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Letter

Editor's choice

Sex chromosome turnover plays an important role in the maintenance of barriers to post-speciation introgression in willows

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Abstract

Almost all species in the genus *Salix* (willow) are dioecious and willows have variable sex-determining systems, the role of this variation in maintaining species barriers is relatively untested. We first analyzed the sex determination systems (SDS) of two species, *Salix cardiophylla* and *Salix interior*, whose positions in the *Salix* phylogeny make them important for understanding a sex chromosome turnover that has been detected in their relatives, and that changed the system from male (XX/XY) to female (ZW/ZZ) heterogamety. We show that both species have male heterogamety, with sex-linked regions (SLRs) on chromosome 15 (termed a 15XY system). The SLRs occupy 21.3% and 22.8% of the entire reference chromosome, respectively. By constructing phylogenetic trees, we determined the phylogenetic positions of all the species with known SDSs. Reconstruction of ancestral SDS character states revealed that the 15XY system is likely the ancestral state in willows. Turnovers of 15XY to 15ZW and 15XY to 7XY likely contributed to early speciation in *Salix* and gave rise to major groups of the *Vetrix* and *Salix* clades. Finally, we tested introgression among species in the phylogenetic trees based on both autosomes and SLRs separately. Frequent introgression was observed among species with 15XY, 15ZW, and 7XY on autosomes, in contrast to the SLR datasets, which showed less introgression, and in particular no gene flow between 15ZW and 7XY species. We argue that, although SDS turnovers in willow speciation may not create complete reproductive barriers, the evolution of SLRs plays important roles in preventing introgression and maintaining species boundaries.

Keywords: *Salix*, sex determination, speciation, sex chromosome turnover, genome evolution

Lay Summary

Sex chromosomes could accumulate reproductive barriers faster than autosomes (chromosomes that do not determine sex) and thus play important roles during speciation. Sex determination systems (SDS) include both XY (males have a pair of different chromosomes) and ZW (females have a pair of different chromosomes) systems. Three SDSs are found in different lineages of willows (genus *Salix*): species of the *Salix* clade have an XY system on chromosome 7 (termed 7XY), early-diverging species of *Vetrix* clade have a 15XY system, while the remaining species of *Vetrix* clade have a 15ZW system. The diverse SDSs and frequent turnovers within *Salix*, even in closely related lineages, provide an ideal example to study the role of sex chromosome evolution in speciation. This study takes *Salix cardiophylla* and *Salix interior*, both in the early-diverging portion of the *Vetrix* clade, as examples and reveals that both have 15XY systems. Phylogenetic analysis suggests that possible turnovers (i.e., 15XY to 7XY and 15XY to 15ZW) have accelerated speciation in *Salix*. Finally, gene flow was more reduced among sex-linked regions than among autosomes, indicating that sex chromosomes maintain reproductive barriers among species. In conclusion, sex chromosome evolution in willows may shed light on the multiple roles of sex chromosomes in the speciation process of dioecious plants.

Introduction

Sex determination systems (SDS) can involve male heterogamety (often termed XX/XY system) or female heterogamety (ZW/ZZ). Sex chromosomes in plants have evolved in many lineages and can differ between closely related species (Slancarova et al., 2013; Tennessen et al., 2018), similar to the relatively recent sex chromosome systems known in some animal groups, including amphibia and fish (Dufresnes et al., 2020; Franchini et al., 2018). In angiosperms, shifts between XY and ZW systems are known in several genera, including *Dioscorea* (Girma et al., 2019), *Silene* (Balounova et al., 2019), *Populus* (Zhang et al., 2022), and *Salix* (Wang et al., 2023). Such changes, and the changes of chromosomal location, known as turnover events, could be important for speciation (Tennessen et al., 2018). Sex chromosome turnovers are termed "homologous" when the sex chromosome pair remains unchanged (as in the change from male to female heterogamety on chromosome 19 in the genus *Populus* (Müller et al., 2020). "Nonhomologous transitions" (or "trans-heterogamety

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transition") refer to changes in which a sex-determining gene emerges on an autosome (Kondo et al., 2006), including transposition of an existing male-determination locus to an autosome (Yang et al., 2020). In *Salix*, two different chromosomes (7 and 15) are known to carry sex-determining loci (Wang et al., 2023). In addition, chromosomal fusions can also cause sex chromosome turnover, as in the plant *Rumex hastatulus*, an X-autosome fusion created a new sex chromosome morphology and probably affected recombination on the chromosomes (Beaudry et al., 2020). It has been proposed that this contributed to reproductive isolation, since the neo-sex chromosome origin is inferred to have coincided temporally with the cessation of gene flow between the two sex chromosome cytotypes. Such nonhomologous transitions might promote speciation especially if genes that control reproductive isolation between populations accumulate differentially on neo-sex chromosomes and on the ancestral sex chromosomes (Kitano & Peichel, 2012).

These arguments suggest that turnovers could lead to accelerated speciation. Hybridization and admixture among lineages with different SDSs have rarely been studied in plants but have been widely studied in animals (Dixon et al., 2019; Kuwana et al., 2021; Miura et al., 2012; Natri et al., 2019). Species of toads (Bufonidae) with different SDSs (*Bufo bufo* with ZW system and *B. spinosus* with XY system) could hybridize only across a narrow area (10 km wide) and reproductive isolation due to genetic incompatibilities likely prevents their gene pools from merging (Dufresnes et al., 2020). Both XY and ZW SDSs were found in East African cichlids on multiple different chromosomes, and rapid turnovers could be associated with rapid adaptive radiations in different lakes (Feller et al., 2021). Sex chromosomes are involved in Haldane's Rule in animals that the heterogametic sex (XY or ZW) is more likely to have reduced fitness in an interspecific cross, and large X effects that the disproportionately large role of the X chromosome in reducing hybrid fitness have been detected in many taxa (Faria & Navarro, 2010; Graves & O'Neill, 1997; Presgraves, 2018).

In plant species of *Silene* section *Otites* with both XY (*S. colpophylla*) and ZW systems (*S. otites* and *S. borysthenica*), the change in heterogamety might have happened through hybridization events; whereby the male-determining chromosome was introgressed into another species and replaced the old sex-determining system (Balounova et al., 2019). The effects of sex chromosome turnover events on phenotypic diversification and speciation are not yet clear (El Taher et al., 2021).

Sex chromosome pairs in plants are usually homomorphic and young (Wang et al., 2022a). Homomorphic sex chromosomes do not differ in size at the karyotype level and usually have a relatively small non-recombining region. However, recent studies showed high diversity in the size of sex-linked regions (SLRs) across plants, from a single locus in poplar (Müller et al., 2020) to a small differentiated region in strawberry (Tennessen et al., 2018) to heteromorphic sex chromosomes with large non-recombinant regions in *Silene* (e.g., Filatov, 2022). During the process of divergence from initially identical to heteromorphic sex chromosomes, the size of the SLR will change (e.g., through gene loss and accumulation of repetitive elements, etc.) and mutations (e.g., inversions, etc.) will accumulate in X, Y, W, and Z chromosomes (Long et al., 2023; Ming et al., 2011). These changes may enhance reproductive isolation in closely related species (Baack et al., 2015).

Taxonomic groups with diverse sex chromosomes, with both male (XY) and female (ZW) heterogamety, can help in understanding whether, and how, SLRs may affect reproductive isolation and speciation (Filatov, 2018; Ogata et al., 2021). The willow genus, *Salix* (Salicaceae), which is closely related to *Populus*, includes two major clades, *Salix* and *Vetrix*. Species of the *Salix* clade usually have an XY system on chromosome 7, while those of clade *Vetrix* have both XY and ZW systems on chromosome 15. The SLR has therefore shifted between chromosomes and changed heterogamy (Gulyaev et al., 2022; Wang et al., 2023), making *Salix* ideal for studying the relationship between sex chromosome turnovers and speciation. In particular, in the *Salix* clade, *Salix nigra*, *S. chaenomeloides*, and *S. dunnii* have XY a system on chromosome 7, henceforth abbreviated to 7XY (He et al., 2021a; Sanderson et al., 2021; Wang et al., 2022a). Most studied species of the *Vetrix* clade, *S. suchowensis* (Hou et al., 2015), *S. viminalis* (Almeida et al., 2020), *S. purpurea* (Zhou et al., 2020), *S. polyclona* (He et al., 2023), *S. koriyanagi*, *S. integra*, and *S. udensis* (Wilkerson et al., 2022), have a ZW system on chromosome 15. However, three early-diverging species in the *Vetrix* clade (*S. arbutifolia*, *S. triandra*, and *S. exigua*) have a 15XY system (Hu et al., 2023; Wang et al., 2022a, 2023). The SDSs of other early-diverging species, including *S. cardiophylla* and *S. interior*, are still unknown (Gulyaev et al., 2022; Wu et al., 2015).

Hybridization events in *Salix* are common (Percy et al., 2014), but natural hybridization happens usually between species in the same clade (Wagner et al., 2021). Several species can produce seeds in crossing experiments. For example, *S. cardiophylla* and *S. arbutifolia* (15XY) in the *Vetrix* clade can produce fertile offspring, which are named *S. kamikotica* (Kimura, 1931, 1937). In addition, *Salix exigua*, a diploid species (15XY), showed no pollination barrier in crosses with other diploid representatives of the same *Vetrix* clade (*S. eriocephala* and *S. petiolaris*), and produced fertile F_1 offspring (Boufford, 1993; Mosseler, 1990). Fertile crosses have also been recorded between *S. triandra* (15XY) and *S. viminalis* (15ZW) and produced F1 hybrids named *S. ×mollissima* (Gulyaev et al., 2022; Karp et al., 2011). By contrast, hybridization between different clades is less common. For example, seed abortion occurred when *S. exigua* and *S. interior* in clade *Vetrix* were crossed with members of clade *Salix* (Mosseler, 1990). The reproductive barriers could be caused by pollen–pistil incongruity (Mosseler, 1989), and Gulyaev et al. (2022) proposed that different SDSs may also be responsible. More recently, genomic approaches were applied to detect hybridization. Sanderson et al. (2023) identified several cases of hybridization between species from different branches among the ancestors of subgenera *Longifoliae*, *Vetrix*, and *Chamaetia* (*Vetrix* clade) of *Salix* based on the ABBA-BABA statistics.

According to the Bateson–Dobzhansky–Muller model (BDM model), speciation is expected to happen when reproductive isolation is mostly completed in the absence of gene flow (Hollinger & Hermisson, 2017). However, genetic incompatibilities can be incomplete, allowing some hybridization, or the extent of intrinsic postzygotic reproductive isolation effects may be sensitive to extrinsic environmental circumstances (Cutter, 2023). *Salix* is a case with low genetic divergence between species within the major clades, resulting in unresolved phylogenies based on DNA barcode markers (Chen et al., 2010), and relationships within clades can be resolved only with genome-wide data (Gulyaev et al., 2022; Wagner et al., 2020). In such systems where reproductive isolation is still incomplete, the extent of introgression can allow us to uncover whether sex chromosome rearrangements affect the evolution of nascent species boundaries (Wang et al., 2022b). Previous studies of the role of sex chromosome turnovers in genome differentiation have mostly used hybrid swarms created by multi-generation experimental crosses (Volz & Renner, 2008; Wang et al., 2022b). Gene flow studies can be used in a genome-wide phylogenetic framework.

In this study, we sequenced populations of both sexes from *Salix cardiophylla* and *S. interior* to (a) discover their SDSs and determine whether they have XY or ZW system on chromosome 15 or 7; (b) reconstruct phylogenetic relationships using the single nucleotide polymorphism (SNP) data from 15 willow species with known SDS, and explore whether there are turnover events in the subclades of *Salix* and identify the possible ancestral SDS of *Salix*; and (c) identify willow species with known SDS that can and cannot undergo gene flow both on autosomes and SLRs, to get insights into whether reproductive isolation has evolved between species that differ in these characteristics.

Methods

Taxon sampling

For the whole-genome resequencing, we collected 42 individuals for diploid *Salix cardiophylla*, including 19 female and 23 male individuals from Japan (Supplementary Table S1). For diploid *S. interior*, we sampled 38 individuals, including 20 female and 18 male individuals from the United States of America (Supplementary Table S1). The two species were identified based on descriptions in relevant floras and taxonomic studies (Argus, 2010; Ohashi, 2006). For each individual, leaves were dried with silica gel and voucher specimens were deposited at the herbarium of Shanghai Chenshan Botanical Garden (CSH).

To confirm the systematic position of the two species in our study, we downloaded whole genome sequence data of 13 representative species, with known SDSs, of *Salix* genus (Supplementary Table S2), which have available genome data. The species *Populus euphratica* (SRR13324572) was also included as an outgroup in this study.

Sequencing, reads mapping, and variant calling

Total genomic DNA of *Salix cardiophylla* and *S. interior* individuals were isolated from silica-dried leaf samples using Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The whole-genome paired-end sequencing library was constructed and sequenced on Illumina NovaSeq 6000 by Beijing Novogene Bioinformatics Technology, China.

The raw sequenced reads were filtered by using fastp 0.20.0 (Chen et al., 2018) with parameters "- - n_base_limit 3, - - length_required 60." Clean reads of *S. cardiophylla* and *S. interior* were aligned to the closely related published reference genome of *Salix purpurea* (Zhou et al., 2020, chromosome 15 W excluded but 15Z kept) by BWA 0.7.12 with default settings (Li & Durbin, 2009). We then extracted primary alignments, sorted, and merged the mapped files in SAMtools 0.1.19 (Li et al., 2009). Sambamba 0.7.1 (Tarasov et al., 2015) was performed to discard clonal duplicates during the library preparation.

We called the variants using the program "HaplotypeCaller" and "GenotypeGVCFs" in Genome Analysis Toolkit (GATK) v. 4.1.8.1 (McKenna et al., 2010). We used the setting "-sample-ploidy 2" in "HaplotypeCaller" for the chromosome regions.

Hard filtering was used for further SNP calling, with the setting (QD < 2.0, FS > 60.0, MQ < 40.0, MQRankSum < −12.5, ReadPosRankSum < −8.0, and SOR > 3.0). We only kept the biallelic sites in the chromosome regions. For the subsequent filtering, sites with coverage more than twice the average depth at variant sites in all samples were excluded. Genotypes with depth < 4 × were treated as no-call, sites with no-call genotypes in more than 10% of samples were removed, and sites with minor allele frequency < 0.05 were further discarded. We also used the same method to call SNPs for phylogenetic analysis.

Identification of the SDS of *S. cardiophylla* and *S. interior*

To identify the SDS of the two species, we first extracted high-quality SNPs, from 42 *S. cardiophylla* and 38 *S. interior* individuals, respectively. The two datasets were analyzed with a standard case-control genome-wide association study of allele frequencies and sex phenotypes with PLINK v1.90b6.18 (Chang et al., 2015). SNPs with $\alpha < 0.05$ after Bonferroni correction for multiple testing yielded 78 and 40 sex-linked SNPs on chromosome 15Z, respectively, in *S. cardiophylla* and *S. interior*.

We also calculated the genetic differentiation (F_{ST}) between the male and female individuals in VCFtools 0.1.16 (Danecek et al., 2011) using the Weir and Cockerham (1984) estimator with 100-kb windows and 10-kb steps. A R package called "Changepoint" (Killick & Eckley, 2014) was used to assess the 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. Regions with significantly higher F_{ST} values than other parts of the chromosome are considered candidate SLRs (He et al., 2021a).

VCFtools was used to calculate heterozygote frequencies of sex-linked SNPs detected by genome-wide association study (GWAS) on a per-individual basis. High heterozygosity in males compared with females suggests male heterogamety, and higher heterozygosity in females indicates female heterogamety.

Plastid genome assembly and alignment

We assembled 16 plastid genomes using Getorganelle v 1.7.6.1 (Jin et al., 2020) from the clean sequence data with default parameters. Homblocks v 1.0 (Bi et al., 2018) was then employed to align the sequences for the following phylogenetic analysis.

Phylogenetic analysis and ancestral SDS reconstruction

To infer the phylogenetic relationships of *S. cardiophylla* and *S. interior* within *Salix*, we included the genomic sequences of another 13 representative *Salix* taxa and one species of *Populus* as outgroup. We used the genome of *S. purpurea* as the reference again and called the SNPs after excluding the putative sex chromosomes 7 and 15. This yielded out 10,665,405 SNPs. The SNPs that are fourfold degenerate sites in the reference genome were detected by a python script (https://github.com/zhangrengang/degeneracy) based on the gene annotation of *S. purpurea*, and only 821,062 SNPs at fourfold degenerate sites were kept. Finally, phylogenomic relationships based on the chromosome dataset were reconstructed by a maximum likelihood approach using RAxML v.8.2.4 (Stamatakis, 2014). Support values were calculated using 1,000 rapid bootstrap replicates based on the GTR + GAMMA nucleotide substitution model. We applied the same settings as the chromosome dataset to infer the plastid RAxML tree.

To infer the relationship among the 16 species, we also estimated species trees. We used OrthoFinder 2.5.2 (Emms & Kelly, 2019) to identify single-copy genes in all five diploid genome assemblies of *S. brachista* (Chen et al., 2019), *S. dunnii* (He et al., 2021a), *S. purpurea* (Zhou et al., 2020), *S. suchowensis* (Dai et al., 2014), and *S. viminalis* (Almeida et al., 2020). We chose the more variable genes with at least 50 SNPs in the coding regions of each gene from the 10,665,405 SNPs dataset. Modelfinder (Kalyaanamoorthy et al., 2017) was then used to select the best model based on the Bayesian information criterion, and IQ-TREE v. 2.1.4 (Minh et al., 2020) was applied to reconstruct individual gene trees based on the selected best model and ascertainment bias correction model. A species tree was estimated using

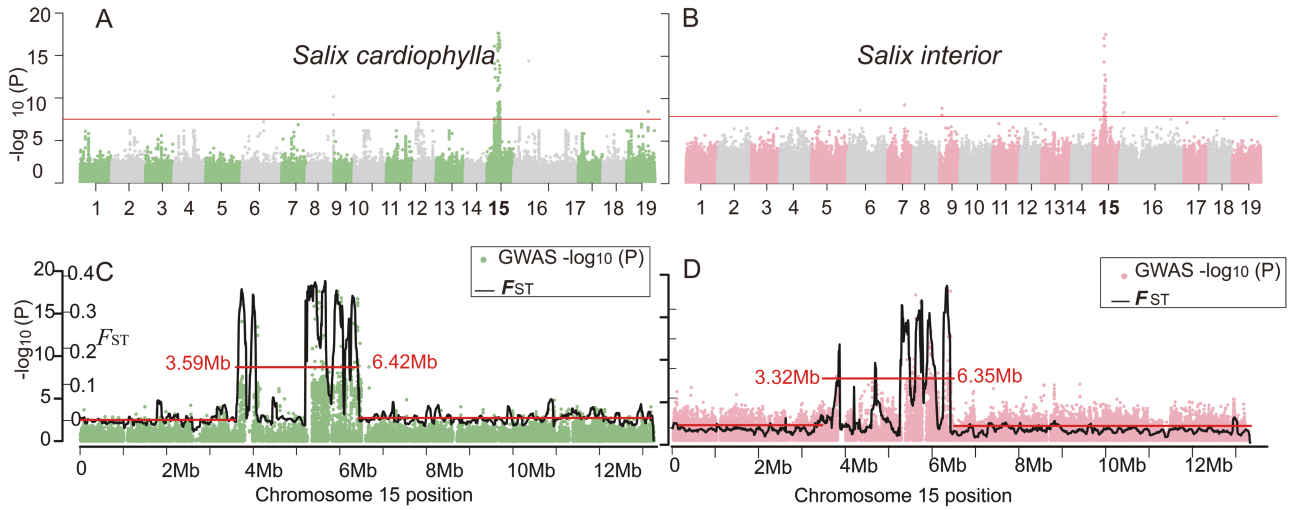

Figure 1. The sex determination systems of *Salix cardiophylla* and *S. interior*. (A) Genome-wide association study (GWAS) results between SNPs and sexes in 42 individuals for *S. cardiophylla*. (B) GWAS results between SNPs and sexes in 38 individuals for *S. interior*. The horizontal lines in A and B show the Bonferroni-corrected significance level corresponding to $\alpha < 0.05$ and the y-axis shows the negative logarithm of *p* values. (C) *p* values of GWAS results and F_{ST} values between the sexes on chromosome 15 for *S. cardiophylla*. (D) *p* values of GWAS results and F_{ST} values between the sexes on chromosome 15 for *S. interior*. Horizontal lines represent the significant sex-associated regions analyzed by changepoint analysis based on F_{ST} values, both in C and D.

ASTRAL v. 5.7.8 (Zhang et al., 2018), and clade support was calculated using local posterior probabilities (Sayyari & Mirarab, 2016). The species tree was then used for Dsuite analysis.

To test the possible ancestral states of SDSs in willows, we reconstructed character evolution in Mesquite v.2.0 (Maddison, 2007) based on the RAxML tree from the chromosome dataset. The 7XY system was coded as 0, the 15XY system was coded as 1, and the 15ZW system was coded as 2 in the probability model.

Gene flow in the genus *Salix*

To test for gene flow among related species in *Salix*, we used ABBA-BABA tests (also known as D-statistics), which detect imbalances in the number of discordant SNPs in species quartets (Patterson et al., 2012). We also applied f_4-ratio for gene flow testing, which was developed to estimate admixture history. We did the analysis in Dsuite 0.5 package (Malinsky et al., 2021), which specifically applied the f_{branch} statistic to assign gene flow results to the external (current) branches and possibly internal branches (the hypothetical ancestral lineages) on the phylogenetic tree. The VCF file and the species tree (see above) were used as input files for Dsuite analysis. We used three VCF datasets generated by GATK using *S. purpurea* as reference: (a) the autosome regions after excluding 7 and 15Z chromosomes (10,665,405 SNPs); (b) SLR extracted from chromosome 7 (Chr07: 5,115,466–7,308,458 of *S. purpurea*, He et al., 2021a; Zhou et al., 2020), which is homologous to the 7X-linked region of *S. dunnii* (23,994 SNPs); and (c) SLR extracted from chromosome 15Z (14,408 SNPs; Chr15Z: 2,346,797–6,704,703 of *S. purpurea*; Zhou et al., 2020). 15Z-linked region was homologous to SLR of 15XY species of *S. arbutifolia* (Wang et al., unpublished). The significance of each test was assessed using 100 jackknife resampling runs.

Additionally, as F_{ST} can be regarded as an indicator for predicting and assessing the degree of introgression, we estimated the weighted F_{ST} between species with different SDSs (7XY vs 15XY species; 7XY vs 15ZW species; and 15XY vs 15ZW species), in 100-kb windows with 10-kb steps on the autosomes/SLRs in VCFtools 0.1.16. We tested the significance of differences in autosomes/SLRs using the Wilcox test.

Results

Whole genome re-sequencing

After filtering the low-quality reads, we obtained 2,928. 19 million reads from a natural population of 42 *Salix cardiophylla* individuals of known sex (32.19–37.20 million reads per sample, mean 34.86; Supplementary Table S3) and 2,658.38 million reads from 38 *S. interior* individuals (32.10–41.31 million reads per sample, mean 34.98; Supplementary Table S4). The average mapping rate varied from 74% to 89.6% (average rate: 85.79%), and the average depth was 29.51 × (Supplementary Table S5) for *S. cardiophylla*. For *S. interior*, the mapping ratio varied from 89.8% to 92.7% (average rate: 91.53%) and the average depth was 33.71 × (Supplementary Table S6). We extracted 1,613,125 and 4,060,148 high-quality SNPs from the *S. cardiophylla* and *S. interior* samples, respectively, using *S. purpurea* (chromosome 15W excluded but 15Z kept, because W was degenerated but Z relatively conserved) as reference (Zhou et al., 2020).

SDS in *S. cardiophylla* and *S. interior*

GWAS and F_{ST} results between the male and female samples revealed an SLR on chromosome 15 (15Z of *S. purpurea*) in both *S. cardiophylla* and *S. interior* (Figure 1A and B; Supplementary Figures S1–S4). Changepoint analysis of the F_{ST} values detected candidate SLRs between 3.59 and 6.42 Mb in the *S. purpurea* 15Z assembly (2.83 Mb, about 21.3% of the chromosome) in *S. cardiophylla*, and 3.32 and 6.35 Mb (3.03 Mb, about 22.8% of the chromosome) in *S. interior* (Figure 1C and D).

GWAS analysis recovered totals of 78 and 40 SNPs with significant associations with sex on chromosome 15 (15Z of *S. purpurea*) in *S. cardiophylla* and *S. interior*, respectively (Supplementary Tables S7 and S8). In *S. cardiophylla*, 1.29% of these SNPs are heterozygous in females and 33.38% in males. In *S. interior*, 44.61% are heterozygous in the males, and all are homozygous in the females (Table 1). These results suggest that both *S. cardiophylla*

and *S. interior* have a SLR on chromosome 15, and male heterogamety (XY).

Phylogenetic trees based on nuclear and plastid data

Both maximum likelihood (ML) and species tree approaches were applied for the nuclear phylogeny reconstruction: (a) A ML phylogenetic tree of 15 willow species with known SDSs and the outgroup poplar species (*Populus euphratica*) was based on concatenation of 821,062 high-quality nuclear variants at fourfold degenerate sites. The tree (Figure 2A) resolved the two main clades, *Salix* and *Vetrix*, with high bootstrap support (100%). The *Salix* clade includes *S. nigra*, *S. chaenomeloides*, and *S. dunnii*. The remaining species fall into the *Vetrix* clade, with *S. triandra* on a basal branch. *Salix cardiophylla* and *S. arbutifolia* formed a subclade on the following branch. *Salix interior* and *S. exigua* also formed a subclade, which was sister to the other *Vetrix* species, including *S. polyclona*, *S. suchowensis*, *S. koriyanagi*, *S. integra*, *S. purpurea*, *S. viminalis*, and *S. udensis* (Figure 2A). (b) For the species tree construction, we reconstructed individual gene trees based on 6,315 variable genes that each had at least 50 SNPs. The species tree based on all the gene trees revealed the same topology as the ML tree (Supplementary Figure S5).

Trees based on plastid genomes (Figure 2B) yielded two major well-supported clades, but the subclade containing *S. interior* and *S. exigua* belonged to the *Salix* clade, other than in the nuclear phylogeny. *Salix cardiophylla* and *S. arbutifolia* appeared to be paraphyletic, and basal to a subclade comprising seven other *Vetrix* species (Figure 2B).

The ancestral character reconstruction analysis with SDSs indicated that the most likely ancestral SDS state for the genus *Salix* is 15XY (the relative likelihood: 53%), while the likelihood of ancestral 7XY is 35% (Figure 2A, Supplementary Figure S6). For the *Vetrix* clade, with both 15XY and 15ZW systems, the most likely ancestral state is 15XY with a proportional likelihood of 81% compared to 15ZW with a proportional likelihood of 11% (Figure 2A, Supplementary Figure S6).

Gene flow

We measured the level of interspecific gene flow along internal and external branches in *Salix* based on three datasets (autosomal, 15Z-linked region, and 7X-linked region datasets) (Figure 3; Supplementary Tables S9–S11). The internal nodes represent hypothetical last common ancestor lineages.

Firstly, the autosomal dataset revealed frequent introgression events among the species with 15XY, 15ZW, and 7XY systems (Figure 3A). In contrast, the sex-linked datasets showed fewer gene flow events among *Salix* species with different SDSs, in particular, no gene flow between 15ZW and 7XY species (Figure 3B and C), suggesting strong selection against introgression of SLRs.

In detail, for the autosomal dataset, the f_{branch} metrics yielded evidence for gene flow between *Salix exigua* and species of both

Table 1. Summary of estimated nucleotide homozygosity and heterozygosity rates on sex-linked SNPs based on GWAS results for *Salix cardiophylla* and *S. interior* using VCFtools.

Species	Sex	Number	Homozygous(%)	Heterozygous(%)
Salix cardiophylla	Female	19	98.71	1.29
Salix cardiophylla	Male	23	66.62	33.38
Salix interior	Female	20	100	0
Salix interior	Male	18	55.39	44.61

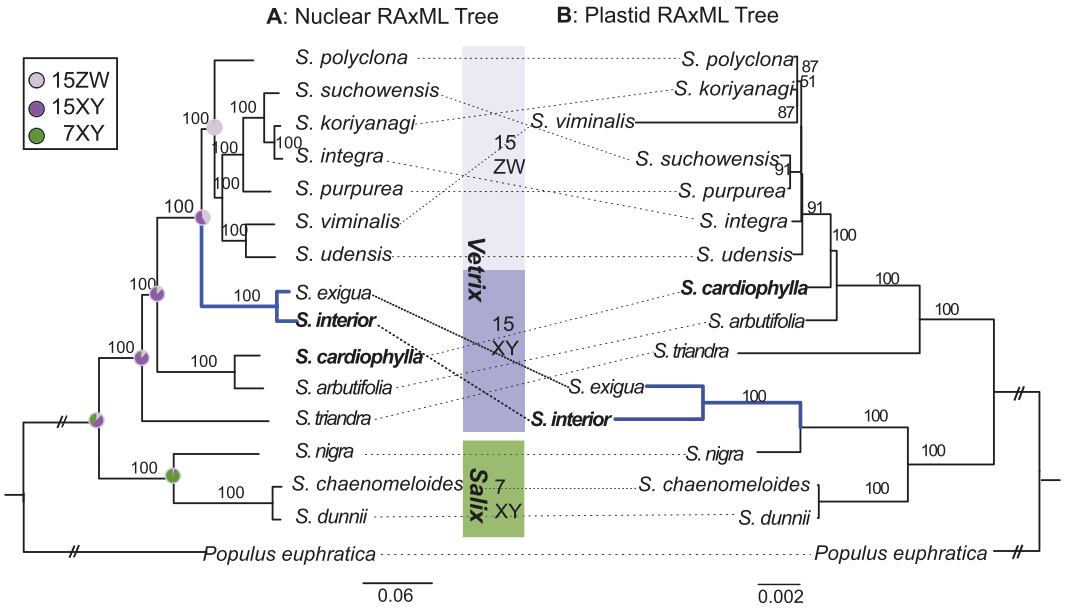

Figure 2. Phylogenetic relationship based on (A) nuclear SNPs and (B) plastid genomes of *S. cardiophylla* and *S. interior* and other *Salix* species with known sex determination systems, using *Populus euphratica* as an outgroup. The numbers at the nodes indicate support values based on 1,000 bootstrap replications. Bold branches for *S. interior/exigua* clade in the plastid tree indicate that the topology is not consistent with the nuclear tree. The tree is marked with the ancestral character-state reconstruction of the sex-determining system from Supplementary Figure S6.

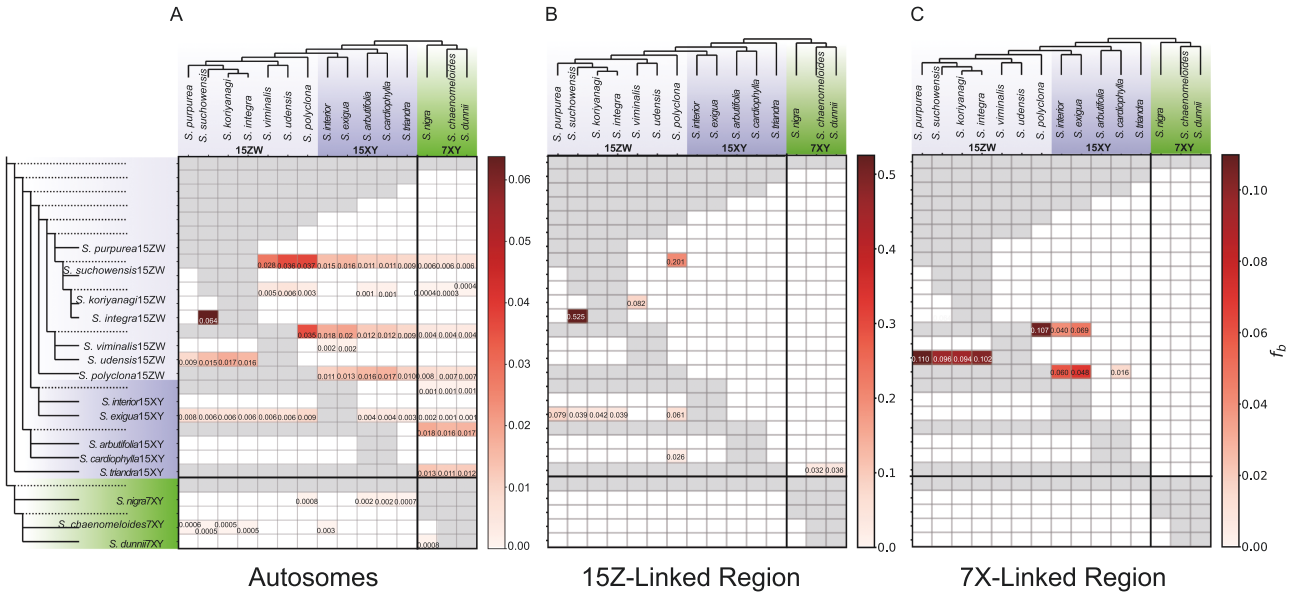

Figure 3. Heuristic f_{branch} analysis results of ongoing and ancient gene flow inferred for species in *Salix* genus with known sex-determination systems using Dsuite based on three SNP datasets. (A) Autosomal dataset; (B) 15Z-linked dataset; and (C) 7X-linked dataset. The nuclear species tree is shown at the top of the matrix. The tree is displayed in an "expanded" form along the y-axis points to a corresponding row in the matrix with ancestral branches. The values in the matrix refer to excess allele sharing between the expanded tree on the y-axis and the species tree on the x-axis.

the *Vetrix* clade (f_{branch} = 0.003–0.009) and the *Salix* clade (f_{branch} = 0.001–0.002). Considering gene flow between different SDSs, the analyses indicate: (a) stronger gene flow between 7XY and 15XY species (f_{branch} = 0.012–0.018) than between 7XY and 15ZW species (f_{branch} = 0.0003–0.008, Wilcox test $p < 0.01$); (b) gene flow was stronger between species with 15XY and 15ZW (f_{branch} = 0.009–0.018) than between 7XY and 15ZW species (f_{branch} = 0.0003–0.008, Wilcox test, $p < 0.01$); (c) gene flow was slightly weaker between 15XY and 15ZW species (f_{branch} = 0.009–0.018) than between 15XY and 7XY species (f_{branch} = 0.012–0.018), though the p value was not significant (p = 0.367). However, we cannot rule out that incompatibilities between the *Salix* clade and the *Vetrix* clade are due to overall genetic divergence, which is much larger between the major clades than within them (Figure 2). Finally, we detected stronger gene flow among 15ZW species of the *Vetrix* subclade (0.0009–0.064) than among either 15XY *Vetrix* clade species (0.006–0.009) or 7XY *Salix* clade ones (0.0005–0.0006, Supplementary Figure S7, Figure 3A).

Fewer gene flow events were detected for both sex-linked datasets. For the 15Z-linked region dataset, (a) gene flow signals were found between *S. exigua* and several 15ZW species including *S. purpurea* (0.079), *S. suchowensis* (0.039), *S. koriyanagi* (0.042), *S. integra* (0.039), and *S. polyclona* (0.061); (b) gene flow between *S. polyclona and S. cardiophylla* (0.026) were detected. For the 15XY and 7XY species, gene flow signals were only found between *S. triandra* (15XY) and two 7XY species, *S. dunnii* (0.036) and *S. chaenomeloides* (0.032, Figure 3B). The analysis of the 7X-linked region dataset revealed similar gene flow events as observed in the 15Z-linked region dataset, while no gene exchange was identified between 7XY and 15XY species (Figure 3C).

Window-based F_{ST} detected significantly higher F_{ST} values in SLRs compared to autosomes in all three datasets (F_{ST} values in 7X-SLR > F_{ST} values in autosomes in 7XY vs 15XY and 7XY vs 15ZW; F_{ST} values in 15Z-SLR > F_{ST} values in autosomes 15XY vs 15ZW), consistent with the gene flow results obtained from Dsuite analysis. The tests of significance of differences (Wilcox test) were also all significant (Supplementary Figure S8). At the same time, we must consider that F_{ST} can also be elevated due to the effects of sex-linked selection (Charlesworth et al., 1997), which may also play a role in the higher F_{ST} in the SLRs of our results.

Discussion

Our study confirms that SDSs are flexible between the major subgroups within *Salix*, while heterogamety and SLR locations are phylogenetically conservative within the subgroups (Figure 2). SLRs showed less genomic introgression compared to autosomes (Figure 3). This indicates that gene flow detection based on genomic analysis can reliably detect isolation between species, or lack of isolation.

XY system on chromosome 15 in *Salix cardiophylla* and *S. interior*

Previous studies showed that three of the earlier-branching species in the *Vetrix* clade have a 15XY system, including *Salix exigua* (Hu et al., 2023), *S. arbutifolia*, and *S. triandra* (Wang et al., 2022a, 2023). Wang et al. (2023) suggested that the species *S. arbutifolia* and *S. triandra* (15XY) are in a transitional position between 15ZW and 7XY systems. Our results provided two further early-branching species of the *Vetrix* clade having the 15XY system (Figure 2).

The American species *S. interior* and *S. exigua* are sister taxa, and both species have a 15XY system. The two species have been classified in section *Longifoliae*, which was considered to be a section of the *Salix* clade (Skvortsov, 1968; Wu et al., 2015). Chen et al. (2010) thought that they belonged to the New World subclade of the *Salix* clade. Previous hybridization experiments showed pollen–pistil incongruity of hybrids from *S. exigua/interior* and members of the *Salix* clade. By contrast, *S. exigua/interior* can hybridize with species of the *Vetrix* clade and produced fertile F_1 offspring (Mosseler, 1989, 1990). This indicates a closer relationship of *S. exigua/interior* with *Vetrix* than with *Salix* clade, which is consistent with Gulyaev et al. (2022) based on phylogenies of nuclear genomic sequences. *Salix interior* grouped with *S. exigua*

in our nuclear and plastid genome trees, but the nuclear data place them both in the *Vetrix* clade whereas the plastid sequences assign them to the *Salix* clade in the phylogenetic tree (Figure 2). Either incomplete lineage sorting (ILS) or an ancient hybridization event between ancestors of the *Salix* and *Vetrix* clades could cause these incongruent topologies. In the latter case, the phylogeny based on plastid genomes indicates that the maternal lineage may have come from the *Salix* clade, which would imply that a 7XX maternal parent was fertilized by a 15XY or 15ZZ paternal plant from the *Vetrix* clade. However, phased 15X and 15Y genes of *S. interior* and/or *S. exigua* are needed to validate this hypothesis.

The species *Salix cardiophylla* was previously segregated into a separate genus, *Toisusu*, based on its unique morphological characters, including deciduous styles (Kimura, 1928), but molecular phylogenetic analyses assigned it to the genus *Salix* (Chen et al., 2010). Until now, most phylogenetic studies accepted that *S. cardiophylla* falls in a clade with *S. arbutifolia* (Chen et al., 2010; Wu et al., 2015). We also found that *S. cardiophylla* and *S. arbutifolia* form a subclade in the *Vetrix* clade. Wang et al. (2023) showed that *S. arbutifolia* has male heterogamety and its SDS is on chromosome 15. Our study confirmed a 15XY system in *S. cardiophylla*. Although the SDSs are quite changeable in the genus *Salix*, the shared 15XY system in the *S. cardiophylla–S. arbutifolia* and *S. interior–S. exigua* subclades indicate that SDSs within subclades are usually conserved.

The role of sex chromosome evolution in speciation of *Salix*

Sex chromosome formation causally drives the speciation of different lineages. It has been suggested that neo-sex chromosomes from turnover events contributed to the speciation process in *Drosophila* (Yu et al., 1997). In the current datasets, the heterogamety and location of the SLR are conserved in related species, with 7XY systems in the *Salix* clade and the species in the *Vetrix* clade having SLRs on chromosome 15. We revealed turnovers in *Salix*, i.e., 15XY to 7XY and 15XY to 15ZW, consistent with the finding in (Wang et al., unpublished). The 15Y-linked region of *S. arbutifolia* lost 11.5% of its genes, while 7Y-SLR of *S. dunnii* and 15W-SLR of *S. purpurea* only lost 1.2% and 2.5%, respectively (He et al., unpublished; Wang et al., unpublished). The highest degradation rate of 15Y supports the hypothesis that 15XY is the ancestral state of genus *Salix*. Turnover of 15XY to 15ZW changed the heterogamety and appeared at the origin of a major group of the *Vetrix* clade that includes more than 300 species (He et al., 2021b). A burst of diversification near the origin of the *Vetrix* clade was found in Sanderson et al. (2023), which is likely to match the 15XY to 15ZW transition in this clade. Furthermore, a turnover of 15XY to 7XY likely triggered the speciation of the *Salix* clade, which includes ~60 species (He et al., 2021b).

After the initial stage of speciation, the evolution of strong reproductive barriers between species is important in maintaining species boundaries. Species in nature are often incompletely isolated after millions of years of species formation. At least 25% of plant species are involved in hybridization and potential introgression with other species (Mallet, 2005). Differentiation of sex chromosomes plays a major role as an introgression barrier during secondary contact between hybridizing species, as found in interspecific hybridization experiments of stickleback species (Kitano & Peichel, 2012) and in the plant genus *Rumex* in natural hybrid zones (Beaudry et al., 2020). Our *Salix* gene flow results further suggested that different SDSs in *Salix* genus possibly act as the barrier to introgression. In the autosomal dataset, frequent introgressions were observed among the species with 15XY, 15ZW, and 7XY (Figure 3A). The results suggested that there are incomplete reproductive barriers between species pairs, at least in the early generations of their separation. Similar incomplete reproductive isolation is also observed in a long-term evolutionary crossing experiment of two *Xiphophorus* fish with ZW and XY SDSs (Franchini et al., 2018). However, our sex-linked datasets showed gene flow between only a few of the species with 15ZW and 15XY SDSs, while no gene flow was found between 15ZW and 7XY species (Figure 3B and C), suggesting strong selection against introgression of SLRs (Sætre et al., 2003).

The SLRs varied among different species of *Salix*, which may relate to their sex chromosome systems. Willow species with known SLR lengths, using *S. purpurea* as a reference, include: *Salix triandra* (15XY) SLR 2.8 Mb (Wang et al., 2023), *S. arbutifolia* (15XY) SLR 3.33 Mb, *S. cardiophylla* (15XY) SLR 2.83 Mb (this study), *S. interior* (15XY) SLR 3.03 Mb (this study), *S. purpurea* (15ZW) Z-SLR 4.4 Mb (Zhou et al., 2020), and *S. polyclona* (15ZW) SLR 4.68 Mb (He et al., 2023). This variation of the SLRs in *Salix* may indicate independent evolution in the sex chromosomes although they share a common ancestry in an earlier stage, like in the true frogs (Jeffries et al., 2018) and sticklebacks (Yoshida et al., 2014).

With available phased genomes, heteromorphism between X/W and Y/Z was detected in three species distributed in different clades covering all three kinds of willow SDSs (Table 2): *S. dunnii* 7XY (He et al., unpublished), *S. arbutifolia* 15XY (Wang et al., unpublished), and *S. purpurea* 15ZW (Zhou et al., 2020). In detail, 7X-SLR of *S. dunnii*, 15X-SLR of *S. arbutifolia*, and 15W-SLR of *S. purpurea* accumulated more repeat sequences compared to their homologous Y- or Z-SLR, and inversions were identified on the X-Y and Z-W pairs (Wang et al., unpublished). In Salicaceae, *Salix*'s sister genus *Populus* has relatively small SLRs in species with known SDSs (0.08–2 Mb, Wang et al., 2023), which probably facilitates hybridizations between species with ZW and XY systems, e.g., *P. alba* ZW × *P. tremula* XY = *P.* × *canescens*, which is fully fertile (Meikle, 1984; Zhang et al., 2023). In *Salix*, hybridization between different clades (*Salix* and *Vetrix*) is less common (Mosseler, 1990), which may be due to the long SLRs and the heteromorphic chromosomes (see above). Previous studies revealed that incompatible loci/heteromorphism for hybrid male/female sterility likely accumulated on the SLRs, which likely facilitates reproductive isolation in different subclades of *Salix* and thus maintains species barriers (He et al., unpublished; Wang et al., unpublished; Zhou et al., 2020). However, breeding experiments between species with different SDSs are urgently required to

Table 2. *Salix* species with phased chromosomes.

Species	SDS systems	X/W length (Mb)	SLR length (Mb)	SLR percent	Y/Z length (Mb)	SLR length (Mb)	SLR percent	References
S. dunnii	7XX/XY	19.21	5.87	30.56%	16.39	2.95	18.00%	He et al., unpublished
S. arbutifolia	15XX/XY	16.16	7.06	43.69%	10.94	1.81	16.54%	Wang et al., unpublished
S. purpurea	15ZZ/ZW	15.65	6.7	42.81%	13.3	4.4	33.08%	Zhou et al., 2020

further test the contribution of sex chromosome evolution to speciation.

Supplementary material

Supplementary material is available online at *Evolution Letters*.

Data and code availability

Sequence data presented in this article can be downloaded from the NCBI database under BioProject accession PRJNA984197 for *Salix cardiophylla* and PRJNA984212 for *Salix interior*.

Author contributions

L.H. designed the study; L.H. and Z.Q.X. performed the analysis and wrote the manuscript with input from W.L.A. (who supplied identified *S. interior*) and E.H.; all authors contributed to later versions of the manuscript.

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