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Title: **Plant contributions to our understanding of sex chromosome evolution**

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Summary

A minority of angiosperms have male and female flowers separated in distinct individuals (dioecy), and most dioecious plants do not have cytologically different (heteromorphic) sex chromosomes. Plants nevertheless have several advantages for studying sex chromosome evolution, as genetic sex determination has evolved repeatedly and is often absent in close relatives. I review sex-determining regions in non-model plant species, which may help us understand when and how (and, potentially, test hypotheses about why) recombination suppression evolves within young sex chromosomes. I emphasise high throughput sequencing approaches that are increasingly being applied to plants to test for non-recombining regions. These data are particularly illuminating when combined with sequence data that allow phylogenetic analyses, and estimates of when these regions evolved. Together with comparative genetic mapping, this has revealed that sex-determining loci and sex-linked regions evolved independently in many plant lineages, sometimes in closely related dioecious species, and often within the past few million years. In reviewing recent progress, I suggest areas for future work, such as using phylogenies to allow informed choice of outgroup species suitable for inferring the directions of changes, including testing whether Y chromosome-like regions are undergoing genetic degeneration, a predicted consequence of losing recombination.

Introduction: Advantages of plants for studying sex chromosome evolution

Most flowering plants have hermaphroditic flowers, and only a minority have separate male and female flowers (monoecy or dioecy). Among dioecious plants, with male and female flowers separated in distinct individuals, some species have environmental, not genetic, control of sex determination (Policansky, 1981; Zimmerman, 1991; Pannell, 1997), and those with genetic sex determination often do not have cytologically differentiated sex chromosomes (Westergaard, 1958; Ming *et al.*, 2011; Renner, 2014). In contrast, separate sexes and heteromorphic sex chromosomes are common in many familiar animal groups (Bachtrog, 2012). Nevertheless, plants have several advantages for research on sex chromosomes, because genetic sex determination has evolved repeatedly among angiosperms, and independently in different families (Charlesworth, 1985; Ming *et al.*, 2011; Renner, 2014). Compared with the best-studied animal systems (Bellott *et al.*, 2014; Cortez *et al.*, 2014; Zhou *et al.*, 2014), many flowering plant sex chromosomes probably evolved very recently (Marais *et al.*, 2011; Renner, 2014), yet, as will be illustrated below, similarities with animal systems are striking. Table 1 summarises the main advantages of using dioecious plants to study sex chromosome evolution, and test hypotheses about sex chromosome evolution derived from theoretical modelling.

Table 1 about here

There is now too much published work on plant sex chromosomes and their evolution to include in a single article. I therefore focus on recombination suppression, the defining characteristic of sex chromosomes, which leads to the evolution of sex chromosomes' other unusual characteristics, genetic degeneration and accumulation of repetitive sequences on the sex chromosomes, which I mention only briefly. I review progress that has come through genetic and molecular evolutionary studies, illustrating how this has involved combining approaches, including DNA sequencing, and resequencing of multiple individuals of the same sex and species for genetic and population genetic tests of sex-linkage, together with sequencing for phylogenetic studies, and to place events in well-established time frames.

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79 **Sex chromosomes and estimating their ages from sequence divergence**

80 I define plant sex chromosomes as genome regions of these species that carry
81 the "SEX" locus that controls individuals' sexes, and that do not recombine. Rather
82 than using the term sex chromosomes, I shall often use "fully sex-linked regions" (and
83 full sex linkage), because of the diversity among plants with genetically controlled
84 dioecy — some have extensive non-pairing regions that show heteromorphism
85 between the sexes, like many animal sex chromosomes, but many have no detectable
86 cytological differences (recently reviewed by Renner, 2014). *Silene latifolia* is an
87 example of sex chromosome heteromorphism. It has an XY system, and males are the
88 heterozygous sex, and, as in mammals (Bellott *et al.*, 2014; Cortez *et al.*, 2014). The
89 Y is largely non-recombining, with XY pairing only in a small pseudo-autosomal
90 region (PAR) region at one tip (Westergaard, 1958; Filatov *et al.*, 2008); mapping of
91 genic markers suggests a single PAR (Bergero *et al.*, 2013), though an AFLP map
92 suggests two (Scotti & Delph, 2006). However, unlike many animal Y chromosomes,
93 the fully sex-linked region still carries hundreds of genes (Bergero & Charlesworth,
94 2011; Chibalina & Filatov, 2011; Muyle *et al.*, 2012). In contrast, the fully Y-linked
95 region in papaya is only about 10% of chromosome 1 (Liu *et al.*, 2004; Wang *et al.*,
96 2012). Some diploid plants have ZW systems, in which females are ZW heterozygotes
97 and males are ZZ homozygotes (Westergaard, 1958), as in birds (Zhou *et al.*, 2014)
98 and Lepidoptera (Suetsugu *et al.*, 2013); these include *Fragaria* (strawberry) species
99 (Spigler *et al.*, 2008; Goldberg *et al.*, 2010) and *Salix* (Alstrom-Rapaport *et al.*, 1998).
100 Other systems, including those in haploid plants, will be described below.
101 The time when recombination stopped can be estimated using DNA sequence
102 divergence between genes present on the Y as well as the X, together with a
103 "molecular clock" for synonymous or silent site divergence per year. Higher X-Y
104 divergence values correspond to greater times since recombination suppression. In
105 both humans (Lahn & Page, 1999) and the plant *S. latifolia* (Bergero *et al.*, 2007),
106 divergence increases with the distance from the PAR (in X chromosome genetic or
107 physical maps; (as these Y chromosomes are extensively rearranged, distances on the
108 Y are not informative Skaletsky *et al.*, 2003; Bergero *et al.*, 2008). Therefore,
109 suppressed recombination must have spread from an early non-recombining region,
110 the oldest "evolutionary stratum" (Lahn & Page, 1999), towards younger "strata"

closer to the current PAR. X-Y divergence in the older *S. latifolia* stratum is similar to that in the youngest of the five strata in humans (Skaletsky *et al.*, 2003), and the *Silene* XY pair probably evolved about 5-10 MYA (Nicolas *et al.*, 2005).

[Figure 1 about here]

A sex chromosome system may be older than its oldest stratum, because recombination suppression in a sex-determining region usually takes time to evolve (see below). On the other hand, recombination suppression may pre-date evolution of separate sexes. In several well-studied plants, results from combining genetic and physical mapping reveal large genome regions with infrequent crossing over surrounding the centromeres, with crossovers restricted to the ends of chromosomes, for example in maize (Rodgers-Melnicka *et al.*, 2015)**.** These regions may include substantial proportions of genes; in barley, for example, about 20% of genes are estimated to be located in such regions (Baker *et al.*, 2014). If sex-determining loci evolve in such a region, the oldest stratum will be contemporaneous with the sex-determination system (Figure 1).

In what follows, I stress the importance of estimating ages of non-recombining regions for understanding several important aspects of sex chromosome evolution. Young sex chromosome systems are well suited for studying the early stages of evolution of recombination suppression and the evolution of these characteristics; in older animal systems, these processes can only be studied over a coarse time scale that cannot reveal much detail. Young evolutionary strata in plant sex chromosomes are also of interest for studying the time course of genetic degeneration, including gene losses from Y chromosomes.

Which plants have sex chromosomes?

Genetic maps can detect the presence of sex-linked regions in dioecious species. In papaya, for example, a large set of AFLP molecular variants was first mapped in a full-sib family (Liu *et al.*, 2004). The completely sex-linked region is small, making BAC sequencing of the region possible, which showed that the X-linked region includes only 3.5 Mb (it is flanked by much larger PARs), and carries about 50 genes with apparently functional Y-linked copies (Wang *et al.*, 2012). Assembly of the physical map of the homologous Y-like region suggests that part of the sex-linked

region is probably in a pericentromeric region (Yu *et al.*, 2007; Zhang *et al.*, 2008). Silent site divergence values for XY pairs suggests that inversions occurred, suppressing recombination, about 7 and 1.7 million years ago, implying that a new recombination-suppressed region has formed since the older stratum evolved (Figure 1).

Ideally, sex-linkage in a family should be confirmed by showing that Y-linked variants are found only in males in a wider sample of genotypes (from natural population samples, or from multiple cultivars of crop species) to exclude partially sex-linked genes in the PAR that did not yield recombinants in the particular cross studied. This is unnecessary for papaya, because, although this is clearly a young system, the sequence divergence across part of the X-Y region is around 7% for silent sites, much higher than between alleles in recombining regions of the genome, including the collinear regions adjoining the fully sex-linked sequences (Wang *et al.*, 2012). This strongly suggests complete sex-linkage.

Now that large numbers of genetic markers can be developed in non-model organisms, using high-throughput approaches, it will be possible to discover how many other plant cases like papaya exist, without major cytologically detectable sex chromosome heteromorphism, but with fully sex-linked regions carrying multiple genes, and assess how many plants evolved dioecy so recently that their sex-determining loci have not yet evolved non-recombining sex chromosome-like regions. I next outline other approaches that have demonstrated that sex-linked regions have evolved in dioecious plants.

When divergence data are not available, genetic mapping in related species can help test whether recombination suppression has evolved in dioecious plants. If the SEX locus of a dioecious species is in a genome region of suppressed recombination, but the homologous region of a non-dioecious relative recombines, this would suggest that recombination suppression evolved following the evolution of genetically controlled dioecy, rather than being the ancestral state (unless the SEX locus is in a pericentromeric region whose extent or location has changed between the species). A dioecious close relative of papaya, *Vasconcellea parviflora*, has been shown to have a homologous SEX locus, based on cytogenetic detection of heterochromatin in the centromere-proximal regions of the homologous chromosomes of the two species. This result also shows that the papaya and *V. parviflora* sex chromosomes are not truly homomorphic (Iovene *et al.*, 2015) — their heteromorphism is minor, but

detectable with refined modern cytological methods, consistent with the sequencing results showing that the papaya Y--linked region is larger than the X region (Wang *et al.*, 2012).

These examples illustrate the value of genetic mapping within families. However, studies of wider population samples can also be used to discover sex-linked regions and establish which is the heterozygous sex. In an XY system, all males should be heterozygotes for fully sex-linked alleles, and population surveys can reveal such male-specific variants (which can be confirmed by data on segregation patterns within families). This approach has established that the date palm (*Phoenix dactylifera*) has at least several fully sex-linked genes (Cherif *et al.*, 2012). Neither the physical size of the SEX region, nor the age of this system has yet been estimated. It is a heteromorphic XY system (Siljak-Yakovlev *et al.*, 1996) that may be ancient, as many other species in the palm family are also dioecious (Renner, 2014).

Haploid plants

Many haploid plants have sex chromosomes, as determined either from morphology differences in the karyotypes of male and female gametophytes (Bull, 1983; Ming *et al.*, 2011), or from the existence of one or more sex-linked genetic markers (Immler & Otto, 2015). The male- and female-determining chromosomes of haploids are now often called V and U, respectively (Bachtrog *et al.*, 2011), to emphasize that the SEX region is never homozygous and can therefore never recombine; until physical maps are produced, it is not possible to relate sequence divergence to the genetic map position in the sex-linked region. The older literature, including studies in *Marchantia polymorpha*, called them X/Y systems (Okada *et al.*, 2001; Yamato *et al.*, 2007). *M. polymorpha* has highly heteromorphic sex chromosomes, and divergence between alleles of the few sex-linked gene pairs studied is extremely high, indicating an ancient system.

In contrast, genetic mapping in the moss *C. purpureus* found that, as in papaya, only markers in the middle of the linkage group with the SEX locus show full sex-linkage (McDaniel *et al.*, 2007). Sequence data for all site types in coding plus (predominantly) non-coding regions of U- and V- linked allele sequences suggests that evolutionary strata may exist in *C. purpureus*. Divergence between four of 8 U-V gene pairs studied is only around 1-3%, but two genes have divergence of almost 7%

for (McDaniel *et al.*, 2013); one of them is long enough to reliably suggest high divergence, either indicating a longer time since recombination stopped between the U and V regions, or a higher mutation rate and/or lesser selective constraint (divergence from the related species is also high for the high U-V divergence gene, consistent with the two latter possibilities). Even the highest silent site divergence currently found suggests, however, that this is not an ancient system (although genes with higher divergence may be discovered when more genes are analysed).

Plants with very small, or no, non-recombining regions.

Genetic mapping (or related methods that can detect such regions even if they are small, such as bulk segregant analysis) has yet to be applied in many plants with genetic sex determination, and they could reveal non-recombining regions in many plants not currently classified as having sex chromosomes. Indeed, a major currently unanswered question is whether the number of plants with sex-linked regions is currently under-estimated. Such studies are, however, limited by marker density, and very small non-recombining regions may be missed due to insufficient marker density. Indeed, in several plants, genetic sex determination has been established, and a SEX locus controlling gender has been mapped, but no fully sex-linked marker has been found. In kiwifruit (*Actinidia chinensis*), for example, mapping with 644 microsatellite markers still failed to detect any fully sex-linked markers. Other species where the recombination status of the SEX locus is currently uncertain include spinach (Khattak *et al.*, 2006), asparagus (Telgmann-Rauber *et al.*, 2007), and *Populus* species (Yin *et al.*, 2008; Pakull *et al.*, 2011). Such species may, of course, truly lack non-recombining SEX regions. They may either not yet have evolved fully sex-linked regions, or may be single gene systems, which can evolve when a new gene takes over control of flower sex determination after dioecy has become established, replacing an existing sex-determining gene (Bull, 1983; van Doorn & Kirkpatrick, 2007; Vuilleumier *et al.*, 2007; Blaser *et al.*, 2013); Figure 1C illustrates such an event. Takeovers are known in several animal taxa, including insects (Wilkins, 1995; Beye *et al.*, 2003) and fish (Ross *et al.*, 2009; Myosho *et al.*, 2012).

To map SEX loci, high throughput methods including RAD-Seq (Baird *et al.*, 2008) or RNA-Seq transcriptome sequencing (Bergero & Charlesworth, 2011; Chibalina & Filatov, 2011; Muyle *et al.*, 2012; Hough *et al.*, 2014) can now generate

large numbers of markers, overcoming the problem of low marker density. Alternatively, given the large resources sometimes available in crop plants, high density linkage maps can be obtained by "target-sequence capture" (Mamanova *et al.*, 2010). In *Fragaria vesca*, for example, a map was made by first obtaining a low coverage genome sequence, then identifying polymorphisms, and then using an enrichment approach to obtain short sequences (200bp) surrounding each polymorphism. This allowed genotyping of 5417 genes in a mapping family (Tennessen *et al.*, 2013). Another recently developed approach that may be helpful in plants ascertains fully sex-linked sequences by searching short read genome sequence data from multiple individuals for *k*-mers (short sequences of length *k*) that appear only in one sex (Carvalho & Clark, 2013). In the section on sex-determining genes below, I outline how this approach has ascertained Y-linked sequences in persimmon (*Diospyros lotus*), in the Ebenaceae (Akagi *et al.*, 2014).

A major difference between ancient animal sex chromosomes and the Y-linked regions of the plants just discussed (with the possible exception of *M. polymorpha*) is the minor extent of gene loss in plants (see section on genetic degeneration below). In plants, sex-linked genes therefore cannot be ascertained by genome sequencing, using their lower coverage in the heterogametic sex. Moreover, assembly of short-read sequences will be difficult, due to sequence divergence and accumulation of repetitive sequences, which occur in non-recombining genome regions (Charlesworth *et al.*, 1994), including plant sex chromosomes (Kubat *et al.*, 2014). Assemblies of the human and papaya Y chromosomes involved deep sequencing of single males, avoiding variants that might confuse assembly, and are restricted to non-heterochromatic regions (Hughes *et al.*, 2010; Wang *et al.*, 2012).

Finally, multiple individuals of each sex are needed to determine sex-specific sequences and distinguish fully and partially sex-linked regions. Even with large family sizes, lack of recombinants does not exclude rare recombination. Population genetic approaches can, however, detect recombination in past generations, even if it occurs very rarely. In papaya, for example, the SEX region adjoins a "collinear region" where the sequenced X and Y chromosomes appear to have the same genes in the same order, unlike the older Y-linked strata, whose assembly includes many rearrangements (Wang *et al.*, 2012). Divergence between the single X and Y sequences so far available is low in the collinear region, indicating that recombination must have continued after it had stopped in the two strata defined by the Y region

inversions described above. Sequence differences between the single X- and Y-linked region so far sequenced may merely be variants that happens to be carried on those particular chromosomes, and not sex-linked in the species as a whole, so this region may prove to be partially sex-linked.

An approach that does include multiple individuals is bulk segregant analysis. This has recently been successful in the grape vine (*V. vinifera*), an XY system. The fully sex-linked genome region is only about 150 kb, and includes only a few fully sex-linked genes, so recombination suppression has clearly not yet extended across any substantial genome region (Picq *et al.*, 2014), consistent with the lack of chromosomal heteromorphism.

The observation that much of the chromosome carrying the SEX locus recombines does not necessarily mean that it is a young system. Instead, it may be an ancient system whose non-recombining region has remained limited to just a part of the chromosome. This is the case in the Ratite birds (Pigozzi, 2011; Vicoso *et al.*, 2013; Zhou *et al.*, 2014). In *Vitis*, most wild species are dioecious, suggesting that dioecy is ancestral, so this could be a plant example of an old-established system that has not evolved recombination suppression. However, divergence between *Vitis* sex-linked genes has not yet been estimated, so the time when the sex-determining region evolved is not yet known.

Comparative genetic mapping

Genetic mapping is also important for detecting differences in the chromosome carrying the SEX region. This can occur when genetic sex-determination evolves independently (or re-evolves after loss of dioecy). For example, genes that are sex-linked in *S. latifolia* do not show sex-linkage in *Silene otites* and *S. colpohpylla* (Mrackova et al., 2008). Phylogenetic analyses of sex systems in *Silene* (Mrackova *et al.*, 2008; Marais *et al.*, 2011) suggest that these species do not have a dioecious common ancestor, so dioecy probably evolved *de novo* in the two lineages, and involved different genes.

Events in which a new gene takes over control of gender, can also cause the SEX loci of related species to be on non-homologous chromosomes (Figure 1C), or to a new location on the same chromosome, as in some animal cases of takeovers (Uno *et al.*, 2008). In *Populus*, as in papaya, the SEX loci appear to be within

pericentromeric regions (Geraldes *et al.*, 2011), but, although these regions map to the same linkage group, their locations differ greatly in the physical maps of different *Populus* species (Yin *et al.*, 2008; Pakull *et al.*, 2011). If confirmed, this suggests a takeover event by a new sex-determining gene on the same chromosome. Independent evolution of separate sexes in different *Populus* lineages is not yet excluded, however, even though almost all Salicaceae are dioecious. Data on the ages of the systems, phylogenetic analysis, and genome sequencing, should help to distinguish between the possibilities. Takeovers or independent evolution both predict that different species should have different sets of genes at their SEX loci, unlike a chromosome rearrangement. Independent evolution predicts that the times since recombination stopped should differ (though this might not be detectable if all species have young systems), while takeover events generating single-locus systems may not have been followed by recombination suppression in the surrounding genome region.

Why does suppressed recombination evolve?

The repeated evolution of regions without crossing over between sex chromosomes strongly suggests a causal connection with the evolution of sex-determining regions (only the centromeric and pericentromeric regions of autosomes generally have suppressed crossing over). The evolutionary strata of sex chromosomes discussed above prove that suppressed recombination often evolves after a sex-determining system is established. Some disadvantage to recombinant genotypes must clearly be involved. Such situations probably occur both during the initial evolution of dioecy, and also later, as males and females evolve in the absence of constraints imposed by the other sex functions. Briefly, as illustrated in Figure 2, separate sexes in plants probably often evolved from hermaphroditic or monoecious ancestors, often called “cosexual” species (Lloyd, 1982).

The change from cosexuality to dioecy probably involves a mutation creating females (a male-sterility mutation in an initially hermaphroditic species, or a mutation suppressing some or all female flowers in an initially monoecious species, or replacing them with male flowers), and then one or more female-suppressing mutations, creating males or male-biased plants (Westergaard, 1958; Charlesworth & Charlesworth, 1978). (I can find no cases where dioecy in plants evolved from environmental sex determination, though this seems possible in principle).

[Figure 2 about here]

In this scenario, male-promoting mutations (suppressing femaleness) clearly reduce females' fitness, and are therefore most likely to spread if linked to the gene causing femaleness, which minimises the conflict. If a two-gene polymorphism results, selection against recombinants will generate linkage disequilibrium (with the X associated with male-sterility and the Y with female-suppressor alleles, see Figure 2). Suppressed recombination is therefore favoured, and may evolve (Charlesworth & Charlesworth, 1980; Bull, 1983), creating a male-determining Y chromosome.

If however, a male-specific female-suppressing mutation occurs, no harm is caused to females; if sufficiently advantageous in males, such a mutation can spread, even if unlinked to the femaleness gene, yielding a single gene sex-determining system (Figure 2A), and no selection for closer linkage with the gene causing femaleness (Muller, 1932; Charlesworth & Charlesworth, 1978).

Much genetic evidence supports the two-gene model for plant sex-determination, rather than one with sex-specific gene actions. First, in several species (or intercrosses of dioecious plants with close non-dioecious relatives) three allelic types at the sex-determining locus control whether individuals are (i) females, (ii) males, or (iii) hermaphrodites or monoecious functional hermaphrodites (Westergaard, 1958). Second, in papaya and grape vine, humans have selected individuals that have Y-linked regions that do not suppress female functions (Wang *et al.*, 2012; Picq *et al.*, 2014). Similarly, in *Silene latifolia*, deletions detectable through loss of Y-linked markers can create hermaphrodites and neuter plants (Fujita *et al.*, 2011 and references therein). These plants' Y-linked regions must therefore carry suppressors of female functions whose loss does not affect male functions, and distinct maleness factor(s) elsewhere on the chromosome. Thirdly, in the strawberry species *Fragaria virginiana*, two closely, but not completely, linked genes with the expected phenotypes have been found (Spigler *et al.*, 2008), while a related *Fragaria* species has suppressed recombination (Goldberg *et al.*, 2010). These species' sex-determining regions probably evolved independently (Goldberg *et al.*, 2010), but the results nevertheless suggest recombination suppression evolving in response to a two-locus polymorphism.

The evolution of dioecy probably often involves further sexually antagonistic mutations, leading to further selection to suppress recombination, and potentially generating younger strata. For example, dioecy has often evolved from monoecy (Renner, 2014), and full maleness may involve successive increases in the proportion of investment in male flowers (Figure 2B), each involving sexually antagonistic "trade-offs", because each must decrease the proportion invested in female flowers. Variable degrees of maleness are indeed seen in the monocotyledon *Sagittaria latifolia*, in the Alismataceae (Dorken & Barrett, 2004), *Spinacia oleracea* in the Chenopodiaceae (Onodera *et al.*, 2011) and *Urtica dioica* (Glawe & Jong, 2009). Similarly, when the ancestral state is hermaphroditism, evolution of dioecy often involves "inconstant males" with partial female function (for example, producing some fruits in favourable conditions). Species where genetic variation in male functions seems likely include *Antennaria dioica* in the Asteraceae (Ubisch, 1936) and *Euonymus europaeus* in the Celastraceae (Webb, 1979), but these have not been investigated using genetic markers to map the factors. Even after complete unisexuality has evolved, male and female functioning may be sub-optimal, and improvements to each sex may often reduce functions of the other. In *S. latifolia*, for example, female fecundity is enhanced by making large flowers, but fertility is highest for males with many small flowers (Delph & Herlihy, 2012). Just as outlined above for sterility mutations, a mutation benefitting one sex at the expense of the other is most likely to invade but not spread throughout the population; if such a polymorphism is established, it creates selection for reduced recombination with the sex-determining locus, if it arises at a locus closely linked to the SEX locus (Rice, 1987; Jordan & Charlesworth, 2012).

Testing for the trade-offs and conflicts assumed in these scenarios, and for involvement of sexually antagonistic polymorphisms in the PAR regions of sex chromosomes is clearly a major task for future work. An approach that can potentially detect sexually antagonistic variation is QTL analysis within the two sexes separately, as proposed and implemented in *Silene latifolia* (Scotti & Delph, 2006; Delph *et al.*, 2010)**.** This detected several autosomal and PAR QTLs, and, interestingly, the latter appeared only in the analysis of males, implying that their phenotypic effects are not expressed in females. Such male-specific expression is consistent with a past conflict between the sexes that has been resolved in later evolution, as seems to have occurred for some sexually selected male coloration genes in the PAR of a fish, the guppy,

Poecilia reticulata (Lindholm & Breden, 2002). Male benefit alleles with male-specific expression no longer harm females, and will spread throughout the population; some other selection is therefore required to maintain the QTL variation, perhaps environmental differences (Scotti & Delph, 2006). In *S. latifolia*, for example, thin leaves appear to be disadvantageous to males only in dry years (Delph *et al.*, 2011). The *S. latifolia* QTL analysis used dominant AFLP markers, but codominant markers now available in this plant's PAR, and obtainable in other plants, will permit future analyses of variation in natural populations. This may detect factors whose conflict has not been resolved, corresponding to the situation that creates selection for reduced recombination in the theoretical models of sexually antagonistic PAR genes.

Recombination suppression: mechanisms

Non-recombining regions may eventually evolve to encompass a large region of the chromosome carrying the sex-determining loci or locus. Studies of young plant sex chromosomes may be valuable for studying the mechanistic basis of recombination suppression, and whether it generally involves infrequent, large-scale events like inversions, or smaller shifts in the position of the PAR boundary.

If chromosome inversions cause recombination suppression in SEX regions (Lahn & Page, 1999), the region will often include many non-sex-determining genes. In papaya, two Y chromosome inversions indeed seem to be involved (including 10 genes with both X and Y copies in the older stratum includes, and 16 in the newer one,Wang *et al.*, 2012). In closely related dioecious *Vasconcellea* species, alleles of several papaya fully sex-linked genes are not associated with gender (Gschwend *et al.*, 2011). Unlike the *Silene* situation described above, this probably does not reflect independent evolution of dioecy in *Carica* and *Vasconcellea*, as BAC-FISH experiments found sex-linked regions including several homologous sequences in similar locations on the largest chromosome of both species (Iovene et al., 2015). Recombination suppression has therefore probably remained restricted in *V. parviflora* to a genome region near the SEX locus, whereas it has spread across a wider region in *C. papaya*. This is testable by sequencing to ask whether *V. parviflora* genes homologous to *C. papaya* genes in the older sex-linked stratum have distinct X and Y haplotypes like those of papaya. The alternative that the *V. parviflora* long arm has become a new recombination-suppressed stratum seems unlikely, because the

chromosomal positions of all nine relevant BACs in an outgroup, *Jacaratia spinosa*, were found to be similar to those in *V. parviflora*, so the inversions probably occurred in the *C. papaya* lineage.

Recombination suppression mechanisms other than inversions may, however, exist, including modifiers controlling the number of crossover events, restricting them to certain genome regions, or restricting crossing over to only one sex. In one of the two human PARs, for example, crossovers are localised very differently in male and female meiosis (Hinch *et al.*, 2014). Some young sex chromosome systems may still be in the process of undergoing recombination suppression. If recombination varies between individuals, or between closely related species that can be interbred, genetic studies can potentially identify the factors involved. In some populations of frog species, male-specificity of microsatellite alleles differs between populations, implying that the XY pair shows suppressed recombination only in some populations (Dufresnes *et al.*, 2014). Apparently similar variation was inferred for an anonymous sequence marker within the plant species *Bryonia dioica* (Oyama *et al.*, 2009), which should be studied further. In *Silene latifolia*, recombination suppression appears to vary between families for several genes (Bergero *et al.*, 2013).

Old-established sex chromosome systems

Old sex chromosomes also exist among plants, for example among liverworts (Okada *et al.*, 2001), but have been less studied than young plant systems. As molecular approaches and phylogenetic analyses are extended to studying more plant sex chromosome systems, it will be interesting to include taxa with high proportions of dioecious species, such as the palm, Vitaceae and Ebenaceae (including *Diospyros lotus*, see below) families, to test whether dioecy is ancestral and estimate the time when recombination stopped, or has evolved several times (as may be the case in the Salicaceae discussed above).

Old systems are particularly interesting for investigating genetic degeneration and repetitive sequence accumulation, which occur over large evolutionary timescales. The potentially large range of ages of dioecious plant sex chromosome systems will allow the time-course of sex chromosome evolution to be studied. Old plant systems may also help us understand why recombination suppression sometimes fails to evolve.

The evidence for old-established systems is currently incomplete, and age estimates based on sequence divergence are lacking. There are currently no dense genetic maps for plants that seem likely to have old XY systems, and, so far, genetic mapping in these systems has largely used non-genic markers such as AFLPs and microsatellites. These are excellent for testing for a non-recombining (sex chromosome-like) region, determining which is the heterozygous sex, and estimating the proportion of the chromosome that is fully sex-linked. However, as explained above, estimating the age of a sex chromosome system, and the time when recombination stopped, requires X-Y sequence divergence estimates, based on ascertaining sex-linked genes and sequencing them.

In the absence of divergence data, the observation that a sex chromosome system is heterochromatic and heteromorphic might be thought to suggest that it is old-established, especially in plant families that include distantly related dioecious species, such as date palms (Al-Mahmoud *et al.*, 2012). For example, *Rumex acetosa* belongs to a clade that may have been dioecious for 15–16 MYA (Navajas-Pérez *et al.*, 2005), but X-Y divergence has not been estimated. Its Y chromosomes are heterochromatic (Shibata *et al.*, 2000; Mariotti *et al.*, 2008), unlike those of other cytologically well-studied plants such as *Silene latifolia* and *S. dioica* (Grabowska-Joachimiak & Joachimiak, 2002; Kubat *et al.*, 2014), which are estimated to be younger (see above). However, heterochromatin can evolve rapidly, as in papaya. Another example is *Coccinia grandis*, within a wholly dioecious genus of 27 species (in the Cucurbitaceae, another family with many dioecious species, often with XY heteromorphism). Its male genome C-value is 10% larger than that of females, indicating that the Y chromosome is much larger than the X, and the entire Y is heterochromatic (Sousa *et al.*, 2012), yet phylogenetic analysis suggests that these characteristics evolved recently (Holstein & Renner, 2011).

Sex chromosome heteromorphism can also arise in young systems, for example through fusions with autosomes, as in *Rumex hastatulus* (Smith, 1964) and possibly spinach (Araratjan, 1939). The systems in *Cannabis sativa* (Peil *et al.*, 2003; Sakamoto *et al.*, 2005) and *Humulus lupulus* (hops) in the Cannabaceae, whose Y chromosome is heterochromatic (Westergaard, 1958) are probably much older.

Studies of old systems are also needed to test the prediction that other sex-determining system are derived from XY systems (Charlesworth & Charlesworth, 1978). Again plants may be very helpful, as systems with male-determining Y

chromosomes probably evolve first, as outlined above, but ZW systems also exist, and it can be tested whether the frequencies of such systems increases over time. X-autosome balance systems are also probably derived from XY systems (and potentially allow loss of the Y chromosome, and evolution of an X0 male genotype). However, it has again not yet been demonstrated that such species tend to be older than other plant sex-determining systems. Absence of carpel development in males or stamen development in females, as in hops, may also indicate an ancient system (but might simply be due to a long history of unisexual flowers, for example because dioecy has evolved from monoecy); so far, only one fully sex-linked genetic marker locus has been found in hops (Jakse *et al.*, 2008).

Genetic degeneration: the need for empirical data in a phylogenetic setting

Ancient systems are also of great interest for studying genetic degeneration (gene loss or loss of function). In diploid organisms, only the Y chromosomes are predicted to degenerate, because X chromosomes recombine in the XX females, whereas Y-linked regions do not, and are subject to several processes that allow detrimental mutations to increase in frequency in the population of Y-linked alleles, or even to become fixed in this population, as recently reviewed (Bachtrog, 2008). In haploid plants, however, the complete lack of recombination across the entire sex-linked region predicts similar degeneration of both U and V chromosomes (Bull, 1983). Genes affecting non-sex functions should not degenerate or become lost, so the female-determining U region should lose only male function genes, and the male-determining V region only female function genes (Figure 3C).

Figure 3 about here

Haploid plants with separate sexes of gametophytes are ideal for studying this prediction. In *Marchantia polymorpha*, a species whose sex chromosomes carry highly diverged sequences, the V has been studied in detail, but analysis of the U chromosome is currently incomplete (Okada *et al.*, 2001). In the brown alga, *Ectocarpus siliculosus*, however, about 24 genes were found in the fully sex-linked regions (either the U or V regions, or both), of which 7 were not detected in the V and 9 in the U (Ahmed *et al.*, 2014). This is in apparent agreement with Bull's prediction;

however, without an outgroup, gene movements onto one sex chromosome, but not the other, cannot be excluded. To determine whether suitably close non-dioecious relatives exist (and avoid species that might have reverted from dioecy to a non-dioecious state), phylogenetic relationships of the species must be known. This is often difficult for closely related species, a frequent situation relevant to the evolution of sex chromosomes. Nevertheless, among plants, sets of species should exist with good phylogenies well suited for future work estimating ancestral character states, and changes in states, (Maddison & Leduc-Robert, 2013).

Diploid dioecious plants also have extended haploid life cycle stages, which may also cause genetic degeneration of non-recombining sex chromosomal regions to be minor (Figure 3B). Around 2/3 of plant genes are expressed in male gametophytes of angiosperms (Tanksley *et al.*, 1981; Gorla *et al.*, 1986; Honys & Twell, 2003). Therefore, only genes with no important pollen functions should be lost from plant SEX regions, or lose their functions; the limited evidence so far about loss of genes from the *S. latifolia* Y chromosome is consistent with this expectation (Guttman & Charlesworth, 1998; Chibalina & Filatov, 2011). Degeneration might be thus restricted to around 1/3 of genes (or possibly somewhat higher, if expression of some pollen-expressed genes is not important, and purifying selection maintaining their functions is consequently weak). The few current estimates, from the unrelated plants *S. latifolia* and *Rumex hastatulus*, suggest that fewer than 30% of Y-linked genes have lost expression (Bergero & Charlesworth, 2011; Chibalina & Filatov, 2011; Hough *et al.*, 2014). In contrast, such regions are almost completely degenerated in the best studied animals, such as species of *Drosophila* (Muller, 1950), mammals (Skaletsky *et al.*, 2003) and those birds that have extensive fully W-linked regions (Zhou *et al.*, 2014), and possibly in part of the much younger Y chromosome of the threespine stickleback (Ross & Peichel, 2008; Yoshida *et al.*, 2014). Large genome regions that stopped recombining recently and carry many genes driving the degeneration processes, such as the neo-Y chromosome of *Drosophila miranda*, have quickly lost functions of large fractions of genes (Bachtrog *et al.*, 2008). However, the regions of the two plants so far studied that recently became fully sex-linked probably include many fewer genes than the *D. miranda* region, so that the small extent of gene losses in these young systems is not surprising. It will be interesting to study older plant systems.

Genetic degeneration in young plant sex chromosomes, and in young evolutionary strata in older systems, is also of interest. The first step after recombination is suppressed between Y- and X-linked regions may be accumulation of repetitive sequences, including transposable elements. Such insertions may decrease expression of Y-linked alleles, even before mutations in the coding regions, or in non-coding regions that control the gene's expression. This appears to be the case in *Drosophila albomicans* (Zhou & Bachtrog, 2012).

However, plants with sex-determining loci within rarely recombining pericentromeric regions, such as papaya and *Populus* species, are not well suited for studying genetic degeneration, because accumulation of maladaptive sequence changes and of repetitive sequences are also expected in pericentromeric genome regions (Charlesworth *et al.*, 1986). It will therefore be difficult to detect extra effects of the evolution of sex-determining genes in the region. For example, in papaya, gene density is low in the sex-linked region, but this is not wholly due to loss of genes; accumulation of repeated sequences has also reduced gene density (Wang *et al.*, 2012).

Dosage compensation

In sex chromosome systems where Y-linked gene expression is reduced, or Y-linked genes have been lost, dosage compensation has sometimes evolved, and it is therefore interesting to test whether X-linked alleles of plant genes whose Y-linked copies have lost function are expressed at higher levels in males than females. There is currently no clear evidence that this occurs in *Silene latifolia* or *Rumex hastatulus*, but partial compensation cannot yet be excluded (Chibalina & Filatov, 2011; Muyle *et al.*, 2012; Hough *et al.*, 2014; Bergero *et al.*, 2015).

Plant sex-determining loci

To identify sex-linked regions and determine whether males or females are the heterozygous sex, it is not necessary to find the gene(s) controlling male or female development. As explained above, it suffices to find genetic markers, even anonymous ones, such as AFLPs, that co-segregate with sex. However, plant sex-determining loci are interesting in several ways, including for identifying the hypothesised two or more genes causing male- and female-sterility during the evolution of dioecy. If sex-determining genes can be discovered, sequence divergence

between their sex-linked alleles may also help estimate the time when recombination first stopped. With the possibility of dense marker development and genome sequences, renewed efforts are being made to identify plant sex-determining genes, and progress can be expected in the next few years.

The approach of testing known flower development genes has been largely superseded by high-throughput sequencing methods. Searches have found MADs-box and ABC(DE) genes involved in flower whorl development on the sex chromosomes of *Silene latifolia* and *Asparagus officinalis* (Matsunaga *et al.*, 2003; Park *et al.*, 2003; Cegan *et al.*, 2010; Nishiyama *et al.*, 2010; Penny *et al.*, 2011). However, genes that control floral organ identity are not generally promising candidates. They might be involved in species with complete absence of one sex organs ("Type I" of Mitchell & Diggle, 2005; Ramos *et al.*, 2014). In many dioecious plants, however, both male and female floral organs are initiated in flowers of both sexes, and the development of opposite sex organs is later interrupted.

Alternative approaches also encounter difficulties due to the numerous candidates whose loss of function can produce male or female sterility. For example, as mentioned above, deletion mapping of the *S. latifolia* Y chromosome has established that separate loci exist whose deletion causes abortion or incomplete development of stamens, or removes the suppression of pistils that occurs in wild-type males, creating hermaphrodite flowers (Farbos *et al.*, 1999; Lardon *et al.*, 1999; Zluvova *et al.*, 2005; Bergero *et al.*, 2008; Fujita *et al.*, 2011). However, these deletions probably involve loss of many fully sex-linked genes other than the ones causing these phenotypes, and this is supported by the observation that pollen carrying deleted Y chromosome regions often has low ability to fertilise ovules (Lardon *et al.*, 1999). When the sex-linked region is large, it will be difficult to identify the genes responsible for the evolution of dioecy unless small deletions can be generated and identified using dense mapping of sequences lost from deleted genotypes.

This problem also hinders attempts to identify genes involved in gender determination using mutations, including mutations induced by EMS or irradiation (Ohnishi, 1985; Christensen *et al.*, 1998; Honys & Twell, 2004; Wellmer *et al.*, 2006; Chang *et al.*, 2011), or by studying genes with different expression in flower buds of the two sexes. Moreover, many genes have stamen- or pistil-specific expression, and will be non-expressed in buds of one sex purely because the relevant structures are

absent. Distinguishing such downstream acting genes from the sex-determiners themselves requires establishing sex linkage. If, however, the fully sex-linked region includes many genes, the problem of having too many candidates with suitable function is not eliminated. In addition, expression differences may not be involved (for example, male sterility can involve mutations in coding sequences, and the mutant alleles may be present in mRNA).

Small sex-linked regions may offer the best prospects for identifying the sex-determining genes, because fewer candidates need to be considered. A candidate Y-linked gene has been proposed in persimmon (Akagi *et al.*, 2014). This study started by identifying sex-linked genes, using pools of males and females from a full-sib family, and their sex-linkage was confirmed in samples of unrelated males and females. Efforts were made to ensure that most Y-linked genes present in transcripts were detected, by employing RNA-Seq, and 22 expressed sequences were identified. The total length of sex-specific sequences was only 1Mb, suggesting a small fully sex-linked region. One candidate for involvement in sex determination was found. This gene (named *OGI*) is expressed only in male flower buds. *OGI* is a duplication onto the Y-linked region of an autosomal gene called *MeGI* that expresses a male-suppressing regulatory RNA in females. Because no X copy exists, X-Y divergence cannot be estimated, but divergence from the presumed autosomal progenitor is high, and Y-linked *OGI* sequences were detected in other species of Ebenaceae, suggesting an old-established Y-linked duplication. Low divergence was found between the X- and Y-linked alleles of other sex-linked genes (silent site divergence of 12 XY allele pairs was below 2%), suggesting that a younger stratum evolved recently.

The proposed scenario for sex-determination in persimmon is that the Y-linked *OGI* gene opposes *MeGI's* male-suppressing action. This form of gene action that could act in the heterozygous state, and should increase male functions, and the processes in the two sexes may indeed conflict, as proposed for the female suppressor in the two-gene model outlined in Figure 2A above. It is currently unclear how females evolved. The *MeGI* male suppressing factor is autosomal, and is therefore unlikely to represent the male-sterility gene in the two-gene model. *OGI* could therefore be an example of a single-locus sex-determining gene that evolved by a take-over event, if searches fail to find a femaleness factor.

Although, as mentioned already, reversals and re-evolution of dioecy can complicate comparative studies and hinder inferences of the ages of the origins of

dioecy, they may also be very helpful in revealing the genetic basis of dioecy (Westergaard, 1958), and molecular studies of such hermaphrodites could help identify plant sex-determining genes. In papaya and *Vitis*, hermaphrodites are commercially successful crop plants. The Y chromosomes in these hermaphrodites do not suppress female functions, but their sequences are very similar to those of males (Picq *et al.*, 2014; Van Buren *et al.*, 2015), and they probably have no large deletions, making them ideal for identifying the gene whose loss causes reversion to hermaphroditism, a good candidate for the female suppressor involved in the evolution of dioecy.

The hypothesised X-linked genes responsible for the male sterility of females in dioecious plant species are likely to be even harder to identify, but this may be possible in systems where suppressed recombination has not yet evolved. If two incompletely linked sex determining genes exist, hermaphrodite recombinants, and recombinants with the male sterility allele of females and the female suppressor of males, should arise. With the modern ability to identify the region, and genotype closely linked markers, as in *Fragaria* species (Tennessen *et al.*, 2013), it should be possible to check that these phenotypes are indeed associated with recombination, and to pinpoint both genes.

Once the genes are identified in some plant species, this will open the way for testing whether the same genes are sex-linked in other dioecious plants. Given that large numbers of genes affect flower and inflorescence development, different genes may be involved in different angiosperm lineages, rather than the same genes being repeatedly involved. If so, plants will differ from major animal groups such as insects, which share sex-determination pathways across major taxa (Saccone *et al.*, 2002; Beye *et al.*, 2003; Pomiankowski *et al.*, 2004; Pane *et al.*, 2005). In plants, sterility factors may have to be identified, and their actions investigated, in individual genera and species. Moreover, it should not be assumed that the sex-determining genes necessarily function during flower development, or cause sterility. In monoecious plants, a state that is ancestral to many dioecious species (Renner & Ricklefs, 1995; Renner & Won, 2001), they might instead control the proportions of male and female flowers, perhaps at developmental stages before flower parts are initiated (Figure 2B). Unisexuality may be much more ancient than dioecy, and early, complete abortion of male or female parts may be ancestral.

Conclusions

It is now technically feasible to use young sex chromosomes in non-model plants to test hypotheses about the initial evolution of suppressed recombination, and to study the time course of later evolution of sex chromosomes in older systems, as has been initiated in some animal systems (Bachtrog *et al.*, 2009). Young plant sex-linked systems should also be suitable for studying the earliest adaptations to dioecy, which have so far been little studied. The change from cosexuality to unisexuality may be accompanied by considerable expression changes, if unisexuals are released from conflicts between the two sex functions, so that changes can occur to optimise expression in each sex. For example, the non-dioecious *S. vulgaris* appears suitable as an outgroup for studying the evolution of changes in expression in the dioecious species *S. latifolia* (Marais *et al.*, 2011). Because hermaphrodite *S. vulgaris* individuals have both stamens and pistils, differences in unisexual individuals of the dioecious species that are caused directly by loss of these structures should be distinguishable from changes in expression of genes that are expressed in non-sex-specific structures. Such studies can potentially discover genes that can be expressed in both sexes, but that change when dioecy evolves, and evaluate whether, as has been predicted, the sex chromosomes, including the PAR, carry unexpectedly large numbers of such genes (Vicoso & Charlesworth, 2006; Vicoso *et al.*, 2013).

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Figure legends

Figure 1. Possible histories of sex-linked regions. A. Sex-determining genome region with no prior history of suppressed recombination, in which recombination suppression evolves in response to a polymorphism at two sex-determining loci. B. Pericentromeric region with suppressed recombination, in which sex-determining loci evolve. The sex-linked region may later become extended by evolution of a further region of suppressed recombination, perhaps due to the establishment of a sexually antagonistic polymorphism on the same chromosome (see text). C. A take-over event in which a new male-determining gene arises in a genome region not previously involved in sex-determination; the figure illustrates the case when the new sex-determining gene is on a chromosome (indicated by a dashed line) different from the previously existing one, but a changed location on the same chromosome is also possible.

Figure 2. Evolution of sex-determining and sex-linked genome regions. A. Evolution of sex-determining genes in a genome region starting from an hermaphrodite ancestor, and of suppressed recombination in the region, forming a sex chromosome-like region, showing disadvantageous recombinants between the proto-Y and the proto-X chromosomes (the reciprocal recombinant would be hermaphroditic, and is not shown). If the female suppressor has male-specific expression (or evolves expression restricted to males), it can spread through the entire population, and create a single-gene sex-determining system (bottom left). B. Evolution of sex-determining genes in a monoecious ancestor.

Figure 3. Loss of genes from sex-linked regions. A. Chromosome before gene loss. The region that will evolve sex linkage includes four genes essential for vegetative functions in both sexes (green), and other genes essential only in males (blue) or females (pink), or affecting both sex functions (both colours). B. Loss of female function genes from a Y chromosome in a diploid plant, and loss of the third gene essential vegetative functions

(which is non-lethal due to the presence of the copy on the X chromosome). C. Loss of female and male function genes from male- and female-determining chromosomes in a haploid plant. Some genes with functions in the diploid vegetative stage could also be lost from either the U or the V.

Table 1. Some characteristics favourable for studying the genetics and evolution of plant sex chromosomes. The main text provides examples from plant studies.

Characteristic	Advantages	Specific evolutionary questions
A range of sex chromosome ages exist, including recently evolved ones and old-established systems	The time when recombination stopped can be estimated using a molecular clock, as it is often not long, and sequence differences will not be saturated, but will reflect times when recombination was suppressed	1. Which species without cytologically visible heteromorphism have sex-linked regions that include genes other than the sex-determining genes?
		2. Does recombination suppression always evolve, even in old-established systems, or does it sometimes fail to evolve (and, if so, why)?
	The earliest stages of sex chromosome evolution can be studied	1. Is there a tendency for chromosomal heteromorphism, heterochromatinisation, ZW systems, and X-autosome balance systems to be associated with older-established systems?
		2. How did recombination suppression evolve (gradually, or in distinct recombination suppression events affecting genome regions with many genes), and how often do such events happen?
		3. Did repetitive sequences accumulate before genes started to lose functions, or does their accumulation contribute to loss of functions?
Closely related non-dioecious outgroup species often exist	The directions of changes during sex chromosome evolution can be studied	1. Have plant X and/or Y chromosomes adapted to the new dioecious state?
		2. Have plant Y chromosomes degenerated genetically? If so, what is the time course?
Dioecy evolved repeatedly	The phylogenetic context is often available, so that he directions of changes during sex chromosome evolution can be studied	In taxa with many dioecious species, is dioecy ancestral or has it evolved several times; if the latter, are the same sex-determining genes involved, or different genes in different species?

Figure 1

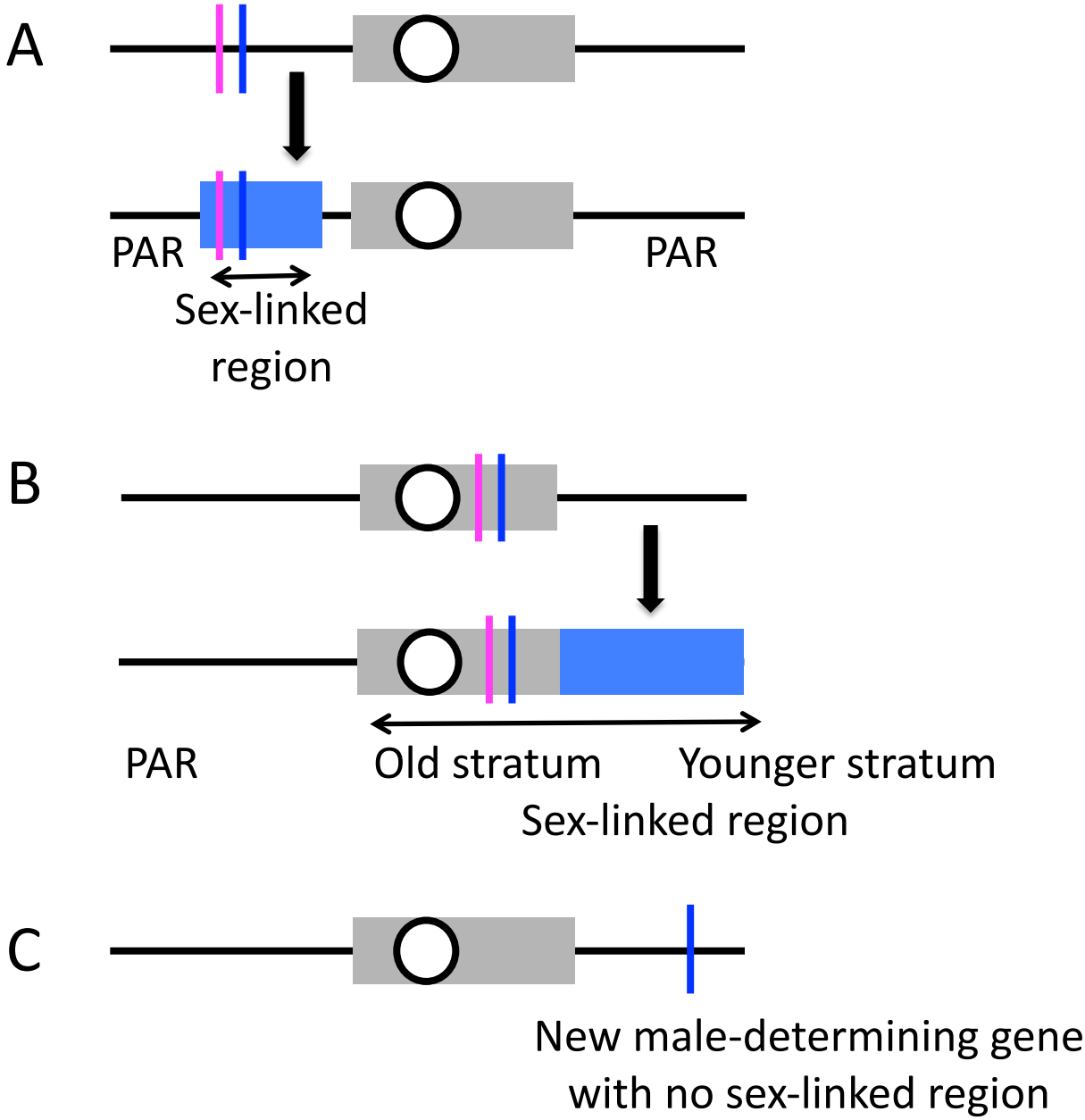

A
PAR
PAR
Sex-linked region
B
PAR
Old stratum
Younger stratum
Sex-linked region
C
New male-determining gene with no sex-linked region
KEY
Centromere
Sex determining genes
Regions with suppressed recombination
Pericentromeric region
Male-specific region

Figure 2

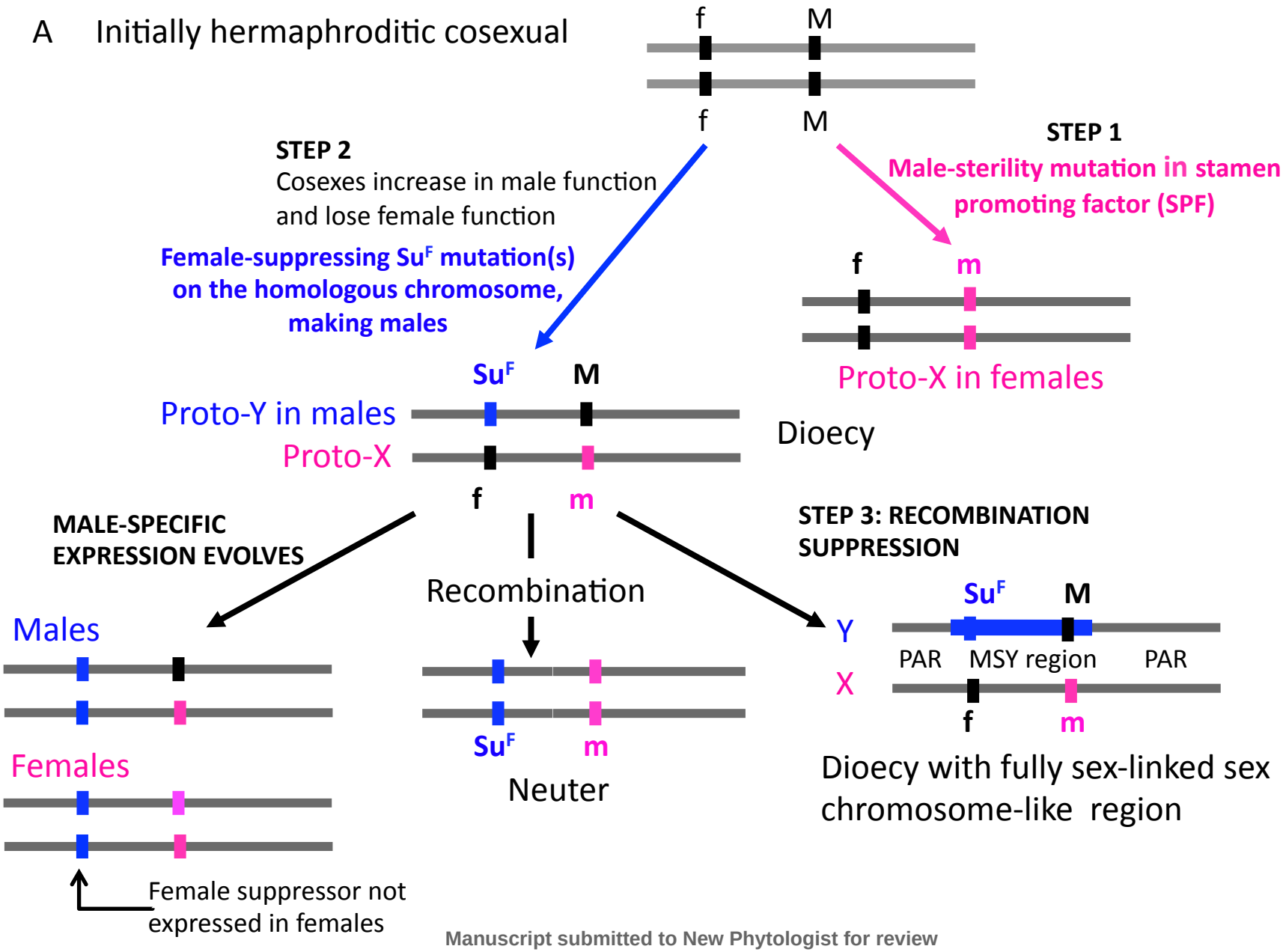

A Initially hermaphroditic cosexual
f M
f M
STEP 1
Male-sterility mutation in stamen promoting factor (SPF)
f m
Proto-X in females
STEP 2
Cosexes increase in male function and lose female function
Female-suppressing SuF mutation(s) on the homologous chromosome, making males
SuF M
Proto-Y in males
Proto-X
f m
Dioecy
MALE-SPECIFIC EXPRESSION EVOLVES
Recombination
STEP 3: RECOMBINATION SUPPRESSION
Males
Females
SuF m
Neuter
Y
X
SuF M
PAR MSY region PAR
f m
Dioecy with fully sex-linked sex chromosome-like region
Female suppressor not expressed in females

Figure 2, continued

B Initially monoecious cosexual

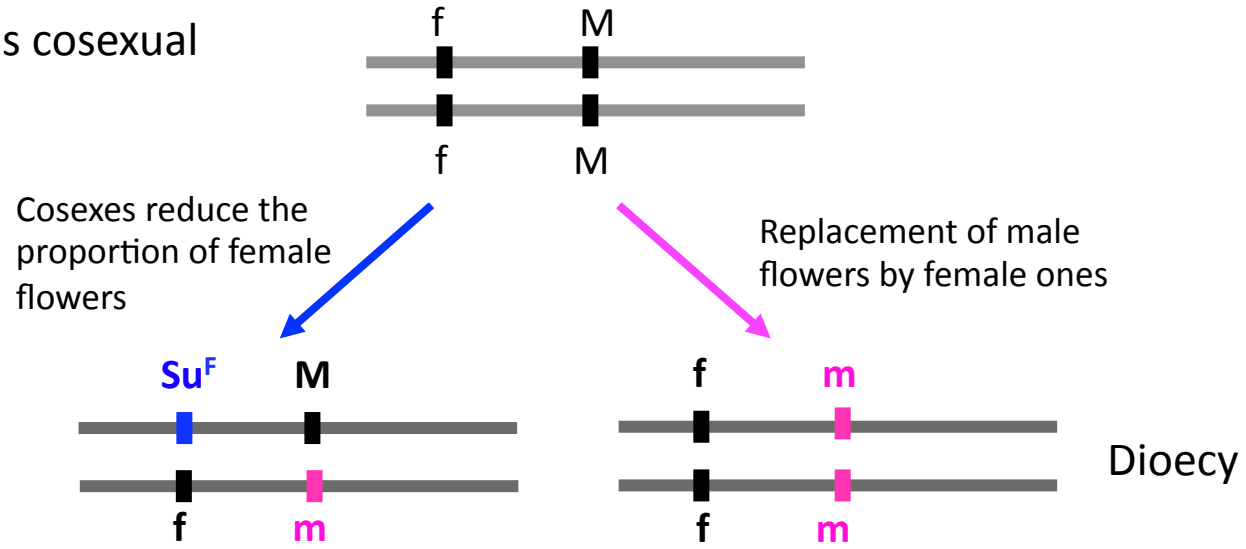
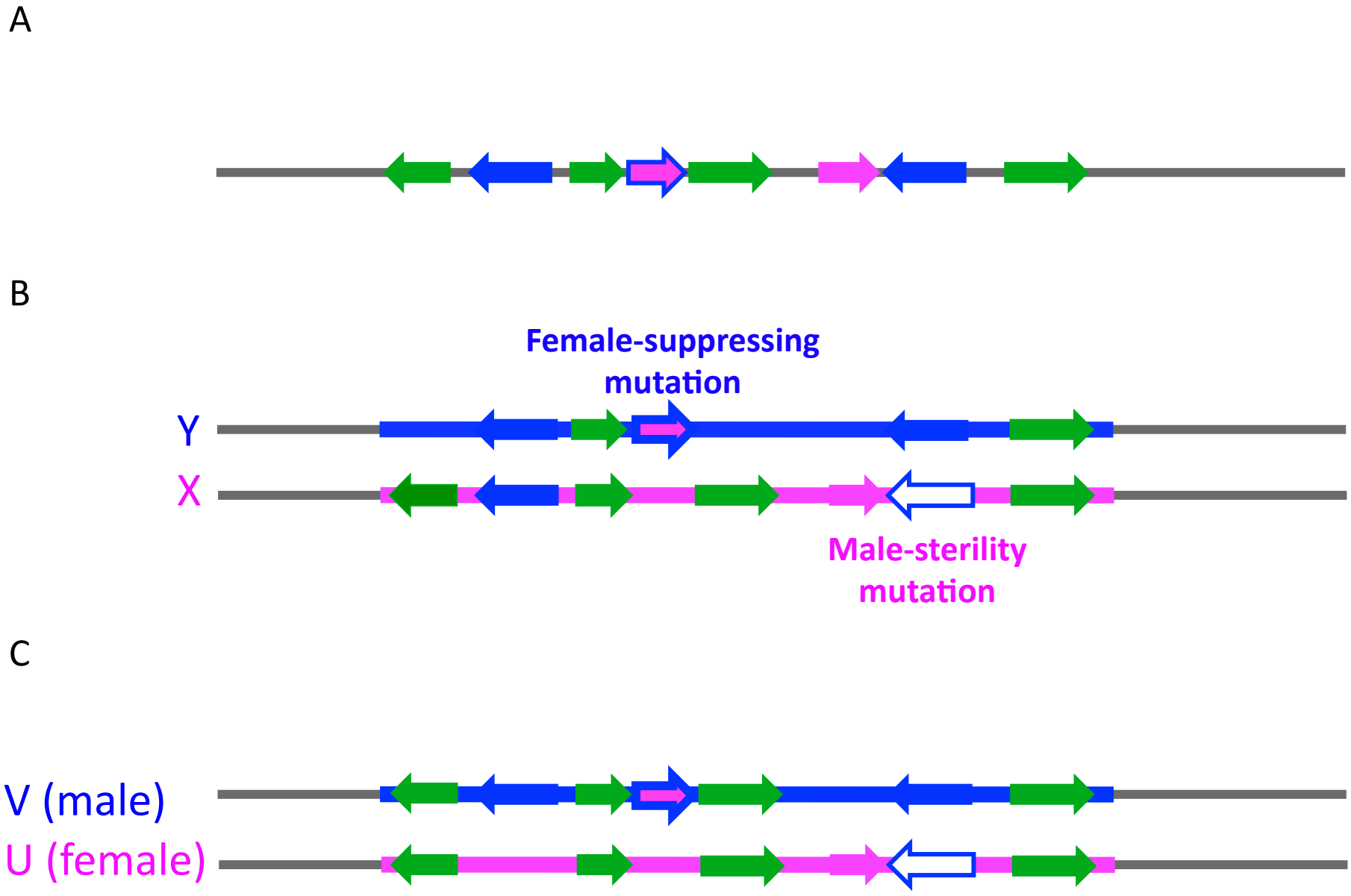


Figure 3


A
B
Female-suppressing mutation
Y
X
Male-sterility mutation
C
V (male)
U (female)