Sex chromosomes in willows

Evolutionary studies of the ZW sex chromosome system in *Salix viminalis*

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Cover: “The sequence of sex determination”. Colored dots, representing genomic sequence from the *Salix viminalis* sex determination region (Green: A, Red: T, Blue: G, Yellow: C), form – by variation of dot size and color intensity – the image of a single male (left) and female (right) flower of *S. viminalis*. The flower images are extracted from Thomé & Müller (1886, Tafel 171, public domain).

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Abstract

Sexual reproduction is found in most eukaryotes and has in the majority of animal species led to the evolution of separate sexes. In contrast, only 5-6% of all angiospersms are dioecious with female and male flowers on separate individuals. Interestingly, dioecy has evolved hundreds of times independently and at different timepoints in angiosperms. The development of separate sexes requires a sex determination mechanism, which often is located on sex chromosomes. The independent evolution of numerous sex determination and sex chromosome systems in angiosperms allows for studies of processes involved in different stages of their evolution. The ratio between male and female individuals in a dioecious population is expected to be equal due to frequency dependent selection. Distorted sex ratios are however common in many plant and animal species.

The overall aim of my thesis was to investigate the sex chromosome system in the dioecious, perennial willow species *Salix viminalis* that both in natural populations as well as in lab populations often displays female biased sex ratios. Although dioecy evolved from hermaphroditic ancestors before the split between *Salix* and its sister genus *Populus*, we found that the two lineages have different sex chromosomes (*Populus*: Chr. 19, *Salix*: Chr. 15). As we found no evidence for translocations between these chromosomes, it is most likely that two different sex determination mechanims are present in the two lineages, meaning that sex chromosome turnover has occurred recently. We furthermore determined that *S. viminalis* is female heterogametic (females Z/W, males Z/Z) and has a single sex determination locus on chromosome 15. The W homolog of the sex determination region contains hemizygous, female specific sequences and the SNP density in this region is increased in females relative to males, witnessing of lost recombination between the Z/W homologs. We did not find a Fast-Z effect or major degeneration of the W chromosome, suggesting a recent evolution. In fact this sex chromosome system is among the youngest observed so far. Based on our data, the insertion of repetitive sequence and sex specific gene expression appear to be among the first processes to happen in sex chromosome evolution. We also determined that female biased sex ratios in *S. viminalis* are likely caused by an allelic incompatibility between Z homologs which results in the lack of one expected male genotype, reducing the male frequency in the population.

My studies thus extended our knowledge on processes involved in sex chromosome evolution and evolution of biased sex ratios in *S. viminalis*. However, given how common these phenomena are, results from my research can be applied to most organisms with genetic sex determination.

Keywords: sex chromosomes, Salicaceae, sex determination, sex ratio, GWAS, QTL, GBS, *Salix*

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Könskromosomer i viden. Evolutionära studier av ZW-könskromosomsystemet i *Salix viminalis*

Sammanfattning

Sexuell reproduktion finns i de flesta eukaryoter och har lett till att majoriteten av alla djurarter har evolverat separata kön. Dock är separata kön ett relativt sällsynt fenomen bland fröväxter (angiospermer) och förekommer i endast 5-6% av alla arter (växten kallas då för dioik eller tvåbyggare). Bland angiospermer har dock separata kön utvecklats hundratals gånger oberoende av varandra och vid olika tidpunkter. Utvecklingen av honor och hanar hos dioika arter kräver som regel en könsbestämningsmekanism som ofta är positionerad på specifika könskromosomer. Då olika könsbestämnings- och könskromosomsystem har uppstått så många gånger bland angiospermer utgör dessa ett enastående system för studier av evolutionära processer involverade i evolutionen av könskromosomer i olika utvecklingsstadier. Hos arter med separata kön förväntar man sig att en population har ungefär lika många honor och hanar, det vill säga att könskvoten är lika med 1. Det antas att en jämn könskvot är ett resultat av frekvensberoende selektion. Trots detta så förekommer skeva könskvoter i många djur- och växtarter.

Det huvudsakliga syftet med min avhandling var att studera könskromosomsystemet i den dioika, perenna videarten *Salix viminalis* som intressant nog ofta både i naturliga populationer och i labbpopulationer uppvisar en skev könskvot (då med flera honor än hanar). Trots att separata kön evolverade från hermafroditiska anfäder innan Salixsläktet separerade från systersläktet Populus, har arter i dessa två släkten olika könskromosomer (Populus: Chr. 19, Salix: Chr. 15). Eftersom vi inte hittade några tecken på translokationer mellan dessa två kromosomer är det rimligt att anta att dessa två släkten har olika könsbestämningsmekanismer, vilket pekar på att dessa nyss evolverade (ett fenomen som kallas för snabb “turnover”). Vi har också visat att honor hos *S. viminalis* är heterogametisk och har en Z- och en W-homolog i ett könsbestämningslokus på kromosom 15 (hanar har istället två Z-homologer). W-homologen består bl.a. av en hemizygotisk, honspecifik sekvens och mängden av den genetiska variationen är förhöjd i honor jämfört med hanar, vilket tyder på en avsaknad av rekombination mellan W- och Z-homologen i honor. Vi fann dock inga tecken på “Fast-Z-effekten” eller storskalig förfall av W-homologen, vilket tyder på att könskromosomsystemet i *S. viminalis* är bland de yngsta som hittills har beskrivits. Våra resultat visar att en ansamling av repetitiva DNA-sekvenser och en förändring mot könsspecifika genuttryck är bland de första processer som sker när könskromosomer evolverar från ett autosomalt kromosompar. Vi visade också att den honliga skeva könskvoten i *S. viminalis* troligtvis uppstår på grund av allelinkompatibilitet mellan två Z-homologer, vilket resultarar i att en förväntat hangenotyp saknas, vilket leder till en reduktion av hanfrekvensen i populationen.

Mina studier har ökat kunskapen om mekanismer involverade i könskromosomevolution och evolutionen av skeva könskvoter i *S. viminalis*. Men då dessa fenomen är så pass vanligt förekommande, kan resultat från min forskning tillämpas på de flesta organismer med genetisk könsbestämning.

What makes it difficult is that research is immersion in the unknown. We just don't know what we're doing. [...] If we don't feel stupid it means we're not really trying.

— Martin A. Schwartz (2008)

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Pascal Pucholt**[⋆], Ann-Christin Rönnberg-Wästljung[⋆] and Sofia Berlin (2015): Single locus sex determination and female heterogamety in the basket willow (*Salix viminalis* L.). *Heredity* 114, 575-583

II Henrik R. Hallingbäck[⋆], **Pascal Pucholt**[⋆], Estelle Proux-Wéra, Allison Churcher, Björn Nystedt, Ann-Christin Rönnberg-Wästljung[◇] and Sofia Berlin[◇]: Genome-wide association mapping uncovers female hemizygous regions on the W chromosome in willows. (manuscript).

III **Pascal Pucholt**, Alison E. Wright, Lei Liu Conze, Judith E. Mank and Sofia Berlin: Early stages of sex chromosome evolution in the ancient dioecious willow *Salix viminalis*. (under review).

IV **Pascal Pucholt**, Henrik R. Hallingbäck and Sofia Berlin: Allelic incompatibility can explain female biased sex ratios in dioecious plants. (under review).

⋆,◇: equal contribution

The contribution of Pascal Pucholt to the papers included in this thesis was as follows:

I Pascal analyzed the data on recombination rates, performed syntheny analyses between the *Salix viminalis* genetic map and physical maps of other species, created the figures and was taking part in the interpretation of the results and the manuscript writing.

II Pascal analyzed the linkage by Fisher's exact test, performed lab work, did genome analysis and took part in writing the manuscript.

III Pascal contributed to the design of the research, performed the lab work, analyzed the data and wrote the manuscript in collaboration with the other authors.

IV Pascal contributed to the design of the research, performed the experiments, analyzed data on marker segregation, generated the linkage map and wrote the manuscript in collaboration with the other authors.

These additional papers were written during the course of my doctoral studies but are not included in this thesis:

- Johan Fogelqvist, Alla V. Verkhozina, Alexander I. Katyshev, **Pascal Pucholt**, Christina Dixelius, Ann Christin Rönnberg-Wästljung, Martin Lascoux and Sofia Berlin (2015): Genetic and morphological evidence for introgression between three species of willows. *BMC Evolutionary Biology* 15:193

- **Pascal Pucholt**, Per Sjödin, Martin Weih, Ann Christin Rönnberg-Wästljung and Sofia Berlin (2015): Genome-wide transcriptional and physiological responses to drought stress in leaves and roots of two willow genotypes. *BMC Plant Biology* 15:244

- Srilakshmy L. Harikrishnan*, **Pascal Pucholt*** and Sofia Berlin (2015): Sequence and gene expression evolution of paralogous genes in willows. *Scientific Reports* 5:18662

*: equal contribution

Abbreviations

AFLP amplified fragment length polymorphism
CMS cytoplasmic male sterility
GBS Genotyping-by-Sequencing
GWAS genome-wide association study
LAMP loop mediated isothermal amplification
NGS next generation sequencing
PAR pseudoautosomal region
RAPD randomly amplified polymorphic DNA
SNP single nucleotide polymorphism

1 Introduction

Sexual reproduction is found in most eukaryotes and it is based on separate female and male gametes (Bachtrog *et al.*, 2014). This separation is extended – so to say – to an organism level in species with separate sexes with either male or female organs in different individuals. Separate sexes are the predominant state in most species of the animal kingdom (Jarne & Auld, 2006) and the functional sex of individuals is in many cases regulated by genetic factors located on sex chromosomes with a sex specific segregation pattern.

In flowering plants (angiosperms), separate sexes (dioecy) are the exception, found in only 5-6% of all species (Renner, 2014). In contrast, most angiosperm species have hermaphroditic flowers with both male and female functions which is considered to be the ancestral state. Phylogenetic data indicates that dioecy has evolved many times (851 - 5000 (Renner, 2014)) independently from hermaphroditic ancestors and sex chromosomes, which are found in several dioecious species, are often evolutionary young (Charlesworth, 2015, and references therein). Angiosperms constitute therefore exceptional systems to study the mechanisms underlying the evolution of separate sexes, sex chromosomes and sex determination mechanisms.

Without other selective pressures it is expected that frequency dependent selection maintains an equal ratio between male and female individuals (Fisher, 1930; Lloyd, 1974; Hardy, 2002). Thus, understanding the background to distorted sex ratios – which are found in many plant and animal species (Field *et al.*, 2013; Pipoly *et al.*, 2015) – will reveal selective forces and molecular mechanisms that shape the interaction between the sexes. Non-Mendelian inheritance of sex chromosomes can be the driving force behind biased sex ratios but alternative mechanisms are presented as well.

Willows (*Salix*) are woody angiosperm trees and shrubs that are nearly universally dioecious with homomorphic sex chromosomes. The *Salix* genus comprises a large number of species (400-550 (The Plant List, 2013; Renner, 2014)) of which many can be easily propagated clonally and high seed counts in a number of species allow the generation of large study populations. In multiple species the sex ratio is uneven, often with a surplus of females. *Salix* and *Populus* are sister genera in the Salicaceae plant family and show high genetic and genomic similarity. Interestingly, the sex determination systems are not shared between the genera and different chromosome pairs have evolved into sex chromosomes in the two taxa, indicating turnover of the sex chromosomes since the split of the genera. Therefore studies on the sex chromosomes in willows are highly interesting and can give insights into their complex evolutionary history and the background to sex chromosome turnover as well as the genetic basis for biased sex ratios.

This thesis starts with an introductory part, providing a broad background to the evolutionary context in which sex chromosomes evolve and an overview over mechanisms that can lead to biased sex ratios. The results of my studies are thereafter summarized in an overview over the articles which are included in the thesis in the form of separate scientific manuscripts in the last section.

1.1 Sexual reproduction

Sexual reproduction can be defined as any means of reproduction that involves a step of meiosis (Beukeboom & Perrin, 2014, p. 3). Meiosis is thought to be an evolutionary adaption for DNA repair (Bernstein *et al.*, 2011; Mirzaghaderi & Hörandl, 2016) and a way to reset epigenetic signaling and thus rejuvanating the cell line (Gorelick & Carpinone, 2009). The recombination of genetic elements from different genomes that generates new allelic combinations and thus provides the basis for selection is, however, usually considered the most important effect of meiosis (Weismann, 1889; Burt, 2000).

In the light of the Red Queen Hypothesis (Jaenike, 1978) and in the context of an ever changing environment the rapid generation of new genetic combinations is beneficial. It also counteracts the Hill-Robertson interference (Hill & Robertson, 1966) in finite populations. Experimental evolution in yeast (*Saccharomyces cerevisiae*) confirms the importance of sex for adaption to new, challenging environments (Goddard *et al.*, 2005).

However, recombination through sexual reproduction can also disrupt favorable genetic combinations and in this way it might reduce the fitness of a population under the rare condition of constant selection pressure (de Visser & Elena, 2007; Otto, 2009).

Importantly, sexual reproduction has, in most animal species and in some plant species, led to the evolution of separate sexes, and if sex determination is genetically controlled, sex chromosomes often evolve. In plants, the evolution of sex chromosomes is according to theory (Charlesworth & Charlesworth, 1978) tightly linked with the evolution of dioecy.

1.2 Dioecy and the evolution of separate sexes in plants

Dioecy, the complete separation of sexes into different individuals with unisexual flowers, is found in only 5-6% of angiosperm species (Renner, 2014) which are however spread over a large part of the angiosperm phylogenetic tree (43% of all families) (Renner, 2014) and thus dioecy must have evolved from an ancestral hermaphrodictic stage hundreds or even thousands of times (Charlesworth, 2002; Renner, 2014).

There are multiple selective forces that are speculated to be involved in the evolution of dioecy. Obviously, dioecy ensures cross pollination to 100% and thus inbreeding avoidance is likely to be a driving force in its evolution (Baker, 1959; Maynard Smith, 1978). At the same time the separation of sexes allows for a specialization for only one reproductive function, and selection for optimal resource allocation might thus be involved in the evolution of dioecy (Charnov *et al.*, 1976; Freeman *et al.*, 1997). Additionally pollen discounting can reduce the paternal fitness of a (partially) selfing plant (Kohn & Barrett, 1994; Harder & Barrett, 1995) by retaining pollen at structures in the same individual (e.g. stigma) which is therefore not released for cross pollination. This effect is reduced by dioecy. All of these forces might be involved simultaneously in the evolution of dioecy and do not exclude each other (Baker, 1984; Charlesworth & Guttman, 1999).

The evolution of complete sexual separation from hermaphrodites requires two major changes in floral structure. On the one hand co-sexual flowers need to change to unisexuality and on the other hand those unisexual flowers need to be separated to different individuals. For flowers to become unisexual, they need to abandon the development of either their male or female organs, a process that can happen during different stages in the development and that likely involves changes in at least two genes (see below). It is unlikely that all these changes happen simultaneously (Ainsworth, 2000) and thus dioecy is expected to evolved through intermediate stages. Both monoecy and gynodioecy are likely candidates for such intermediate stages (Barrett, 2002).

1.2.1 Dioecy via gynodioecy

A transition from hermaphroditic to one type of unisexual individuals is possible with one single mutational change (Charlesworth & Charlesworth, 1978). If a mutation leads to male (or female) sterility in all flowers, this individual will be functionally female (or male). However, the individuals in the population that do not express this new mutation will still be hermaphroditic. A population with both female and hermaphroditic individuals is gynodioecious while a population with both males and hermaphroditic individuals is androdioecious. Due to resource limitations, high pollen output will limit the number of ovules produced by a cosexual plant and *vice versa*. But siring opportunities are presumably limited mainly by ovule availability (Charlesworth & Guttman, 1999, p. 34) which means that higher pollen output from a pure male individual – gained by resource reallocation – might provide less fitness benefit than higher seed output gained by a pure female plant. Therefore gynodioecy is expected to be selectively more advantageous than androdioecy. This theoretical prediction is supported by the observation that many more gynodioecious than androdioecious species exist (Charlesworth & Guttman, 1999, p. 32; Renner, 2014) and that the phylogenetic distribution of the androdioecious species indicate that it is likely a state derived from dioecy (Charlesworth & Guttman, 1999, p. 33; Barrett, 2002; Pannell, 2002). Field tests could show that the increased fitness of females over hermaphrodites can be strong enough to grant them selective advantage (Schultz & Ganders, 1996; Sakai *et al.*, 1997).

If the fraction of females in a population increases over time, pollen can become limited and thus the remaining hermaphrodictic individuals can gain higher fitness by reallocating resources to pollen production and increase their male role in reproduction. This process can then lead to the complete separation of the sexes and to the evolution of dioecy (Barrett, 2002). The combination of female sterilizing and male sterilizing mutations on a homologous chromosome pair can initialize the evolution of sex chromosomes (see below).

As the evolution of dioecy through gynodioecy is based on a mutation with a strong effect leading to male sterility and potentially multiple subsequent mutations with weaker effects, gradually leading to female sterility, inconstant sex expression (e.g. (genetic) males with few female flowers) is expected to be found more often in males (Lloyd, 1980). Additionally, inconstant sex expression in females might lead to the total loss of outbreeding fitness gain (i.e. few pollen-producing flowers might self-fertilize most ovules of an inconstant female) while (selfed) seed set of a small number of seeds in a male plant might not be under strong negative selection (Lloyd, 1980; Webb, 1999). Thus, gynodioecy as an intermediate stage can be assumed if the molecular mechanism identified through the analysis of the sex determination system of a dioecious species fit these predictions.

In principal, dioecy can evolve through a very similar pathway from a monoecious (separate male and female flowers on the same individual) population. In this case a single mutation with strong effect can lead to the abortion of all male flowers and thus to pure female plants. Subsequently the remaining monoecious individuals undergo masculinization by reducing the number of female flowers (Webb, 1999; Dorken & Barrett, 2004).

1.2.2 Dioecy via monoecy-paradioecy

Both early studies (Lewis, 1942) and recent analyses of a large dataset (Renner, 2014) found a strong correlation between the presence of dioecy and monoecy in plant families. This might indicate that monoecy (separate male and female flowers on the same individual) could be a transitional state in the evolution of dioecy and since in monoecy unisexual flowers have already evolved, the transition to dioecy might be facilitated. In the monoecy-paradioecy pathway the transition from monoecy to dioecy is expected to happen gradually by changing the male to female flower ratio within individuals (Barrett, 2002; Dorken & Barrett, 2004). In this scenario a single key gene is thought to regulate the flower sex ratio and diversifying selection can lead to alternative alleles of this gene to produce pure males or pure females (Renner, 2016; Charlesworth, 2016). The chromosome with this key gene can develop as sex chromosome (see below).

Due to the incremental change towards dioecy, in this pathway the fitness gains through resource reallocation or inbreeding avoidance can be incremental as well and the immediate two-fold fitness increase required for the gynodioecy pathway is not needed (Lloyd, 1980).

There are several examples where dioecy likely has occurred from monoecy through the intermediate paradioecy state (Lloyd, 1975, 1981; Webb, 1999) indicating the relevance of this pathway in at least some cases. But dioecy can also evolve from monoecy by mechanisms similar to the gynodioecy pathway (see above) (Webb, 1999; Dorken & Barrett, 2004) and the strong correlation of monoecy and dioecy found on family level does not hold on a genus level, indicating that the correlation might be partly due to a common potential for the formation of unisexual flowers (Baker, 1984). It is therefore not sure how common this pathway is.

1.2.3 Other paths to dioecy

Plants have developed different mechanisms to prevent self fertilization and some of them might give rise to dioecy by increased diversification. Both temporal separation of the ripening of male and female organs (dichogamy / duodichogamy / heterodichogay) (Bertin & Newman, 1993; Webb, 1999; Renner *et al.*, 2007) and spatial separation of sexual organs within a flower (herkogamy) in different morphs (distyly/heterostyly) (Webb & Lloyd, 1986; Belaoussoff & Shore, 1995; Pailler *et al.*, 1998; Webb, 1999) reduce selfing and could incrementally evolve into female and male morphs specialized for pollen or seed production. Some evidence for such pathways is found (Pailler *et al.*, 1998; Webb, 1999), however, little is know about the selective mechanisms and the genetic basis involved in these transition (Barrett, 2002).

While studies on the evolution of dioecy often focus predominantly on the selective forces and morphological changes that are involved, these changes must be based on molecular mechanisms and genetic or environmental factors that regulate the distinct morphology of males and females and thus determine sex. Genetic sex determination factors are the basis for the evolution of sex chromosomes.

1.3 Sex determination

1.3.1 Sex determination systems and sex chromosomes

The mechanisms of sex determination has fascinated scientists and philosophers for a long time from Aristotle (384-322 BC) (cited in Mittwoch, 2005) to today.

Many of the speculations in the early times were aimed to understand how sex is determined in humans. Here, for a long time, environmental factors such as diet during pregnancy, but also for example the side of the womb in which the embryo develops were postulated as causative factors of sex determination (Mittwoch, 2005, and references therein).

In 1891 Herman Henking described for the first time a sex chromosome in the fire wasp *Pyrrhocoris apterus* as an element that behaved strangely during meiotic divisions (Henking, 1891). As he was unaware of its function he termed it the “element X” which lead to the naming of a type of sex chromosomes as “X chromosomes”. In 1905 Netty Stevens then described the heterochromosomes of the mealworm *Tenebrio molitor* and demonstrated that in this species one out of 20 chromosomes in males is smaller than the other 19 while females posses 20 chromosomes of equal size. She concludes that “it seems certain that an egg fertilized by a spermatozoön which contains the small chromosome must produce a male” (Stevens, 1905) and thus she discovered the common genetic X/Y male heterogametic sex determination system. It was only several years after this finding that Painter (1921) described the human X/Y sex chromosome systems and laid the grounds to understand the genetic basis of sex in our species and the first sex chromosome system in a dioecious plant (*Silene latifolia*) was discovered soon thereafter (Blackburn, 1923).

The discovery of heteromorphic X/Y sex chromosomes was a major step in understanding sex determination systems. But over time it became obvious that there are many different and variable sex determination systems in different animal and plant species and not one single sex determination system universal to all species with separate sexes. Even within apparently similar systems as the male heterogametic X/Y system, found in many species, either the Y chromosome can play the active role and contain a dominant factor that leads to maleness of the bearer as for example in mammals (Goodfellow & Lovell-Badge, 1993) or papaya (*Carica papaya*) (Wang *et al.*, 2012; Wu & Moore, 2015) or the absence of a second X chromosome decides over the development as a male, and the dose between X and autosomes is the actual determining factor as in *Drosophila melanogaster* (Hodgkin, 1990).

Similarly, an active factor on one of the sex chromosomes can lead to femaleness as for example in the silkworm (*Bombys mori*) (Fujii & Shimada, 2007) and thus individuals with two identical sex chromosomes without this factor (denoted as Z/Z) develop as males and females are the heterogametic sex (Z/W). Alternatively, also in female heterogametic systems the Z to autosome ratio can determine sex, a model found for example in chicken (Smith *et al.*, 2009).

While genetic sex determination is most common in those species with known mechanisms (Bachtrog *et al.*, 2014), there is even some truths to be found in the ancient hypotheses that attributed sex determination to conditions during pregnancy. Environmental stimuli like temperature, photoperiod and social factors can indeed determine offspring sex in different species (e.g. Bull, 1980; Warner *et al.*, 1996; Walker, 2005; Guler *et al.*, 2012; Kobayashi *et al.*, 2012; Merchant-Larios & Díaz-Hernández, 2012; Czerwinski *et al.*, 2016). And even random expression variance of key genes in the sex determination pathway could be enough to determine an individuals functional sex and thus sex determination might even have a random component in some species (Perrin, 2016).

In plants, environmental effects on the sex ratio (i.e. the relative number of males and females in a population) are well documented (see below), stable determination of offspring sex by environmental factors might be however of minor relevance since in this kingdom sex specific phenotypes – in most species – are limited to flowers which are generated from the meristems late in life and thus sex is – so to say – determined many times in environmental diverse conditions during a plants lifetime. Environmental sex determination should therefore not give rise to individuals with the same sexual function in every reproductive season. In some cases however transitions from one sex to another are observed during the lifetime of plants. Such labile sex expression can be regulated by the environment (Freeman *et al.*, 1980; Zimmerman, 1991; Korpelainen, 1998; Dorken & Barrett, 2004) as well as the extend to which hermaphrodite individuals invest in their female vs. male function (Freeman *et al.*, 1980; Korpelainen, 1998; Sánchez Vilas & Pannell, 2014).

But also with genetic sex determination variations from the system of distinct nuclear sex chromosomes are observed. Cytoplasmic male sterility (CMS) is caused by genes transmitted through cytoplasmic organelles, often mitochondria, and thus shows always a non-Mendelian maternal segregation pattern which facilitate its spread in the population (Dufay *et al.*, 2009). The associated spread of pure female individuals in the population can create the selective environment for a (nuclear) male fertility restorer gene to emerge (Frank, 1989; van Damme *et al.*, 2004; Delph *et al.*, 2007). In fact CMS is speculated to be the most common mechanism underlying gynodioecy (Frank, 1989; Hanson, 1991; Klaas & Olson, 2006; Dufay *et al.*, 2009) and it is found in many plant species (van Damme *et al.*, 2004; Klaas & Olson, 2006; Dufay *et al.*, 2009; Chen & Liu, 2014; Ashman *et al.*, 2015) with some indications of a cryptic CMS locus even in *A. thaliana*, suggesting a gynodioecious past of this highly selfing hermaphroditic species (Gobron *et al.*, 2013). But importantly, due to the maternal segregation of the cytoplasms, cytoplasmic female-sterile mutations would prevent the spread of themselves and thus pure cytoplasmic sex determination can not lead to complete separation of sexes.

Sex can also be determined by multiple alleles in one locus or multiple loci on different chromosomes where one factor can be dominant over other factors or through additive and epistatic effects (Kosswig, 1964; Moore & Roberts, 2013; Bachtrog *et al.*, 2014). Such systems are found for example in pygmy mice (*Mus minutoides*) (Veyrunes *et al.*, 2010) and cichlid fish (*Metriaclima* spp.) (Ser *et al.*, 2010) and polygenic sex determination was also described for the model species *Danio rerio* (zebrafish) (Bradley *et al.*, 2011; Liew *et al.*, 2012; Nagabhushana & Mishra, 2016). However, a study on wild zebrafish lines indicate one strong sex determination locus that was potentially lost during domestication (Wilson *et al.*, 2014; Nagabhushana & Mishra, 2016). In several plant species, multiple nuclear loci are described to interact to restore CMS (Belhassen *et al.*, 1991; Koelewijn & van Damme, 1995; Ashman *et al.*, 2015). However, it is not clear if polygenic sex determination systems are evolutionary stable or if they reflect a transition in sex determination systems (Moore & Roberts, 2013).

Finally sex can be determined by the absence or presence of the whole sex chromosome (XX/X0 system) as found for example in nematods (Hodgkin, 1987) or by the ploidy (haploid or diploid) as for example found in hymnopteran insects (Dzierzon, 1845, as cited in Gempe *et al.*, 2009; Verhulst *et al.*, 2010).

1.3.2 Molecular mechanisms of sex determination

While the mode of sex determination (e.g. X/Y or Z/W) is identified in an ever increasing number of species (Renner, 2014; The Tree of Sex Consortium, 2014; Gamble *et al.*, 2015) the detection of the underlying molecular mechanism and regulatory pathways of sex determination are known in much fewer species. In well established model systems, sex determining genes were detected years ago (mammals *SRY* (Gubbay *et al.*, 1990; Sinclair *et al.*, 1990; Li *et al.*, 2014), birds *DMRT1* (Smith *et al.*, 2009)) but the molecular sex determining mechanisms in dioecious plant species start to emerge only now.

Akagi *et al.* (2014) studied for example the male heterogametic (X/Y) sex determination system of Caucasian persimmon *Diospyros lotus* and revealed the key regulator for purely male flowers *OGI*. The genus *Diospyros* contains ca. 475 species of subtropical and tropical trees that, based on current knowledge, all are dioecious. Male flowers have stamens that are fertile but the pistils are rudimentary and arrested. In female flowers the anthers are developed but defective and do normally not produce pollen grains. By screening pooled sequencing data for male specific genomic regions and analyzing RNA expression data, the authors could establish a list of candidate genes and demonstrated that the small RNA OGI is the female sterilizing factor and suppresses the expression of the MeGI mRNA.

For the naturally monoecious melon plant *Cucumis melo*, Boualem *et al.* (2015) unraveled a cascade of several transcription factors – namely *CmACS11*, *CmWIP1* and *CmACS-7* – mediating ethylene production and they could show that the controlled expression of these factors regulates the expressed sex of flowers. Deactivation of *CmWIP1* due to epigenetic changes – triggered by the insertion of a transposable element into the promoter region of the gene – leads to plants with only female flowers (Martin *et al.*, 2009) while a non-functional *CmACS-7* leads to plants with only male flowers (Boualem *et al.*, 2008). Based on this knowledge and together with the discovery of the key-regulator *CmACS11*, Boualem *et al.* (2015) could generate an artificial but stable dioecious line of *C. melo* with homozygous recessive *Cmacs11*/*Cmacs11* in all plants and heterozygous *CmWIP1*/*Cmwip1* males and homozygous *Cmwip1*/*Cmwip1* females.

These examples show that in dioecious plants, different regulatory pathways can lead to the separation of sexes. In fact, in plants, any gene that is required for the successful development of stamen or carpel could act as sex determining factor or being a direct target of that factor. Changes in function or expression pattern of such genes could easily lead to male or female sterility (e.g. Lewis, 1941; Robinson-Beers *et al.*, 1992; Byzova *et al.*, 1999;

Stintzi & Browse, 2000). Gene expression studies in *Arabidopis thaliana* revealed hundreds of genes to be specifically expressed in stamen as well as carpels (Wellmer *et al.*, 2004), highlighting the large number of genes involved in the development of these tissues. Thus the number of potential developmental processes leading to sex determination is huge, especially if the flowers start to develop as perfect hermaphroditic flowers and organ abortion happens during the development. It appears that there is not even a preferred stage – from organ initiation to post-meiosis – at which organ abortion is happening among the systems studied so far (Diggle *et al.*, 2011). But as floral organ identity is determined by a conserved set of genes described in the ABC model (Haughn & Somerville, 1988) this set of genes and their regulators are key candidates for sex determination genes in species where the “undesirable” organ is not initiated. Confirmatory for this hypothesis, sex determination in the dioecious species *Thalictrum dioicum* and *Spinacia oleracea*, which only initiate one type of sexual organs, has been linked to the regulation of B class genes by yet unknown factors (Di Stilio *et al.*, 2005; Pfent *et al.*, 2005; Sather *et al.*, 2010).

So with the independent evolution of dioecy in hundreds of cases (Ming *et al.*, 2011; Renner, 2014) in the angiosperm clade it is quite likely that even hundreds of different systems of molecular sex determination exist and knowledge of the sex determination system in one species is only a weak indication of the genes that are involved in sex determination in another species. But by understanding the diversity of plant sex determination systems it will be possible to determine if general patterns are present and to fully understand evolutionary forces that lead to the evolution of separate sexes.

1.4 Sex chromosome evolution

With an enormous variety in sex determination mechanisms, it might appear as if independent evolution of sex determination results in unique systems with little shared characteristics. However, sex chromosome evolution follows a similar pattern in many lineage from different kingdoms and both male and female heterogamety. This is of no surprise as the two phenomena: sex determination mechanism and evolution of sex chromosomes are connected but not identical.

Sex chromosomes are chromosomes in the genome of an organism that contain genetic sex determination factors and they evolved several times independently in different animal taxa (Lahn & Page, 1999; Ross *et al.*, 2005; Rice, 1996) and many times in angiosperm plants (Ming *et al.*, 2011; Papadopulos *et al.*, 2015; Charlesworth, 2016). The evolutionary origin of sex chromosomes are ordinary autosomes (Rice, 1996; Bachtrog, 2013; Bachtrog *et al.*, 2014; Wei & Barbash, 2015). The pathway towards sex chromosomes is, according to an established theory, thought to involve two different mutations on the homologous chromosomes, one leading to male sterility (thus to female individuals) and one leading to female sterility (thus to male individuals) (Charlesworth & Charlesworth, 1978). These can be the same mutations that lead to dioecy in the first place (see above) or they can occur on a new autosomal pair in an already dioecious species and lead to sex chromosome turnover (see below). The mutations are expected to happen subsequent to each other on the same chromosome pair (Charlesworth & Charlesworth, 1978) (see fig. 1). Dependent on the type of these mutations (dominant or recessive) and the order in which they occur, the heterogametic sex can be females (Z/W) or males (X/Y) and the intermediate state of the population can be either gynodioecy or androdioecy (Bachtrog *et al.*, 2014) (fig. 1). Some of the evolutionary pathways are considered more likely than others which can explain the abundance of male heterogametic systems and gynodioecy as intermediate stage. But theoretically male and female heterogamety can be reached through both gynodioecy and androdioecy (Bachtrog *et al.*, 2014).

Alternatively a single key gene might regulate the relative investment in male or female function (either in hermaphrodites or monoecious individuals) and diversifying selection can lead to alternative alleles, producing males or females (Renner, 2016; Charlesworth, 2016).

1.4.1 Reduced recombination

In both cases, the genetic sex determination factor(s) on the incipient sex chromosomes will, however, experience sex specific inheritance patterns, with one homolog being always found in the heterogametic sex (sex limited Y or W chromosome) and the other homolog (shared X or Z chromosome) found in both sexes. This sex-specific inheritance of the sex determination locus opens the possibility for genes located in close linkage to it to evolve in a sex specific way and alleles on the proto-W chromosome to gain female beneficial effects (or proto-Y: male effects). Such effects might be (mildly) deleterious for the opposite sex and thus selection will favor reduced recombination between those sexually antagonistic alleles and the sex determining factor to ensure their linkage (Fisher, 1931; Rice, 1987, 1992; Qiu *et al.*, 2013; Wei & Barbash, 2015). The case of two opposite sterility genes is in this context the most extreme possibility of sexual antagonism and in case of recombination between them, half of the offspring would be neuters which will result in strong negative selection against recombination (Charlesworth & Charlesworth, 1978; Charlesworth, 2016). Different mechanisms to reduce recombination can thus evolve, or the sex determination system can establish in a region of *a priori* low recombination rate for example the centromers

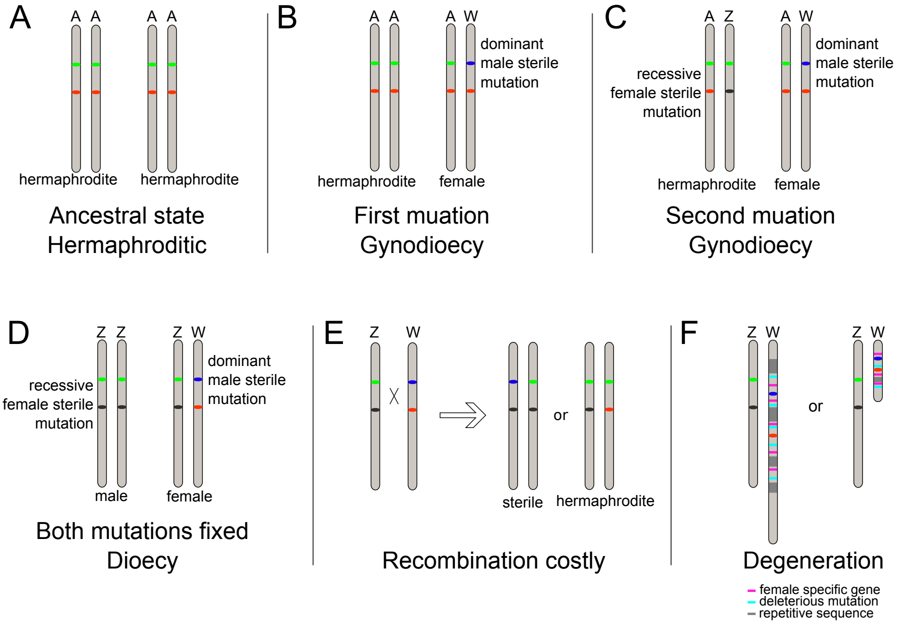

Figure 1: Possible pathway of sex chromosome evolution. Starting from an ancestral hermaphroditic population (A) a dominant male sterility mutation occurs on one chromosome pair (B). As a result individuals carrying this mutation will be female (not producing pollen) and only contributing to the populations reproduction as mothers. The population is in a gynodioecious state. On a chromosome, homologous to the one with the first male sterility mutation, another recessive female sterility mutation occurs (C). Individuals that are homozygous for the second mutation will loose their female function and thus will act as males and contribute only as fathers to the reproduction within the population. If both mutations became fixed the population is dioecious (D). Due to the selective cost to recombination between the two mutations (E) mechanisms to avoid recombination develop and the sex limited sex chromosome will acquire sex specific functions but also degenerate by deleterious mutations, accumulating repetitive sequence or loosing large parts of its content (F). In a similar pathway with a primary recessive male sterile mutation an X/Y sex chromosome system can evolve.

(Telgmann-Rauber *et al.*, 2007; Yu *et al.*, 2007; Kersten *et al.*, 2014). Thus the sex determination locus is expected to be located in a genomic region where recombination has halted.

The molecular mechanism underlying the block of recombination is however not completely understood. The evolutionary strata – spatial clusters of X-Y or Z-W orthologs with similar divergence estimates – found on many sex chromosomes for example in birds (Moghadam *et al.*, 2012; Wright *et al.*, 2012, 2014), mammals (Lahn & Page, 1999; Ross *et al.*, 2005), fish (Roesti *et al.*, 2013; White *et al.*, 2015), snakes (Vicoso *et al.*, 2013a; Yin *et al.*, 2016) as well as plants (Bergero *et al.*, 2007; Hough *et al.*, 2014; Papadopulos *et al.*, 2015) indicate a stepwise extension of non recombining regions on sex chromosomes. It was hypothesized that inversions of parts of the sex chromosomes are the cause for this pattern (Charlesworth *et al.*, 2005; Wright *et al.*, 2016). However, young sex chromosomes show a more gradual expansion of non recombining regions and thus the swift effect of an inversion appears unlikely as the cause for recombination suppression (Pala *et al.*, 2012a; Bergero *et al.*, 2013; Natri *et al.*, 2013). In this scenario inversions, that are found between sex chromosomes, might instead be the consequence of a relaxed selection for retained syntheny but the reduced selection against inversions is caused by other means.

Independent of the underlying cause, reduced recombination allows additional sexually antagonistic genes to evolve in or transposition to the sex determining region which in turn can lead to suppression of recombination in a larger area (Bergero *et al.*, 2013). Often recombination between the homologous sex chromosomes is retained in distal regions which are thus behaving just as autosomes and are termed pseudoautosomal regions(PARs) (Lahn *et al.*, 2001).

1.4.2 Degeneration of sex chromosomes

Recombination is an essential process that separates advantageous and deleterious mutations on the same chromosome and thus allows deleterious mutations to be purged from the population (see the section “Sexual reproduction” above). The cessation of recombination between the sex chromosomes will therefore, in the long run, lead to degeneration of the non-recombining part of the sex limited sex chromosome (W or Y) (Charlesworth, 1996; Berlin & Ellegren, 2006). The effect of degeneration due to lost recombination was well described in old animal sex chromosomes before (Rice, 1996; Skaletsky *et al.*, 2003; Wright *et al.*, 2016) and recent insights from genomic studies in the dioecious plant *Silene latifolia* confirmed its relevance in the plant kingdom (Papadopulos *et al.*, 2015). Results from the plant species *Rumex hastatulus* in which different sex chromosome systems co-occur also demonstrated that sex limited sex chromosomes accumulate deleterious mutations and unpreferred codon usage in a time dependent manner (Hough *et al.*, 2014). Population genetic processes, such as selective sweeps, background selection, and Muller's ratchet, result in a reduction in effective population size (N_e) of the sex limited sex chromosome beyond the 1:3 ratio due to its inherent segregation pattern, which increase the likelihood of fixation of deleterious mutations by drift and thus a lower-than-expected levels of genetic variability can occur (Charlesworth & Charlesworth, 2000; Sachidanandam *et al.*, 2001; Berlin & Ellegren, 2004, 2006; Berlin *et al.*, 2007; Moghadam *et al.*, 2012; Hough *et al.*, 2014). Moreover, following the arrest of recombination, gene expression on the sex chromosomes can change as well, due to transcriptional decay of the sex-limited chromosomes (W or Y) resulting in reduced expression of the W (or Y) allele compared to the corresponding Z (or X) copy. This process has been shown to occur quickly (Bachtrog *et al.*, 2008; Hough *et al.*, 2014; Papadopulos *et al.*, 2015).

Additionally, random insertions of transposable elements and other repetitive sequence might extend the lengths of non-recombining sex limited regions (Hobza *et al.*, 2015; Li *et al.*, 2016) but sequence loss will ultimately take over, leading to reduced size of the sex limited sex chromosome and to the loss of many genes (Rice, 1996; Skaletsky *et al.*, 2003; Lahn *et al.*, 2001; Li *et al.*, 2016; Wright *et al.*, 2016). This leads to high levels of heterochromatic DNA (Blackburn, 1923; Carvalho, 2002; Bachtrog, 2013; Wei & Barbash, 2015) and low gene density (Rice, 1996; Zhou & Bachtrog, 2012; Bachtrog, 2013; Skinner *et al.*, 2016) in many old sex limited sex chromosomes. Genes that are retained on the sex limited sex chromosome can, however, be essential and are found to be concerned mostly with sex determination, fertility or ubiquitous function (Charlesworth *et al.*, 1987; Lahn & Page, 1997; Lahn *et al.*, 2001; Skaletsky *et al.*, 2003).

In the long run, the accumulation of deleterious changes on the sex limited sex chromosome will lead to the evolution of morphological clearly distinct (heteromorphic) sex chromosomes like they are found in most old systems. Some homomorphic sex chromosomes (e.g. European tree frogs (Stöck *et al.*, 2011), snakes (Vicoso *et al.*, 2013a), ratite birds (Mank & Ellegren, 2007; Vicoso *et al.*, 2013b; Yazdi & Ellegren, 2014)) are however old, but display limited levels of differentiation, indicating that loss of recombination has not spread very far from the sex determination locus. In such systems selective factors preventing or slowing down the degeneration of sex chromosomes – and thus the evolution of heteromorphic sex chromosomes – probably exist. These can for example be: a long haploid phase, incomplete dosage compensation, recombination of sex chromosomes in rare sex reversed individuals or low levels of sexual conflict (Wright *et al.*, 2016, and references therein). Tight regulation of the spread of repetitive DNA might also play a role in the fate of individual sex chromosomes (Hobza *et al.*, 2015).

Many homomorphic sex chromosomes are however young and in the early stage of degeneration (e.g. papaya (Liu *et al.*, 2004; Wang *et al.*, 2012), wild strawberry (Spigler *et al.*, 2008; Tennessen *et al.*, 2016), *Silene colpophylla* (Mrackova *et al.*, 2008), *Populus* (Geraldes *et al.*, 2015), *Megaselia scalaris, Megaselia abdita, Calliphora erythrocephala* (Vicoso & Bachtrog, 2015, and references therein)).

1.4.3 Sexualization of the recombining sex chromosome

Like the sex limited sex chromosome (W or Y), also the homologous shared sex chromosome that is recombining in the homogametic sex (Z or X) undergoes specialized evolution. As the W chromosome degenerates, it loses functional genes, rendering the Z chromosome effectively haploid (hemizygous) for these genes in females. The ratio between W linked genes and autosomal genes is halved compared to that of Z/Z males, creating a potentially detrimental imbalance for dosage-sensitive genes. To overcome this problem different dosage compensation systems have evolved, either by up-regulation of hemizygous genes in the heterogametic sex (Straub *et al.*, 2005; Naurin *et al.*, 2012) or by silencing of one allele in the homogametic sex (Senner & Brockdorff, 2009; Splinter *et al.*, 2011; Graves, 2016b) (but see Chen & Zhang (2016)). At the same time newly arisen recessive or partially recessive mutations in hemizygous genes on the shared sex chromosome are more often exposed to selection compared to autosomal genes and thus beneficial mutations can be fixed more rapidly by selection. Additionally, reduced effective population size can lead to higher rate of fixation of novel mutations due to drift. Therefore Z-linked (or X-linked) genes can be more divergent between species compared with autosomal genes, a phenomenon known as the ‘faster-X effect’ (Charlesworth & Charlesworth, 1987; Baines & Harr, 2007; Mank *et al.*, 2010; Connallon *et al.*, 2012; Meisel & Connallon, 2013). Similarly a faster-X (faster-Z) effect for gene expression can be seen (Kayserili *et al.*, 2012; Llopart, 2012; Meisel *et al.*, 2012; Dean *et al.*, 2015) which can be caused by faster evolution of *cis* regulatory non-coding sequence (Meisel & Connallon, 2013).

Dominant alleles on the proto-Z (proto-X) of genes that are retained on both homologs are expressed 2/3 of the time in males (X: females) and thus can similar to genes on the W (Y) chromosome obtain a sex specific effect due to sex biased selection, while recessive changes on the shared sex chromosomes are predominantly under selection in the hemizygous stage in the females (X: males) and thus are selected for benefits in the opposite sex (Rice, 1984; Wright *et al.*, 2012; Jaquiéry *et al.*, 2013). Conflicts arising from this sexualization of the shared sex chromosome can be solved by sex specific expression and thus genes on a sexualized shared sex chromosome are expected to be more commonly expressed in a sex specific way than genes on autosomes (Connallon & Knowles, 2005; Scotti & Delph, 2006; Ellegren & Parsch, 2007). Extensive sex specific expression might be sufficient to mitigate detrimental effects of sexual antagonistic genes and thus might be an alternative to recombination suppression between sex chromosomes in some clades, as for example seen in emus (Vicoso *et al.*, 2013b).

1.4.4 Sex chromosome turnover

The major differentiation and high functional specialization especially of old sex chromosomes might appear to be a dead end and it might seem unlikely that the sex chromosome system changes (Mank & Avise, 2009). None the less, new chromosomes actually can take over the role as sex chromosomes or established sex chromosomes can be extended by fusion events. These sex chromosome turnover events are happening often and in different taxa (Smith, 1964; Phillips *et al.*, 2001; Miura, 2008; Mank & Avise, 2009; Kitano & Peichel, 2012; Slancarova *et al.*, 2013; Bachtrog *et al.*, 2014; Yoshida *et al.*, 2014; Vicoso & Bachtrog, 2015; Mulugeta *et al.*, 2016). Turnover can also change the sex determination system and induce a transition from male to female heterogamety or *vice versa* and therefore turnover provides another evolutionary pathway to these systems than the evolution together with dioecy.

A new pair of autosomes can evolve into sex chromosomes and take over sex determination if a new sex determining mechanism is established on this chromosome pair which sets off a new round of sex chromosome evolution. This can occur through transposition or translocation of existing sex-determination genes, the fusion of sex chromosome and autosome or the evolution of a new sex determining mechanism (fig. 2) (Smith, 1964; Kitano & Peichel, 2012; Hough *et al.*, 2014; Yoshida *et al.*, 2014; Wei & Barbash, 2015). This transition can be facilitated by (partial) environmental sex determination as an intermediate step (Ezaz *et al.*, 2009; Quinn *et al.*, 2011; Holleley *et al.*, 2015; Graves, 2016a).

Different driving forces can theoretically induce sex chromosome turnovers such as sexually antagonistic selection (Charlesworth & Charlesworth, 1980; van Doorn & Kirkpatrick, 2007, 2010), heterozygote advantage (Charlesworth & Wall, 1999), selection for sex ratio bias (Fredga *et al.*, 1977), rescue of deleterious mutation on Y chromosomes (the “Hot-Potato model”) (Blaser *et al.*, 2013, 2014), a combination of genetic drift and heterozygote disadvantage (Lande, 1985; Yoshida *et al.*, 2014), the disruption of sex determination by endosymbionts (Cordaux *et al.*, 2011) or the presence

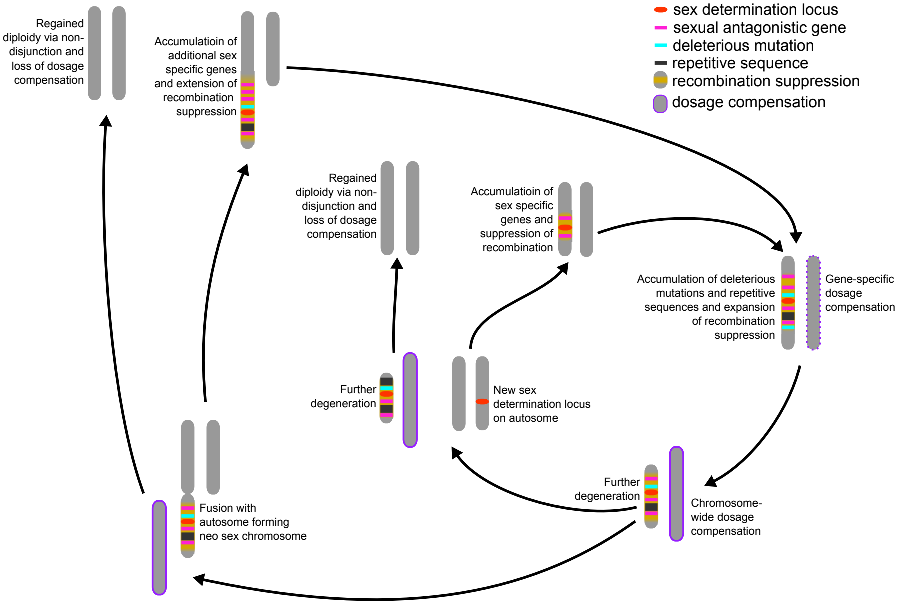

Figure 2: Cycle of sex chromosome turnover. New sex chromosomes can evolve by chromosomal fusion or acquisition of a new sex determination system. Illustration inspired by Wei & Barbash (2015).

of sex chromosome meiotic drive (Maynard Smith, 1978, p. 164,Yoshida & Kitano, 2012).

Sex chromosome turnover can lead to mating barriers between individuals with the old and the new sex determination system respectively (Graves & O'Neill, 1996; Graves, 2016a) and thus sex chromosome turnover might lead to speciation. The three major mammal lineages (Eutheria, Metatheria, Prototheria) are separated by sex chromosome turnover events and it is thus conceivable that the turnover of sex determination systems is causative for those speciation events (Graves, 2016a, and references therein).

Neo sex chromosomes can be the result of the fusion between an autosomal part and the former sex chromosomes or sex determination can be regulated by a new pair of former autosomes (fig. 2). In the first case the evolutionary pattern of sex chromosome divergence can extend to the new parts of the sex chromosome (Waters *et al.*, 2001; Pala *et al.*, 2012a,b; Bergero *et al.*, 2013; Hough *et al.*, 2014). In the case of a new sex chromosome system, emerging directly from an autosome however, the previous sex chromosome can revert to autosomal inheritance (Vicoso & Bachtrog, 2013, 2015; Wei & Barbash, 2015). Especially if the previous sex chromosomes had strongly diverged from each other, the reversion needs to overcome selection barriers which might contribute to the apparent stability of some old sex chromosome systems. As an initial step it is likely that the shared sex chromosome (Z or W) reestablishes diploidy, possibly through meiotic nondisjunction (Vicoso & Bachtrog, 2013), which is than followed by a loss of dosage compensation mechanisms (Wei & Barbash, 2015).

1.5 Deviations from equal sex ratios

By separating sexual functions into different individuals, selection can act differently on them. One effect of this sex specific selection is the evolution of sex chromosomes (see above), another effect can be the higher mortality or a selective advantage of individuals of one sex. Genetic sex determination creates also an environment where genetic elements can be inherited specifically to one sex. Such elements can benefit from a biased sex ratio that facilitates their spread in the population. However, frequency based selection is expected to maintain a balanced offspring sex ratio, provided that the cost of producing male and female offspring and their fitness is equal (Fisher, 1930; Lloyd, 1974; Hardy, 2002). None the less, both in the plant kingdom (Barrett *et al.*, 2010; Sinclair *et al.*, 2012; Field *et al.*, 2013) but also among animals (Westerdahl *et al.*, 1997; Donald, 2007; Neto *et al.*, 2011; Pipoly *et al.*, 2015) uneven sex ratios are common on a family and species-wide level due to often unknown genetic or ecological mechanisms.

Males and females invest different resources into reproduction and thus bear different costs (e.g. fruit formation or high amounts of pollen in wind pollination). This effect can lead to differences in resilience and sex-biased mortality which in turn influences the adult sex ratio (Crawford & Balfour, 1983; Delph, 1999; Donald, 2007; Petry *et al.*, 2016). As an additional factor, in species with heteromorphic sex chromosomes the heterogametic sex will be hemizygous for a large number of loci and thus unable to mask the expression of recessive deleterious alleles. According to the ‘unguarded sex chromosome hypothesis’ this will lead to higher mortality of individuals of the heterogametic sex (Haldane, 1922; Dorken & Barrett, 2004; Donald, 2007; Pipoly *et al.*, 2015).

But especially for plants, determining the sex of an individual that is not flowering (juvenile or non-reproducing) is difficult since sexual dimorphisms are usually limited to floral tissue and therefore there is a lack of knowledge on when in an organism’s life cycle the sex bias is introduced. Not only does adult sex ratio not necessarily reflect seed sex ratio but one sex might flower more often or have an earlier onset of flowering during life history and therefore the measured sex ratios might be biases by data collection (Barrett *et al.*, 2010). Molecular markers or cytological methods can in some cases be used to determine genetic sex without phenotypic differences and in an early life stage (Stehlik & Blattner, 2004; Xu *et al.*, 2004; Stehlik *et al.*, 2008; Abreu *et al.*, 2015; Tsai *et al.*, 2016) and these tools are therefore valuable for the understanding of mechanisms underlying sex ratio biases during the life history (Barrett *et al.*, 2010).

Sex chromosome meiotic drive is a mechanism that – due to non-Mendelian segregation of sex determination alleles – can influence the offspring sex ratio directly. A gene on a sex-determining chromosome, making that chromosome more likely to participate in fertilization, will increase its frequency – until checked by some counterbalancing force (Hamilton, 1967; Meiklejohn & Tao, 2010). An example for such a system is the Y-linked gene M^D in *Aedes aegypti* (Hickey & Craig, 1966; Newton *et al.*, 1976; Shin *et al.*, 2012). The presence of this gene in males results in an over-abundance of males in the offspring generation due to X chromosome breakage during male meiosis. Similar systems have been observed in *Drosophila* species (Gershenson, 1928; Novitski, 1947; Jaenike, 2001) and seem to play a role in the female biased sex ratio in *Silene latifolia* (Taylor, 1994; Taylor & Ingvarsson, 2003; Taylor, 1999).

Uneven transmission of sex chromosomes to the next generation can, in male heterogametic species, also be caused by selection in the haploid pollen stage and thus influence the sex ratio. This can be caused either by differential survival of the gametophyte (Smith, 1963) or different types of pollen can vary in their fertilization success in competitive situation (certation, e.g. differences in pollen tube growth) (Smith, 1963; Taylor, 1994, 1999; Taylor & Ingvarsson, 2003; Stehlik *et al.*, 2008) which can results in a higher abundance of embryos of one sex. Based on this mechanism sex ratios can vary dependent on population density for example in *Rumex nivalis* (Stehlik *et al.*, 2008).

In the androdioecious plant *Phillyrea angustifolia* males and hermaphrodites co-occur at approximately equal ratios (Lepart, 1992) which is of surprise as functionally androdioecious species are expected to exhibit low male frequencies in populations unless males have a strong selective advantage (Saumitou-Laprade *et al.*, 2010). It was shown that the genetic background to this system is a genetic incompatibility between two classes of hermaphrodites. Males are able to sire hermaphrodites of both incompatibility groups due to a “permissive” incompatibility allele in tight linkage to the sex determination locus (Saumitou-Laprade *et al.*, 2010). Detailed studies of pedigree populations based on different types of hermaphrodites and males revealed an additional selfish genetic element in some males, which is also linked to the female-sterility locus and which effectively prevents the formation of hermaphroditic offspring in one type of crosses. All offspring of these crosses inherited exclusively the Y-acting allele of the sex determining region from the father while expected hermaphrodictic siblings are purged from the population by a unknown mechanism that is potentially similar to gametophytic self-incompatibility (Billiard *et al.*, 2015; Pannell & Voillemot, 2015).

Beside nuclear genetic elements also units in the cytosol can influence the sex ratio. Since the cytosol is usually predominantly maternally inherited, symbionts and organells benefit from a female biased offspring sex ratio. Many insects, including *Drosophila* species but also other animals, often are associated with symbiotic bacteria (Moran *et al.*, 2008; McFall-Ngai *et al.*, 2013). Some symbionts like *Wolbachia*, *Spiroplasma*, *Cardinium* and *Arsenophonus* can act through male-killing, feminization, cytoplasmic incompatibility and parthenogenesis to alter the operational sex ratio among offspring of an individual bearing them and thus spread into their host populations in selfish ways (Werren *et al.*, 2008; Cordaux *et al.*, 2011; Hurst & Frost, 2015; Perlman *et al.*, 2015; Harumoto *et al.*, 2016). Similarly mitochondria are known to change the operative sex in different hermaphroditic plant species through cytoplasmic male sterility (CMS) (see above). This effect is sometimes referred to as sex ratio change (Bailey & Delph, 2007; Perlman *et al.*, 2015). Likewise, in species with completely separate sexes, effects on the genetic or operational sex ratio by organells – similar to the effects of symbionts – are conceivable but were not yet described (Matessi & Saino, 2003; Lane, 2006, p. 239; Perlman *et al.*, 2015).

1.6 The Salicaceae family

While this thesis studies aspects of sex determination, sex chromosome evolution and deviations from equal sex ratio which are of very general interest both within the plant kingdom but also beyond, we used the willow species *Salix viminalis* as a model. This plant species is a member of the Salicaceae family which also contains the well studied tree species *Populus trichocarpa* (Tuskan *et al.*, 2006) as well as several other species that are investigated for different aspects.

According to the current molecular evidence the Salicaceae family is a group of about 1000 species in ca. 55 genera (Cronk *et al.*, 2015b) with *Populus* and *Salix* forming a monophyletic clade and *Idesia* and *Bennettiodendron* being the closest sister genera (Leskinen & Alström-Rapaport, 1999; Liu *et al.*, 2016). Based on fossil records the divergence of the *Populus* and *Salix* genus started more than 45 mya (Boucher *et al.*, 2003; Manchester *et al.*, 2006). Species in the Salicaceae family are woody (trees or shrubs, fig. 3) with simple, usually alternate leaves (Cronk *et al.*, 2015b). The flowers of

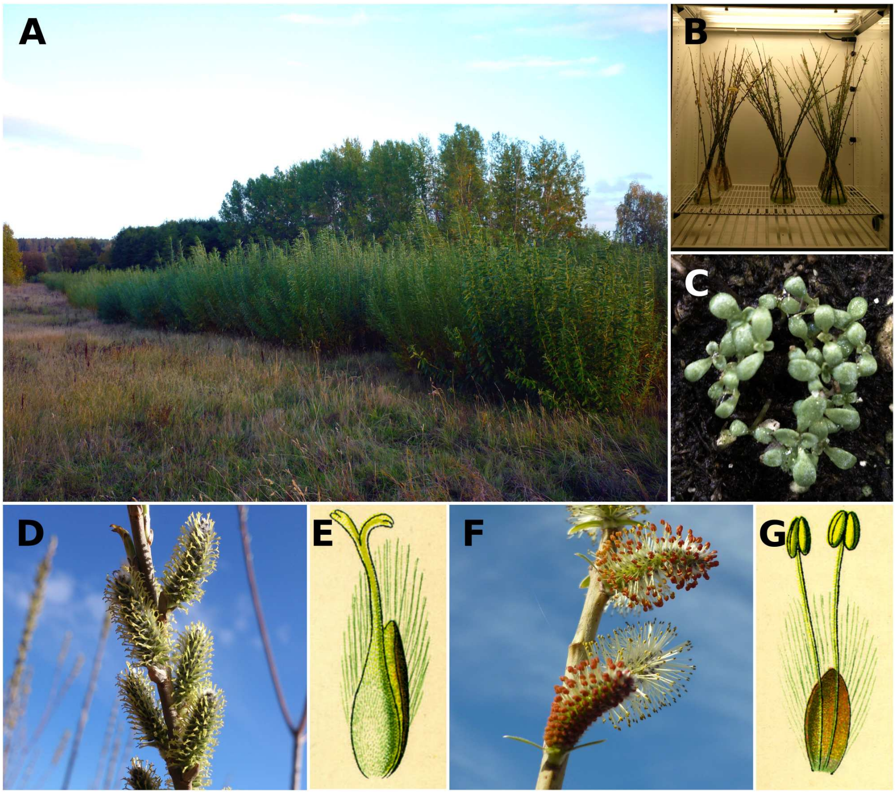

Figure 3: Physical appearance of *S. viminalis*. (A) Field archive with shoots regrown from rootstock during one growing season. (B) Growth in controlled conditions in a climate chamber. (C) Seedlings. (D) Female catkins with protruding gynoecia. (E) Schematic image of female limited reproductive organ (gynoecium) (F) Male catkins with protruding stamens. (G) Schematic image of male limited reproductive organs (filaments and anthers). Panel E and G are reproduced from Thomé & Müller (1886).

Populus and *Salix* species are arranged in catkins (racemose inflorescence, fig. 3) while sister genera usually show branched inflorescences (Cronk *et al.*, 2015b). Most genera in this family are dioecious and thus dioecy is likely an ancient trait in this taxon that evolved at least prior to the split of *Populus* and *Salix* (Cronk *et al.*, 2015b; Geraldes *et al.*, 2015).

The *Salix* genus comprises a large number of species (400-550 (Leskinen & Alström-Rapaport, 1999; Berlin *et al.*, 2010; The Plant List, 2013; Renner, 2014)) of which many can be easily propagated clonally and high seed numbers in some species allow the generation of large study populations. Willows are a “foundation species” and provide an abundant food-source for generalist and specialist animals such as rodents, deer, elk, reindeer and many insects

(Cronk *et al.*, 2015a, and references therein) and thus shape characteristic ecosystems. The genomes of willows and poplars are highly synthenic except for a major interchromosomal rearrangements involving poplar chromosome 1 and 16 and willow LG Ib (S3 map, Berlin *et al.* (2010)) / chromosome 16 (S5 map, paper IV) as a result of a fission or fusion event in one of the lineages. Wild populations and artificial crosses of many *Salix* species display distinct, mostly female biased sex ratios (Crawford & Balfour, 1983; Alliende & Harper, 1989; Dawson & Bliss, 1989; Alström-Rapaport *et al.*, 1997; Predavec & Danell, 2001; de Jong & van der Meijden, 2004; Ueno *et al.*, 2007; Myers-Smith & Hik, 2012; Che-Castaldo *et al.*, 2015).

Both poplars, aspens and willows are used as energy crop in short-rotation coppice systems with positive energy output (Karp & Shield, 2008; Dillen *et al.*, 2013; Murphy *et al.*, 2014) and detailed studies on the genetic background of traits related to breeding goals, for example on growth and phenology (Hallingbäck *et al.*, 2016) and drought resistance (Pucholt *et al.*, 2015b), are undertaken. The shared whole genome duplication between poplars and willows (Tuskan *et al.*, 2006; Berlin *et al.*, 2010) also allows to study evolutionary questions on, for example, the retention of paralogous genes (Berlin *et al.*, 2010; Harikrishnan *et al.*, 2015).

1.6.1 Sex determination in the *Populus* genus

Studies on sex determination in *Populus* species (poplars and aspens) revealed that likely multiple systems exist within this genus. While dioecy is ancestral and thus originated more than 45 mya, sex chromosomes seem not to have evolved into heteromorphic sex chromosomes and the sex determination mechanism(s) have evolved more recently (Geraldes *et al.*, 2015).

Genetic mapping studies have identified a sex-determining region in the proximal telomeric end of chromosome 19 in poplars (*Populus* sections Tacamahaca and Aigeiros) (Gaudet *et al.*, 2008; Yin *et al.*, 2008) and a pericentromeric region in aspens (*Populus* section Populus) (Pakull *et al.*, 2009; Paolucci *et al.*, 2010; Kersten *et al.*, 2012). There are reports on female heterogamety (Z/W system) (Yin *et al.*, 2008; Paolucci *et al.*, 2010) and male heterogamety (X/Y system) (Gaudet *et al.*, 2008; Pakull *et al.*, 2009; Kersten *et al.*, 2014; Pakull *et al.*, 2015) and for *Populus tremuloides* and *Populus tremula* it could be shown that the gene TOZ19 is hemizygous in males and absent in females, possibly indicating a role of this gene in sex determination (Pakull *et al.*, 2015).

The presence of at least two different sex determination systems in different sections of the *Populus* genus at different loci of chromosome 19 is also supported by a genome-wide association study (GWAS) in *P. trichocarpa* and *P. balsamifera* and comparative sequence analysis with *P. tremuloides*

(Geraldes *et al.*, 2015). Based on this data the Y-chromosmal region in *P. trichocarpa* is 6-7 my old and thus the sex determination system in this species is much more recent than dioecy (Geraldes *et al.*, 2015).

However in the context of our own results on the study of sex determination in *Salix viminalis* it is important to stress that in all *Populus* species studied so far chromosome 19 was identified as the sex chromosome (Tuskan *et al.*, 2012; Geraldes *et al.*, 2015, and references therein).

1.6.2 Previous knowledge on sex determination in the *Salix* genus

Sex determination in the *Salix* genus (willows) has previously attracted less attention than in *Populus*, partly due to the absence of a reference genome in this genus. However, a study mainly targeting the biased sex ratios in *S. viminalis* proposed a genetic multi-locus system of sex determination in this species (Alström-Rapaport *et al.*, 1997) and the genetic basis for sex determination was confirmed by the presence of randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers that were associated with sex in some genetic backgrounds (Alström-Rapaport *et al.*, 1998; Gunter *et al.*, 2003b; Semerikov *et al.*, 2003). These markers were linked to the morphological sex marker but the position of the markers in the genome was not resolved (Gunter *et al.*, 2003b; Semerikov *et al.*, 2003). However both the low number of sex linked markers (e.g. 1 in 1080 (Alström-Rapaport *et al.*, 1998)) and the evidence that recombination is present along large parts of the chromosome that contained sex linked markers (Semerikov *et al.*, 2003) indicate that a non-recombining region would be relatively narrow. The complete co-segregation of sex markers and phenotypic sex in a mapping population indicated a single genetic locus determining sex in this family (Semerikov *et al.*, 2003).

Gunter *et al.* (2003b) converted two of the sex linked markers into PCR based SCAR markers: SCAR UBC 354_{520} and SCAR $OPAE08_{780}$ which were also used in subsequent studies. Some attempts were made to amplify these markers in other species and to evaluate their correlation with sex. While unsuccessful amplification of the SCAR 354 marker in some *Salix* species indicate the absence of the marker region in these species (Temmel *et al.*, 2007) successful amplification and linkage with sex for the marker SCAR AE08 (Gunter *et al.*, 2003a) (but not SCAR 354) in *Salix eriocephala* indicate that the sex determination system might be shared over species boundaries. Similarity between the sequence amplified by SCAR 354 and a locus on the *P. trichocarpa* chromosome 15 gave a first indication of a potential genetic position of sex determination in *Salix* (Temmel *et al.*, 2007).

In summary, the knowledge on sex determination in the *Salix* genus prior to this work was limited and mainly focused on a small number of genetic markers with largely unresolved genomic location. They co-segregated with phenotypic sex in some but not all genetic backgrounds.

In parallel to my work, another group was independently studying sex determination in *Salix suchowensis* by applying QTL analysis to a mapping population (similar to our approach). Shortly after our publication of Paper I they could confirm one of our main findings, namely a single sex determination locus that is located centrally on chromosome 15 and thus on a different chromosome than sex determination in poplars but on the same locus as we describe it in *S. viminalis* (Pucholt *et al.*, 2015a; Hou *et al.*, 2015).

1.7 Next generation sequencing and big data as research tool

This work is based to a very large extent on the analysis of genomic and transcriptomic sequencing data from so called next generation (or second and third generation) sequencing (NGS) machines. Compared to previous methods of sequencing (e.g. Sanger sequencing (Sanger *et al.*, 1977)) the new innovation was the extremely high parallelization of the sequencing reaction. This field of technology is developing with an ever increasing speed and during the time of this thesis I witnessed both how previously widely used technologies (Roche 454 (Margulies *et al.*, 2005), ABI SOLiD (Shendure *et al.*, 2005)) were nearly abandoned as well as the rapid adoption of the third generation of sequencing technologies (PacBio (Eid *et al.*, 2009), Oxford Nanopore (Mikheyev & Tin, 2014)) that generate long reads from a large number of single molecules.

In a broader perspective this rapid technological development has led to an enormous rate of sequence data generation with a predicted doubling time of all sequence data ever generated of 7 to 18 month (Stephens *et al.*, 2015). This development has been fueled by a drop in sequencing costs that surpassed Moore's law (Moore, 1965) by orders of magnitude (Wetterstrand, 2016). Future biological research will thus have an even greater use of this type of data than current studies but handling and analyzing these data and their sheer amount requires large computational resources and new skills for the researcher.

As the number of applications of NGS data in all biological fields is enormous I will refrain from trying to give a general overview here and instead exemplify the use of sequencing and genotyping methods in my thesis project.

For Paper I we used Sanger sequencing (Sanger *et al.*, 1977) to identify polymorphisms in the parents of an experimental cross. These single nucleotide polymorphisms(SNPs) were then genotyped in the whole population using the Illumina GoldenGate high throughput method (Fan *et al.*, 2003). This combination of traditional sequencing to identify polymorphic sites and high throughput genotyping delivered a dataset that was large compared to similar studies.

For Paper II 291 individuals of a collection of wild and largely unrelated accessions of *S. viminalis* were genotyped using the technique developed by Elshire *et al.* (2011) called Genotyping-by-Sequencing (GBS). In this process the whole genome of an individual is in a first step reduced to regions in proximity to restriction enzyme cut sides and this reduced library is then sequenced. This technique makes it feasible to analyze a large number of individuals without prior knowledge of polymorphisms and it generates a high density of traceable SNPs in a nearly random distribution over the whole genome. In a GWAS we then analyzed the association between genotypes and the sex of individuals.

The same technique was used for Paper IV on 273 individuals of a mapping population to analyze association between genotypes and sex ratio, and gender and to generate a high density genetic map.

To link markers from paper II to genomic positions we additionally used a *S. viminalis* genome assembly based on PacBio RSII single molecule sequencing reads. This third generation sequencing technique proved to be highly valuable to obtain long genomic sequence scaffolds of the complex genome.

For Paper III we applied the Illumina HiSeq technique (Bentley *et al.*, 2008) to analyze the whole genome sequence of four *S. viminalis* individuals as well as the transcriptome from catkins and leaves of six individuals. The total sequencing data generated for this project was approximately 220 Gbp which is equivalent to 73 times the size of the human genome or 440 times the size of the *S. viminalis* genome.

With this huge amount of data comes an enormous power to detect scientifically interesting phenomena and many of the questions posed in this project would not be possible to answer without large datasets.

The data analysis usually requires the application of several tools/programs in different computational environments to convert raw data via multiple intermediate steps into results that lead to new insights. Tools in this process can be optimized algorithms, performing computationally expensive tasks like read mapping or variant detection, utilities to convert between different file formats or filtering the results based on given parameters or applications for plotting or statistical testing. Every one of these steps typically has a number of adjustable parameters which often need to be optimized to obtain the best analysis of the data at hand.

Such analyses can be seen as a workflow which van der Aalst & van Hee (2004) defined as a collection of tasks that are interdependent and thus need to be completed in a specific order to obtain a result or to create a product. In order to ensure reproducibility, to document the flow of data, the applied methods and parameters and to automate and optimize the task execution, workflow management systems are widely used in bioinformatics. Simple systems of workflow management can be shell scripts that execute a defined sequence of tasks. There are also browser based systems available that facilitate the analysis through simplified interfaces – as for example the Galaxy framework (Giardine *et al.*, 2005; Afgan *et al.*, 2016) that integrates many widely used analysis tools.

However, in many cases an increased flexibility and higher parallelization in the analysis of multiple samples with different sets of parameters can be achieved through a workflow definition that resembles a programming language. A popular system for workflow management that is based on the python language and allows for extremely flexible execution of widely variable workflows in diverse computational environments is the Snakemake system (Köster & Rahmann, 2012).

Current biological research does already now – and will do so even more in the future – use and depend on large datasets and thanks to the rapid development in sequencing technologies, but also the increased usage of imaging techniques etc. these datasets will become even larger. Therefore a modern research lab requires equipment and strategies to handle and analyze big data. In my thesis work I followed the whole process from planning and execution of the biological experiment, through DNA/RNA extraction to data generation, analysis and insights for several large collections of sequencing and genotyping data.

2 Aims of the study

Sexual reproduction is omnipresent in the eukaryote lineage and separate sexes are found in most animal and some plant species. Sex chromosomes are found in many of the species with separate sexes and they represent a unique evolutionary system. This thesis aims to contribute to the knowledge of sex chromosome evolution, sex determination and sex ratio distortion. We used the plant species *S. viminalis* as a model to gain knowledge about these processes. Therefore in a first stage we aimed to unravel the genomic architecture of sex determination in this species and to compare it to the system in the related species *P. trichocarpa* to then analyze the evolutionary history of the sex determination locus in greater detail. A specific feature of the model system *S. viminalis* is its pronounced but variable biased sex ratio. We thus aimed to identify mechanisms underlying this trait and to determine if sex determination and sex ratio distortion are controlled by the same or different mechanism.

The work had the following specific objectives:

- To determine the mode of sex determination in *S. viminalis*: genetic, environmental, single locus, multi locus
- To identify the genomic location of the sex determination factor(s)
- To analyze the mode of inheritance of sex determination (female / male heterogametic)
- To describe the effects that evolutionary processes had on the sex chromosomes and by this, to identify processes that happen early during sex chromosome evolution
- To elucidate factors regulating the biased sex ratios in *S. viminalis* and their relation to sex determination factors

3 Results and discussion

The results of this thesis are reported in the four articles reproduced in a thematic order at the end of this book. These four parts of the thesis each contain a detailed presentation of the findings and a discussion of the results in their context. Here I will give a short summary and highlight some results to present a more general picture of the work and the interconnection of the sub-projects. Articles that are part of this thesis are referred to as roman numerals.

3.1 Female heterogamety and chromosome 15 as sex chromosome in *Salix viminalis* (I, II & IV)

One important aspect of this thesis was to characterize the sex determination system of *S. viminalis* on a chromosomal level. Prior to this work it was speculated that the sex determination in willows is based on a multi locus sex determination system with dominance of certain loci over other (Alström-Rapaport *et al.*, 1997). These results were based on observed segregation patterns but the assumed loci were not located in the genome. In paper I we thus applied an open approach to identify any region in the genome that was linked to sex determination through a QTL study in a population with even sex ratio. Results from this analysis confirmed the genetic sex determination and did show that there was a single locus on chromosome 15 that did explain 97.9% of the variation for the trait "sex". Other than this, only marginally significant signals were found. We could also show that one haplotype of three markers on chromosome 15 was completely coupled to femaleness and that these markers were maternally segregating and that females were always heterzygous in those markers. Based on these results we concluded that females are the heterogametic sex (Z/W) in *S. viminalis* and that there is a single locus on chromosome 15 (fig. 4) that determines the sex in this pedigree population.

Even though the main aim of paper IV was to study mechanisms behind uneven sex ratios, data from this population was also very valuable to analyze the sex determination system further. The pedigree populations in

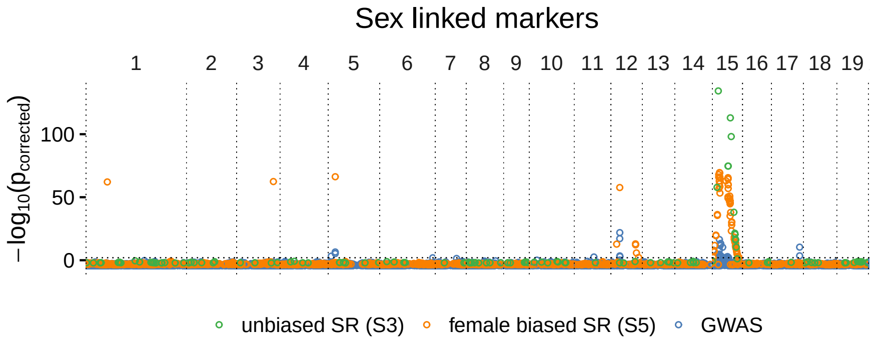

Figure 4: Association between genetic markers and sex. Markers from three populations (S3: even sex ratio, paper I; S5: female biased sex ratio, paper IV; GWAS: unrelated individuals, paper II) were located in the *P. trichocarpa* genome (x-axis) and the significance of their association with the trait “sex” was calculated (Fisher’s exact test, y-axis). Data from all populations highlight chromosome 15 as the sex chromosome.

paper I and IV share the same father but differ in the female parent. As the results from paper I showed that females are the heterogametic sex, this second population was ideal to test if there was any variance in the location of the sex determination locus between female individuals or if different loci in a potential multi-locus sex determination system would be dominant. The results from paper IV showed however that sex determination was based on the same single locus on chromosome 15 (fig. 4) also in this population and here as well, female heterozygosity was evident.

We could then extend these observations to a species wide level by applying GWAS to a large association mapping population with a high number of genetic markers in paper II. We detected 48 SNP markers that were significantly associated with sex and we could link the majority of these markers to chromosome 15 (fig. 4). Even here we demonstrated that females are the heterogametic sex and we found a correlation between heterozygous genotypes in the sex determination region and femaleness.

These findings are highly interesting as they clearly demonstrate the difference in sex chromosome systems (*Salix*: Z/W, Chr 15, *Populus*: X/Y or Z/W, Chr 19) between *Salix* and *Populus* species, while dioecy is ancestral to the Salicaceae family that contains both clades. It is thus likely that one (or both) clades underwent sex chromosome turnover events during their evolution.

Additionally we could show in paper II that some parts of the W sequence in *S. viminalis* are hemizygous and – at least partially – are paralogous to sequence on chromosome 9. The split between the paralogous sequences happened before the split from other *Salix* species (fig. 5) and thus the hem-

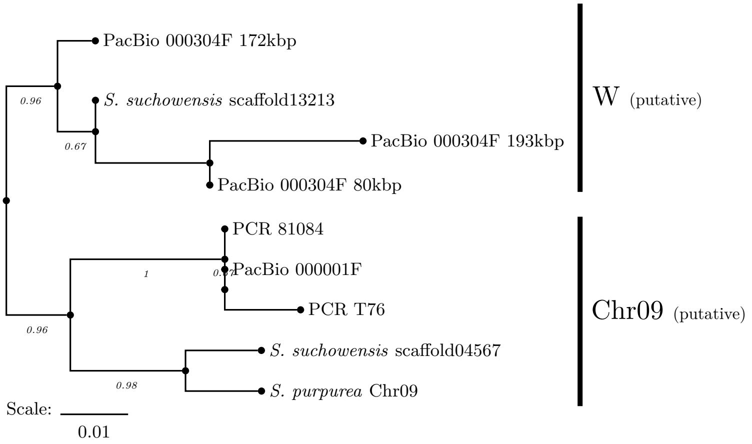

Figure 5: Phylogenetic tree of homologous sequences to the W-hemizygous region detected on chromosome 15. Details on the methods how this tree was generated and a discussion of its content is found in paper II.

izygous region is expected to be share over species boundaries suggesting that also the sex determination system is shared by other *Salix* species.

This insight, purely based on our own data, is supported by the observation that the same locus on chromosome 15 is determining sex in some other *Salix* species as well: *S. suchowensis* (Hou *et al.*, 2015), *S. purpurea* (personal communication). Contrary, a study in *S. herbacea* indicates the existence of additional loci associated with sex (Cortés, 2015). More studies in different *Salix* species would allow an analysis on how universal the sex chromosome system observed in *S. viminalis* is and to what phylogenetic level it is shared.

A possible mechanism in sex chromosome turnover would be the translocation of the sex determining region from one chromosome (e.g. Chr19) to another chromosome (e.g. Chr15). If this had happened, some genetic markers that are associated with sex might be located on the *S. viminalis* chromosome 15 while they have a location on chromosome 19 in *P. trichocarpa*. We specifically analyzed our datasets for this possibility but did not find any indications for a translocation between these two chromosomes. It is thus likely that different genetic elements underlay the sex determination in the *Populus* and *Salix* lineage respectively.

3.2 Sex chromosome divergence and the evolutionary history of the *S. viminalis* sex determination system (III)

Both theoretical predictions (Fisher, 1931; Charlesworth & Charlesworth, 1978; Rice, 1987, 1992; Qiu *et al.*, 2013) and actual observations in paper I and II suggest that some part of the *S. viminalis* sex chromosome experiences reduced or absent recombination. Analyzing the sequence divergence of this region allows for an approximate estimation of the age of the sex chromosome system which is of high interest in the context of the presumed sex chromosome turnover events in the Salicaceae family. Using *S. viminalis* as a model system however also allows new insights into the evolutionary processes involved in sex chromosome evolution in general.

In paper III we used DNA and RNA sequencing data from multiple female and male individuals and could show that a small non-recombining region exists at the sex determination locus. The level of sequence divergence and the absence of a Fast-Z effect indicate however a very recent evolutionary origin. The existence of genes in this region that are expressed in a sex specific way together with the absence of allele specifically expressed genes indicate that sex chromosome sexualization presumably predates severe W chromosome degeneration. We could also show that insertion of repetitive sequence into the sex limited sex chromosome likely happens early in sex chromosome evolution, a finding that is confirmed by results from paper II.

3.3 Z chromosome incompatibility as a basis for biased sex ratios (IV)

Biased sex ratios are common in different *Salix* species, both in wild populations and in pedigree populations (Crawford & Balfour, 1983; Alliende & Harper, 1989; Dawson & Bliss, 1989; Alström-Rapaport *et al.*, 1997; Predavec & Danell, 2001; de Jong & van der Meijden, 2004; Ueno *et al.*, 2007; Myers-Smith & Hik, 2012; Che-Castaldo *et al.*, 2015; Paper II). In paper IV we studied a population with a strong female bias (~66% females in population) and could demonstrate that individuals with a certain genotype are missing completely from the population and we attribute this to a form of genetic incompatibility. Since this incompatibility locus is coupled to the sex locus in such a way that the W-linked female determining region and the Z-linked incompatibility region are located on different homologous sex chromosomes, the incompatibility only affects males and thus leads to a female bias in the population. A similar incompatibility system was reported for the androdioecious species *P. angustifolia* were a selfish genetic element benefits from biased sex ratios (Billiard *et al.*, 2015; Pannell & Voillemot, 2015). Even though the incompatibility system, as we found it in this population, will lead to reduced spread of Z-chromosomes and thus should not benefit a selfish element, we could show that such an allelic incompatibility could be maintained in the species through a system of overdominance or pseudooverdominance.

Such a mechanism for sex ratio distortion is reported in very few systems, however it will only be detectable through high marker density in a reasonably large population. Detailed studies in other non-model species might therefore detect similar mechanism that went un-noted up to now. This mechanism can also explain biased sex ratios previously described in *S. viminalis* that were attributed to a multi-locus sex determination system.

3.4 Genome structure of *S. viminalis* (IV)

Based on the “S5” population, we generated a new genetic map for *S. viminalis* with a high marker density of GBS based genetic markers. This map confirmed previous findings of high syntheny between the genome of *P. trichocarpa* and the fission/fusion event between the chromosomes 1 and 16 since the split of the species. We used this tool also to anchor scaffolds containing sex associated markers to the genome in paper II and it will be of great use in the ongoing work to assemble the *S. viminalis* genome and for upcoming QTL studies in the S5 pedigree population.

4 Conclusion

During the course of this project we gained a multitude of new insights into the sex determination system, the evolution of sex chromosomes and the mechanism underlying biased sex ratio in *S. viminalis*. The open approach to identify factors involved in these processes has been exceptionally successful and extended the previous knowledge on sex determination in this species greatly. We analyzed, for the first time, the evolutionary history of *Salix* sex chromosomes and we suggest that the basis for biased sex ratio is incompatibility between certain alleles.

Some of the conclusion from this work are:

- In *S. viminalis* sex is determined by a single genetic locus on chromosome 15. The locus may however include multiple genes or other genetic elements. We confirmed this finding in multiple independent datasets and results from other *Salix* species, as well as phylogenetic analyses, indicate that it is shared over species boundaries.

- Females are the heterogametic sex in *S. viminalis* and thus carry two different homologous sequences (Z and W) in the sex determination region, while males carry two alleles of the Z type.

- The sex determination systems in the related species *S. viminalis* and *P. trichocarpa* are based on different genomic regions and sex chromosome turnover – likely with the emergence of a new sex determination mechanism – has happened at least once between the species.

- The sex determination region contains W-specific hemizygous sequence that is limited to females. Phylogenetic analyses indicate that the origin of this sequence predates the speciation.

- Within the sex determination region, recombination is reduced. The observed increased SNP density in females in the region is likely a result of this.

- The sex chromosomes show little sequence differentiation and no signs of extensive degradation or the Fast-Z effects. This indicates that they are evolutionary young.

- Insertion of repetitive sequence and sexualization of the sex chromosomes appear to be among the first processes to happen in sex chromosome evolution.

- Biased sex ratios in a *S. viminalis* pedigree population could be traced back to the absence of an expected male genotype. This observation indicates a role of allelic incompatibility as base for sex ratio distortion.

5 Future perspectives

In this work we demonstrated that *S. viminalis* has a single locus sex determination system in chromosome 15. While some data indicate that also other *Salix* species share this mechanism, it is not clear how universal the system that we describe here is in the genus. To investigate this question further, either unbiased mapping approaches, similar to this work could be undertaken in other species or the presence of the W-specific hemizygous region could be tested specifically. Such analyses should be accompanied by a phylogenetic analysis of the genus since the current classification in sub-genera and even species is partially contradictory in the literature (Leskinen & Alström-Rapaport, 1999; Azuma *et al.*, 2000; Cronk *et al.*, 2015a; Fogelqvist *et al.*, 2015) but a well resolved phylogeny is crucial to determine the evolutionary trajectory of the sex chromosome system found here.

The hemizygous regions in the genome of *S. viminalis* demonstrate the beginning of differentiation between the sex chromosomes. More work is however needed to understand if these hemizygous regions are actually causative in the sex determination or if they appeared as a consequence of stalled recombination between sex chromosomes that occurred due to other sex determining factors and sexually antagonistic genes. The currently ongoing project to assemble the *S. viminalis* genome with a specific focus to separate W and Z-linked sequences with the help of third generation sequencing data will prove useful in identifying candidate genes or other genetic elements that are causative in sex determination and will also clarify the extend and function of hemizygous regions.

To further characterize the genetic factors that are causatively involved in sex determination, expression studies during the flower development – especially during the specialization phase at the time were female and male floral tissue starts to develop differently – can give new insights. In a time series of RNA seq data the regulatory pathways can be traced back and in this way the factors that regulate the development into male or female flowers can be identified. To confirm the involvement of these candidates, transformation studies in *Salix*, *Populus* or *A. thaliana* could potentially be used.

To study the genetic basis of biased sex ratios more, we have already generated new pedigree populations as back-crosses, sibling-crosses and out-crosses to other individuals based on the genetic material from the “S5” population (paper IV). Due to the extensive set of well characterized markers for these populations, it will be possible to use molecular methods to determine the genetic sex of the offspring and we can thus analyze the sex ratios and the presence of genotypes based purely on molecular data from seedlings. The genome assembly can then be used to identify candidate genes that might be involved in the incompatibility system.

To be able to analyze sex ratios in vegetative populations of any age, and from a breeding perspective an easy to use universal molecular sex marker is desirable. The PCR primers we designed in paper II do already separate between females and males, presumably on a species wide level, but in some cases the banding pattern alone might not be sufficient to predict an individuals sex. Other binary markers, either based on PCR or loop mediated isothermal amplification (LAMP) (Tsai *et al.*, 2016) should be effortless to create based on the results from our studies and can be used to determine (and select on) the sex of individuals already in the seedling stage.

This work has thus laid the foundation for many more, highly interesting research projects.

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