The male-heterogametic sex determination system on chromosome 15 of *Salix arbutifolia* and *Salix triandra* reveals ancestral male heterogamety and subsequent turnover events in the genus *Salix*

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Abstract

Dioecious *Salix* evolved more than 45 million years ago, but have homomorphic sex chromosomes, suggesting that turnover event(s) prevented major differentiation. Sex chromosome turnover events have been inferred in the sister genus, *Populus*. The genus *Salix* includes two main clades, *Salix* and *Vetrix*, with several previously studied *Vetrix* clade species having female-heterogametic (ZW) sex-determining systems (SDSs) on chromosome 15, while two *Salix* clade species have male-heterogametic (XY) SDSs on chromosome 7. We here studied two basal taxa of the *Vetrix clade*, *S. arbutifolia* and *S. triandra*. Analyses of whole genome resequencing data for genome-wide associations (GWAS) with the sexes and genetic differentiation between the sexes (F_{ST} values) showed that both species have male heterogamety with a sex-determining locus on chromosome 15, suggesting an early turnover event within the *Vetrix* clade, perhaps promoted by sexually antagonistic or (and) sex-ratio selection. Changepoint analysis based on F_{ST} values identified small sex-linked regions of ~3.33 Mb and ~2.80 Mb in *S. arbutifolia* and *S. triandra*, respectively. The molecular mechanism of sex-determination remains unknown. Ancestral state reconstruction of SDS and stamen numbers suggests that at least two turnover events occurred in *Salix*. Willows with XY SDS usually have multiple stamens, while ZW species have two stamens.

Introduction

Angiosperms have sexual systems including hermaphroditism, monoecy, and dioecy, among others (reviewed by Baránková et al. 2020), and approximately 5-6% of flowering plant species are dioecious plants (Charlesworth 1985; Renner 2014). Sex determination systems (SDSs) have evolved independently in different dioecious plant lineages (Westergaard 1958; Bull 1985; Charlesworth 1985; Ming et al. 2011), and are classified into male (XX/XY) and female heterogametic (ZW/ZZ) systems. The chromosomes carrying sex determining regions (SDRs) may differ cytologically (heteromorphic) and include extensive non-recombining regions (Westergaard 1958; Bull 1985), but are often indistinguishable (homomorphic). It is thought that suppressed recombination has sometimes evolved to produce the heteromorphic state. Sex chromosomes often have recombining regions at one or both ends, termed pseudo-autosomal regions (PARs, reviewed by Otto et al. 2011). Genetic maps of dioecious plants are scarce, but the species for which maps or genomic data provide evidence about recombination suggest that plant sex chromosomes also have PARs, and that these are sometimes physically extensive.

In some taxa, sex chromosomes have been maintained for long evolutionary times. However, there are many examples of changes, termed “sex chromosome turnovers”, between closely related species (reviewed by Vicoso 2019 and Kuhl et al. 2021), including in fish, amphibia, and plants (reviewed in Renner and Müller 2021). Two major kinds of turnovers are possible. Either a new sex determining gene arises on an autosome (or in a different gene on the same chromosome as the previous sex-determining locus), replacing the ancestral one, in a process termed “allelic diversification” by Pan et al. (2021) and “SNP” or “expression” differences by Tao et al. (2021, see also Saunders et al. 2018), or a pre-existing sex determining gene (or set of genes) is transposed to a new genome location (termed “duplication” in Tao et al. and Pan et al. 2021); the new region created will be hemizygous in the heterozygous sex, and not present in the homozygous sex. Either type of turnover can create a new, non-degenerated sex-linked region (reviewed by Kuhl et al.

2021). Changes between female and male heterogamety also fall into two categories (Bull 1983; van Doorn and Kirkpatrick 2010). In “nonhomologous” transitions, the ancestral and the new sex determining loci are on different linkage groups, and the turnover converts the ancestral sex chromosome pair into an autosomal pair, as in the fish taxa Tilapia and *Oryzias* (reviewed in Pan et al. 2021). In homologous transitions, the chromosome that carries the sex determining gene does not change. A change from female and male heterogamety may, for example, occur, if a Z chromosome (whose sex-determining function is recessive to that of the W) acquires a masculinizing effect gene that is dominant to the W, and this Y chromosome replaces its homolog. This situation was found in Platyfish (Kallman 1984).

Recent studies have detected turnovers in plant lineages (Charlesworth 2015; Moore et al. 2016; Henry et al. 2018). A change from female to male heterogamety, or vice versa, involving different chromosomes, was discovered in section *Otites* of the genus *Silene* (Balounova et al. 2019; Martin et al. 2019). In *Populus* (in the family Salicaceae), heterogamety has apparently changed from female to male, or vice versa (Tuskan et al. 2012; Renner and Müller 2021). Whether these turnovers involved duplication or allelic diversification is not known, though, in both *Silene* and the Salicaceae, they involve changes of the sex determining locus to different chromosomes. In the wild North American octoploid strawberries (*Fragaria*) with female heterogamety, the “duplication” process is involved, as the sex-determining locus maps to three different chromosomes in different taxa; these turnovers therefore involve translocations to different chromosomes (Tennessen et al. 2018).

The ecological or genetic factors involved in observed turnovers are still unknown. Four hypotheses have been proposed: 1) accumulation of deleterious mutation on the sex chromosomes (Blaser et al. 2013, 2014), 2) neutrality, in which a new sex determining allele can undergo genetic drift (random variations of allelic frequencies) and become ‘fixed’ (Bull and Charnov 1977; Veller et al. 2017; Saunders et al. 2018), 3) selection for a new sex-determining system to equalise the sex ratio (Jaenike 2001; Werren and Beukeboom 1998; Scott et al. 2018), and 4) presence of a sexually antagonistic polymorphism on an autosome favouring the fixation of a new sex-determining locus in a genome region closely linked to it, as modelled by van Doorn and Kirkpatrick (2007, 2010).

The recent development of whole genome re-sequencing has created the possibility of identifying SDSs in non-model species, sometimes even if they are physically small, using genome-wide association study (GWAS) and analysis of sequence differentiation between the sexes (often using F_{ST}) (Gammerdinger et al. 2020; Franchini et al. 2018; Darolti et al. 2019; He et al. 2021a). This can reveal differences in SDS locations.

Populus and *Salix* are closely related genera in the family Salicaceae, almost all members of which are dioecious (Fang et al. 1999; Argus 2010). The two genera are estimated to have diverged about 45 million years ago, and to share a common dioecious ancestor (Dai et al. 2014; Tuskan et al. 2006). Despite this long history of dioecy, their sex chromosomes are homomorphic (Zhou et al. 2020; He et al. 2021a). The sex-determining locus is on different chromosome in different *Populus* species (reviewed in Renner and Müller 2021), and both XY and ZW systems exist in both genera (He et al. 2021a) (Table 1). These observations suggest the possibility of sex chromosome turnovers, and that at least four turnover events have been suggested in the Salicaceae (Manchester et al. 2006; Mank 2014, Yang et al. 2021). More recent studies

detected at least four turnovers in *Populus* alone; two translocation events may have occurred in *P. alba* (Li et al. 2022; Müller et al. 2020). Here, we study diploid *Salix* species.

The genus *Salix* includes ~450 species, in two main groups *Salix* and *Vetrix* (He et al. 2021b; Gulyaev et al. 2022). In studies of diploid species, male and female heterogamety have been found in the *Vetrix* and *Salix* clades, respectively (He et al. 2021a; Gulyaev et al. 2022). The *Salix* clade includes species from subgenera *Salix*, *Longifoliae* and *Protitea*, and the *Vetrix* clade includes subgenera *Chamaetia* and *Vetrix* (*Chamaetia*/ *Vetrix* clade), sect. *Amygdalinae* and the formerly recognized genera *Chosenia* (*S. arbutifolia*) and *Toisusu* (*S. cardiophylla*) (Wu et al. 2015; Lauron-Moreau et al. 2015; Gulyaev et al. 2022). Two *Salix* clade species have XY SDS on chromosome 7, including *S. nigra* (Sanderson et al. 2021) and *S. dunnii* (He et al. 2021a), whereas all five *Vetrix* clade species so far studied (*S. suchowensis*, *S. viminalis*, *S. purpurea*, *S. triandra*, and *S. polyclona*) have ZW SDS on chromosome 15 (Hou et al. 2015; Almeida et al. 2020; Zhou et al. 2020; Li et al. 2020; He et al. 2022). Willows usually have physically longer sex-linked regions than poplars (He et al. 2021a), and these may be slightly degenerated (Almeida et al. 2020), though more data are needed; if so, willows' sex chromosomes could be more stable, as degenerated sex chromosomes are predicted to be less likely to undergo turnovers, which can generate low fitness of individuals homozygotes for these chromosomes (Bull 1983). Our present study adds information about two further species, *Salix arbutifolia* and *S. triandra*, whose relationships within the genus *Salix* have been debated. Our results resolve some of the uncertainties, and illuminate the evolution of *Salix* sex chromosomes.

Salix arbutifolia is a diploid dioecious species, mainly distributed in northeastern Asia (Skvortsov 1999; Ohashi 2006). It was treated as a member of the genus *Chosenia* by Nakai (1920), based on its morphological characteristics, drooping male catkins like poplars and single bud scale like willows, which suggested that it is an intermediate phenotype between genus *Salix* and *Populus*. Some molecular systematic studies supported merging *Chosenia* into the genus *Salix* (Kimura 1988; Chen et al. 2010; Hardig et al. 2010), though its phylogenetic position has remained controversial. Lauron-Moreau et al. (2015) concluded that *S. arbutifolia* belongs to the subg. *Vetrix* (*Vetrix* clade), while Wu et al. (2015) proposed that it belongs to subg. *Pleuradenia*, and is sister to *Chamaetia*/ *Vetrix* clade. Gulyaev et al. (2022) hypothesized that its SDS might be on chromosome 15, like other *Vetrix* clade species.

Salix triandra is a species of sect. *Amygdalinae* native to Europe and Western and Central Asia, and its male flowers usually have three stamens (Skvortsov 1999; Ohashi 2006). Chen et al. (2010), Wu et al. (2015) and Abdollahzadeh et al. (2011) concluded that *S. triandra* and *S. triandroides* formed a distinct clade (subg. *Triandrae*) sister to *Chamaetia*/ *Vetrix*, but Lauron-Moreau et al. (2015) and Gulyaev et al. (2022) suggested that *S. triandra* belongs to the *Vetrix* clade. Li et al. (2020) suggested that *S. triandra* may have a ZW SDS on chromosome 15, based on linkage analysis. However, they used female *Salix purpurea* v1.0 (https://phytozome-next.jgi.doe.gov/info/Spurpurea_v1_0) as the reference genome. Chromosome 15 in this assembly contains unphased W- and Z-linked regions (Zhou et al. 2020). Hence, the SDS of *S. triandra* need further investigation.

We use whole genome re-sequencing data to reconstruct the phylogeny of *S. arbutifolia, S. triandra* and 6 willow species with known SDSs, with *P. euphratica* as an outgroup, (i) to investigate the phylogenetic position of *S. arbutifolia* and *S. triandra*; (ii) test whether *S. arbutifolia*'s sex-determining locus is on chromosome 15, and, if so, whether this is a ZW system, like other *Vetrix* clade species; (iii) investigate the SDS of *S. triandra* to test the results of previous research; (iv) test the association between sex determining systems and morphological characters of male flowers; (v) investigate turnover events in Salicaceae, to explore the possible role of turnover in homomorphic sex chromosomes in Salicaceae species.

Materials And Methods

Taxa sampling

We sampled 41 individuals (21 females and 20 males, Table S1) from two wild populations of *S. arbutifolia* and 39 individuals (20 females and 19 males, Table S1) from a wild population of *S. triandra* for whole genome re-sequencing. The two species were identified according to the descriptions of Fang et al. (1999) and Ohashi (2006). Leaves of each individual were dried with silica gel. Voucher specimens of these samples are deposited in the Beijing Forestry University herbarium (BJFC) and Shanghai Chenshan Botanical Garden (CSH). To estimate the phylogeny, we also downloaded whole genome sequencing data for six *Salix* species whose SDSs are known, covering the two main clades (Gulyaev et al. 2022): *Salix* clade *S. dunnii* (SRR12893418) and *S. nigra* (SRR16018717); and *Vetrix* clade *S. polyclona* (SRR16018674), *S. purpurea* (SRR3927002), *S. suchowensis* (SRR10197854), and *S. viminalis* (ERR1558612). We chose *Populus euphratica* (SRR13324572) as outgroup.

Sequencing, Reads Mapping, and Variant Calling

Total genomic DNA was extracted from leaves of *Salix arbutifolia* and *S. triandra* using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer instructions. Whole genome re-sequencing using paired-end libraries were performed on Illumina NovaSeq by Beijing BerryGenomics and Beijing Novogene Bioinformatics Technology. The *S. purpurea* genome assembly v5.1 (Zhou et al. 2020), excluding chromosome 15W, was used as a reference genome for mapping *S. arbutifolia* and *S. triandra* reads and subsequent variant calling. The genome of *S. purpurea* v5.1 (Zhou et al. 2020), again excluding chromosome 15W, was used as the reference genome for mapping the downloaded samples of reads from the seven other species.

The sequenced reads were filtered and trimmed by fastp 0.20.0 (Chen et al. 2018). Then clean reads were aligned to the reference genomes using the BWA-MEM algorithm from BWA 0.7.12 (Li and Durbin 2009; Li 2013). We used SAMTOOLS 0.1.19 (Li et al. 2009) to extract primary alignments, sort, and merge the mapped data. Sambamba 0.7.1 (Tarasov et al. 2015) was used to mark the potential duplications which from the PCR amplification step of library preparation. The variants were called using 'HaplotypeCaller' and 'GenotypeGVCFs' (GATK (Genome Analysis Toolkit) v. 4.1.8.1). 'HaplotypeCaller' was run with '–sample-ploidy 2'. Joint genotyping was conducted using 'GenotypeGVCFs'. Hard filtering of the SNP calls was

carried out with best practices quality recommendations (QD < 2.0, FS > 60.0, MQ < 40.0, MQRankSum < -12.5, ReadPosRankSum < -8.0, SOR > 3.0). We kept the biallelic sites for subsequently filtering. We excluded sites with coverage exceeding twice the mean depth at variant sites across all samples. Genotypes with depth less than four in a sample were treated as no-call, and sites with no-call genotypes in more than 10 % samples were filtered out, and sites with minor allele frequency (maf) less than 0.05 were discarded.

Phylogenetic analysis

We analysed sequences of one individual from each of the 9 specie. The dataset included 870,166 single nucleotide variants at sites that are four-fold degenerate in the *S. purpurea* genome sequence, detected using a python script (https://github.com/zhangrengang/degeneracy) based on the gene annotation (*S. purpurea* GFF3 files, Zhou et al. 2020). Phylogenetic relationships were inferred by a maximum likelihood approach using RAXML v.8.2.4 (Stamatakis 2014). Support values were calculated using 100 rapid bootstrap replicates (-f a option) based on the GTR+GAMMA nucleotide substitution model as used by He et al. (2021b) and Gulyaev et al. (2022) for the phylogenomics of willows.

Genome wide association studies between SNPs and sexes

We extracted 1,619,524 and 1,467,556 high-quality SNPs, respectively, from the 41 *Salix arbutifolia* samples and 39 *S. triandra* samples. These two datasets were used in a standard case-control GWAS of allele frequencies and sex phenotypes using PLINK v1.90b6.18 (Chang et al. 2015). SNPs with $\alpha < 0.05$ after Bonferroni correction for multiple testing yielded 91 and 5 sex linked-SNPs, respectively, in *S. arbutifolia* and *S. triandra*. All were on chromosome 15Z.

BLASTP (Madden 2002) was used to identify the *Arabidopsis thaliana* homologs of the genes containing these putatively sex linked-SNPs), with “blastp -evalue -e-5 -max_target-seqs 1”. Since ARR17-like genes (Müller et al. 2020; Xue et al. 2020) have been identified as playing a role in *Populus* sex determination, we also blasted the Potri.019G133600 sequence (of Müller et al. 2020; Xue et al. 2020) against the *S. purpurea* reference genome to search for possible homologs within the putative sex-linked regions (S-LRs) identified by our GWAS.

Divergence of female and male populations

We also used the two SNP datasets to evaluate genetic differentiation (estimated as F_{ST}) between the male and female individuals. Weighted F_{ST} values between the sexes were calculated using the Weir and Cockerham (1984) estimator with 100-kb windows and 10-kb steps using VCFtools (Danecek et al. 2011). Regions with significantly higher F_{ST} values than other parts of the chromosome are considered candidate sex-linked regions (He et al. 2021a). A changepoint package (Killick and Eckley 2014) was used to assess significance of differences in the mean and variance of the F_{ST} values in chromosome 15 windows, using the function cpt.meanvar, algorithm PELT and penalty CROPS. We extracted SNPs in windows with F_{ST}

values > 0.3 in the S-LRs identified from the two SNP datasets. VCFtools (Danecek et al. 2011) was then used to calculate heterozygote frequencies of these SNPs on a per-individual basis. High heterozygosity in males compared with females suggests male heterogamety, and higher heterozygosity in females suggests female heterogamety.

Ancestral character and SDS reconstruction

We recorded the stamen numbers of the eight selected diploid willows described in floras and papers (Fang et al 1999; Skvortsov 1999; Ohashi 2006; Argus 2010; Liu et al. 2020), and the SDSs of these species using the relevant papers (reviewed in Gulyaev et al. 2022). To test the possible association between these two characters, we reconstructed the characters' ancestral states. Willows with two (filament connate or distinct) stamens per flower were coded as 0, and those at least three stamens per flower as 1. An XY system on chromosome 7 was coded as 0, a ZW system on chromosome 15 as 1, and an XY system on chromosome 15 as 2. To reconstruct the ancestral states, we used a current probability model in Mesquite v. 2.75 (Maddison and Maddison 2014) based on the ML tree (Fig. 1).

The sex-linked region and proportion on sex chromosomes of willows in *Vetrix* clade

To test whether the S-LR sizes of *S. arbutifolia* and *S. triandra* were affected by the turnovers (see below), we compared the proportion of the sex chromosome that our analyses above found to be completely sex-linked in each *Vetrix* clade species, using *S. purpurea* as the reference genome. These were compared with the Z- and W-linked regions in *S. purpurea* (Zhou et al. 2020) and Z-linked region of *S. polyclona* (He et al. 2022). One-way analysis of variance (ANOVA, performed with SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) was used to analyze the significance of the size differences.

Results

DNA resequencing data and variant calling

After quality control, a total of 2,684.69 Mb and 2,737.71Mb clean reads were obtained for 41 and 39 individuals of *S. arbutifolia* (from 23.06 Mb to 68.27 Mb per individual, mean 32.74 Mb; Table S2) and *S. triandra* (from 31.98 Mb to 38.97 Mb per individual, mean 35.10 Mb; Table S3), respectively. High-quality reads were mapped to the reference genomes of *S. purpurea*; the mapping ratio ranged from 84.50% to 90.00% in *S. arbutifolia* (mean 88.07%; Table S4), and from 79.2% to 85.2% in *S. triandra* (mean 82.85%; Table S5). The average sequencing depths were 28.2× and 25.0×, respectively. Using *S. purpurea* as the reference genome, we detected 1,619,524 and 1,467,556 high-quality SNPs (Table 2).

Phylogenetic analysis

We reconstructed phylogenetic tree of willows with known SDSs based on 870,166 four-fold site variants (Fig. 1). This resolved four clades with high bootstrap support. *Salix arbutifolia* (clade 2) and *S. triandra* (clade 3) sister to clade 1 (*S. polyclona*, *S. viminalis*, *S. suchowensis*, and *S. purpurea*) formed a monophyletic *Vetrix* clade. Clade 4 (the *Salix* clade) includes *S. nigra* and *S. dunnii*. This topology is consistent with previous conclusions (Wu et al. 2015; Gulyaev et al. 2022).

Identification the sex determination system of *Salix arbutifolia* and *Salix triandra*

Using *Salix purpurea* as the reference genome, GWAS analysis in *S. arbutifolia* and *S. triandra* recovered a total of 91 and 5 SNPs on chromosome 15Z, respectively, with significant associations with sex (Fig. 2a, b, S1a; Fig.3a, b, S1b, Table S6; Table S7). Changepoint analysis of F_{ST} between male and female individuals of *S. arbutifolia* and *S. triandra* detected candidate fully sex-linked regions between 3.21 and 6.54 Mb (about 24.9% of chromosome 15Z; Fig. 2c and d; S2) and 3.67 and 6.47 Mb (about 20.9% of chromosome 15Z; Fig. 3c and d; S3). A total of 8,026 and 748 SNPs, respectively, were within windows with F_{ST} values > 0.3 in *S. arbutifolia* and *S. triandra*. More than 74 % and 89 % of these SNPs are heterozygous in *S. arbutifolia* and *S. triandra* males, while only 18.80 % and 15.53 % of these SNPs are heterozygous for the females, respectively, suggesting that both species have XY systems on chromosome 15.

Our GWAS identified only a single likely fully sex-linked gene in each of *S. arbutifolia* (Sapur.15ZG052500) and *S. triandra* (Sapur.15ZG056900). The Sapur.15ZG052500 gene is involved in unidimensional cell growth (reciprocal best hits found the *A. thaliana* genes ATGUS2, which is involved cell elongation and beta-glucuronidase activity), and the Sapur.15ZG056900 homolog is involved in positive regulation of abscisic acid-activated signaling pathway (reciprocal best hits found the *A. thaliana* ATPPRT3 genes). Sapur.15ZG071500 (not within the S-LRs identified in this study) is the Potri.019G133600 homolog (at 8.12 Mb in the *S. purpurea* reference genome, identity = 64.8; Zhou et al. 2020).

Ancestral state and SDS reconstruction

The SDS of previously studied *Vetrix* clade species is on chromosome 15, while the *Salix* clade SDS is on chromosome 7 (Gulyaev et al. 2022). Our analysis suggests an equivocal state for ancestors of *S. triandra* and *S. arbutifolia*, but a transition to the XY system on chromosome 15 that is the ancestral state to clade 1 (Fig. 4a). The relative likelihoods of ancestral SDS states for the genus *Salix* are 0.395 for XY on chromosome 7, 0.356 for XY on chromosome 15, and 0.249 ZW on chromosome 15 (Fig. 4a).

The ancestral character state reconstructions of stamen number and SDS imply that species with multiple stamens (≥ 3) usually have XY systems, while species with less than three stamens usually have a ZW system (Fig. 4b).

Sex-linked region sizes of willows of *Vetrix* clade

The S-LR of *S. purpurea* 15W is the largest (6.7 M, or 42.7% of chromosome 15 in the reference *S. purpurea* v5.1 sequence), followed by that of *S. polyclona* (4.68 Mb, or 35.2% of the sex chromosome). The sex-linked regions of *S. arbutifolia* and *S. triandra* are smaller, accounting, respectively, for 24.9% and 20.9% of the sex chromosome 15Z (reference *S. purpurea* v5.1), significantly smaller ($p < 0.01$) than those of other willows in the *Vetrix* clade (Table 2).

Discussion

A XY system on chromosome 15 of *Salix arbutifolia* and *Salix triandra*, and possible turnovers in willows

The two species studied in detail here, *Salix arbutifolia* and *S. triandra*, both have SDSs on chromosome 15 (Fig. 2, Fig. 3) as Gulyaev et al. (2022) hypothesized for species of the *Vetrix* clade. Our heterozygosity analyses suggest that both have male heterogamety. Li et al. (2020) suggested *S. triandra* has a ZW SDS on chromosome 15, based on linkage mapping, but they used the female *S. purpurea* v1.0 reference genome sequence, whose Z and W were not phased, and represent a chimera (https://phytozome-next.jgi.doe.gov/info/Spurpurea_v1_0; Li et al. 2020; Zhou et al. 2020), which can cause false inferences. The chromosome 15 of *S. purpurea* v1.0 is 22.6 Mb in length, and after phasing (*S. purpurea* v5.1), its 15W and 15Z sequences are 15.7 Mb and 13.3 Mb, respectively (Zhou et al. 2020). It is however worth considering that the SDSs are quite flexible in *Salix*, it be possible that there is a change within *S. triandra*. It is a very widespread species and different evolution could happen in different areas.

Our ancestral state reconstruction suggests that *Salix arbutifolia* and *S. triandra* are in a transitional stage between clades 1 and 4, whereas a Y-linked region on chromosome 7 appears to be the ancestral state for clade 4 (*Salix*) (Fig.1; 4a). Since our ancestral state analysis yielded similar probabilities for XY on chromosome 7 (0.395) or chromosome 15 (0.356) as the ancestral state for the genus *Salix*, we cannot rule out the possibility that XY on chromosome 15 is the ancestral state of the genus *Salix*. Two scenarios are thus possible, both involving at least two turnover events (Fig. 4a): 1) if Y-linked region on chromosome 7 is the ancestral state for the genus *Salix*, at least two turnover events may have occurred in the *Vetrix* clade (XY on chromosome 7 to XY on chromosome 15, and to ZW on chromosome 15), while the entire *Salix* clade maintained the chromosome 7 ancestral state (Fig. 4a, blue arrow), or 2) if an XY system on chromosome 15 is the ancestral state for genus *Salix*, at least two turnover events must have occurred within the genus *Salix* (XY to ZW on chromosome 15 in the *Vetrix* clade, and XY on chromosome 15 to XY on chromosome 7 in the *Salix* clade, Fig. 4a, gray arrow). As the sex-linked regions of *Populus*, the sister genus of willows, are mostly on chromosome 19, the two groups of willows with sex-linked regions on different chromosome probably evolved independently from chromosome 19 (He et al. 2021a) (Table 1), perhaps involving different sex determination gene(s). This remains to be tested, as willow sex determination gene(s) have not yet been identified.

Our phylogenomic analyses and the sex-linked region identified in *S. arbutifolia* and *S. triandra* supports their assignment to the *Vetrix* clade (Fig. 1), consistent with previously studies (Lauron-Moreau et al. 2015; Wu et al. 2015; Gulyaev et al. 2022).

Possible reasons for turnovers in the genus *Salix*

Bull (1983) and Vicoso (2019) proposed that some clades have extremely conserved sex chromosomes because they have become genetically degenerated, while others have not degenerated and can undergo sex chromosome turnovers. The physical sizes of sex-linked regions of willows are relatively large (Table 1), but their S-LRs are, at most, only slightly degenerated (Almeida et al. 2020; He et al. 2021a). Hence, turnover events should be possible in *Salix* and *Populus* (He et al. 2021a; Gulyaev et al. 2022). It has, however, been proposed that genetic degeneration can promote turnovers, replacing degenerated Y with new, non-degenerated sex chromosomes (Blaser et al. 2014). Accumulation of deleterious mutations on the ancestral sex chromosomes seems unlikely to explain the XY to ZW change on chromosome 15 in the *Vetrix* clade, because such “homologous” turnovers are predicted to maintain the ancestral heterozygous sex (Bull, 1983; Blaser et al. 2014; van Doorn and Kirkpatrick 2010; Jeffries et al. 2018; Scott, Osmond and Otto 2018). Drift-induced turnovers are also generally expected to maintain the ancestral heterogamety (Veller et al. 2017).

The sex-ratio selection and sexually antagonistic selection models are more likely to lead to changes in the heterogametic sex (Jeffries et al. 2018) like those inferred in willows. To explain the XY to ZW transition in the *Vetrix* clade, the W-linked factor must be dominant and cause femaleness in the presence of the Y. Sexual selection in plants, such as pollinator attraction, is very similar to some forms of sexual selection in many animals (Charlesworth et al. 1987). In *S. dunnii*, *Apis cerana*'s significant preference for visiting female flowers suggests that sexual selection pressure can be placed on male flowers (Zeng et al. 2022). Likewise, evidence of sexual dimorphism was also found in *S. purpurea* (Gouker et al. 2021). Hence, sexually antagonistic selection may play a role in the turnover of the genus *Salix*.

Sex ratio selection is, however, likely in plants, and could be involved in the turnover events of the genus *Salix*. Sex ratio imbalances are common in nature (Scott et al. 2018), and can favour a new sex determining locus that restores an equal sex ratio (Beukeboom and Perrin 2014; Bull 1983; Mank, Hosken and Wedell 2014). In willows, female biased sex ratios are associated with ZW systems (Table S8), possibly reflecting incompatibility between maternally and paternally inherited Z-linked alleles (Pucholt et al. 2017a). Willows with XY SDS may generally have male-biased or balanced sex ratios (Table S8). If the ancestor of the *Vetrix* clade had male heterogamety and a male biased sex ratio, a feminizing gene might be able to invade and cause a transition to ZW SDS (Kozielska et al. 2010). On the other hand, as many willows with female biased sex ratios have ZW systems. Male or female bias can be also introduced during polyploidization, and many willows are polyploids (He and Hörandl 2022). Our ancestral state reconstruction supports changes in both the heterogamety and the stamen number, with a reduction to two stamens accompanying the change from male to female heterogamety. However, it is worth noting that in *Salix* group there are some polyploid species with just 2 stamens, eg. *S. chienii* (tetraploid) and *S. matsudana* (tetraploid) (Gulyaev et al. 2022), whether

such a pattern exists in polyploidy requires further investigation. Hence, the relationship between turnover events and biased sex ratios remains unclear.

Can turnovers explain homomorphic sex chromosomes in Salicaceae?

The sex chromosomes in the Salicaceae *stricto sensu* (*Populus* and *Salix*) show no signs of major genetic degeneration, and most have remained homomorphic (Pucholt et al. 2017b), despite almost all species being dioecious, and dioecy having evolved more than 45 million years ago in the genus *Salix*. The rate at which genetic degeneration occurs after loss of recombination between sex-linked regions is not yet understood (reviewed by Charlesworth 2021). However, a turnover event will evidently create a new non-degenerated sex-linked region (Beukeboom and Perrin 2014; Gamble et al. 2015; Bachtrog et al. 2014; Jeffries et al. 2018; Kuhl et al., 2021; Vicoso 2019). Turnovers via chromosomal mutations (translocations) are in *Salix* probably feasible because the chromosomes are morphologically very similar to each other (Pucholt et al. 2017b). Hence, turnovers and also the return of previous SDS-bearing chromosomes to autosomes would be probably easier than in genera with more differentiated karyotypes. Turnovers, in turn, would inhibit degeneration and loss of size of sex chromosomes. Turnovers, including transitions between XY and ZW systems, may be associated with a lack of sex chromosome heteromorphism (Bachtrog et al. 2014, Zhou et al. 2018). Poplars have more known turnover events than willows (Table 1). The estimated sizes of *Salix* sex-linked regions are generally significantly larger than in *Populus*, possibly due to more frequent turnover events in *Populus*. Another possibility is that the S-LRs of *Salix* species are within pericentromeric regions that recombine very rarely (Chen et al. 2016; Zhou et al. 2018; He et al. 2021a; Wikerson et al. 2022), creating larger fully sex-linked regions than in *Populus*.

The S-LRs in *Salix arbutifolia* and *S. triandra* were estimated above smaller than those of other willows of the *Vetrix* clade (see Table 3). This might seem to be evidence against the turnover events inferred here, since, if the ZW on chromosome 15 is the derived state, it might be expected to be smaller than that in species with a male-determining region on chromosome 15. However, our estimates used the *S. purpurea* reference genome, and may reflect the actual sizes in *S. arbutifolia* and *S. triandra*.

We only identified two sex-link genes in S-LRs, among which Sapur.15ZG052500 (best hit in the *A. thaliana* genes: ATGUS2) is involved cell elongation and beta-glucuronidase activity, and Sapur.15ZG056900 (reciprocal best hits found the *A. thaliana* genes ATPPRT3) in positive regulation of the abscisic acid-activated signaling pathway. The homolog of Sapur.15ZG052500 also shows female-biased expression in shoot tip of *S. purpurea* (Carlson et al. 2017).

In poplars, turnovers have also been inferred, and different partial duplications of ARR17-like genes have been shown to be involved in different species (Yang et al. 2020; Li et al. 2022; Xue et al. 2020). We searched for ARR17-like genes in the S-LRs of *S. arbutifolia* and *S. triandra*, but did not find any sex-linked copies in either species. We speculate that willows probably do not share the same sex-determining gene as the one found in poplars (He et al. 2021a), or duplicated copy of ARR17-like gene could be found in their own genomes (*S. arbutifolia* and *S. triandra*). However, turnovers that involve a change from male to female

heterogamety, or vice versa, also seem unlikely to involve the same gene (for example, a dominant male-determining factor in a population with male heterogamety cannot cause a turnover by simply being duplicated to a different location, as a dominant factor is required, that can convert individuals to females even if they carry the ancestral male-determining factor). Thus such turnovers seem unlikely to involve the duplication mechanism, and this is consistent with different genes being involved in *Populus* and *Salix*. The results in are also consistent with the expectation that, when heterogamety changes, female heterogamety is generally the derived state. This is because, if the first step in de novo evolution of separate sexes in plants is probably a loss of function sterility mutation, which is likely to be recessive. A recessive male sterility mutation, a common kind of mutation, can initiate evolution of an XY system, but a ZW system requires an unusual type of mutation causing dominant male sterility. A turnover can, however, readily produce a ZW system through a mutation that dominantly suppresses male fertility, for example a duplication that leads to production of an RNA sequence that suppresses a function essential for anther or pollen development.

Declarations

Data Archiving

Sequence data presented in this article can be downloaded from the NCBI database under BioProject accession ######.

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Author Contributions

Wang and He were responsible for the conception and writing of the paper, experimental design, production of pictures and tables and data analysis; Cai was responsible for data analysis; Zhang and Zhang was responsible for field sample collection and the overall direction of the paper, respectively. Hörandl was responsible for the paper conception and revision.

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Conflict of Interest

The authors declare there are no competing interests.

References

1. Abdollahzadeh A, OsaloO SK, Maassoumi AA (2011). Molecular phylogeny of the genus *Salix* (Salicaceae) with an emphasize to its species in Iran. Iran J Bot. 17:244-53.

2. Almeida P, Proux-Wera E, Churcher A, Soler L, Dainat J, Pucholt P et al (2020). Genome assembly of the basket willow, *Salix viminalis*, reveals earliest stages of sex chromosome expansion. BMC Biol 18(1): 78. https://doi.org/10.1186/s12915-020-00808-1
3. Argus GW, Eckenwalder JE, Kiger RW (2010). Salicaceae. In: Flora of North America Editorial Committee, editor. Flora of North America. Oxford and New York: Oxford University Press. 7: 23-162.
4. Bachtrog D, Mank JE, Peichel CL, Kirkpatrick M, Otto SP, Ashman TL et al (2014). Sex determination: why so many ways of doing it? PLoS Biol 12(7): e1001899. https://doi.org/10.1371/journal.pbio.1001899
5. Balounova V, Gogela R, Cegan R et al. (2019). Evolution of sex determination and heterogamety changes in section *Otites* of the genus *Silene*. Sci Rep 9, 1045. https://doi.org/10.1038/s41598-018-37412-x
6. Baránková S, Pascual-Díaz JP, Sultana N, Alonso-Lifante MP, Balant M, Barros K, D'Ambrosio U, Malinská H, Peska V, Pérez Lorenzo I, Kovařík A, Vyskot B, Janoušek B, Garcia S (2020). Sex-chrom, a database on plant sex chromosomes. New Phytol, 227(6):1594-1604. https://doi.org/10.1111/nph.16635
7. Beukeboom LW, Perrin N (2014). The Evolution of Sex Determination Oxford University Press, Oxford, UK.
8. Blaser O, Grossen C, Neuenschwander S, Perrin N (2013). Sex-chromosome turnovers induced by deleterious mutation load. Evolution 67, 635-645. https://doi.org/10.1111/j.1558-5646.2012.01810.x.
9. Blaser O, Neuenschwander S, Perrin N (2014). Sex-chromosome turnovers: the hot-potato model. Am. Nat. 183, 140-146. https://doi.org/10.1086/674026
10. Bull J J (1985). Sex determining mechanisms: an evolutionary perspective. Experientia. 15;41(10):1285-96. https://doi.org/10.1007/BF01952071
11. Bull J J (1983). Evolution of sex determining mechanisms. San Francisco, CA: Benjamin-Cummings Publishing Company.
12. Bull JJ, Charnov EL (1977). Changes in the heterogametic mechanism of sex determination. Heredity, 39(1), 1-14. https://doi.org/10.1038/hdy.1977.38
13. Carlson CH, Choi Y, Chan AP, Serapiglia MJ, Town CD, Smart LB (2017). Dominance and Sexual Dimorphism Pervade the *Salix purpurea* L.Transcriptome. Genome Biol Evol 9(9): 2377-2394.https://doi.org/10.1093/gbe/evx174
14. Chang CC, Chow CC, Tellier LC, Vattikuti S, Purcell SM, Lee JJ (2015). Second-generation PLINK: rising to the challenge of larger and richer datasets. Gigascience. 25: 4:7. https://doi.org/10.1186/s13742-015-0047-8
15. Charlesworth D (1985). Distribution of dioecy and self-incompatibility in angiosperms. In P. J. Greenwood, & M. Slatkin (Eds.), Evolution essays in honour of John Maynard Smith. Cambridge University Press 237-268.
16. Charlesworth D (2015). Plant contributions to our understanding of sex chromosome evolution. New Phytol. 208(1):52-65. https://doi.org/10.1111/nph.13497
17. Charlesworth D (2021) The timing of genetic degeneration of sex chromosomes. Philos Trans R Soc Lond B Biol Sci 376: 20200093.https://doi.org/10.1098/rstb.2020.0093

18. Charlesworth D, Schemske D, Sork V (1987). The evolution of plant reproductive characters; sexual versus natural selection. Experientia 55: 317-335. https://doi.org/10.1007/978-3-0348-6273-8_14
19. Charlesworth D, Bergero R, Graham C, Gardner J, Keegan K (2021). How did the guppy Y chromosome evolve? PLoS Genet 17: e1009704. https://doi.org/10.1371/journal.pgen.1009704
20. Chen JH, Sun H, Wen J et al. (2010). Molecular phylogeny of *Salix* L.(Salicaceae) inferred from three chloroplast datasets and its systematic implications. Taxon. 59(1):29-37. https://doi.org/10.2307/27757048
21. Chen S, Zhou Y, Chen Y, Gu J (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34: i884-i890. https://doi.org/10.1093/bioinformatics/bty560
22. Chen Y, Wang T, Fang L, Li X, Yin T (2016). Confirmation of Single-Locus Sex Determination and Female Heterogamety in Willow Based on Linkage Analysis. PLoS One 11(2): e0147671.
23. https://doi.org/10.1371/journal.pone.0147671
24. Dai X, Hu Q, Cai Q, Feng K, Ye N, Tuskan GA et al (2014). The willow genome and divergent evolution from poplar after the common genome duplication. Cell Res 24(10): 1274-1277.https://doi.org/10.1038/cr.2014.83
25. Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA et al (2011). The variant call format and VCFtools. Bioinformatics 27(15): 2156-2158.https://doi.org/10.1093/bioinformatics/btr330
26. Darolti I, Wright AE, Sandkam BA, Morris J, Bloch NI, Farre M et al (2019). Extreme heterogeneity in sex chromosome differentiation and dosage compensation in livebearers. Proc Natl Acad Sci USA 116(38): 19031-19036. https://doi.org/10.1073/pnas.1905298116
27. Fang CF, Zhao SD, Skvortsov AK (1999). Salicaceae. In: Wu ZY., Raven PH., eds. Flora of China. Beijing and St. Louis: Science Press and Missouri Botanical Garden Press 4: 139-274.
28. Franchini P, Jones JC, Xiong P, Kneitz S, Gompert Z, Warren WC et al (2018). Long-term experimental hybridisation results in the evolution of a new sex chromosome in swordtail fish. Nat Commun 9(1): 5136. https://doi.org/10.1038/s41467-018-07648-2
29. Gamble T, Coryell J, Ezaz T, Lynch J, Scantlebury DP, Zarkower D (2015). Restriction Site-Associated DNA Sequencing (RAD-seq) Reveals an Extraordinary Number of Transitions among Gecko Sex-Determining Systems. Mol Biol Evol 32(5): 1296-1309.
30. https://doi.org/10.1093/molbev/msv023
31. Gammerdinger WJ, Toups MA, Vicoso B (2020). Disagreement in FST estimators: A case study from sex chromosomes. Mol Ecol Resour 20(6): 1517-1525. https://doi.org/10.1111/1755-0998.13210
32. Gaudet M, Jorge V, Paolucci I, Beritognolo I, Mugnozza GS, Sabatti, MJ (2008). Genetic linkage maps of *Populus nigra* L. including AFLPs, SSRs, SNPs, and sex trait. Tree Genetics and Genomes, 4(1), 25-36. https://doi.org/10.1007/s11295-007-0085-1
33. Geraldes A, Hefer CA, Capron A, Kolosova N, Martinez-Nu EF, Soolanayakanahally RF, Stanton B, Guy RD, Mansfield SD, Douglas CJ, Cronk QC (2015). Recent Y chromosome divergence despite ancient origin of dioecy in poplars (*Populus*). Molecular Ecology, 24(13), 3243-3256. https://doi.org/10.1111/mec.13126

34. Gouker FE, Carlson CH, Zou J, Evans L, Crowell CR, Smart CD et al (2021). Sexual dimorphism in the dioecious willow *Salix purpurea*. Am J Bot 108(8): 1374-1387. https://doi.org/10.1002/ajb2.1704
35. Gulyaev S, Cai XJ, Guo FY, Kikuchi S, Applequist WL, Zhang ZX et al (2022). The phylogeny of *Salix* revealed by whole genome re-sequencing suggests different sex-determination systems in major groups of the genus. Ann Bot 129(4): 485-498. https://doi.org/10.1093/aob/mcac012
36. Haldane J (1922). Sex ratio and unisexual sterility in animal hybrids. Journal of Genetics, 12, 101-109. https://doi.org/10.1007/bf02983075
37. Hardig TM, Anttila CK, Brunsfeld SJ (2010). A Phylogenetic Analysis of *Salix* (Salicaceae) Based on matK and Ribosomal DNA Sequence Data. Journal of Botany 2010: 1-12. https://doi.org/10.1155/2010/197696
38. He L, Hörandl E (2022). Does polyploidy inhibit sex chromosome evolution in angiosperms? Frontiers in Plant Science. https://doi.org/10.3389/fpls.2022.976765
39. He L, Jia KH, Zhang RG, Wang Y, Shi TL, Li ZC et al (2021). Chromosome-scale assembly of the genome of *Salix dunnii* reveals a male-heterogametic sex determination system on chromosome 7. Mol Ecol Resour 21(6): 1966-1982.https://doi.org/10.1111/1755-0998.13362
40. He L, Wagner ND, Hörandl E (2020). Restriction-site associated DNA sequencing data reveal a radiation of willow species (*Salix* L., Salicaceae) in the Hengduan Mountains and adjacent areas. Journal of Systematics and Evolution 59 (1): 44-57.https://doi.org/10.1111/jse.12593
41. He L, Guo FY, Cai XJ, Chen HP, Lian CL, Wang Y, et al. (2022). Evolutionary origin and establishment of the diploid-tetraploid complex in *Salix polyclona*. Authorea. https://doi.org/10.22541/au.165769042.25880844/v1
42. Henry IM, Akagi T, Tao R, Comai L (2018). One hundred ways to invent the sexes: theoretical and observed paths to dioecy in plants. Annu Rev Plant Biol. 69(1):553-575. https://doi.org/10.1146/annurev-arplant-042817-040615
43. Hou J, Ye N, Zhang D, Chen Y, Fang L, Dai X et al (2015). Different autosomes evolved into sex chromosomes in the sister genera of *Salix* and *Populus*. Sci Rep 5: 9076. https://doi.org/10.1038/srep09076
44. Jaenike J (2001). Sex chromosome meiotic drive. Annual Review of Ecology and Systematics, 32, 25-49. https://doi.org/10.1146/annurev.ecolsys.32.081501.113958
45. Jeffries DL, Lavanchy G, Sermier R, Sredl MJ, Miura I, Borzee A et al (2018). A rapid rate of sex-chromosome turnover and non-random transitions in true frogs. Nat Commun 9(1): 4088. https://doi.org/10.1038/s41467-018-06517-2
46. Kallman K (1984). Evolutionary Genetics of Fishes (ed Turner, B. J.) Springer, New York, NY, USA, 95-171.
47. Kersten B, Pakull B, Groppe K, Lueneburg J, Fladung M (2014). The sex-linked region in *Populus tremuloides* Turesson 141 corresponds to a pericentromeric region of about two million base pairs on *P. trichocarpa* chromosome 19. Plant Biol (Stuttg) 16(2): 411-418.
48. https://doi.org/10.1111/plb.12048
49. Killick R, Eckley IA (2014). Changepoint: An R package for changepoint analysis. Journal of Statistical Software, 58(1), 1-19. https://doi.org/10.1093/bioinformatics/btr330

50. Kimura A (1988). De salicis subgenere Pleuradenia commentatio. Sci Rep Tohoku Univ (4 Ser Biol). 39: 143-147.
51. Kozielska M, Weissing FJ, Beukeboom LW, Pen I (2010). Segregation distortion and the evolution of sex-determining mechanisms.Heredity (Edinb) 104(1): 100-112.https://doi.org/10.1038/hdy.2009.104
52. Kuhl H, Guiguen Y, Hohne C, Kreuz E, Du K, Klopp C et al (2021). A 180 Myr-old female-specific genome region in sturgeon reveals the oldest known vertebrate sex determining system with undifferentiated sex chromosomes. Philos Trans R Soc Lond B Biol Sci 376(1832): 20200089. https://doi.org/10.1098/rstb.2020.0089
53. Lauron-Moreau A, Pitre FE, Argus GW, Labrecque M, Brouillet L (2015). Phylogenetic relationships of American willows (*Salix* L., Salicaceae). PLoS One 10(4): e0121965. https://doi.org/10.1371/journal.pone.0138963
54. Li H (2013). Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv, 1303.3997v2. https://doi.org/10.48550/arXiv.1303.3997
55. Li H, Handsaker B, Wysoker A, et al. (2009). The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078-2079. https://doi.org/10.1093/bioinformatics/btp352
56. Li H, Durbin R (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25, 1754-1760. https://doi.org/10.1093/bioinformatics/btp324
57. Li W, Wu H, Li X, Chen Y, Yin T (2020). Fine mapping of the sex locus in *Salix triandra* confirms a consistent sex determination mechanism in genus *Salix*. Hortic Res 7: 64. https://doi.org/10.1038/s41438-020-0289-1
58. Li Y, Wang D, Wang W, Yang W, Gao J, Zhang W et al (2022). A chromosome-level *Populus qiongdaoensis* genome assembly provides insights into tropical adaptation and a cryptic turnover of sex determination. Mol Ecol. https://doi.org/10.1111/mec.16566
59. Liu L, He L, Applequist WL (2020). Untangling two Chinese *Salix* species (Salicaceae) published by CK Schneider, with lectotypification of four names. Willdenowia, 50(2), 159-163.
60. Ohashi H (2006). Salicaceae. In: Boufford D, Ohba H., eds. Flora of Japan. Tokyo: Kodansha, IIa: 7-25.
61. Madden T (2002). The BLAST Sequence Analysis Tool - The NCBI Handbook - NCBI Bookshelf. Handbook.
62. Maddison W, Maddison D (2014). Mesquite: a modular system for evolutionary analysis. Version 2.73. http://mesquiteproject.org.
63. Manchester SR, Judd WS, Handley B (206). Foliage and fruits of early poplars (Salicaceae: *Populus*) from the Eocene of Utah, Colorado, and Wyoming. Int J Plant Sci.167:897-908. https://doi.org/10.1086/503918
64. Mank JE, Hosken DJ, Wedell N (2014). Conflict on the sex chromosomes: Cause, effect, and complexity. Cold Spring Harbor Perspectives in Biology, 6(12), a017715. https://doi.org/10.1101/cshperspect.a017715
65. Martin H, Carpentier F, Gallina S, Gode C, Schmitt E, Muyle A et al (2019). Evolution of Young Sex Chromosomes in Two Dioecious Sister Plant Species with Distinct Sex Determination Systems. Genome Biol Evol 11(2): 35-361. https://doi.org/10.1093/gbe/evz001

66. Melnikova NV, Pushkova EN, Dvorianinova EM, Beniaminov AD, Novakovskiy RO, Povkhova LV et al (2021). Genome Assembly and Sex-Determining Region of Male and Female *Populus* x *sibirica*. Front Plant Sci 12: 625416. https://doi.org/10.3389/fpls.2021.625416
67. Ming R, Bendahmane A, Renner S (2011). Sex chromosomes in land plants. Annual Review of Plant Biology 62: 485-514. https://doi.org/10.1146/annurev-arplant-042110-103914
68. Moore RC, Harkess AE, Weingartner LA (2016). How to be a seXY plant model: a holistic view of sex-chromosome research. Am J Bot. 103(8):1379-1382. https://doi.org/10.3732/ajb.1600054
69. Müller NA, Kersten B, Leite Montalvao AP, Mahler N, Bernhardsson C et al. (2020). A single gene underlies the dynamic evolution of poplar sex determination. Nat Plants 6: 630-637. https:/doi.org/10.1038/s41477-020-0672-9
70. Nakai T (1920). Chosenia, a new genus of Salicaceae. Botanical Magazine (Tokyo). 34:66-69. https://doi.org/10.15281/jplantres1887.34.401_66
71. Otto SP, Pannell JR, Peichel CL, Ashman TL, Charlesworth D, Chippindale AK, Delph LF, Guerrero RF, Scarpino SV, McAllister BF (2011). About PAR: the distinct evolutionary dynamics of the pseudoautosomal region. Trends Genet. 27(9):358-67. https://doi.org/10.1016/j.tig.2011.05.001
72. Pakull B, Groppe K, Meyer M, Markussen T, Fladung M (2009). Genetic linkage mapping in aspen (*Populus tremula* L. and *Populus tremuloides* Michx.). Tree Genetics and Genomes, 5(3), 505-515. https://doi.org/10.1007/s11295-009-0204-2
73. Pan Q, Kay T, Depince A, Adolfi M, Schartl M, Guiguen Y et al (2021). Evolution of master sex determiners: TGF-beta signalling pathways at regulatory crossroads. Philos Trans R Soc Lond B Biol Sci 376(1832): 20200091. https://doi.org/10.1098/rstb.2020.0091
74. Paolucci I, Gaudet M, Jorge V et al, (2010). Genetic linkage maps of *Populus alba* L. and comparative mapping analysis of sex determination across *Populus* species. Tree Genetics & Genomes 6, 863-875. https://doi.org/10.1007/s11295-010-0297-7
75. Pucholt P, Wright AE, Conze LL, Mank JE, Berlin S (2017). Recent Sex Chromosome Divergence despite Ancient Dioecy in the Willow *Salix v iminalis*. Mol Biol Evol 34(8): 1991-2001.
76. https://doi.org/10.1093/molbev/msx144
77. Pucholt P, Hallingback HR, Berlin S (2017). Allelic incompatibility can explain female biased sex ratios in dioecious plants. BMC Genomics 18(1): 251. https://doi.org/10.1186/s12864-017-3634-5
78. Renner SS (2014). The relative and absolute frequencies of angiosperm sexual systems: Dioecy, monoecy, gynodioecy, and an updated online database. American Journal of Botany, 101(10), 1588-1596. https://doi.org/10.3732/ajb.1400196
79. Renner SS, Muller NA (2021). Plant sex chromosomes defy evolutionary models of expanding recombination suppression and genetic degeneration. Nat Plants 7(4): 392-402. https://doi.org/10.1038/s41477-021-00884-3
80. Sanderson BJ, Feng G, Hu N, Carlson CH, Smart LB, Keefover-Ring K et al (2021). Sex determination through X-Y heterogamety in *Salix nigra*. Heredity (Edinb) 126(4): 630-639. https://doi.org/10.1038/s41437-020-00397-3

81. Saunders PA, Neuenschwander S, Perrin N (2018). Sex chromosome turnovers and genetic drift: a simulation study. J Evol Biol 31(9): 1413-1419. https://doi.org/10.1111/jeb.13336
82. Scott MF, Osmond MM, Otto SP (2018). Haploid selection, sex ratio bias, and transitions between sex-determining systems. PLoS Biol 16(6): e2005609. https://doi.org/10.1371/journal.pbio.2005609
83. Skvortsov AK (1999). Willows of Russia and Adjacent Countries: Taxonom-ical and Geographical Revision. Joensuu: University of Joen-suu. 1-307
84. Stamatakis A (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30: 1312-1313. https://doi.org/10.1093/bioinformatics/btu033
85. Tarasov A, Vilella AJ, Cuppen E, Nijman IJ, Prins P (2015). Sambamba: fast processing of NGS alignment formats. Bioinformatics 31(12):2032-2034. https://doi.org/10.1093/bioinformatics/btv098
86. Tao W, Conte MA, Wang D, Kocher TD (2021). Network architecture and sex chromosome turnovers: Do epistatic interactions shape patterns of sex chromosome replacement? Bioessays 43(3): e2000161.https://doi.org/10.1002/bies.202000161
87. Tennessen JA, Wei N, Straub SCK, Govindarajulu R, Liston A, Ashman TL (2018). Repeated translocation of a gene cassette drives sex-chromosome turnover in strawberries. PLoS Biol 16(8): e2006062. https://doi.org/10.1371/journal.pbio.2006062
88. Tuskan GA, DiFazio S, Faivre-Rampant P, Gaudet M, Harfouche A, Jorge V et al (2012). The obscure events contributing to the evolution of an incipient sex chromosome in Populus: a retrospective working hypothesis. Tree Genetics & Genomes 8(3): 559-571.https://doi.org/10.1007/s11295-012-0495-6
89. Tuskan GA, Difazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U et al (2006). The genome of black cottonwood, Populus trichocarpa (Torr. & Gray). Science 313(5793): 1596-1604. https://doi.org/10.1126/science.1128691
90. van Doorn GS, Kirkpatrick M (2007). Turnover of sex chromosomes induced by sexual conflict. Nature. 449(7164), 909-912. https://doi.org/10.1038/nature06178
91. van Doorn GS, Kirkpatrick M (2010). Transitions between male and female heterogamety caused by sex-antagonistic selection. Genetics 186(2): 629-645. https://doi.org/10.1534/genetics.110.118596
92. Veller C, Muralidhar P, Constable GWA, Nowak MA (2017). Drift-induced selection between male and female heterogamety. Genetics 207, 711-727. https://doi.org/10.1534/genetics.117.300151
93. Vicoso B (2019). Molecular and evolutionary dynamics of animal sex-chromosome turnover. Nat Ecol Evol 3(12): 1632-1641.https://doi.org/10.1038/s41559-019-1050-8
94. Weir BS, Cockerham CC (1984). ESTIMATING F-STATISTICS FOR THE ANALYSIS OF POPULATION STRUCTURE. Evolution.38(6):1358-1370. https://doi.org/10.1111/j.1558-5646.1984.tb05657.x
95. Werren JH, Beukeboom LW (1998) Sex determination, sex ratios, and genetic conflict. Annual Review of Ecology and Systematics. 29, 233-261. https://doi.org/10.1146/annurev.ecolsys.29.1.233
96. Westergaard M (1958). The mechanism of sex determination in dioecious flowering plants. Advances in genetics, Demerec M, Academic Press 9, 217-281. https://doi.org/10.1016/S0065-2660(08)60163-7

97. Wu J, Nyman T, Wang DC, Argus GW, Yang YP, Chen JH (2015). Phylogeny of *Salix* subgenus *Salix* s.l. (Salicaceae): delimitation, biogeography, and reticulate evolution. BMC Evol Biol 15: 31.https://doi.org/10.1186/s12862-015-0311-7
98. Xue L, Wu H, Chen Y, Li X, Hou J, Lu J et al (2020). Evidences for a role of two Y-specific genes in sex determination in *Populus deltoides*.Nat Commun 11(1): 5893. https://doi.org/10.1038/s41467-020-19559-2
99. Yang W, Wang D, Li Y, Zhang Z, Tong S, Li M et al (2021). A General Model to Explain Repeated Turnovers of Sex Determination in the Salicaceae. Mol Biol Evol 38(3): 968-980. https://doi.org/10.1093/molbev/msaa261
100. Yin T, Difazio SP, Gunter LE, Zhang X, Sewell MM, Woolbright SA et al (2008). Genome structure and emerging evidence of an incipient sex chromosome in *Populus*. Genome Res 18(3): 422-430.
101. https://doi.org/10.1101/gr.7076308
102. Zeng SW, Liang T, Li LX et al. (2022). Pollination System and Reproductive Allocation Strategies of Dioecious Tree *Salix dunnii*. Journal of Tropical and Subtropical Botany 30(3): 357-366. https://kns.cnki.net/kcms/detail/44.1374.
103. Zhou R, Macaya-Sanz D, Carlson CH, Schmutz J, Jenkins JW, Kudrna D et al (2020). A willow sex chromosome reveals convergent evolution of complex palindromic repeats. Genome Biol 21(1): 38.https://doi.org/10.1186/s13059-020-1952-4
104. Zhou R, Macaya-Sanz D, Rodgers-Melnick E *et al.*, 2018 Characterization of a large sex determination region in *Salix purpurea* L. (Salicaceae). Mol Genet Genomics 293, 1437-1452. https://doi.org/10.1007/s00438-018-1473-y

Tables

Table 1 Possibile transitions in sex determination and sex chromosomes in Poplar and willows. The number in parentheses indicates the number of turnover events, while the “?” indicates that turnover events was considered as speculation and not confirmed in the published paper.

Taxon	Species	Male or female heterogamety	Chromosome carrying the sex-determining locus	Estimated size of the sex-linked regions (kb)	Possibile transition	References
Populus						
	P. euphratica	male	14	~84 (X), 658 (Y)	19 XY to 14 XY or maintained 14 XY (1)	Yang et al. 2020
	P . pruinosa	male	14	unknown	19 XY to 14 XY or maintained 14 XY (1)	Müller et al. 2020
	P. qiongdaoensis	female	19	~1710(W), ~1280(Z)	14 XY or 19 XY to 19 ZW ?	Li et al. 2022; Renner et al. 2021
	P. adenopoda	female	19	unknown	14 XY or 19 XY to 19 ZW (2)	Renner et al. 2021
	P. alba	female	19	~140 (W), 33 (Z)	14 XY or 19 XY to 19 ZW (2) to 19 ZW (3)	Li et al. 2022; Müller et al. 2020
	P. tremula	male	19	~1000 (Y)	19 ZW to 19 XY (4)	Li et al. 2022; Renner et al 2021
	P. tremuloides	male	19	2000 (Y)	19 ZW to 19 XY ?	Pakull et al. 2009; Kersten et al. 2014; Renner et al 2021
	P. nigra	male	19	unknown	14 XY to 19 XY or maintained 19 XY (1)	Gaudet et al. 2008;
	P. deltoides	male	19	~300(X, Y)	14 XY to 19 XY or maintained 19 XY (1)	Xue et al. 2020
	P. balsamifera	male	19	~100(Y)	14 XY to 19 XY or maintained 19 XY (1)	Geraldes et al. 2015; McKown et al. 2017
	P. × sibirica	male	19	unknown	14 XY to 19	Melnikova

					XY or maintained 19 XY (1)	et al. 2021
	P. trichocarpa	male	19	~100 (Y)	14 XY to 19 XY or maintained 19 XY (1)	Geraldes et al. 2015; McKown et al. 2017
	P. trichocarpa	female	19	~1000 (W)		Yin et al. 2008
Salix						
	***Salix* clade**					
	S. dunnii	male	7	3205 (X)	15 XY to 7 XY (1) or maintained 7 XY	He et al. 2021a
	S. nigra	male	7	2000	15 XY to 7 XY (1) or maintained 7 XY	Sanderson et al. 2020
	***Vetrix* clade**					
	S. triandra	male	15	~2800	7 XY to 15 XY (1) or maintained 15 XY	this study
	S. triandra	female	15	~6500		Li et al. 2020
	S. arbutifolia	male	15	~3330	7 XY to 15 XY (1) or maintained 15 XY	this study
	S. purpurea	female	15	6700 (W), 4400 (Z)	15 XY to 15 ZW (2)	Zhou et al. 2020
	S. suchowensis	female	15	unknown	15 XY to 15 ZW (2)	Hou et al. 2015
	S. viminalis	female	15	3100–3400 (W, Z)	15 XY to 15 ZW (2)	Almeida et al. 2020
	S. polyclona	female	15	46800	15 XY to 15 ZW (2)	He et al. 2022

Numbers indicate turnover events.

Table 2 List of the high-quality SNP datasets used for relevant analyses.

The high-quality SNP datasets that used for relevant analyses

Analytical method	SNP datasets
Phylogenetic analysis	870,166 (genome sequences of *S. purpurea*, 9 samples)
F_{ST}	1,619,524 (*S. arbutifolia*, *S. purpurea* as reference); 1,467,556 (*S. triandra*, *S. purpurea* as reference)
GWAS	1,619,524 (*S. arbutifolia*, *S. purpurea* as reference); 1,467,556 (*S. triandra*, *S. purpurea* as reference)
Heterozygote frequencies	8,026 (*S. arbutifolia*, *S. purpurea* as reference); 748 (*S. triandra*, *S. purpurea* as reference)
Ancestral state reconstruction	870,166 (*S. purpurea* as reference)

Table 3 The sex-linked region and proportion on sex chromosomes of willows in *Vetrix* clade.

Species	Sex/reference chromosome size (Mb)	Sex linked regions (Mb) and proportion on sex chromosomes
Salix arbutifolia	13.3 (Ref *Salix purpurea* v5.1, chromosome 15Z)	3.33 (24.9%)
Salix triandra	13.3 (Ref *Salix purpurea* v5.1, chromosome 15Z)	2.80 (20.9%)
Salix purpurea	15.7 (Ref *Salix purpurea* v5.1, chromosome 15W)	6.7 (42.7%) *
Salix purpurea	13.3 (Ref *Salix purpurea* v5.1, chromosome 15Z)	4.4 (33.1%) *
Salix polyclona	13.3 (Ref *Salix purpurea*, v5.1, chromosome 15Z)	4.68 (35.2 %) *

Note: * indicated that the proportion of SLR on sex chromosome was significantly different from *Salix arbutifolia* according to ANOVA ($p < 0.01$). Here we only used the longer SLR of *Salix arbutifolia* based on *S. purpurea* genome, because the two species are both from clade *Vetrix*.

Figures

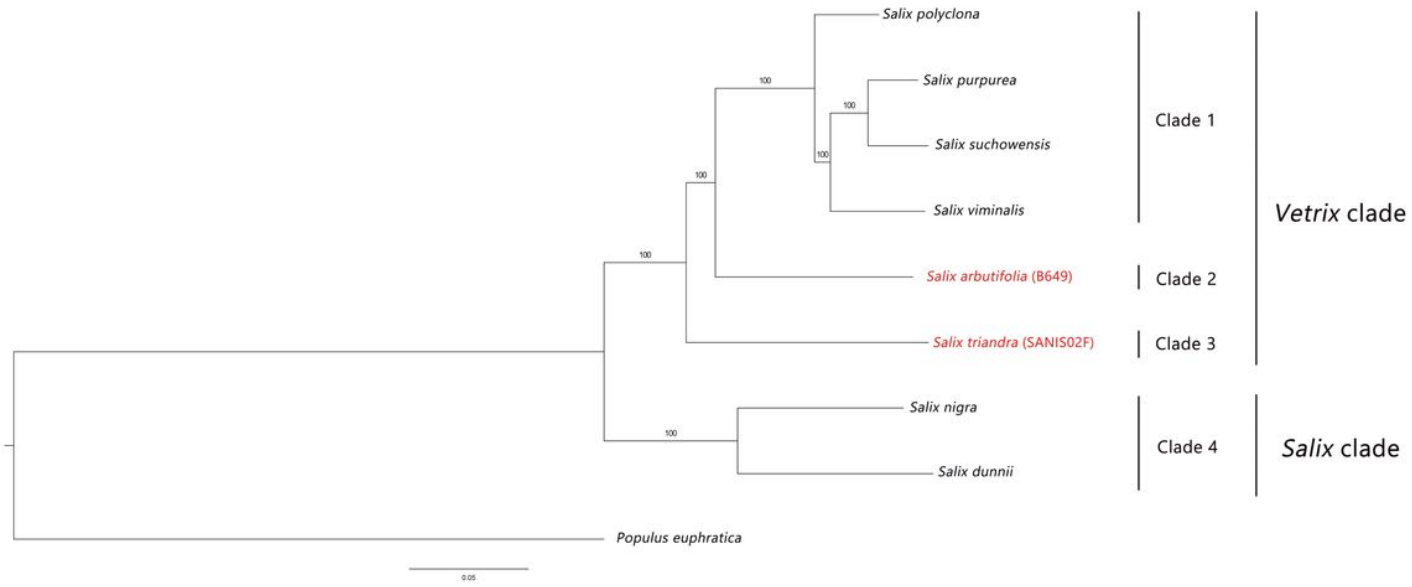

Figure 1

Phylogenetic tree of all willow species with known sex systems and the outgroup *Populus euphratica*.Values above branches refer to bootstrap. The scale bar refers the mean number of nucleotide substitution per site. Species in red font are studied in this study.

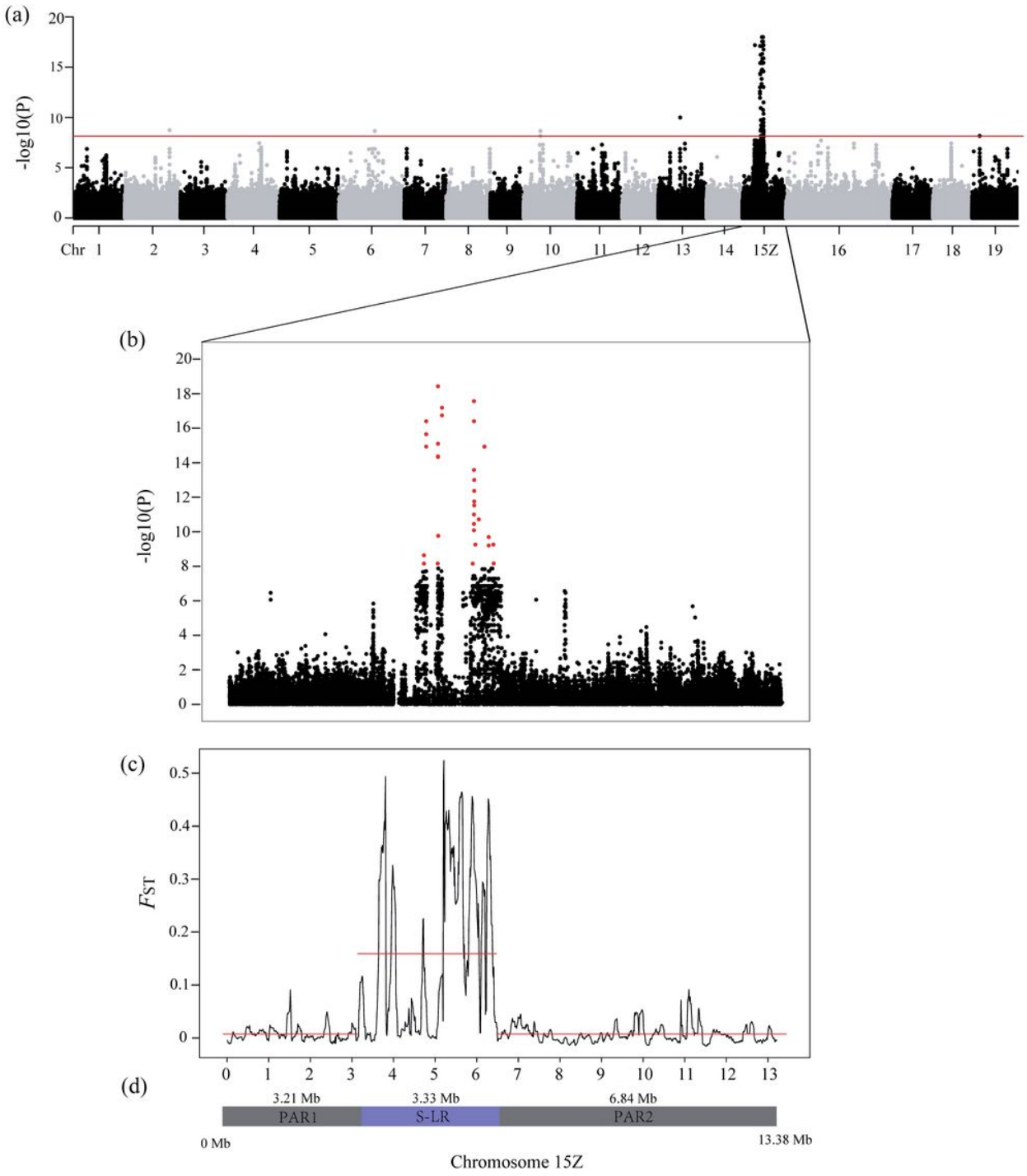

Figure 2

Identification of the sex-determination system of *Salix arbutifolia*. (a): Results of genome wide association studies (GWAS) between SNPs and the sexes of 41 individuals. The *y* axis is the negative logarithm of *p* values, and the red line shows the Bonferroni-corrected significance level corresponding to $\alpha < 0.05$. (b): Plot for GWAS p-values of all SNPs of chromosome 15Z. Red dots show significantly sex-associated SNPs. (c): *F*ST values between the sexes for 100-kb overlapping windows of chromosome 15Z with 10-kb steps. Red

lines represent significant regions on chromosome 15Z suggested by changepoint analysis. (d): The positions of the PAR1, S-LR and PAR2 regions of chromosome 15.

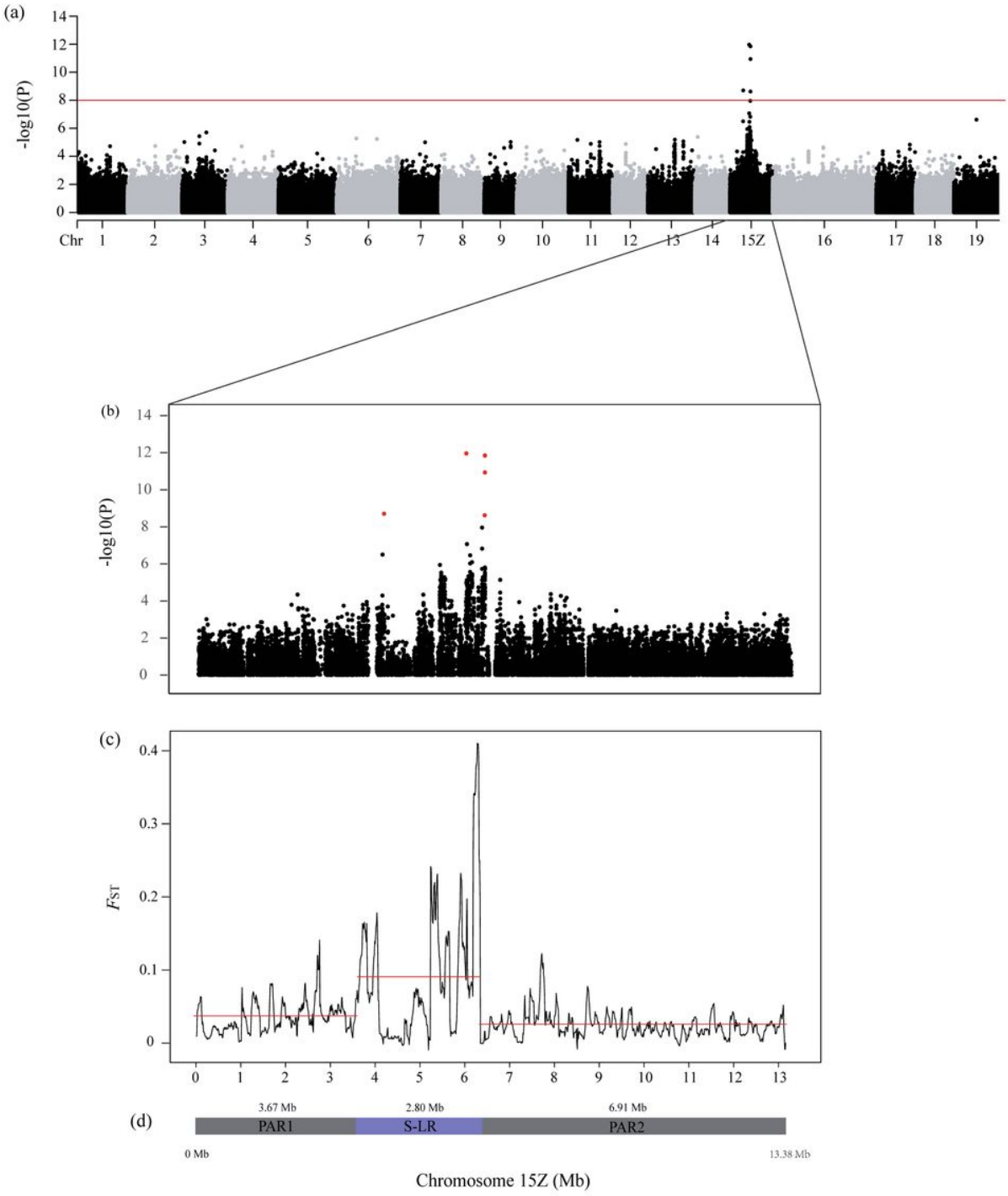

Figure 3

Identification of the sex-determination systems of *Salix triandra*. (a): Results of genome wide association studies (GWAS) between SNPs and the sexes of 39 individuals. The *y* axis is the negative logarithm of *p* values, and the red line shows the Bonferroni-corrected significance level corresponding to $\alpha < 0.05$. (b): Plot

for GWAS p-values of all SNPs of chromosome 15Z. Red dots show significantly sex-associated SNPs. (c): *F*ST values between the sexes for 100-kb overlapping windows of chromosome 15Z with 10-kb steps. Red lines represent significant regions on chromosome 15Z suggested by changepoint analysis. (d): The positions of the PAR1, S-LR and PAR2 regions of chromosome 15Z.

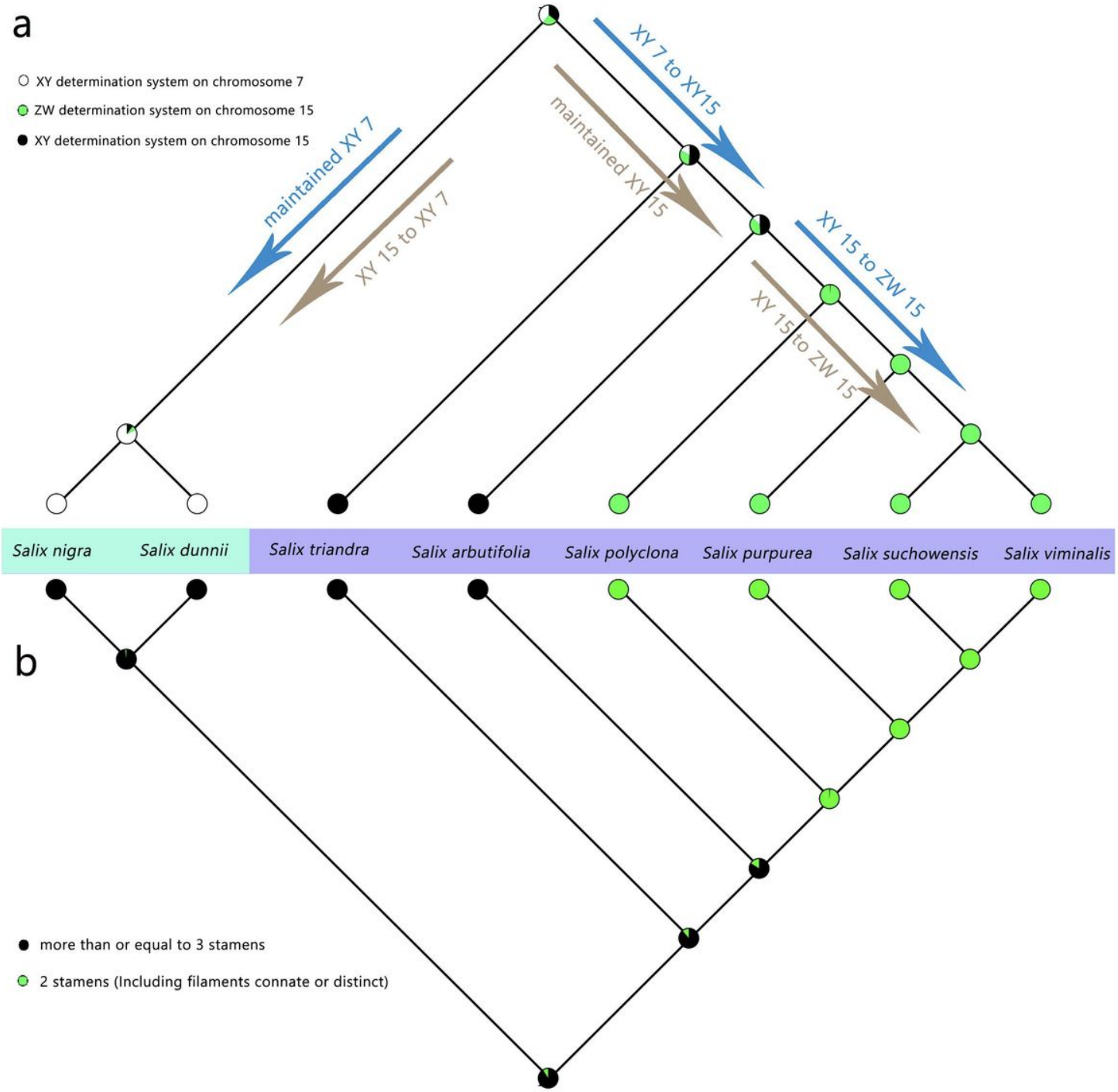

Figure 4

Ancestral state reconstruction of (a) the sex determination system of all willows with known sex determination systems and (b) stamen numbers based on a ML tree (Figure 1).

Supplementary Files

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