Title: The diversity of plant sex chromosomes highlighted through advances in genome sequencing

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Abstract: For centuries scientists have been intrigued by the origin of dioecy in plants, characterizing sex-specific development, uncovering cytological differences between the sexes, and developing theoretical models. However, through the invention and continued improvements in genomic technologies, we have truly begun to unlock the genetic basis of dioecy in many species. Here we broadly review the advances in research on dioecy and sex chromosomes. We start by first discussing the early works that built the foundation for current studies and the advances in genome sequencing that have facilitated more-recent findings. We next discuss the analyses of sex chromosomes and sex-determination genes uncovered by genome sequencing. We synthesize these results to find some patterns are emerging, such as the role of duplications, the involvement of hormones in sex-determination, and support for the two-locus model for the origin of dioecy. Though across systems, there also many novel insights into how sex chromosomes evolve, including different sex-determining genes and routes to suppressed recombination. We propose the future of research in plant sex chromosomes should involve interdisciplinary approaches, combining cutting-edge technologies with the classics to unravel the patterns that can be found across the hundreds of independent origins.

Keywords: Dioecy, sex determination, seed plants, bryophytes, whole-genome sequencing, two-gene model

Introduction

Across land plants exists an amazing variety of strategies for sexual reproduction [1]. Species have independently evolved self-incompatibility loci [2], temporal variation in flower development [3,4], and spatial distancing of male and female organs on the same plant [5–7], among many others [1]. Perhaps the most extreme case, however, is dioecy, where sex-specific structures develop on separate plants. In angiosperms, dioecy is rare, found in an estimated 5% of species, but has hundreds of independent origins across more than half of the families [5]. In the other land plant lineages most species are dioecious, at approximately 65% of gymnosperms, 68% of liverworts, 57% of mosses, and 40% of hornworts (technically dioicous in bryophytes because they are haploid when expressing gametic sex, but here we will use dioecy for simplicity) [8,9]. The frequency and phylogenetic breadth of dioecy across plants provides an unparalleled opportunity to examine the key forces involved in its repeated evolution.

Early models theorized how dioecy can evolve from a hermaphroditic ancestor [10–12], invoking the need for two-linked mutations: one that causes male-sterility and another female-sterility. Recombination within this region can result in offspring that are either hermaphroditic or sterile. Thus, selection is strong to suppress recombination in the region containing these two mutations, forming a sex chromosome pair. For dioecious species that express gametic sex in the diploid stage, like in seed plants, the sex chromosomes are referred to as XY or ZW depending on which is the karyotypically heterogametic sex [13,14]. In haploid-dominant plants, like bryophytes, dioecious species with genetic sex determination have UV sex chromosomes, with the inheritance of a U correlating with female gametic sex expression and a V with male [13,15]. Though some species have multiple sex chromosomes (e.g., XY_1Y_2 or U_1U_2V) [8,16–18], which can occur through structural changes like chromosomal fusions and fissions or through polyploidy. These differences in heterogamety and ploidy of sex chromosomes found across land plants also provide power for contrasting the evolutionary processes that impact these genomic regions, especially as the mechanisms of sex determination on sex chromosomes have now expanded beyond the classic “two-locus” model [19].

Here we review the recent advances in sex chromosome evolution across land plants. We start by covering a brief history of identifying dioecy and sex chromosomes, and the advances in genome sequencing that have made new discoveries possible. We next broadly review new findings in plant sex chromosomes, paying particular focus on how the sex-determining region (SDR) evolves, both with the diversity of genes that are involved in sex determination and other processes that shape these complex regions of the genome. We conclude with future directions in plant sex chromosome evolution research.

The history of identifying plant sex chromosomes

Analyses of dioecy and sex chromosomes start with the remarkable work of naturalists whom, with a careful eye, characterize reproductive structures throughout development. Categorizing plants as dioecious can be traced back to Linnaeus’ *Systema Naturae* (1735), where angiosperms were classified by their floral characteristics, such as number of anthers, pistils, or sexual condition [20]. Darwin even discussed the curiosities of dioecy in *The Different Forms of Flowers of the Same Species* (1877) [21]. In some species dioecy is easily observable. One example is hops, where female inflorescences develop the characteristic “cones” used in beer production, while males have a completely different floral architecture [22]. Another example is found in the classic dioecy model white campion (*Silene latifolia*), studied intensively since the 19th century [23], where a suite of sexually dimorphic traits is obvious at early stages of flower development. However, in other species dioecy can be more subtle. In garden asparagus, both sexes phenotypically appear similar in early stages of floral development, but ultimately the stamens degenerate in females and the ovary is non-functional in males [24]. In some species, like *Solanum appendiculatum* or kiwifruit, dioecy is even more cryptic, where females even produce pollen grains, but they are non-viable [25,26]. In non-flowering groups, like the mosses, early naturalists searched for the “hidden flowers” (reviewed in [27]), which are called antheridia and archegonia (male and female gametangia, respectively) to identify dioecious species. Antheridia are easily visible during their development, however, archegonia are more challenging to locate because they are largely enclosed in modified leaves [28,29]. It is also common in mosses to not develop gametangia [30–32] and disentangling individual (i.e., genetically distinct) plants from their densely-grown patches can be challenging. As such, some of the first confirmations of dioecy in species like *Ceratodon purpureus* and *Bryum argenteum* were done by growing individuals from spores [30]. It is unequivocal that these kinds of taxonomic observations form the critical basis of our understanding of dioecy, in addition to other sexual systems (for databases in angiosperms see [5,33]).

Uncovering the genetic basis for sex determination began with early cytological analyses (reviewed in [34]). Dr. Nettie Stevens first discovered the correlation between the inheritance of a smaller chromosome in a meiotic pair (which we now know as the Y chromosome) with male gametic sex expression in mealworms [35]. Indeed, this clear heteromorphy between sex chromosomes was critical to their identification in further cytological studies. The first plant sex chromosomes were identified in the liverwort *Sphaerocarpos donellii* [36] and subsequently many other heteromorphic pairs were found in *Humulus*, *Rumex*, and *Silene* [16,37,38] among others [14]. However, in many plants the sex chromosomes are cytologically homomorphic, or nearly so, making identifying them through classical microscopy a challenge.

Dioecious species with sex chromosomes played a pivotal role in the modern synthesis, in particular with regard to the inheritance of sex. In the early 1900s, after the re-discovery of Mendel’s foundational work on pea plants [39,40], dioecious flowering plant *Silene latifolia* (formerly *Melandrium album*) became a cornerstone for understanding the genetic basis of sex and sex-linked traits. This is partly because it has such obvious flowers and a particularly large Y chromosome that is nearly 1.5 times the size of the X [41]. In fact, the first sex-linked gene in plants was discovered in *S. latifolia*; the X-linked recessive lethal *angustifolia* mutation produced narrow leaves that were only found in XY male plants and never led to viable XX females [42–44]. Early irradiation studies on *S. latifolia* pollen and seeds produced higher than expected sex ratios of female plants, suggesting that Y chromosome deletions led to sex-switching [45]. Such large-scale sex chromosome irradiation experiments are still immensely useful today, and have been leveraged to map sex-determining genes on the Y chromosome in *S. latifolia* [46] and in garden asparagus [47,48].

Today genomic approaches have unlocked other previously intractable analyses of plant sex chromosomes. Some of the first genome references for dioecious species include the liverwort *Marchantia polymorpha* [49], grape [50], papaya [51,52], and poplar [53], published only a few years after the first plant genome (*Arabidopsis thaliana* [54]). More than two decades later, reference genomes for over 50 dioecious species have been published (Table 1). Though there are many characteristics about sex chromosomes that have made them challenging to assemble. Because of suppressed recombination, natural selection is less effective in these regions [55,56] and they often accumulate repeats [57]. This makes assembly of large contigs using short reads improbable [58] because reads often do not span the entire repeat, causing these regions to collapse [59,60]. Linkage maps, which use recombination rates across the genome, can help pull low-contiguity assemblies into linkage groups [61], but very small sex-determining regions (SDR) (e.g., ~59 kilobases (Kb) in *Morella rubra* [62]) are hard to reliably identify and very large SDRs (e.g., >100 megabases (Mb) in *Ceratodon purpureus* [63]) are hard to put in linear order due to the inherent lack of recombination. The use of Bacterial Artificial Chromosomes (BACs) has helped to resolve some sex chromosome assemblies [64,65], but like linkage maps, this approach is labor intensive. Adding to assembly issues, sequencing the heterogametic sex in diploids can result in chimeric contigs that contain a mixture of the X and the Y (or Z and W), especially if there is low divergence between homologous regions, as is expected if suppressed recombination has recently evolved [66,67]. These issues with assembling sex chromosomes are compounded by the fact that plant genomes are overall inherently complex, with many species having high heterozygosity and abundant repeats genome-wide, in addition to frequent polyploidy [68]. Despite these complications, through much tenacity, a lot of headway has been made on plant sex chromosomes using these short-read assembly approaches.

More recently, long-read technologies, like PacBio and Oxford Nanopore, have made phenomenal strides for assembling complex regions, like sex chromosomes. Because they span an average of 10-15 Kb, as opposed to 100-300 basepairs with short reads, they are better at spanning repeat regions [68,69]. Not to mention longer reads mean fewer pieces of the genomic puzzle need to be put together. Although depending on the size and complexity of the genome, even with long reads, the assembly may not be pulled into pseudomolecules and may still contain mis-joins. However, in these cases, with the addition of chromatin conformation data, like Hi-C, which does not rely on linkage, genomes can now readily be assembled to chromosome-scale, including the sex chromosomes [70–72]. Indeed, the telomere-to-telomere, gapless assembly of a human X chromosome, including the centromeres, [73] represents the future (or really the present) for genome assembly. The most-recent improvements in longer-read technologies (e.g., PacBio HiFi), including lower error rates, and novel computational tools for assembling these data (e.g., HiCanu and HiFiAsm [74,75]) also mean phasing heterogametic sex chromosomes may now be possible. Though there are also downsides to long-read technologies, the foremost being the requisite high-molecular weight DNA, which often ideally comes from fresh, young, flash frozen tissue. This inherently precludes the incredible taxonomic resources maintained in herbaria, as well as any other avenues that could cause DNA degradation. As such, one of the biggest bottlenecks for genomic studies of most taxa today is identifying viable (and properly permitted) tissue that can be used for the genome reference, gene annotation, and maintained for future studies.

Critical for analyses in sex chromosomes, is identifying the non-recombining SDR. Currently, it is with a combination of both long and short read technologies that is best suited for high-quality assemblies to accomplish this. Long reads are ideal for assembling genomes into fewer contigs, but short reads are still valuable for genome polishing (even with lower error rates in long reads; e.g., with Racon [76]), Hi-C data for additional genome scaffolding (e.g., with JUICER [71]), genome annotation (e.g., with BRAKER2 [77]), and identifying the SDR (reviewed in [78]), in addition to gene expression analyses [68]. In fact, genes annotated to the SDR that have sex-specific expression are strong candidates for being involved in sex determination.

Advances in sex-determination gene identification

Yam: Most species in the genus *Dioscorea* (Dioscoreaceae) are dioecious [79] and have XY sex chromosomes [80–82], suggesting dioecy may have evolved ~80 million years old (MYA) [83]. In *D. alata* a recent genetic map uncovered a ~10 Mb male-specific Y (MSY) [81]. However, in *D. rotundata*, data support a ZW system with a small SDR (~161 Kb) [84], suggesting a recent turnover in this species. A candidate list of floral genes has been developed in *D. rotundata* [85], but more in-depth analyses are needed to identify those involved in sex determination.

Asparagus: Several species of *Asparagus* (Asparagaceae) are dioecious including garden asparagus (*A. officinalis*) [86]. *Asparagus officinalis* has XY sex chromosomes, with a ~1 Mb MSY [47] that contains 13 genes with no homologs on the X (and only one X-specific gene), suggesting suppressed recombination is through a deletion on the X [47,48]. Two of the Y-linked genes have functionally been shown to be involved in the development of the sexes through gamma ray and Ethyl methanesulfonate (EMS) mutagenesis in XY males (Figure 1) [48]. Knockouts of Suppressor of Female Function (*SOFF*), which contains a *DUF247* domain, develop hermaphroditic flowers, with both functional anthers and ovules [48]. Knockouts of Tapetal Development and Function 1 (*TDF1*), an *R2R3-MYB*, make sterile individuals, where neither functional carpels nor stamens develop [48]. Furthermore, knockouts of both *SOFF* and

TDF1 develop functional ovaries, but non-functional anthers [48]. Together these results show that *SOFF* and *TDF1* are the female and male-sterility genes, respectively, in *A. officinalis*. Further comparative analyses will uncover whether this sex-determination mechanism is a shared across the other dioecious species in *Asparagus* or if other genes are involved.

Date palm: In the genus *Phoenix* (Arecaceae), phylogenetic analyses of a *MYB1* gene suggest the XY sex chromosomes may have an ancient origin, prior to the diversification of the species [87,88]. In the date palm, *P. dactylifera,* the MSY is ~13 Mb [89,90]. Comparative analyses across all 14 species of the genus identified three potential sex-determining genes [91]. Y-linked Cytochrome P450 (*CYP703*) and glycerol-3-phosphate acyltransferase 6-like (*GPAT3*) genes are expressed only in male flowers and are likely critical for pollen and/or anther development (Figure 1) [91]. The third gene, a Y-linked, Lonely Guy-like gene (*LOG*), which is involved with cytokinins, is also largely expressed in male flowers, and may have a role in suppressing carpel development [91]. While these genes seem like ideal candidates for sex determination, functional follow ups are necessary to validate these putative roles in *Phoenix*.

Grape: All wild species of *Vitis* (Vitaceae) are dioecious, however, similar to papaya (described below) domestic grapes have transitioned back to hermaphroditism [92,93]. Males are the heterogametic sex in *Vitis* and in *V. vinifera sylvestris* the MSY is small at ~150 Kb and contains 20 genes [94,95]. More recent analyses show grapes also support a two-gene model of sex determination. A Y-linked copy of Inaperaturate pollen1 (*VviINP1*) likely plays a role in pollen aperture formation [96] and thus male fertility. An X-linked copy of *YABBY3* (*VviYABBY3*), genes of which have been shown to play a role in flower and lateral organ development [97], is strongly supported as the female-promoting gene [98] (Figure 1). However, functional follow-ups are necessary to confirm these roles in grapes.

Poplar: Nearly all species in *Populus* (Salicaeae) are dioecious [99,100] and across the genera, both XY (*P. deltoides*, *P. euphratica*, *P. tremula*) and ZW (*P. alba*) sex chromosomes have been identified, suggesting at least one turnover event has occurred [101]. In *P. tremula* the MSY is ~1.5 Mb and contains a type-A cytokinin response regulator (*RR*), homologous to Arabidopsis *RR* 17 (*ARR17*), that is found in inverted repeats [102]. CRISPR knockouts of *ARR17* in karyotypic females developed functional stamens and mostly did not develop carpels, whereas in karyotypic males, *ARR17* knockouts showed no difference in development [102] (Figure 1). Some evidence suggests gene silencing of *ARR17* in males is through RNA-directed DNA methylation, however, this has not been formally been tested [102]. In *P. alba*, the W also contains *ARR17*, that is lacking from the Z. This intriguing result highlights how a single gene can determine sex on both diploid sex chromosome types. Although interestingly, within the same genus, is recent evidence of two genes involved in sex determination. In *P. deltoides* one of the sex-determining genes is also related to *ARR17,* though they call it female-specifically expressed *RESPONSE REGULATOR* (*FERR*) [103]. The ~300 Kb MSY has a duplication of *FERR* that represses it (*FERR-R*), inhibiting carpel development. The second gene, a male-specific lncRNA (*MSL*), is likely involved in promoting male function [103].

Willow: The genus *Salix* is sister to poplars in the Salicaceae family and most species are also dioecious [100]. *Salix purpurea* and *S. viminalis* both have a ZW sex-determination system that share an evolutionary origin having arisen ~8.6 MYA [67,104]. The *S. purpurea* female-specific W (FSW) is ~6.8 Mb and interestingly contains palindromic repeats, similar to those found in humans [104,105]. Within these repeats are five genes, that may be associated with sex determination, however, the cytokinin *RR* is particularly of interest as this gene is homologous with the sex-determining *ARR17* gene in poplar [102,104]. The *S. viminalis* FSW (~3.1 Mb) also contains *ARR17*, further supporting the putative role of this cytokinin-related gene in sex determination in willows and poplars, although this has not yet been confirmed with functional analyses in *Salix* [67]. Interestingly, >100 additional genes are found on the *S. viminalis* FSW, which show evidence of two strata, however, there is no evidence of chromosomal inversions involved in their capture, suggesting instead the buildup of transposable elements may be involved in suppressing recombination [67]. *Salix nigra*, contrastingly, has XY sex chromosomes with a ~2 Mb MSY on a different chromosome than in the other *Salix* species examined, suggesting a translocation of the SDR (i.e., turnover) [106]. Though with current analyses it is unclear if *RR* is also sex-linked in this species [106]. Given the many turnovers and changes in heterogamety found in Salicaceae, often involving the same *RR* gene, a general model has been developed to explain this pattern [101]. Consistent with results described in Müller [102], in species with ZW sex chromosomes, *RR* acts as a dominant female promotor, but in XY systems *RR* duplicates target and repress *RR* by RNA-directed DNA methylation [101].

Strawberry: In *Fragaria* (Rosaceae) several species are dioecious, octoploids that are nested within a diploid, hermaphroditic clade [107], highlighting the role polyploidy can play in the evolution of dioecy [108]. Strawberries have ZW sex chromosomes that arose ~1 MYA [109]. In *F. chiloensis* the FSW is small at ~280 Kb [110], though in other *Fragaria* there is evidence the SDR is in different locations, suggesting either independent evolutions or translocations [111]. Recent evidence supports the latter, where the FSW has translocated at least twice, each time capturing more DNA into the region of suppressed recombination [112]. In *F. virginiana* ssp. *virginiana*, which has the smallest SDR cassette, there are two genes, a GDP-mannose 3,5 epimerase 2 gene and a 60S ribosomal protein P0 [112]. These two genes are also located in the *F. virginiana* ssp. *platypetala* and *F. chiloensis* SDRs [112], although functional analyses will highlight whether they play a role in sex determination across these species.

Red bayberry: In the genus *Morella* (Myricaceae), most species are dioecious, including *Morella rubra*, the red bayberry [113]. Recent genome sequencing found *M. rubra* has ZW sex chromosomes with a ~59 Kb FSW that contains seven genes. Three of these have putative roles in flower development (Mr*CKA2*, Mr*ASP2*, Mr*FT2*) and two are related to hormones (Mr*CPS2*, Mr*SAUR2*; [62]), however, more functional work will help uncover which are involved in sex determination. All genes in the FSW have a paralogous copy on the same chromosome, suggesting gene duplication may have also played a role in the evolution of the sex chromosomes in this species [62].

Papaya: Papaya (*Carica papaya*) is the sole species in the genus *Carica* of the family Caricaceae that comprises 35 species in 6 genera [114]. Caricaceae species are sexually dimorphic, 32 of them are dioecious, two are trioecious, and one is monoecious [14]. Multiple lines of evidence suggested that sex chromosomes have evolved multiple times independently in Caricaceae and sex chromosomes in *Carica* and *Vasconcellea* may have originated from the same ancestral autosomes after the divergence of these two genera [115,116]. Papaya is one of the two trioecious species in the family Caricaceae, and sex determination of papaya is controlled by an XY system with two slightly different Y chromosomes, a male-specific Y and a hermaphrodite-specific Y^h [117]. The papaya X- and Y-linked regions were sequenced and annotated [64,93,118]. Two large inversions in the Y-linked region caused recombination suppression with the X and initiated sex chromosome evolution [64]. No hermaphrodite papayas have been found in wild populations and the Y^h chromosome exhibits lower nucleotide diversity than the Y, suggesting that hermaphrodite papaya is likely a product of human domestication [93]. Several candidate genes showing functional and/or structural association with sex types were identified based on sequence comparison and gene expression analysis [119,120]. Further functional validation of candidate genes is still needed, although several independent studies point towards SVP (SHORT VEGETATIVE PHASE) as being involved in male flower development [121,122], though this putative gene does not have a sex-related function in other species.

Palmer amaranth: Most species are monoecious in the genus *Amaranthus* (Amaranthaceae), however, dioecy is thought to have evolved multiple times independently [123]. The recent genome sequences of *A. palmerii* identified an XY sex chromosome system with a ~1.3-2 Mb MSY containing 121 gene models [124–126]. *Amaranthus tuberculatus* has a larger MSY (~4.6 Mb) with 147 genes [126]. Despite being in separate dioecious clades [123], two genes are found in the MSY of both species (Disintegrin and metalloproteinase domain-containing protein 9, *ADAM9*, and *FLOWERING LOCUS T*, *FT*) [126], making them candidates for sex determination or male-specific development.

Spinach: All three species of *Spinacia* (Amaranthaceae) are dioecious, and though *S. oleracea* and *S. tetrandra* diverged ~5.7 MYA, analyses of sex-linked homologs suggest suppressed recombination occurred after their divergence [127]. Recent analyses in *S. oleracea* have found the SDR to be between 10-19 Mb, with a 10 Mb MSY that has 210 genes [128,129]. These genes have been captured into the region of suppressed recombination through chromosomal inversions, making two strata of divergence between the X and the Y [129]. The 12 MSY genes with putative floral functions [129] and additional transcriptomic analyses of female and male flowers [130] have narrowed in potential sex-determining genes, though none so far are clear candidates.

Persimmon: Most species in Ebenaceae are dioecious including *Diospyros* [131]*. Diospyros lotus* has XY sex chromosomes with a ~1.3 Mb MSY [132]. Expression of an autosomal HD-Zip1 family gene, Male Growth Inhibitor (*MeGI*), results in the development of female flowers, with functional carpels, but not functional stamens. However, a Y-linked pseudogene, Oppressor of MeGI (*OGI*), encodes a small RNA that suppresses *MeGI*, resulting in male flowers [133] (Figure 1). Moreover, the male-determining role of *OGI* is stable in the hexaploid persimmon, *D. kaki*, which has both monoecious and female flowers [134,135]. These data, like in poplar, support a single gene is involved in sex-determination in persimmons. This sex-determination system evolved through a recent whole-genome duplication, making two copies of *MeGI*. Functional analyses of these genes in tobacco suggests *SiMeGI* (sister copy of *MeGI*) may have maintained the original gene function, while *MeGI* neofunctionalized as a repressor of anther development [132]. A second duplication of *MeGI* resulted in the Y-linked *OGI*.

Kiwifruit: Most species in *Actinidia* (Actinidiaceae) are dioecious [136] and the sex chromosomes arose ~20 MYA [137]. Although kiwifruit is in a different family than persimmons, they are in the same order (Ericales), representing at least two independent origins of sex chromosomes. *Actinidia chinensis* var. *chinensis* have XY sex chromosomes and the MSY is ~0.8 Mb, containing 30 genes [138]. Two of these have been identified as sex determining, additionally supporting the “two-locus” model for the evolution of dioecy. One gene, a type-C cytokinin *RR*, suppresses ovary formation (*SyGl*) and the other has a fasciclin domain that contributes to tapetum degradation resulting in male fertility (*FyBy*) [137,138] (Figure 1). The function of these genes was validated through several approaches [138]. First, analyses of the genome of the hermaphroditic species, *A. deliciosa*, showed no evidence of a copy *SyGl*, but did have *FrBy* [138]. This suggests either the loss of *SyGl* or the gain of *FrBy* caused transition to hermaphroditism [138]. Moreover, knock-ins of *FrBy* into an XX female were hermaphroditic, with both functional carpels and stamens that produced fertile seeds after self-pollination [138]. Current work is in progress to also functionally validate *SyGl* [139].

Solanum: Dioecy evolved at least four times across the genus *Solanum* (Solanaceae) [140]. In *S. appendiculatum*, the XY system arose (<4 MYA) [141] and the MSY contains at least 20 genes [142]. Consistent with female flowers producing inaperature pollen, many sex-biased and

Y-linked genes are involved in pectin development [142], though more analyses will undoubtedly uncover genes involved in sex determination.

Amborella: *Amborella trichopoda* is a monotypic species in Amborellaceae that is sister to the rest of flowering plants [143,144]. Although the *Amborella* lineage diverged from the rest of angiosperms ~200 MYA [145], the ZW sex chromosomes are estimated at 9.5 to 14.5 MYA [146]. This recent origin of *A. trichopoda* sex chromosomes is consistent with the ancestral flower of all angiosperms being reconstructed as hermaphroditic [147]. The FSW is ~4 Mb and has ~150 genes [146] though which are involved in sex determination is unknown.

Maidenhair tree: The dioecious gymnosperm, *Ginkgo biloba* (Ginkgoaceae) [148], is a monotypic species. Two recent genomes suggest *Ginkgo* has an XY system [149,150] that arose ~14 MYA [149]. The MSY is ~27 Mb, with 241 genes, including 4 MADs-box genes expressed in staminate (male) cones [150]. Given the clear role MADs-box genes play in flower development in angiosperms [151], these genes are interesting candidates for sex-determination in *Ginkgo* as well.

Fire moss: The moss *Ceratodon purpureus* (Ditrichaceae) UV sex chromosomes provide an interesting contrast to the XY/ZW systems in seed plants. The *C. purpureus* U and V are large with each >100 Mb and have >3400 annotated genes, totaling ~30% of the 360 Mb genome and ~12% of the gene content [63]. The moss sex chromosomes evolved at least 300 MYA in the ancestor to ~95% of extant mosses, making them among the oldest known sex chromosomes across Eukarya [63]. Compared to angiosperms, much less is known about the functions of genes in bryophytes, so narrowing in on candidate sex determiners is a challenge. However, some genes have been identified that are potentially of interest in sex-specific development. For example, the *C. purpureus* female-specific U chromosome contains an *RWP-RK* transcription factor [63], which are involved in egg cell formation across land plants [152,153] and in the same gene family as the *MID* mating-type loci in green algaes [154]. Other notable genes on the *C. purpureus* U and V [63] are orthologs to the cis-acting sexual dimorphism switch found in *Marchantia polymorpha* (described below; [155]).

Common liverwort: The liverwort *M. polymorpha* (Marchantiaceae) also has a UV sex-determination system with an ancestral origin [63,156]. The male-specific V is ~7.5 Mb and the female-specific U ~4.3 Mb, with 247 and 74 genes annotated, respectively [126,156], though the U has not been fully assembled, which may explain some of the difference in size. Similar to *C. purpureus*, it is unclear which genes on the U or V are involved in sex determination in *M. polymorpha*. However, intriguingly, an autosomal *MYB* transcription factor has a clear role in sex-specific development. Expression of *FEMALE GAMETOPHYTE MYB* (Mp*FGMYB*) results in archegonia development, whereas expression of its cis-acting antisense gene suppresses Mp*FGMYB* resulting in antheridia development and sperm production, though the sperm lack motility [155]. Several other dioecious bryophyte genomes have recently been published or are in progress [157–160], commencing an era for comparative analyses to uncover sex determination and further insights on sex chromosomes in this predominantly dioecious clade.

The diversity of proposed mechanisms of sex determination

The plant sex chromosomes analyzed to date vary in age, size, and overall gene content, but what may be most striking is how many different genes have evolved to be the sex-determiners (Figure 1). This stands in contrast to animal systems where the same gene(s) have been shown to be involved in sex determination across many taxa (e.g., *SRY/SOX3; DRMT1* [161]). For the genes identified in plants, some necessary similarities exist: they must be involved at some stage of sex-specific structure development (e.g., anther or carpel). Whether certain genes in these developmental pathways are more likely to evolve sex determination than others is unknown. Genes with broad-expression patterns seem to be unlikely candidates, as sex-linkage, and any subsequent molecular evolutionary consequences like protein evolution, may be deleterious to other functions. Although duplications, whether by doubling of the whole genome or through single genes, free genes from such constraints, allowing for neofunctionalization [108]. In fact a common theme in recent studies has been that duplications play a role in sex-determining genes (e.g., *Asparagus*, strawberry, persimmon, red bayberry, date palm, and kiwifruit [47,62,91,112,132,137]) or subsequent translocations to the SDR (e.g., *Ceratodon* [63]). Though not all of the sex-determining genes in these systems show evidence of a recent duplication (e.g., *Asparagus TDF1* [47]). In these latter cases, genes with tissue-specific or narrower expression may be more likely to evolve a sex-determining role.

Although several different genes have evolved to be sex-determining, in other dioecious species where they remain autosomal, they often instead show sex-biased expression, suggesting they play a conserved, sex-specific role or may be regulated by the sex-determining (or other sex-linked) genes [162]. For example, in kiwifruit, *FrBy* is the Y-linked, male-fertility gene, but *TDF1* also shows male-biased expression [138], which makes sense given its role in tapetum development [48,163,164]. One pattern shared across many of these systems is the role many of these genes play in the cytokinin pathway (e.g., poplar, willow, date palm, and kiwifruit [67,91,102,137]), which are involved in floral development, particularly in the carpel and female gametophyte (reviewed in [165]). As we characterize the SDRs of more independent evolutions of dioecy, we will gain more insight on what genes are more likely to be involved, if any.

Another notable pattern emerging is the empirical support for the “two-gene” model for dioecy. In *Asparagus*, kiwifruit, and grape [48,98,138], the SDRs all have two genes involved in female and in male sterility (Figure 1). Though some differences do exist between these groups, aside from which genes evolved to be sex-determining. In asparagus and kiwifruit, both genes are Y-linked, where one promotes male development (*TDF1* and *FrBy*, respectively) and another one suppresses female development (*SOFF* and *SyGI*, respectively) [48,138]. In contrast, in grapes, the functional copy of *YABBY3* is X-linked (recessive, female promoter), while *INP1* is Y-linked (dominant, male promoter) [98]. In fact, it is likely recombination between this tightly-linked region of the X and Y that caused the transition back to hermaphroditism seen in domestic grapes [98]. It will be interesting if similar patterns of occasional recombination are involved in other transitions back to hermaphroditism (e.g., papaya) or if other processes like whole-genome duplications are involved [108]. Contrastingly, in other systems, a single gene has been shown to be a sex-determining switch, like in poplar and persimmon [102,133]. Though this result does not dispute the two-gene model, as the putatively ancestral hermaphroditic population had to first transition to gyno- or androdioecious [103,166].

Recent genome assemblies in dioecious plants have revealed more than sex-determining genes. Some studies have uncovered similar patterns in the evolution of the sex-determining region that have been found in animal systems. The ancestral origins of sex chromosomes in the bryophytes more-closely resembles that of mammalian, bird, and some insect lineages [63,167–169]. Moreover, similar patterns of gene gain have been found such as evolutionary strata [67,129,170,171], inversions [64,129,172], and chromosomal fusions [63,173,174]. Sex chromosome turnovers (here we are referring to a sex-determining gene or cassette translocating to a new autosome [175]) have also occurred [84,101,102,112,175]. While some other striking convergent patterns, like palindromes, have been found [104,105]. But with as many similarities that have been found there are just as many differences. In some species, suppressed recombination can evolve before the SDR [174] with several evolving in close proximity to centromeres [51,65]. In other systems hemizygosity between the SDR caused by a deletion on the X suppresses recombination, rather than other structural changes like chromosomal inversions [48,91,103], and in others suppressed recombination can occur without structural changes likely through the build-up of transposable elements [67]. Even the characteristic patterns of degeneration and gene loss thought to affect sex-specific chromosomes, or at the very least the tempo of these processes, are questioned in several recent analyses [63,171,176]. Together this begs the question of whether the proposed linear model for the stages of sex chromosome evolution is overall applicable to plants (see also [177]) or if a more-encompassing one can be developed once we gain insight from more systems.

The future of plant sex chromosome research

Combined, plants provide many independent tests for the evolution of sex chromosomes. And while here we have focused on land plants, algaes also provide other exciting, independent evolutions [15,178]. Although, despite the many recent publications, we have only just begun to uncover what plant sex chromosomes can illuminate. Assuming 5% of the 300,000 species of angiosperms are dioecious (using conservative numbers), only ~0.3% of these species have had their genomes sequenced to date, with an order of magnitude fewer in the other major clades (Table 1). Thus, one clear path moving forward is to increase the number and phylogenetic breadth of high-quality genome assemblies and annotations of dioecious species. While this has traditionally meant assembling a single exemplar genome for a species, the future of sex chromosome genomics should encompass pangenomes [179] that incorporate within-species variation, as well as closely-related non-dioecious sister taxa that serve as outgroups. As sequencing technologies continue to improve, and the costs decrease, this becomes more tractable. Adding gene co-expression analyses will uncover downstream regulatory pathways [135,180] and whether these are more conserved than the sex-determining genes [162]. In addition to gene annotations, we should move to consistently annotate non-coding sequences, like small RNAs, [102,133,181] and uncover their targets to better understand their role in floral development and sex determination. Moreover, as technologies like CRISPR improve, and protocols are established for more species, functional validations of these results will likely become standard [182]. These discoveries are all valuable for breeding programs of dioecious and closely-related hermaphroditic crops. In fact, most of the species described in this review are economically important species. There are also applications for controlling invasive species, like in palmer amaranth [126]. And from a conservation perspective, focusing on dioecious species is especially pressing, as the sexes often respond to stressors differently, meaning that due to climate change these species may be especially at risk for extinction [183].

In addition to comparative and functional genomics, a lot more interdisciplinary work in dioecy and sex chromosome research awaits. We need to focus on many classic (albeit also constantly improving) analyses rather than just the so-called “cutting-edge”. We need to fund more field work to identify new, potentially dioecious species and common-garden analyses to characterize development (e.g., [184]). We need better-supported, species-level phylogenies to infer the number of evolutions of dioecy, for example using Angiosperm353 [185] and GoFlag (Genealogy of Flagellate plants) [186] probe sets. We need more cytological analyses, to uncover how these chromosomes behave in the cell (e.g., [187,188]) or verifying in what tissues genes are expressed (e.g., [48]). Together through these many approaches we can discover a wealth of untapped knowledge to better understand the rules at play in these complex and dynamic regions of the genome.

Figures

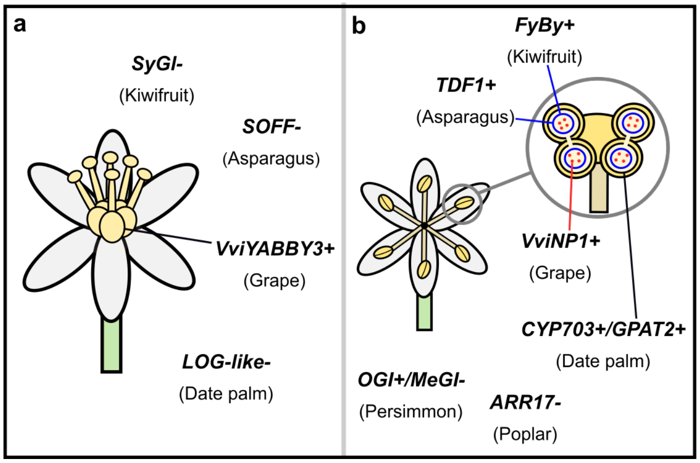

Figure 1. Recently discovered angiosperm sex-determination genes. Most sex-determining genes recently identified that are involved with carpel development (a) have been dominant Y-linked genes. These genes, including *SyGI*, *SOFF*, and *LOG-like*, when expressed (+) in males suppress the function or development of the carpel, however, the lack of expression (-) in females allows for functional carpel development. *VviYABBY3*, in contrast, is X-linked and promotes carpel development in grapes. Several Y-linked genes are also involved promoting stamen function (b). *FyBy* and *TDF1* both promote tapetum development (in blue) and *VviNP1* promotes pollen development (in red). It is unknown yet whether CYP703 or GPAT2 is the male-determining gene in date palm, however, both are involved in pollen and/or anther development. In persimmon and poplar, a single gene is involved in sex determination (*OGI* and *ARR17*, respectively).

Tables

Table 1. Published dioecious nuclear genomes. The species listed here are dioecious, though for many others closely-related hermaphroditic or monoecious references may be available.

Lineage	Family	Species	Sex chromosome type	Citation
Moss	Ditrichaceae	*Ceratodon purpureus*	UV	[63]
Moss	Pottiaceae	*Syntrichia princeps*	UV	[160]
Moss	Fontinalaceae	*Fontinalis antipyretica*	UV	[189]
Moss	Hylocomiaceae	*Pleurozium schreberi*	UV	[159]
Liverwort	Marchantiaceae	*Marchantia polymorpha*	UV	[156,190]
Liverwort	Marchantiaceae	*Marchantia inflexa*	UV	[158]
Gymnosperm	Ginkgoaceae	*Ginkgo biloba*	XY or ZW	[149,150]
Gymnosperm	Gnetaceae	*Gnetum montanum*	Possibly XY	[191]
Angiosperm	Amborellaceae	*Amborella trichopoda*	ZW	[143]
Angiosperm	Dioscoreaceae	*Dioscorea alata*	XY	[81]
Angiosperm	Dioscoreaceae	*Dioscorea rotundata*	ZW	[84]
Angiosperm	Asparagaceae	*Asparagus officinalis*	XY	[47,48]
Angiosperm	Arecaceae	*Phoenix dactylifera*	XY	[91]
Angiosperm	Vitaceae	*Vitis arizonica*	XY	[98]
Angiosperm	Vitaceae	*Vitis amurensis*	XY	[192]
Angiosperm	Vitaceae	*Vitis riparia*	XY	[193]
Angiosperm	Vitaceae	*Vitis vinifera sylvestris*	XY	[98]
Angiosperm	Vitaceae	*Muscadinia rotundifolia*	XY	[98]

Angiosperm	Euphorbiaceae	*Mercurialis annua*	XY	[171]
Angiosperm	Salicaceae	*Populus alba*	ZW	[102]
Angiosperm	Salicaceae	*Populus deltoides*	XY	[102]
Angiosperm	Salicaceae	*Populus euphratica*	XY	[194]
Angiosperm	Salicaceae	*Populus ilicifolia*	XY	[195]
Angiosperm	Salicaceae	*Populus tremula*	XY	[102]
Angiosperm	Salicaceae	*Populus trichocarpa*	XY	[196]
Angiosperm	Salicaceae	*Salix brachista*	Possibly ZW	[197]
Angiosperm	Salicaceae	*Salix matsudana*	Possibly ZW	[198]
Angiosperm	Salicaceae	*Salix purpurea*	ZW	[104]
Angiosperm	Salicaceae	*Salix suchowensis*	ZW	[199]
Angiosperm	Salicaceae	*Salix viminalis*	ZW	[67]
Angiosperm	Rosaceae	*Fragaria x ananassa*	ZW	[200]
Angiosperm	Moraceae	*Ficus carica*	XY	[201]
Angiosperm	Moraceae	*Ficus erecta*	Possibly XY	[202]
Angiosperm	Moraceae	*Ficus hispida*	XY	[203]
Angiosperm	Cannabaceae	*Cannabis sativa*	XY	[204]
Angiosperm	Cannabaceae	*Humulus lupulus*	XY	[205]
Angiosperm	Myricaceae	*Morella rubra*	ZW	[62]
Angiosperm	Myricaceae	*Morus alba*	XY	[206]
Angiosperm	Myricaceae	*Morus notabilis*	Possibly XY	[207]
Angiosperm	Anacardiaceae	*Pistacia vera*	ZW	[208]
Angiosperm	Caricaceae	*Carica papaya*	XY	[52,209]
Angiosperm	Polygonaceae	*Rumex hastatulus*	XY	[174]
Angiosperm	Amaranthaceae	*Amaranthus palmeri*	XY	[125,126]

Angiosperm	Amaranthaceae	*Amaranthus tuberculatus*	XY	[126]
Angiosperm	Amaranthaceae	*Spinacia oleracea*	XY	[129]
Angiosperm	Simmondsiaceae	*Simmondsia chinensis*	XY	[210]
Angiosperm	Ebenaceae	*Diospyros lotus*	XY	[132]
Angiosperm	Actinidiaceae	*Actinidia chinensis*	XY	[211]
Angiosperm	Actinidiaceae	*Actinidia eriantha*	XY	[212]
Angiosperm	Solanaceae	*Solanum appendiculatum*	XY	[142]

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