



Review

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The sexual determination system in *Salix* L. (Salicaceae)Hernán Ariel López^{1,2*}

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Abstract

Current knowledge of the sex determination system that has been analyzed in different species of the genus *Salix* L. is reviewed. In this genus, sexual chromosomes have been recognized and it was proposed that a gene for sexual determination must be present. In the genus *Salix* there are two systems of sexual determination: one in which the homogametic system is female and the heterogametic is male (XX/XY system) and another in which the homogametic system is male and the heterogametic is female (ZZ/ZW). The evidence is analyzed in relation to phylogenetic considerations and the need to include the analysis to species of other subgenera is raised. The role of the determination system in the hybridization of species with a mixed determination system is discussed, while it is suggested that this characteristic be investigated in the rest of the members of *Salicaceae*. Both sex determination systems (SDSs) are found in *Salix* and are of phylogenetic importance because each type of system is found in different phylogenetic groups. The SDS can influence the expression of the sexes in individuals, especially in polyploid species, and at a population level by influencing the sex ratio related to ploidy levels.

Key words: polyploidy, *Salix*, sex determination system (SDS), sex linked region (SLR), sex ratio, XY, ZW.

Introduction

The reproductive strategies of plants can be highly variable, employing hermaphroditism, dioecy or polygamy to ensure cross-pollination and their genetic variability (Cronk, 2022). Within the strict dioecy, sex determination systems (SDS) have evolved repeatedly among flowering plants and independently across lineages. Some plants have differences between their sex chromosomes (heteromorphism), while others show no differences in them (homomorphism). Sex-linked regions (SLR) have recently been identified for several plants with homomorphic sex chromosomes, and some of these have been found to be small parts of chromosome pairs, allowing sex-determining genes to be identified

(Semerikov, *et al.*, 2003; Yin *et al.*, 2008; Chen *et al.*, 2016; Akagi *et al.*, 2019; Harkess *et al.*, 2017, 2020; Cronk and Müller, 2020; Müller *et al.*, 2020; Zhou *et al.*, 2020). These genes are often involved in hormonal response pathways, mainly associated with cytokinin and ethylene response pathways (Feng *et al.*, 2020; Leite Montalvão *et al.*, 2022). XX/XY (heterogametic male) and ZW/ZZ (heterogametic female) sex determination systems have been found in related species (Carlson *et al.*, 2017; Martin *et al.*, 2019; Müller *et al.*, 2020; Zhou, Macaya-Sanz, Carlson, *et al.*, 2020). Similarly, in the genus *Salix* it has been possible to determine both SDSs. According to recent phylogeny studies of *Salix* through whole genome re-sequencing (Gulyaev *et al.*, 2022), two main groups or clades are recognized, *Vetrix* and *Salix* although these groups do not necessarily represent taxonomic groups or categories. The *Vetrix* clade comprises four subclades, endemic Asian species, Eurasian and North American species, two species of section *Longifoliae* and one species from section *Amygdalinae*. The *Salix* clade becomes paraphyletic by inclusion of mainly polyploid species and includes a group with mostly species of the subgenus *Protitea* (Gulyaev *et al.*, 2022). The species analyzed so far show that two SDS mechanisms exist in separate clades of the genus, *Vetrix* and *Salix*. In the *Vetrix* clade, the species analyzed so far present the ZW system while in the *Salix* clade, the species that have been analyzed belong to the XY system (Hu *et al.*, 2019; Sanderson *et al.*, 2021; He *et al.*, 2021; Gulyaev *et al.*, 2022). This separation of SLR in specific clades could be the result of their independent evolution (Yang *et al.*, 2021).

The implications of the occurrence of SDSs in the genus *Salix*, in relation to phylogeny, ploidy and the balance of the sexes, as well as the possible consequence of hybridization between species with different sex determination systems has not been discussed in depth. In the current review taxonomic and reproductive details are provided, in order to recognize certain patterns in the influence of SDS, that could guide future research on the subject.

The objectives of this work are: (1) to review the knowledge about chromosomal sex determination in the genus *Salix*; (2) to relate the presence of SLR to its implication in the phylogeny of the genus; (3) to highlight the effect of SDS on the balance of the sexes; (4) to analyze the possible phenotypic effect of sex determination in relation to ploidy; (5) to discuss the effects of SDS under hybridization conditions; (6) to relate the effect of SDS to the sexual expression of individuals of *Salix* species.

The information on sexual determination reported in the scientific literature related to the genus *Salix* is reviewed, discussed and analyzed. The bibliographic searches have been carried out on the Google Scholar site, using keywords including willows, *Salix*, dioecy, pseudodioecy, subdioecy, hermaphroditism, sex determination system, SDS, sex linked region,

SLR, ploidy, sex ratio 1:1. Chromosome numbers have been consulted in the Index to Plant Chromosome Numbers, (Goldblatt and Johnson 1979-).

Discussion

Evidence of sex determination systems in *Salix*.

To date, the SLRs of only 9 *Salix* species have been evaluated, 5 of which belong to the subgenus *Vetrix* Dumort. (*S. koriyanagi* Kimura ex Goerz, *S. integra* Thunb., *S. purpurea* L., *S. udensis* Trautv. & C.A.Mey. and *S. polyclona* C.K.Schneid.), where the SLR has been detected on chromosome 15, the female being the heterogametic sex. (ZW/ZZ system). The taxonomic position of *S. triandra*, initially located in the subgenus *Salix* (section *Amygdalinae* W.D.J.Koch), was the subject of controversy, until Gulyaev and his co-authors (2022) placed it within the *Vetrix* clade. The rest of the analyzed species belong to the subgenera *Protitea* Kimura and *Longifoliae* Argus, where the SLR is found on chromosome 7, determining a male heterogametic system (XY/XX). The presence of both systems in different chromosomes has promoted interest in the study of this character within the genus (He *et al.*, 2021) and its possible implications and applications (Gulyaev *et al.*, 2022).

Mosseler and Zsuffa (1989), while carrying out studies of inter- and intra-specific crossing, observed certain sexual expressions *S. amygdaloides* Andersson and *S. lucida* Muhl, which suggested that they could have a male heterogametic sexual determination mechanism (considered here as XX/YY). the first report of such a possibility.

Nagamitsu and Futamura, (2014), using intraspecific crosses, proposed that *S. subfragilis* Andersson, could have a male heterogametic system (XY), although, in genetic studies the SLR was placed in chromosome 15. In the same way, *S. exigua* Nutt. which belongs to the subgenus *Longifoliae*, has a sex determination system XX/XY (Mosseler and Zsuffa, 1989), but with an SLR located on chromosome 15 (Hu *et al.*, 2019). In both species the SDS is XX/XY and the SLR is located on chromosome 15 where a different SDS system, ZZ/ZW, is usually found.

The species analyzed in this regard represent only 1.3% of the total species of the genus *Salix*, which shows how little evidence is available and how much remains to be known before a full understanding of the functioning of the SDS in the genus.

Implications of the presence of SLR on the phylogeny of the genus

The genus has a complex phylogeny that is constantly being updated as new phylogenetic analyses are published. The difficulty in clarifying the phylogeny within the

genus is due to the lack of prezygotic and postzygotic barriers that prevent gene flow, cross-linked hybridization, reticulate evolution and polyploidization (Wu *et al.*, 2015; Wagner *et al.*, 2020).

Two clades are currently recognized in the genus, *Salix* and *Vetrix*. The *Salix* clade has been the subject of several phylogenetic studies to establish its subgroups in the subgenus *Salix* (Skvortsov, 1999; Argus, 2010; Abdollahzadeh *et al.*, 2011; Wu *et al.*, 2015; Lauron-Moreau *et al.*, 2015, Acar *et al.*, 2022). Lauron-Moreau and co-workers (2015) subdivide the genus *Salix* into 4 subgenera: *Chamaetia/Vetrix*, *Longifoliae*, *Protitea* and *Salix*.

Table 1. Sex determinant systems in species of *Salix* (adapted from He *et al.*, 2021)

Species	Subgenus ¹	Section	Chromosome	Ploidy level	SD S	References
<i>S. nigra</i> Marshall	<i>Protitea</i>	Humboldtianae	7	2x	XY	Sanderson <i>et al.</i> , 2021
<i>S. dunnii</i> C.K.Schneid.	<i>Protitea</i> ²	Humboldtianae	7	2x	XY	He <i>et al.</i> , 2021
<i>S. exigua</i> Nuttall	<i>Longifoliae</i>	<i>Longifoliatae</i>	15	2x	XY	Hu <i>et al.</i> , 2019; Mosseler and Zsuffa, 1989.
<i>S. subfragilis</i> Andersson	<i>Salix</i>	<i>Salix</i>	15	n.d.	XY	Nagamitsu and Futamura, 2014, Wang <i>et al.</i> , 2023
<i>S. triandra</i> L.	<i>Vetrix</i> ³	n.d.	15	2x	ZW	Li <i>et al.</i> , 2020
<i>S. arbutifolia</i> Pall.	<i>Vetrix</i>	n.d.	15		XY	Wang <i>et al.</i> , 2023
<i>S. suchowensis</i> W.C.Cheng ex G.Zhu	<i>Vetrix</i>	n.d.	15	n.d.	ZW	Hou <i>et al.</i> , 2015; Wilkerson <i>et al.</i> , 2022
<i>S. polyclona</i> C.K.Schneid.	<i>Vetrix</i>	n.d.	15	2x, 4x	ZW	He <i>et al.</i> , 2023
<i>S. purpurea</i> L.	<i>Vetrix</i>	<i>Helix</i>	15	2x	ZW	Zhou <i>et al.</i> , 2018; Zhou, Sanz, Carlson <i>et al.</i> , 2020
<i>S. integra</i> Thunb.	<i>Vetrix</i>	<i>Helix</i>	15	n.d.	ZW	Wilkerson <i>et al.</i> , 2022
<i>S. koriyanagi</i> Kimura ex Goerz	<i>Vetrix</i>	<i>Helix</i>	15	n.d.	ZW	Wilkerson <i>et al.</i> , 2022
<i>S. viminalis</i> L.	<i>Vetrix</i>	<i>Viminiella</i>	15	2x	ZW	Alstrom-Rapaport <i>et al.</i> , 1998; Pucholt <i>et al.</i> , 2015; 2017; Almeida <i>et al.</i> , 2020; Hallingbäck <i>et al.</i> , 2021
<i>S. udensis</i> Trautv. & C.A.Mey.	<i>Vetrix</i>	<i>Viminiella</i>	15	2x	ZW	Wilkerson <i>et al.</i> , 2022
<i>S. herbacea</i> L.	<i>Chamaetia</i>	n.d.	15	2x	n.d.	Cortés, 2015

¹ Classification system according to Argus (2010). ² Position according to Wu *et al.*, 2015. ³ Position according to Gulyaev *et al.*, 2022, n.d.= not determined

The species studied are representatives of the two major clades or subgenera, *Salix* and *Vetrix*. The distribution of SDS among the species studied (Table 1) shows that there are different patterns in terms of the SDS for each one of them. The species of the *Salix* clade present the masculine heteromorphic system (XY/XX), belonging to the subgenera *Protitea* and *Longifoliae*. In the studies on the subject by Mosseler and Zsuffa (1989) and Almeida and co-authors (2020) it is assumed that the dominant system in the *Salix* clade is XY/XX. It has

been observed that in species of the *Vetrix* clade the SDS is female heteromorphic (ZW/ZZ) (Table 2), with no differences between the sections *Helix* Dumort. and *Viminella* Ser. The different SDSs may support a biologically important subdivision of *Salix* which could be used as a character in subgeneric classification (Gulyaev *et al.*, 2022). However, it is necessary to confirm with future studies if this pattern of sexual determination is also present in the remaining species of the *Salix* clade, especially those that are allopolyploid and occurred in more recent evolutionary times (Wu *et al.*, 2015; Gulyaev *et al.*, 2022).

The determination of an XX/XY system on chromosome 15 of *S. exigua* (Hu *et al.*, 2019) is of special importance because it could represent an intermediate evolutionary stage in sexual determination in *Salix*, given that the remaining species that have an SDR on chromosome 15 have a different SDS system (ZZ/ZW) and belong to a different clade (*Vetrix*). Likewise, a similar case is observed in *S. triandra*, the SLR of which has been found on chromosome 15 (He *et al.*, 2020), which could mean that it would have a ZZ/ZW SDS. However, in intraspecific crossbreeding experiments in unusually hermaphroditic individuals, the results suggest that it would have an SDS XX/XY, with the male being the heterogametic sex (Nagamitsu and Futamura, 2014). *S. exigua*, *S. triandra* and *S. subfragilis* with intermediate phylogenetic positions between the sections (Azuma *et al.*, 2000) could be taxa in which the SDS have diverged in the evolution of the genus. It is possible that this group represents the transition from an XY system present in the most basal species to a ZW system with a change in the position of the SDR from chromosome 7 to chromosome 15, without the intervention of a regulatory or suppressor zone (see later for model of gene regulation). Yang and co-authors (2021) proposed that in *Salix* the change from XY to ZW has been relatively recent and was mediated by long-terminal repeat retrotransposons (LTR-RTS) in a different way to that in *Populus*.

Influence of SDS and ploidy level on sex ratio

Willow species may show a different pattern in the balance of the sexes in a population. The expected 1:1 ratio of the sexes in a population of a dioecious species may vary in relation to species with different types of SDS. The ratio of the sexes can be balanced with equivalent numbers of each sex in the population or unbalanced, with a predominance of the number of individuals of one sex over another. Table 2 summarizes the evidence found for 38 species of *Salix* where the sex ratio was obtained.

Table 2. Sex ratio ($\sigma^{\text{♂}}:\text{♀}$) reported in *Salix* species, ordered by taxonomic position, SDS and ploidy

Species	Subgenus	Section	Ploidy level	SDS	Sex ratio ($\sigma^{\text{♂}}:\text{♀}$)	References
<i>S. exigua</i> Nutt.	<i>Longifoliae</i>	<i>Longifoliae</i>	2x	XY	$\sigma^{\text{♂}}=\text{♀}$ $\sigma^{\text{♂}}>\text{♀}$	Mosseler and Zsuffa, 1989; Ottenbreit and Staniforth, 1992; Aravanopoulos, 1998
<i>S. hukaoana</i> Kimura	<i>Longifoliae</i>	<i>Longifoliae</i>	2x	XY*	$\sigma^{\text{♂}}=\text{♀}$	Ban and Ide, 2004
<i>S. dunnii</i> C.K.Schneid.	<i>Protitea</i>	<i>Humboldtianae</i>	2x	XY	$\sigma^{\text{♂}}=\text{♀}$ $\sigma^{\text{♂}}>\text{♀}$	Zeng <i>et al.</i> , 2022
<i>S. nigra</i> Marshall	<i>Protitea</i>	<i>Humboldtianae</i>	2x	XY	$\sigma^{\text{♂}}=\text{♀}$	Pitcher and McKnight, 1990 (* sex ratio taken)
<i>S. acmophylla</i> Boiss.	<i>Protitea</i>	<i>Humboldtianae</i>	n.d	XY*	$\sigma^{\text{♂}}=\text{♀}$ $\sigma^{\text{♂}}<\text{♀}$	Rottenberg, 1998
<i>S. amygdaloides</i> Andersson	<i>Protitea</i>	<i>Humboldtianae</i>	2x	XY*	$\sigma^{\text{♂}}=\text{♀}$	Kaul and Kaul, 1989
<i>S. eriocephala</i> Michx.	<i>Vetrix</i>	<i>Cordatae</i>	2x	XY	$\sigma^{\text{♂}}=\text{♀}$	Mosseler and Zsuffa, 1989; Elmquist <i>et al.</i> , 1988 (syn. <i>S. rigida</i>)
<i>S. magnifica</i> Hemsl.	<i>Salix</i>	<i>Magnificae</i>	n.d.	XY*	$\sigma^{\text{♂}}<\text{♀}$	Lei <i>et al.</i> , 2017
<i>S. paraplesia</i> C.K.Schneid.	<i>Salix</i>	<i>Pentandrae</i>	4x	XY*	$\sigma^{\text{♂}}<\text{♀}$	Liao <i>et al.</i> , 2019
<i>S. alba</i> L.	<i>Salix</i>	<i>Salix</i>	4x	XY*	$\sigma^{\text{♂}}<\text{♀}$	Rottenberg, 1998
<i>S. × fragilis</i> L.	<i>Salix</i>	<i>Salix</i>	4x,5x	XY*	$\sigma^{\text{♂}}<\text{♀}$	Shafroth <i>et al.</i> , 1994 (syn. <i>S. x rubens</i>)
<i>S. herbacea</i> L.	<i>Chamaetia</i>	<i>Retusae</i>	2x	ZW	$\sigma^{\text{♂}}<\text{♀}$	Crawford and Balfour, 1983; Carbognani <i>et al.</i> , 2019; Cortés, 2015
<i>S. triandra</i> L.	<i>Salix</i> ¹	<i>Amygdalinae</i>	2x, 3x, 4x,	ZW*	$\sigma^{\text{♂}}=\text{♀}$	Belyaeva, 1991
<i>S. purpurea</i> L.	<i>Vetrix</i>	<i>Helix</i>	2x	ZW	$\sigma^{\text{♂}}<\text{♀}$	Gouker <i>et al.</i> , 2021
<i>S. udensis</i> Trautv. & C.A.Mey.	<i>Vetrix</i>	<i>Viminella</i>	2x	ZW	$\sigma^{\text{♂}}<\text{♀}$	Ueno <i>et al.</i> , 2007 (syn. <i>S. sachalinensis</i> F. Schmidt)
<i>S. viminalis</i> L.	<i>Vetrix</i>	<i>Viminella</i>	2x	ZW	$\sigma^{\text{♂}}<\text{♀}$	Alström-Rapaport <i>et al.</i> , 1997
<i>S. gmellini</i> Pall.	<i>Vetrix</i>	<i>Viminiella</i>	2x, 3x, 4x,	n.d.	$\sigma^{\text{♂}}=\text{♀}$	Belyaeva, 1991
<i>S. suchowensis</i> W.C.Cheng ex G.Zhu	<i>Vetrix</i>	<i>Helix</i>	2x,3x	ZW	$\sigma^{\text{♂}}<\text{♀}$	Hou <i>et al.</i> , 2017
<i>S. nipoclada</i> Rydb.	<i>Chamaetia</i>	<i>Glaucae</i>	2x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Myer-Smith and Hik, 2012
<i>S. arctica</i> Pall.	<i>Chamaetia</i>	<i>Glaucae</i>	4x,5x,6x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Tolvanen <i>et al.</i> , 2001
<i>S. glauca</i> L. var. <i>acutifolia</i> (Hook.) C.K.Schneid.	<i>Chamaetia</i>	<i>Glaucae</i>	4x,5x,6x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Myer-Smith and Hik, 2012
<i>S. polaris</i> Wahlenb.	<i>Chamaetia</i>	<i>Retusae</i>	4x,5x,6x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Dromann and Scarpe, 2002
<i>S. variegata</i> Franch.	<i>Vetrix</i>	<i>Variagatae</i>	n.d.	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Su <i>et al.</i> , 2016
<i>S. barrattiana</i> Hook.	<i>Vetrix</i>	<i>Villosae</i>	n.d.	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Myer-Smith and Hik, 2012
<i>S. caprea</i> L.	<i>Vetrix</i>	<i>Cinerella</i>	2x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Danell <i>et al.</i> , 1991
<i>S. myrtilifolia</i> Andersson	<i>Vetrix</i>	<i>Hastatae</i>	2x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Neid <i>et al.</i> , 2006
<i>S. repens</i> L.	<i>Vetrix</i>	<i>Incubaceae</i>	2x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	de Jong and van der Meijden, 2004; Nissinen <i>et al.</i> , 2016
<i>S. lanata</i> L.	<i>Vetrix</i>	<i>Lanatae</i>	2x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Predavec and Danell, 2001
<i>S. richardsonii</i> Hook.	<i>Vetrix</i>	<i>Lanatae</i>	2x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Myer-Smith and Hik, 2012
<i>S. sitchensis</i> Sanson ex Bong.	<i>Vetrix</i>	<i>Sitchenses</i>	2x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Che-Castaldo <i>et al.</i> , 2015
<i>S. starkeana</i> Willd.	<i>Vetrix</i>	<i>Vetrix</i>	2x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Danell <i>et al.</i> , 1991
<i>S. lapponum</i> L.	<i>Vetrix</i>	<i>Villosae</i>	2x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Hrones <i>et al.</i> , 2019
<i>S. alaxensis</i> (Andersson) Coville var. <i>alaxensis</i>	<i>Vetrix</i>	<i>Villosae</i>	2x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Myer-Smith and Hik, 2012
<i>S. cinerea</i> L.	<i>Vetrix</i>	<i>Cinerella</i>	4x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Alliende and Harper, 1989
<i>S. lasiolepis</i> Benth.	<i>Vetrix</i>	<i>Mexicanae</i>	4x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Sacchi and Price, 1988
<i>S. pulchra</i> Cham.	<i>Vetrix</i>	<i>Phyllicifoliae</i>	4x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Myer-Smith and Hik, 2012
<i>S. myrsinifolia</i> Salisb.	<i>Vetrix</i>	<i>Nigricantes</i>	6x	ZW*	$\sigma^{\text{♂}}<\text{♀}$	Danell <i>et al.</i> , 1991
<i>S. acutifolia</i> Willd.	<i>Vetrix</i>	<i>Daphnella</i>	2x	n.d.	$\sigma^{\text{♂}}=\text{♀}$	Belyaeva, 1991

* = Indicates that the SDS is assumed, based on Gulyaev *et al.*, 2022. Species SDS requires confirmation.
n. d.= not determined. ¹. Clade *Vetrix* after Gulyaev *et al.*, 2022.

Balanced sex ratio in diploid XY species of *Salix*

When considering the species in relation to the ploidy level, it can be observed that diploid species can have a balanced sex ratio with 50% of each member of the sexes in a population, as occurs in the species of subgenus *Protitea*, as in *Salix amygdaloides* Andersson (Kaul and Kaul, 1989), *S. dunnii* C.K.Schneid (Zeng *et al.*, 2022) and *S. acmophylla* Boiss. (Rottenberg, 1998). In *S. nigra* Marshall, a sex ratio of 1:1 was found by Pitcher and McKnight (1990). The chromosome number of *S. acmophylla* is not known but in this review it is assumed that the species is diploid like the other species of the same subgenus. In the subgenus *Longifoliae*, of debatable taxonomic position, the sex ratio has been recorded for *S. exigua* Nutt. (Mosseler and Zsuffa, 1989; Ottenbreit and Staniforth, 1992; Aravanopoulos, 1998) and *S. hokaoana* Kimura (Ban and Ide, 2004). *S. exigua*, as previously mentioned, could represent a transition state between the two types of SDS. Surprisingly, *S. eriocephala* Michx, a diploid species of the subgenus *Vetrix* (section *Cordatae*) with an SDS estimated as XY by intraspecific crosses (Mosseler and Zsuffa, 1989), exhibits a 1:1 sex ratio. This is a special case that differs from the rest of the subgenus *Vetrix* species where the ZW system prevails and an unbalanced sex ratio, as discussed later.

Evidence of hybridization between *S. exigua* and *S. eriocephala* have been reported (Mosseler and Papadopol, 1989; Mosseler, 1990), which was used as proof of the absence of reproductive barriers between the *Salix* and *Vetrix* clades (Gulyaev *et al.*, 2022). However, this case does not seem to provide good evidence for a general rule because, exceptionally, both taxa share the same SDS (XY) (Table 2) despite belonging to different subgenera and this could be the reason that hybridization between *S. exigua* and *S. eriocephala* can generate viable offspring.

Male biased sex ratio in diploid XY species of *Salix*

It has been reported for *S. dunnii* (Zeng *et al.*, 2022) and *S. exigua* (Ottenbreit and Staniforth, 1992) that there is a male biased sex ratio in some populations of the species, although populations with a 1:1 sex ratio have also been reported in these species (Mosseler and Zsuffa, 1989; Aravanopoulos, 1998; Zeng *et al.*, 2022). In *S. dunnii*, the male biased sex ratio could be explained by allocated more resource in producing male flowers at flowering stage, which was probably beneficial for wind-pollination, while female plants fought against the “pollinator limitation” by increasing the variety and output of volatile to improve insect-pollination (Zeng *et al.*, 2022).

Female biased sex ratio in polyploid XY species of *Salix*

In species of the subgenus *Salix*, a female biased sex ratio has been found in *S. magnifica* Hemsl (Lei *et al.*, 2017), *S. paraplesia* C.K.Schneid (Liao *et al.*, 2019), *S. alba* L. (Rottenberg, 1998) and *S. × fragilis* L. (Shafroth *et al.*, 1994). In the case of *S. magnifica*, its chromosome number is not known, but due to the tendency found in other species of the same group, it is hypothesized in this review that it is polyploid. Future chromosome studies on the species may clarify this hypothesis. The genetic cause of the imbalance in the sex ratio in polyploid species is unknown, it could possibly be related to the effect of additional copies of the gene that determines femaleness on the X chromosome and/or the lack of inhibition activity by the gene on the Y chromosome.

Unbalanced sex ratio in ZW diploid and polyploid species of *Salix*

In the case of species that present a ZW system, regardless of whether they are diploid or polyploid, they present, in most cases, a female biased sex ratio (Table 2, Figure 1). The sex determination system ZW has been reported in the subgenera *Chamaetia* Dumort. and *Vetrix*, which are phylogenetically close (Gulyaev *et al.*, 2022). In the subgenus *Chamaetia*, for both the diploid species such as *S. herbacea* L. (Crawford and Balfour, 1983; Carbognani *et al.*, 2019 and Cortés, 2015) and *S. niphoclada* Rydb. (Myers-Smith and Hik, 2012) and the polyploid species *S. artica* Pall. (Tolvanen *et al.*, 2001), *S. glauca* L. (Myer-Smith and Hik, 2012) and *S. polaris* Wahlenb. (Dormann and Skarpe, 2002) unbalanced female relationships have been observed. The 27 species of the subgenus *Vetrix*, both diploid and polyploid, present mostly a female biased sex ratio (Table 2). However, in some populations of *S. triandra*, *S. viminalis*, *S. gmelinii* and *S. acutifolia* a sex determination system ZW is present but with a balanced ratio of the sexes (Belyaeva, 1991).

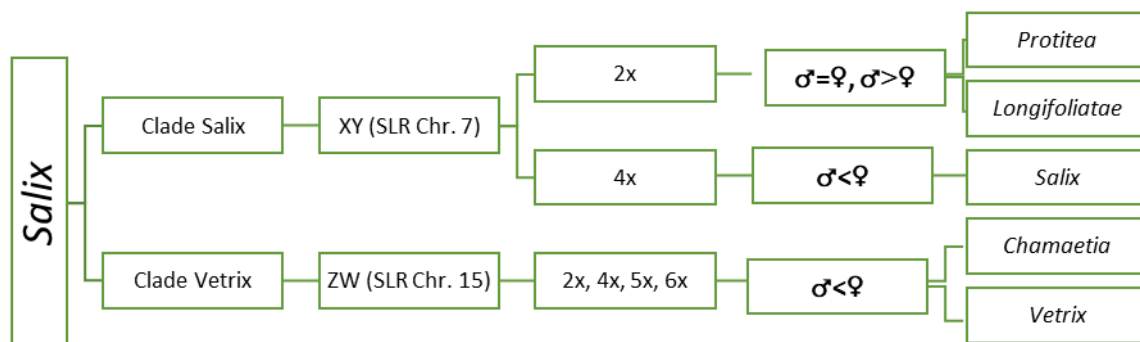


Figure 1: Summary of data. Classification of the *Salix* groups in which the evidence for SDS, ploidy and sex ratio have been related.

In general, the reported species that have a ZW type SDS with the gene that determines femaleness on chromosome 15, present a female biased sex ratio regardless of the ploidy level. The probable causes of these observations could be attributed to the dominant character of this gene.

In *S. purpurea* L. a 1.15 Mb sex-linked region on Chr15W was confirmed to be absent in monoecious plants, the inheritance of which structural variation is responsible for the loss of a male-suppressing function what would otherwise be genetic females (ZW), resulting in monoecy (ZW^H or WW^H), or lethality, if the male phenotype is homozygous ($W^H W^H$) (Hyden *et al.*, 2023). Some of these genotypic variants could also explain the predominance of the female phenotype in ZW plants.

Sex ratio and environmental effect

In certain species there are variations in the relationship of the sexes due to latitudinal variations in their distribution range, which suggests that control of sexual expression at the population level is influenced by environmental variations (Cronk *et al.*, 2015). The variation in sexual expression at the population level could possibly be caused by epigenetic regulation in a similar way to that shown by Cheng and his co-workers (2019), where the DNA methylation, an important epigenetic modification, regulates the expression of genes and is therefore involved in the transitions between floral developmental stages in flowering plants.

Determination of sex in relation to ploidy.

Salix species show an evolutionary pattern towards polyploidy. Polyploidy may be influencing sex determination because of the multiple copies of SLRs that these individuals possess. Until now, the species whose SDS have been studied are the diploid species, *S. nigra*, *S. triandra*, *S. viminalis*, *S. udensis* and *S. purpurea* (Buechler, 2010), although *S. triandra* was shown to be polyploid by several authors (Blackburn and Heslop-Harrison, 1924; Wilkinson, 1944; Fedorova-Sarkisova, 1946; Hörandl *et al.*, 2002; Schanzer *et al.*, 2022). It is of interest to extend the SLR analysis to polyploid species to assess their behaviour. The effect of ploidy on the evolution of sex chromosomes was recently reviewed by He and Hörandl (2023). They analysed studies of ploidy and sex determination in several genera and concluded that the size of regions should be investigated for both XY and ZW systems and the times when they evolved. Ploidy could generate a diversity of phenotypes in relation to the expression of the various combinations of SLR genes, one of the possible effects, as mentioned, being the predominance of female phenotypes (Gulyaev *et al.*, 2022). Until now, SDS and SLR of

polyploid species have not been studied and the phenotypic manifestations of the various combinations of SLR in their dominant states are still unknown. Therefore, this point is of interest for future research to reach an understanding of the manifestation and relationships between the various alleles.

Effect of SDS on hybridization conditions

Within the *Salix* genus, natural hybridization between species of different groups is frequent, which results in the appearance of allopolyploid hybrids (Wagner *et al.*, 2020), mainly in the subgenus *Salix*. However, there may be some barriers to hybridization between the two main clades of *Salix* (*Vetrix* and *Salix*). Species of the *Salix* clade have an XX/XY system, while species of the *Vetrix* clade have a ZW/ZZ system, which can act as a barrier to gene exchange (Gulyaev *et al.*, 2022).

Although there are difficulties in crossing between species of the *Vetrix* and *Salix* clades, in certain circumstances such a crossing may occur. Thus, the diploid species *S. humboldtiana* Willd. of the subgenus *Protitea* has been crossed with diploid species of the subgenus *Vetrix* (*S. viminalis*, *S. purpurea* and *S. daphnoides* Vill.), although the degree of fertility of their progeny has not been determined (Argus, 2010; Förster *et al.*, 2021).

Regarding crosses of different ploidy levels and different SDS, hybrids of *S. triandra* (*Vetrix* clade) have been produced with species of the subgenus *Salix* (*S. alba* and *S. pentandra* L.) (Karp *et al.*, 2011) which do not result in fertile crosses, but *S. triandra* can hybridize with the tetraploid *S. × fragilis* of the *Salix* clade, resulting in a triploid hybrid *S. × speciosa* Host (= *S. × alopecuroides*) (Neumann and Polatschek, 1972; Neumann, 1981; Dobes *et al.*, 1997; Wagner *et al.*, 2020). Probably, in those cases of successful crosses it could have resulted from the parental lineages of the diploid species of the *Vetrix* and *Salix* clades, as also occurs between crosses of *S. exigua* and *S. eriocephala* (Mosseler and Papadopol, 1989; Mosseler, 1990; Gulyaev *et al.*, 2022).

Gene regulation model

A general model for the regulation of SDRs has been proposed for each SDS (Cronk and Müller, 2020; Leite Montalvão *et al.*, 2022). The first, in sex determination system XX/XY, is presented on chromosome 7 and is an SLR that acts as suppressor of the factors that codify for female characters, being the male heterogametic sex. The homologue of an Arabidosis regulatory region (ARR17) gene is a determinant of sexual characteristics and codes for hormonal responses of cytokinins and ethylene (Hyden *et al.*, 2021; Leite Montalvão *et al.*,

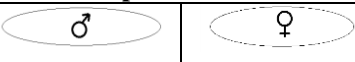

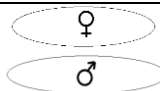




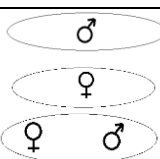
2022). The suppressor consists of an inverted repeat of the ARR17 pseudogene (Ψ ARR17-IR). This structure leads to sRNA production and thus RNA-directed DNA methylation (RdDM) by silencing ARR17 (Müller *et al.*, 2020; Yang *et al.*, 2021; Cronk and Müller, 2020). The second, the ZZ/ZW system, presents an SLR on chromosome 15 that controls femaleness, showing that the heterogametic sex is female. There is no suppressor of female characteristics in this SDS (Yang *et al.*, 2021). This indicates that the sex determination system in *Salix* may have been changed from XY to ZW relatively recently since the suppressing mechanism from the partial duplication (like Ψ ARR17-IR) is still retained (see also Renner and Müller, 2021). Recently in *Salix purpurea* it has been discovered that there is a two-gene sex determination model mediated by ARR17 and GATA15 that is different from the single-gene ARR17-mediated system in the related genus *Populus*. (Hyden *et al.*, 2023).

SDS and sexual expression of individuals

Sexual expression in *Salix* species is grouped into the following 8 conditions (Table 3). All but the first condition are not usual for strict dioecy and appear only in a few individuals or in a particular environment, this state being known as pleogamy (Cronk, 2022). **(1)** Diclinia dioica is the dominant condition in most species where they exist as male and female individuals. **(2)** Monoecious dicliny in the same catkin, where there can be exceptional individuals that have female and male flowers in the same inflorescence. This condition has been frequently reported within *Salix*. Thus, in the triploid hybrid *S. × argentinensis* Ragonese & F.R.Alberti, nom. inval. (*S. babylonica* ♀ × *S. humboldtiana* ♂) it is possible to observe male and female flowers in the same catkin (Ragonese and Rial Alberti, 1958). In *S. aff. alba*, individuals with bisexual catkins have been observed (Lopez *et al.*, pers. comm.). In *S. × speciosa* (= *S. × alopecuroides* nom. superfl.), Neumann (1981) noted that plants often have catkins with male and female flowers. Smith (1940) observed the same in *S. planifolia* Pursh, and Mosseler and Zsuffa (1989) reported this condition in individuals of *S. amygdaloides*, *S. discolor* Muhl. and *S. exigua*. Some individuals of *S. caprea* L. with male and female flowers in the same catkin have recently been reported (Terzioglu *et al.*, 2020). **(3)** Monoecy with flowers in female and male catkins in the same individual. This rare condition has been observed in *S. × pendulina* Wender. nothof. *tristis* (Gaudin) I.V. Belyaeva (Belyaeva *et al.*, 2021), *S. rosmarinifolia* (Talalaj, unpubl., in Mirski, 2014). **(4)** The condition of andromonoecy, where there are plants that develop catkins with male and hermaphroditic flowers in the same inflorescence. This was observed in *S. lucida* by Mosseler and Zsuffa (1989) as a result of intraspecific crosses and in *S. × pendulina* nothof. *tristis* (Belyaeva *et al.*,

2021). In the next season, the plants changed to producing catkins with male flowers in the next reproductive season (Mosseler and Zsuffa (1989). (5) Androdioecy, having hermaphrodite and male flowers on separate plants, has been reported for *S. arctica* by Smith (1940). (6) Intersex is when flowers of one sex change to morphological flowers of another sex, an event that was observed by Smith (1940) in *S. rhamnifolia* Pall. (= *S. podophylla* Andersson), *S. brachycarpa* Nutt. and *S. glauca* L. var. *villosa* Andersson (= *S. pseudolapponum*). (7) Trimonoecy with flowers of different sexes in the same catkin, where there may be individuals with catkins composed of female flowers at the base, hermaphrodite flowers in the center and male flowers at the apex of the inflorescence. In *S. × pendulina* nothof. *tristis* (= *S. pendulina* nothof. *salamonii* (Carrière) I.V. Belyaeva), a form under cultivation, this condition has been observed (Belyaeva *et al.*, 2018; 2021). This seems to be also the case for the diploid variety *S. humboldtiana* var. *martiniana* (Leyb.) Andersson, a variety found in Brazil, Paraguay and Northeast Argentina (Rohwer and Kubitzki, 1984). (8) Trioecy, where catkins occur with unisexual and bisexual flowers, observed in *S. myrsinifolia* Salisb. (Falinski, 1998; Mirski, 2014; Mirski and Brzosko, 2015), *S. acmophylla* (Rottenberg, 2007) and *S. subfragilis* (Kurahashi and Kimura, 2002; Nagamitsu and Futamura, 2014).

Table 3. Types of sexual expressions in *Salix*

Condition	Representation	Frequency	References
1. Diclinia dioica		general	General condition of <i>Salix</i> species
2. Diclinia monoica (andróginia), in the same catkin		exceptional	Falinski, 1998; López <i>et al.</i> , pers. comm.; Ragonese and Rial Alberti, 1958; Mosseler and Zsuffa, 1989; Rottenberg, 2007. Terzioglu <i>et al.</i> , 2020.
3. Diclinia monoica (androginia), in different catkins		very rare	Smith, 1940; Talalaj unpubl. in Mirski, 2014.
4. Andromonoecy		very rare	Mosseler and Zsuffa, 1989; Belyaeva <i>et al.</i> , 2021
5. Androdioecy		very rare	Smith, 1940
6. Intersex		infrequent	Smith, 1940
7. Trimonoecy, flowers of opposite sex in the same catkin		infrequent	Rohwer and Kubitzki, 1989; Belyaeva <i>et al.</i> , 2018, 2021.
8. Trioecy, with unisexual and bisexual catkins		infrequent	Falinski, 1998; Rottenberg, 2007; Mirski and Brzosko, 2015.

In the representation of the different states, each division of the table represents different individuals. The ellipse represents the catkin per individual with the flower-type composition according to its sex.

The interactions between the different alleles, different SDS and ploidy condition could generate infrequent phenotypes that deviate from strict dioecy, although the effect of SDS in polyploid species is still unknown (Gulyaev *et al.*, 2022) as is its phenotypic manifestations, being a point of interest for future research.

In some species growing in natural populations, variations of the dioecy have been seen in relation to the distribution range of the species, trimonoecious specimens appearing at the edge of the distribution, as occurs in the hexaploid species *S. myrsinifolia*, where individuals with these characteristics would have a reproductive advantage for the colonization of new environments (Mirski and Brzosko, 2015; Mirski *et al.*, 2017).

Regarding the sexual lability observed in some species, Mosseler and Zsuffa (1989) through observation and monitoring over time of individuals of *S. amygdaloides*, *S. lucida* and *S. exigua* reported the presence of hermaphroditic flowers that appeared in a reproductive season and later reverted to male flowers, while in *S. discolor*, they reverted to female ones. This seems to suggest that the evolution of the dioecy in *Salix* has resulted from hermaphroditism (Mosseler and Zsuffa, 1989; Cronk, 2022).

The regulatory mechanism of sexual expression in these species with extraordinary hermaphroditism remains to be clarified although it seems that it could be mediated by a genetic mechanism (Mosseler and Zsuffa, 1989; Nagamitsu and Futamura, 2014; Leite Montalvão *et al.*, 2022) and some type of mediated epigenetic regulation could be involved, for example, by methylation patterns in the genes of the SLR region, as has already been documented for *S. viminalis* (Cheng *et al.*, 2019). In many cases, this regulation is influenced by environmental factors (Mirski, 2014). Mutations in regulatory genes have been suggested as the cause of these states. Musseler and Zsuffa (1989) explain the manifestations of hermaphroditism found in *S. amygdaloides*, *S. lucida* and *S. exigua* by mutations in suppressor genes (for example ARR17 that was most recently identified). It also seems to be the same case found in *S. subfragilis* (Nagamitsu and Futamura, 2014). In *S. purpurea* a 1.15 Mb sex-linked region on Chr15W was confirmed to be absent in monoecious plants. (Hyden *et al.*, 2023). In some case (Musseler and Zsuffa, 1989) this gene alteration does not seem to be permanent since it can change the state of hermaphroditism to a male phenotype in the following reproductive period, for which the expression of the gene is apparently subject to environmental conditions. However, Nagamitsu and Futamura (2014) suggested that the extraordinary hermaphroditism of the allelic variant is maintained in the progeny, suggesting that inherited alleles are the cause of this condition rather than gene regulation.

Conclusions

This review brings together what is known about sexual expression and gender determination with the most recent findings at the molecular level concerning the location of SLRs. The data reviewed, so far, establishes that both sex determination systems (XX/YY and ZZ/ZW) are important determinants to differentiate the main biological groups within the genus *Salix*. Both SDSs influence the sex ratio in a population. The interspecific crosses that usually occur naturally in the genus causing allopolyploids with different degrees of ploidy, together with hybridizations between species with different levels of ploidy and artificial hybridizations between different groups of unrelated species, promote the expression of different sexual variants. Such expressions are manifestations of the various combinations of alleles and interactions of genes or their regulation that are influenced by certain environmental conditions, which mechanisms are still unknown. The elucidation of this regulatory mechanism or inheritance type, especially in polyploid species, in future studies should lead to a better understanding of the manifestations and influence of SLR in the reproduction and classification of the species of this complex genus.

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Statements and declarations

The author has no competing interests to declare that are relevant to the content of this article.

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