

Opinion

Dioecy in Flowering Plants: From the First Observations of Prospero Alpini in the XVI Century to the Most Recent Advances in the Genomics Era

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Abstract: Prospero Alpini was an Italian physician, botanist and scientist. Born in Marostica, in the Republic of Venice, in his youth he served in the Milanese army, but in 1574 he decided to study medicine at the University of Padova, where he graduated in 1578. After a short period as a doctor in Camposampiero (Padova, Italy), he became the personal doctor of Giorgio Emo, the appointed consul in Cairo in Egypt. In this way, he was able to devote himself to the study of botany. In this country, from the cultivation practices of the date palm, he described for the first time the sexual dimorphism in plants, later adopted as the basis of Linnaeus' scientific classification system. Since then, this behavior, termed dioecy, has been described in other plant species, and many advances have been made in understanding the molecular mechanisms underlying this phenomenon, especially with the advent of genomics. Starting from a brief description of Prospero's life and his pioneering scientific contribution, we illustrated the two main models explaining dioecism. This was achieved by taking a cue from two plant species, grapevine and poplar, in which genomics and single molecule sequencing technologies played a pivotal role in scientific advance in this field.

Keywords: *Phoenix dactylifera*; *Vitis* spp.; *Populus* spp.; sex-determining region; hermaphroditism



Citation: Vannozzi, A.; Palumbo, F.; Lucchin, M.; Barcaccia, G. Dioecy in Flowering Plants: From the First Observations of Prospero Alpini in the XVI Century to the Most Recent Advances in the Genomics Era. *Agriculture* **2022**, *12*, 364. <https://doi.org/10.3390/agriculture12030364>

Academic Editor: Aimin Zhang

Received: 8 February 2022

Accepted: 2 March 2022

Published: 3 March 2022

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1. Prospero Alpini and the History of Dioecy

Prospero Alpini, also known as Prosper Alpinus, Prospero Alpino or Prosper Alpin, was born in Marostica (Vicenza, Italy) on 23 November 1553. Son of the doctor Francesco Alpini and Bartolomea Tarsia of Padua, he was addressed in his youth to a military career, which after a short time he left to enroll at the University of Philosophers and Doctors in Padua, where he graduated in 1578 in medicine and began to practice the profession in Camposampiero (Padua, Italy). On 21 September 1580, he left for Cairo at the behest of the Venetian patrician Giorgio Emo, who had been sent by the Venetian Republic as a consul in Egypt. Alpini stayed in Egypt for about three years, and in November 1584 he returned to Venice, where the prince of Melfi Giovanni Andrea Doria hired him as his personal doctor and took him to Genoa. In 1590, Alpini found himself in Venice again and his botanical works allowed him to receive the professorship "Ad lecturam simplicium" at the University of Padua in 1594. A few years later, in 1603, he was appointed prefect of the botanical garden. Despite suffering from serious illnesses, he remained dedicated to all his assignments until his death on November 23, 1616. His works deal with his two main passions, medicine and botany. After his trip to Egypt, Alpini became passionate about this branch of biology, so much so that he wrote several texts relating to the native Egyptian flora and its uses in the medical field. In 1592, he published the work *De plantis Aegypti liber*, structured in a dialogical form with the imaginary interlocutor Melchiorre Guilandino, the first professor of botany at the University of Padua (1567). In *De plantis Aegypti*, together

with a detailed description of Egyptian plants and their use in the medical field, we can also read the observations of Alpini regarding the fertilization of some species: in chapter VII he describes how the Egyptians promoted the assisted fertilization of the date palm (*Phoenix dactylifera*) to produce fruit of better size (Figure 1). The Egyptians in fact used to link the male flowers of this dioecious species to the female ones, and the close contact between the two flowers of opposite sex favored a better fertilization. In this sense, Prospero Alpini can be considered the first scientist to write about assisted cross fertilization between dioecious species. Below is a translation of some parts of the text “*De Plantis Aegypti*”.

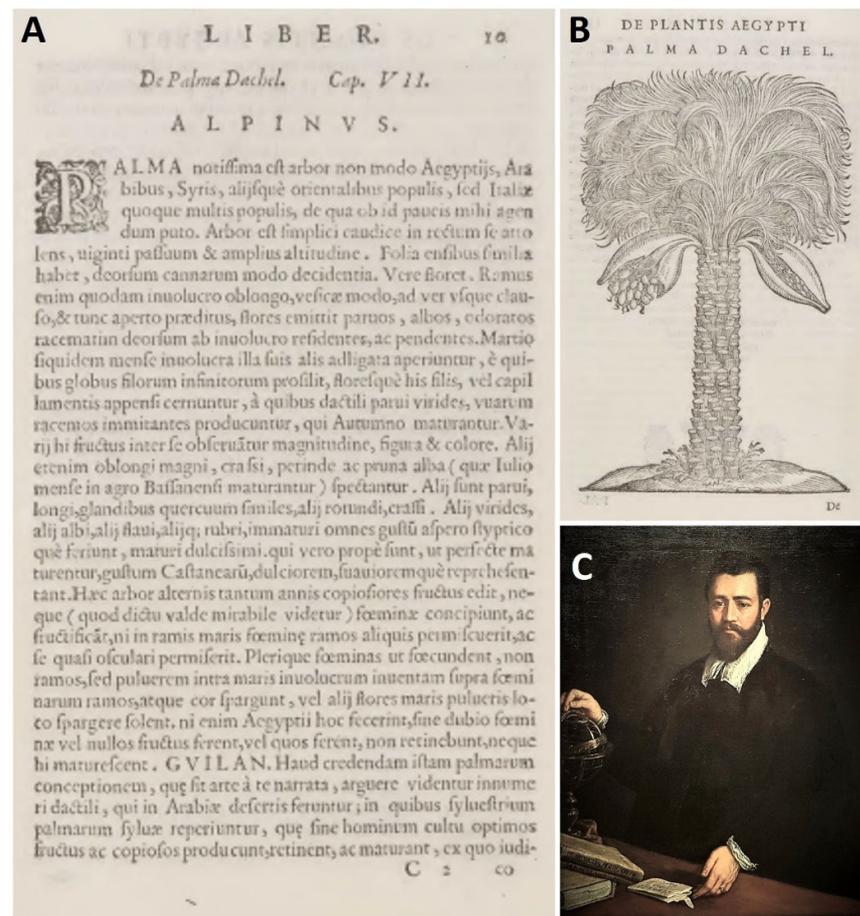


Figure 1. (A) A picture of the first page of Chapter VII of “*De Plantis Aegypti liber*”. Reprinted from ref. [1]; (B) Graphic representation of the date palm as reported by Prospero Alpini. Reprinted from ref. [1]; (C) Portrait of Prospero Alpini from 1586, by Leandro Bassano, Staatsgalerie, Stuttgart.

[...] *Hæc arbor alternis tantum annis copiosiores fructus edit, neque (quod dictu valde mirabile videtur) foeminae concipiunt, ac fructificat, ni in ramis maris foemine ramos aliquis permiscuerit, ac se quasi osculari permiserit. Plerique foeminas ut foecudent, non ramos, sed pulverem intra maris inuolucrum inuentam supra foeminarum ramos, atque cor spargunt, vel alij flores maris pulueris loco spargere solent. ni enim Aegyptii hoc fecerint, sine dubio foeminae vel nullos fructus ferent, vel quos ferent, non retinebunt, neque hi maturescent.* [...] GVLAN. Haud credendam istam palmarum conceptionem, quæ sit arte à te narrata, arguere videntur innumeri dactili, qui in Arabiae desertis feruntur; in quibus syluestrium palmarum sylvarum reperiuntur, quæ sine hominum cultu optimos fructus ac copiosos produunt, retinent, ac maturant, ex quo iudi-

[...] *This tree produces abundant fruit only in alternate years, and (which seems to be very admirable) females conceive and bear fruit only if someone has grafted the branches of the female into the branches of the male and allowed them to kiss. Some do not use the branches to fertilize the females, but the dust found on them, while others usually scatter the flowers of the male instead of the dust. In fact, if in fact the Egyptians did not do this, the females either do not produce any fruit, or those who produce will not keep them and these will not reach maturity.* [...]

2. Dioecy in Angiosperms

According to the World Flora Online (WFO), a compendium of the world's plant species, around 350,000 plant species have been so far cataloged [2]. Among them, several taxa evolved by organizing their reproductive organs (male and female) on distinct individuals. This sexual system takes the name of dioecy if the tissues producing female and male gametes (archegonia and antheridia, respectively) are carried by separate gametophytes (i.e., in the case of bryophyte); the term "dioecy" is instead used if the two reproductive organs are produced on separate sporophytes (i.e., seed plants, including angiosperms and gymnosperms) [3].

The fraction of dioicous/dioecious species is extremely variable. According to the estimates provided by Villarreal and Renner [4], non-vascular land plants (i.e., bryophytes) seem to be characterized by percentages of dioicous species ranging from 40% (Anthocerotophyta, e.g., hornworts) to 68% (Bryophyta, e.g., mosses). Dioecy seems to even be predominant in gymnosperms for which Walas and colleagues estimated 65% of species carrying male and female flowers separately [5]. By contrast, this sexual system is relatively uncommon in angiosperms; a recent review estimated that 15,600 dioecious plants were well distributed in 43% of angiosperm families but represented only the 5–6% of the angiosperms species [6].

The rare occurrence of dioecy in flowering plants is considered the consequence of two main disadvantages: the lack of reproductive assurance of individuals and the fact that virtually only half of the population (female plants) can carry the seeds and produce offspring [7]. This led to the hypothesis that this behavior could be considered an evolutionary dead end of angiosperms with higher probabilities of extinction and lower diversification rates [8]. However, in the last few years some studies started to overturn such hypotheses [7]. The dispute remains open also because it is still unknown whether the ancestral angiosperm had unisexual or bisexual flowers. According to the most accredited hypothesis, all angiosperms (including dioecious) are likely to derive from a hermaphrodite or monoecious ancestor [9,10], as would confirm *Amborella trichopoda*, the living sister lineage to all remaining flowering plants, which is dioecious but with stamen residues in the female flowers [11].

Assuming therefore that dioecy does not represent the ancestral condition of angiosperms, it can be stated, with a certain safety margin, that this sexual system has originated independently multiple times in different families, similarly to other sexual behaviors (e.g., apomixis) [12]. In support of this is not only the fact that dioecy and monoecy coexist within hundreds of families but also that, within the same family, there are simultaneously cases of male and female heterogamy. Heterogametic sex or digametic sex is the condition in which sexual chromosomes are different [13]. We distinguish between male heterogamy (XX/XY)—where male individuals carry different sex chromosomes (XY)—and female heterogamy (ZW/ZZ). Several genera including *Populus* and *Salix* (Salicaceae) [14,15], *Silene* (Caryophyllaceae) [16] and *Dioscorea* (Dioscoreaceae) [17] present both species with XX/XY and ZW/ZZ sexual systems, suggesting the concrete possibility that shifts between different sex chromosome systems ($XY \leftrightarrow ZW$) are feasible not only within the animal kingdom—where multiple cases have been reported in several phyla [18,19]—but also in plants [16,17].

3. Models for Sex Determination in Angiosperms

Studies aimed at investigating the genetic determinants behind the angiosperms dioecy have been greatly facilitated by the recent advances in molecular techniques. Based on this and depending on whether the ancestor was hermaphroditic (perfect flowers in each plant) or monoecious (coexistence of male and female flowers in each plant), different theories have been suggested to explain the origin of dioecy. According to the original hypothesis proposed by Charlesworth and Charlesworth in the late 1970's, to switch from a hermaphrodite to a dioecious state, two distinct mutations affecting male and female gametes production are required [20]. A first recessive mutation affects pollen development and

leads to an intermediate condition of gynodioecy, which is the coexistence of female (i.e., male-sterile individuals) and hermaphrodite individuals. A second dominant mutation affects ovule production in hermaphrodite plants leading to male plants (i.e., female-sterile individuals). The opposite situation (based on an intermediate condition of androdioecy) is possible too, although there is a striking rarity of androdioecy relative to gynodioecy [21]. Clearly, for the correct functioning of a two-gene model (TGM)-based dioecism, the species must set a mechanism capable of preventing recombination between the two independently mutated genes, avoiding the generation of hermaphrodites and asexual individuals. TGM still seems to fit for some species, including kiwis [22], grapevines [23] and the date palm itself [24], the object of the first observations on dioecy by Prospero Alpini.

For other plant taxa, including poplar [25], persimmon [22] and spinach [26], a single gene model (SGM) seems to explain better the switch from hermaphroditism to dioecy. In XX/XY systems, it was shown that a Y chromosome-carried gene can negatively regulate one or more genes triggering female development. On the opposite side, in WZ/ZZ systems-based organisms, it is more likely that a gene carried by the W chromosome determines the female development.

However, Golenberg and West argued that neither SGM nor TGM are able to explain effectively a switch to a dioecious state starting from a gyno/andromonoecious condition (i.e., a single plant carries both hermaphrodite and female or male flowers) or from a unisexual flowers-based monoecious condition. It is in fact unlikely to observe different genotype combinations (e.g., *Aabb* and *aaBb*) within individual floral meristems of the same plant [27]. In this case, it is more likely that either external environmental or internal physiological factors (i.e., hormones) directly or indirectly regulate the expression of one or more genes involved in sex determination. This behavior is also known as sex plasticity and the main masculinizing and feminizing effects produced by environmental stresses or hormonal treatments and have been brilliantly reviewed by Golenberg and West [27].

4. Plant Species Supporting Models

The advent of second and third generation sequencing technologies has made it possible to take giant steps in understanding the molecular and genetic mechanisms underlying the determination of sex in numerous plant species. In fact, the heterogamy that characterizes many sexual systems in dioecious plants requires, as a fundamental prerequisite, the ability to resolve diploid phases in heterozygous genotypes, in order to determine the genomic variants in the male and female SDRs. In the following chapters, we focus on some examples of plant species for which, thanks to advances in genomics, it was possible to finely characterize the SDRs and define the model at the basis of dioecy: *Vitis* spp. for the two-gene system and *Populus* spp. for the one-gene system.

4.1. The Two-Gene Model in the *Vitis* Genus

Vitis, with specific reference to the *vinifera* species, represent a particularly fascinating case as regards the evolution of the mating system, since a first event of switch from hermaphrodite progenitor to dioecious plant was followed, through domestication, by a second event of “reversion” to the hermaphrodite state.

The *Vitis* genus is divided into two subgenera: *Muscadinia*, with chromosomal number $2n = 40$, represented by a limited number of species, and *Euvoitis*, with chromosomal number $2n = 38$, divided into at least 28 species of wild grapevine in North America, 30 in Asia and 1 in Europe, *V. vinifera* L. ssp. *sylvestris*, the wild ancestor of all domesticated species [28]. All wild *Vitis* species are dioecious, showing separate male and female individuals, with known genetic dominance of the male allele (M) over the hermaphroditic one (H) which in turn is dominant over the female one (f). Conversely, *Vitis vinifera* ssp. *vinifera* has been selected, during domestication, in order to develop hermaphroditic flowers [29,30]. This transition of the mating system probably occurred ~8000 years ago [31], following a rare recombination event between male (M) and female (F) haplotypes firstly hypothesized by Picq et al., and Henry et al. [32,33] and recently demonstrated by Zou et al. [34]. The

evolution of perfect flowers as a fundamental trait in the history of *V. vinifera* domestication is related to the higher self-pollination efficiency of hermaphrodite plants with respect to monoicous ones, which in turn guarantees reliable and stable yields.

Therefore, within the genus *Vitis* there are three types of individuals: males with flowers with reduced pistils, devoid of stigma and style, females with flowers containing reflected anthers and stamens that produce sterile pollen, and hermaphrodites, within the *V. vinifera* clade, which have perfect flowers with both functioning sexual organs.

The study of genetic determinants of sex in grapevine represents an emblematic example of how genomics and the evolution of increasingly fine technological and bioinformatic approaches in Next Generation Sequencing, led to the identification of genes involved in the determination of sex and to the constitution of a model explaining the evolution of dioecy and the reversion to hermaphroditism in cultivated varieties.

Amongst the major limitations in the study of sex determination in the *Vitis* genus, is the fact that, until recently, there was a lack of whole genomic data for wild and cultivated grapevine, and the *V. vinifera* PN40024 reference genome represented only a partially assembled f haplotype since it was obtained from a highly homozygous hermaphrodite line, providing no insights into the structural variants (SVs) that distinguish heterozygous chromosomes [35]. The sex-determining region (SDR) in grapes had already been identified in multiple genetic and genomic studies [30,32,34,36,37] in the first decade of 2000, and was described as a nonrecombinant region of chromosome 2 spanning approximately 150 kb and containing between 15 and 20 genes [30,32].

The recent advent of third generation sequencing technologies such as single-molecule real-time sequencing (SMRT), their use in hybrid assemblies for de novo genomes to combine short-read sequence data with long-read sequence data, and the consequent availability of an increasing number of phased diploid genomes of H, M and f genotypes, made it possible to finely analyze the SDR region in different grapevine species taking important steps forward in the comprehension of mechanisms underlining sex determination in grapevine [23,34,38]. Recently, Massonnet et al. [23] analyzed this region comparing 11 wild and cultivated grapevine genomes, revealing structural differences between the M, H and f haplotypes, and suggested candidate genes for both male and female sterility. The most obvious difference between haplotypes was in the length of the nonrecombinant SDR region amongst sexes: all f haplotypes analyzed (12 in total) had a much shorter average length (181.4 ± 10.2 kbp) than M (425.9 ± 274.6 kbp) and H (289.2 ± 7.4 kbp) ones, reflecting the presence of several sex-linked structural variants (SVs). By using sequence alignments to the Cabernet Sauvignon H haplotype, the authors identified SNPs that associate perfectly with sex among *Vitis* spp [23]. In particular, an 8 bp deletion of the gene *VviINP1* (*INAPERTURATE POLLEN1*) causing a premature STOP codon was proposed as the potential male sterility mutation in *Vitis* spp. Conversely, M-linked polymorphisms together with expression evidence, suggested the gene *VviYABBY3*, encoding for a transcriptional regulator, is the female sterility candidate. Following a similar approach, [39] Badouin et al., examined the SDR using a comparative genome approach in *V. sylvestris* and proposed a different candidate for female sterility: *VviAPT3*, a gene involved in the cytokinin pathway. Although functional characterization for the role of both *VvYABBY3* and *VviAPT3* are still lacking in grapevine, a support for the role of *VvYABBY3* as the candidate gene for female sterility came from another pivotal study [34].

In this study, by means of Pacific Biosciences (PacBio) long-read assemblies, whole-genome bulked sequencing, population genetics, transcriptomics and machine-learning approaches, the grape sex-determining region (SDR) was analyzed in 12 *Vitis* genomes and its conservation was demonstrated across 556 genotypes (193 accessions from 47 wild grapevine species and 363 accessions of cultivated grapevine). According to the two-gene model of dioecy, the grapevine SDR, is precisely conserved across the entire *Vitis* genus, and it was confirmed as recombination-free in all wild species, as demonstrated in previous studies [23,32,38]. Conversely, distinct hermaphrodite (H) haplotypes (H1 and H2) were found among *vinifera* cultivars, both chimeras of male (M) and female (f) haplotypes.

These two independent recombination events carry different genetic signatures which long predate the domestication of grapevine, suggesting independent evolutions of this trait in wild European grapevine gene pools prior to human domestication [34]. In addition, the allele-specific expression (ASE) analysis including genotypes with either the H1 or H2 haplotypes, the examination of the patterns of SNPs across hundreds of genotypes from all grapevine gene pools and the discovery of the H2 allele, give greater support to *VviINP1* and *VviYABBY3* as candidate genes for male sterility and female sterility of this species (Figure 2).

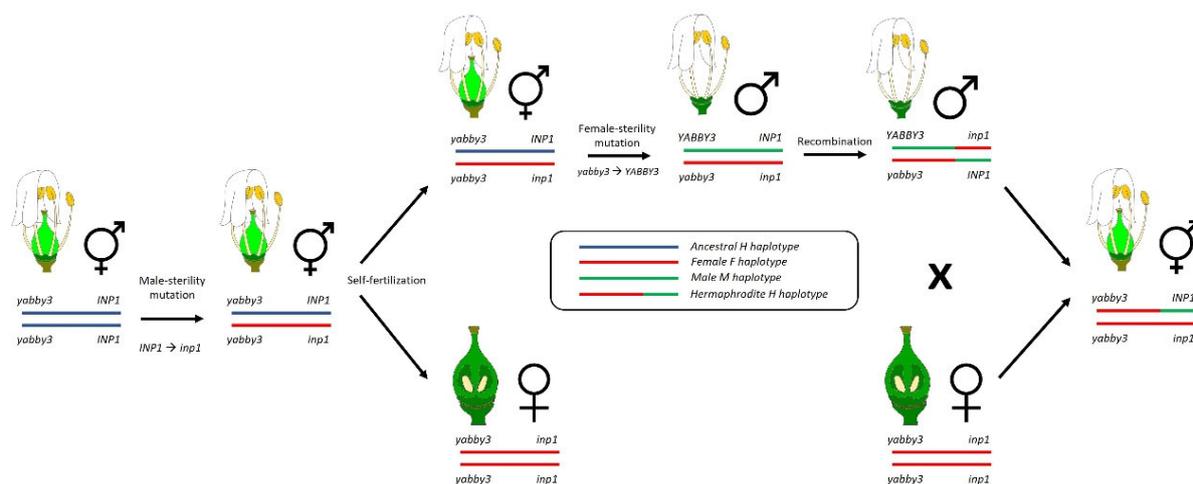


Figure 2. Schematic model of evolution of sex determination in grapevine. From left to right, a hermaphroditic ancestor gave rise to male-sterility mutation at the *INP1* locus to produce gynodioecious individuals. The recessive male-sterility mutation is indicated as *inp1*. Conversely, a dominant female-sterility mutation at the *YABBY3* locus originate male flowers. Based on a recent hypothesis [34], a rare recombination event occurred in a *V. sylvestris* male, leading to H haplotype and hermaphrodite individuals in domesticated *V. vinifera* cultivars. Adapted from ref. [23].

4.2. The One-Gene Model in Poplar Genus

Almost all species belonging to the genus *Populus* are dioic plants, characterized by a male heterogametic (XY) system of sex determination. The only exception is white poplar (*Populus alba*), which presents a ZW system with heterogametic females [40]. In the last 20 years, many studies on sex determination in *Populus* genus were performed and several *Populus* species were reported with a male heterogametic configuration of sex mapped on chromosome 19. Nonetheless, the exact location of the SDR is variable between species: in *P. trichocarpa* and *P. nigra* (sections *Tacamahaca* and *Aigeiros*), it is located in the proximal telomeric end of chr19 [41,42]. Conversely, in *P. tremula*, *P. tremuloides* and *P. alba* (section *Populus*), the SDR in a pericentromeric region of chr19 [40,43,44]. Finally, in *P. euphratica* the SDR regions mapped on the telomeric region of chromosome 14 [45]. Similar to what happened in grapevine, the availability of third generation sequencing technologies enables the production of long reads and thus to solve diploid phases, allowing the genomic determination and comparison of the SD region in *P. trichocarpa*, *P. deltoides*, *P. tremula*, *P. alba*, *P. euphratica* and *P. purpurea* [25,42,45].

Genome-wide association studies (GWAS) together with long read sequencing of the SD region led to the identification of a homolog of the *A. thaliana* gene pair *ARABIDOPSIS RESPONSE REGULATOR 16* (*ARR16/ARR17*), a type A cytokinin response regulator. Amongst those genes located in the SD region *ARR17* represent an excellent candidate for sex determination [41,46]. As a matter of fact, recent studies revealed that genes involved in cytokinin signaling play important roles in the regulation of unisexual flower development in plants [47]. Whereas in all species analyzed the *ARR17* gene on the female SDR is intact, the male-specific region of the Y chromosome carries partial duplicates of *ARR17* arranged as inverted repeats [25]. The observation that (i) CRISPR/Cas9-mediated *arr17* knockout in

early flowering aspen lines, revert females to fully functional males [25] and (ii) long read sequencing and de novo assembly of a female white poplar (ZW) identified three complete copies of *ARR17* in the W chromosome-specific contig and no copies in the Z haplotype, suggests that the *ARR17* genes are more likely to function as a dominant promoter of female function [45]. In contrast, the *ARR* gene fragments on the Y chromosome of *P. euphratica*, *P. trichocarpa*, *P. deltoides* and *P. tremula* may serve as a female suppressor by encoding an siRNA that targets the intact *ARR* gene at the distal end of chromosome 19, possibly by means of RNA-directed DNA methylation (RdDM). Thus, whether in the only female heterogametic species, *P. alba*, sex determination appears to be based on a simple presence/absence of *ARR17*, in all other species it seems to be dependent on the suppression of functional *ARR* gene by means of RNA-directed DNA methylation caused by inverted duplicated *ARR* pseudogenes on the male (Y) SDR haplotype (Figure 3).

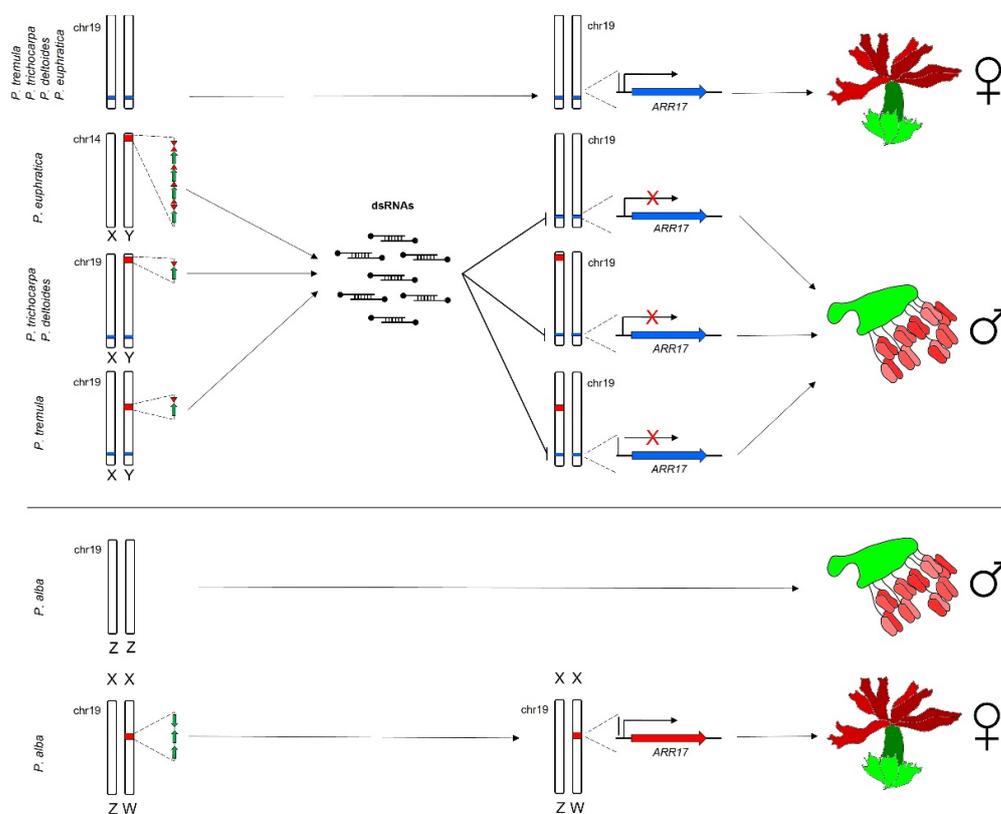


Figure 3. Schematic model of evolution of sex determination in *Populus* genus. In *P. euphratica*, *P. trichocarpa*, *P. deltoides* and *P. tremula*, partial inverted duplicates of the *ARR17* gene on the Y SDR haplotype act as suppressors of female development by an siRNA-mediated silencing of the intact *ARR17* gene located on a different region of chr19. In *P. alba*, several intact *ARR* genes totally absent on the Z chromosome act as a dominant promoter of female function. Adapted from ref. [45].

5. Conclusions

In this brief excursus, which started with a brief tribute to Prospero Alpini, the pioneer of dioecism, we considered two plant species of great agronomic and forestry value, grapevine and poplar, highlighting the contribution that the most recent applications of genomics provided in deciphering this phenomenon. Novel molecular techniques such as third generation sequencing, together with next generation breeding techniques such as CRISPR/Cas9 are speedily becoming accessible, facilitating the identification of SDRs. On the one hand, long-read sequencing technologies such as Pacific Biosciences' (PacBio) single molecule real-time (SMRT) sequencing and Oxford Nanopore Technologies' (ONT) nanopore sequencing allow us to obtain phased, accurate and complete (including base modifications) genomes, *condicio sine qua non* the identification of structural variations (SVs)

within the sex-determining region (SDR) would be hardly feasible. On the other hand, the ability to edit genomes in a targeted and precise manner is crucial for the functional characterization of candidate loci within the SDR and the consequent determination of key genes involved in sex configuration. It is therefore expected that the combination of sequencing and editing techniques, combined with the reduction of their costs and their complexity of application, will likely lead to the accurate determination of the mechanisms underlying dioecy in an increasing number of different plant species, laying the foundations for a unifying model capable of explaining this phenomenon in a transversal way.

Author Contributions: Conceptualization, G.B.; investigation, A.V. and F.P.; writing—original draft preparation, A.V. and F.P.; writing—review and editing, A.V., F.P., G.B. and M.L.; supervision, G.B.; project administration, G.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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