 2020, 2022). Investigating t_{RC} on these added parts would allow us to identify the chromosomal regions, as well as the genes, that have maintained recombination for some time after the fusion. Moreover, further analyses could reveal whether these t_{RC} coincide with the timings of fusion or if the added part had functioned as a PAR after the fusion. In addition, we may gain insights in whether recombination cessation is a prerequisite for the degeneration of sex chromosomes and in the rate of degeneration of the sex-limited sex chromosome (W) by comparing the degree of gene loss with the t_{RC} for different chromosomal regions.

5.2 The dominance of mutations for adaptive evolution

The rate of new mutations under positive selection determines the speed of adaptive evolution (Ellegren, 2007). Notably, the speed of adaptive evolution can also be affected by the chromosome types, because the hemizygous state of a recessive mutation on the X (or Z) chromosomes in the heterogametic sex will expose it to selection. Theoretical studies have found that the different levels of dominance under a male mutation bias scenario affect the rate of evolution between chromosome types, as Z-linked loci have the fastest substitution rate when the additive mutations have a dominance coefficient below 0.32, followed by X-linked and autosomal loci (Kirkpatrick and Hall, 2004). When the mutations have a dominance coefficient above 0.79, the substitution rate on autosomal loci exceeds Z-linked loci and W-linked loci (Kirkpatrick and Hall, 2004). Therefore, evaluating how the male mutation bias could possibly contribute to rapid adaptive evolution entails investigations into the dominance of new mutations.

5.3 Recombination hotspots in birds

The search for genes determining the locations of recombination events continues. So far, it has been found that the PRDM9 determines the locations of recombination events in humans, mice, and some other mammals. There are some other candidate genes identified by phylogenetic analyses. For example, the presence or absence of *ZCWPW1* and its paralog *ZCWPW2* together with *PRDM9* seem to play a role in regulating recombination locations (Cavassim *et al.*, 2022). In birds, although stable recombination hotspots were reported in the zebra finch, there is so far no evidence that *PRDM9* is present, nor *ZCWPW1* and its paralog *ZCWPW2* (Singhal *et al.*, 2015; Cavassim *et al.*, 2022).

The association between PRDM9 and recombination locations was identified by comparing the sequences from recombination hotspots and cold spots to identify the enriched DNA motif, which will be largely facilitated by a fine-scale recombination landscape. In my study of recombination, it is challenging to obtain a fine-scale landscape of changes in recombination rates with only 224 COs being located, considering that there are approximately 1.2 billion sites in the whole genome of great reed warbler. Future studies analysing more pedigrees are therefore warranted.

5.4 GWAS in studying sexual antagonism: larger sample size or improved methods?

GWAS statistically tests the association between a SNP and a trait by quantifying how much phenotypic variation within the population can be explained by the SNP. The power of GWAS lies in the possibility to identify the true positives (causative SNPs) which could be swamped by the false positives (non-causative SNPs) having an association by chance, regardless of the LD decay. Traits governed by a small number of loci with large effect sizes would be amenable for GWAS, and in such cases even a study based on less than 100 samples can produce meaningful results (Atwell *et al.*, 2010). In contrast, GWAS is difficult with traits that have polygenic architecture, *i.e.*, when the traits are controlled by many common variants, but each has a small phenotypic effect. Detecting these loci may need thousands of samples, as seen in many GWAS studies on humans (Sulem *et al.*, 2007; Wellcome Trust Case Control Consortium, 2007). A GWAS will also be hampered when there are many rare causative variants, even if each has a large phenotypic effect.

Using GWAS to search for signs of sexual antagonism in the genome could be similar to analysing a polygenic trait. Increasing the sample size may improve the power of the analysis, but it is also worthwhile to consider conducting GWAS with a potentially better responsive variable. The phenotypic sex differences can be partitioned into a component with the same effect between the sexes, called the sexual concordant index, and into another component showing an antagonistic effect between the sexes, called the sexual antagonistic index. Graphically, it is equivalent to rotating 45° for the Cartesian coordinates where the axes represent the responsive variable in each sex, respectively. As a result, the coordinates on the new horizontal axis represent sexual concordance, and the coordinates on the new vertical axis represent sexual antagonism. Using GWAS to analyse the association between the SNPs and only the sexually antagonistic index may minimise the noise from non-causative SNPs with association just by chance. In practice, this approach was employed to calculate the sexually antagonistic indices from fitness measurements in hemiclonal males and females of lab *Drosophila*, and the GWAS managed to locate 230 chromosomal clusters of sexually antagonistic loci with robust significance (Ruzicka *et al.*, 2019). Nonetheless, this transformation could be difficult to accomplish in a wild population where hemiclonal individuals are scarce.

In addition, GWAS can be hampered by its nature of performing the statistical test at each SNP individually, which disregards any potential correlation structures among different loci. Some alternative approaches incorporating machine learning framework have been proposed to improve the analysis of GWAS (Enoma *et al.*, 2022), for example, the COMBI algorithm uses a machine learning framework to determine a subset of candidate SNPs and next performs standard GWAS on these SNPs (Mieth *et al.*, 2016).

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Additional information

The figures are also accessible on GitHub through the link:
https://github.com/HKyleZhang/Thesis_Figure_and_Supplementary.git
or the QR code:

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性别差异的基因组研究

List of papers

I. Hongkai Zhang, Hanna Sigeman, Bengt Hansson. 2022. Assessment of phylogenetic approaches to study the timing of recombination cessation on sex chromosomes. *Journal of Evolutionary Biology,* 35, pp.1721-1733, doi: 10.1111/jeb.14068.

II. Hongkai Zhang, Max Lundberg, Maja Tarka, Dennis Hasselquist, Bengt Hansson. 2022. Male-driven mutations: a pedigree-based study in the great reed warbler. Manuscript.

III. Hongkai Zhang, Bengt Hansson. 2022. RecView: an interactive R application for viewing and locating recombination positions using pedigree data. *bioRxiv,* doi: 10.1101/2022.12.21.521365.

IV. Hongkai Zhang, Max Lundberg, Dennis Hasselquist, Bengt Hansson. 2022. A genomic analysis of a great reed warbler pedigree reveals sex-specific recombination landscapes. Manuscript.

V. Hongkai Zhang, Max Lundberg, Maja Tarka, Dennis Hasselquist, Bengt Hansson. 2022. The genetics of sex differences: searching for signs of sexual antagonism in the genome of the great reed warbler. Manuscript.

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