The genetic basis of sex determination in *Populus* provides molecular markers across the genus and indicates convergent evolution

Gihwan Kim[1], Ana P. Leite Montalvão[1], Birgit Kersten, Matthias Fladung, Niels A. Müller*

Thünen Institute of Forest Genetics, Sieker Landstr. 2, 22927 Grosshansdorf, Germany.

[1]These authors contributed equally

*Corresponding author: Niels A. Müller, E-mail: niels.mueller@thuenen.de

Abstract

Many dioecious angiosperms are trees, which only flower after years of vegetative development and do not usually exhibit marked secondary sexual dimorphism. Nevertheless, if the genetic basis of sex determination is known, the sex of an individual can be determined using molecular markers. Here, we report that in the genus *Populus* sect. Populus an XY system of sex determination, which is found in *P. tremula* and *P. tremuloides*, likely re-evolved from a ZW system present in *P. alba, P. adenopoda* and *P. qiongdaoensis*. Strikingly, this new XY system is mechanistically identical to the older system found in several species of the *Populus* sections Tacamahaca, Aigeiros and Turanga demonstrating a remarkable example of convergent evolution. In both XY systems, male-specific inversely repeated sequences appear to silence the *ARR17* gene, which functions as a sex switch, via small interfering RNAs and DNA methylation. In the ZW system, female-specific copies of *ARR17* appear to regulate dioecy. With this detailed information on the genetic basis of sex determination it was possible to develop molecular markers that can be utilized to determine the sex in seedlings and non-flowering trees of different poplar species. We used the female-specific *ARR17* gene to develop a sex marker for *P. alba* and *P. adenopoda*. For *P. grandidentata*, we employed the male-specific *ARR17* inverted repeat. Finally, we summarize previously described markers for P. *tremula*, *P. tremuloides*, *P. trichocarpa*, *P. deltoides* and *P. nigra*. These markers can be useful for poplar ecologists, geneticists and breeders.

Keywords: *Populus, poplar breeding, dioecy, sex-determining region, ARR17, sex chromosome evolution, female and male heterogamety, sex markers, convergent evolution*.

Introduction

Unisexuality in plants can be regulated through different molecular mechanisms and has been used as model to study flower development and plant sex determination. Recent studies in different dioecious plant species, such as kiwifruit (Akagi et al., 2019, 2018), garden asparagus (Harkess et al., 2020, 2017), persimmon (Akagi et al., 2016, 2014) or poplar (Müller et al., 2020), have started to clarify some of the molecular mechanisms of sex determination, improving our knowledge about the evolution of dioecy (Carey et al., 2021; Feng et al., 2020; Leite Montalvão et al., 2021; Renner and Müller, 2021). *Populus*, an economically and ecologically important genus of the Salicaceae family, is composed of dioecious species that are collectively called poplars (Slavov and Zhelev, 2010), however, *P. tremula* L., *P. grandidentata* Michx. and *P. tremuloides* Michx. are often termed aspens. Sex determination in poplars and aspens is genetically controlled by sex-determining regions (SDRs) on different chromosomes and with different heterogamety (Geraldes et al., 2015; Kersten et al., 2014; Müller et al., 2020; Yang et al., 2021) and it was demonstrated that at least in aspens it relies on the expression of a single gene (named *ARR17* for

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consistency with previous reports) that functions as a sex switch (Müller et al., 2020).

Interestingly, transitions between heterogametic systems (XY ↔ ZW) are documented for some genera, including *Populus* (Bhat and Bindroo, 1980; Cormier et al., 2019; He et al., 2021; Martin et al., 2019; Müller et al., 2020; Paolucci et al., 2010; Sanderson et al., 2021; Zhou et al., 2018). The same molecular mechanism of sex determination based on the *ARR17* gene appears to be present in *Populus* species with both, XY and ZW systems. *Populus* species presenting an XY system (Müller et al., 2020; Zhou et al., 2020), e.g., *P. tremula* and *P. tremuloides*, have partial *ARR17*-duplicates arranged as inverted repeats located in the sex-determining region (SDR) of the Y chromosome and, apparently, through small RNAs and DNA methylation, the *ARR17* gene is dominantly silenced in males. In contrast, in the poplar ZW system, e.g., *P. alba* L., sex determination is based on a presence/absence variant of the *ARR17* gene (Müller et al., 2020; Yang et al., 2021).

Revealing the genetic basis of sex determination in different *Populus* species allows to explore the possibilities for sex markers, which can be used to assess sex in trees that are not yet sexually mature or not flowering. For species with long life cycles that exhibit no or minimal sexual dimorphism, such as poplars (McKown et al., 2017; Renner and Müller, 2021; Robinson et al., 2014), molecular markers are important tools for research and breeding programs.

In this work we aimed to (i) establish the W-specific *ARR17* as a female-specific sex marker applicable in poplars with ZW system (*P. alba* and *P. adenopoda* Maxim.), (ii) employ the Y-specific *ARR17* inverted repeat as a male-specific marker in *P. grandidentata*, and (iii) summarize the previously described sex markers in *P. tremula* and *P. tremuloides* (Y-specific *TOZ19*) (Pakull et al., 2015) and *P. trichocarpa* Torr. & A. Gray ex Hook., *P. deltoides* Marshall and *P. nigra* L. (Y-specific *HEMA1* partial duplicate) (Geraldes et al., 2015). The molecular sex markers that we describe here can reliably discriminate males from females in non-flowering trees of different species across the *Populus* genus which may be useful for ecologists, geneticists, and breeders.

Materials and Methods

Re-sequencing data analysis

Raw sequencing reads were downloaded from NCBI's SRA (Bioproject PRJNA612655 – accessions SRR11308211 to SRR11308214 and SRR11308190) (Shang et al., 2020) and NGDC's GSA (Bioproject PRJACA001334 – accessions CRR050666 to CRR050668) (Wang et al., 2020) for P. *adenopoda*. From NCBI's SRA (Bioproject PRJNA612655 – accessions SRR11308208 to SRR11308210) (Shang et al., 2020) and NGDC's GSA (Bioproject PRJACA001334 – accessions CRR050678 to CRR050680) (Wang et al., 2020) for *P. qiongdaoensis*. And from NGDC's GSA (Bioproject PRJACA002485 – accessions CRR176864 to CRR176875 and CRR176894 to CRR176905) for *P. alba* (Yang et al., 2021). Reads were trimmed using Trimmomatic (Bolger et al., 2014) with the following parameters: ILLUMINACLIP: TruSeq3-PE-2.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36, and subsequently mapped to the reference genome of female *P. tremula* (Schiffthaler et al., 2019), with bowtie2 (Langmead and Salzberg, 2012) using default parameters. Read coverage was visualized for the previously described sex-linked region using R ("R Core Team," 2018) with the packages Rsamtools (Morgan et al., 2021), GenomicRanges (Lawrence et al., 2013), zoo (Zeileis and Grothendieck, 2005), ggplot2 (Wickham, 2016) and tidyverse (Wickham et al., 2019).

Sex-linked markers

PCR experiments were performed in 25 µl reactions, except the PCR-RFLP assay, which was done in 10 µl. The PCR control TOZ13-1, previously described by Pakull et al. (2015), was used as PCR positive control in combination with the sex markers ARR17, ARR17_IR or TOZ19-2, to confirm the successful PCR amplification. Primer sequences are given in Table 1. Genomic DNA (gDNA) from *Populus alba* (10 individuals), *P. adenopoda* (5), *P.* × *tomentosa* Carrière (6), *P. grandidentata* (10), *P. tremula* (10), *P. tremuloides* (10), *P. trichocarpa* (9), *P. deltoides* (10) and *P. nigra* (10) was extracted from leaves. For all PCR reactions, 100ng of gDNA, 200 µM of dNTP, primers at 0.24 µM (ARR17, ARR17_IR and TOZ19-2) and 0.16 µM (TOZ13-1) and 0.2 units of Taq polymerase were used with the exception that proofreading DNA polymerase (i.e., Phusion™ High-Fidelity DNA Polymerase) and primers at 0.5 µM were used for the PCR-RFLP assay.

P. alba, P. adenopoda and P. × *tomentosa*

For the *ARR17* marker, which can be used to determine the female-specific presence of the W chromosome, the PCR started with a 3 min denaturation at 95 °C, followed by 30 cycles of 30 sec denaturation at 95 °C, 30 sec annealing at 55 °C, 1 min extension at 72 °C and a final extension of 2 min at 72 °C.

P. grandidentata

For the *ARR17* inverted repeat marker (ARR17_IR), which is based on the Y chromosome-specific *ARR17* inverted repeat sequence of *P. grandidentata*, the following program was used: 3 min denaturation at 94 °C, followed by 38 cycles of 30 sec denaturation at 94 °C, 45 sec annealing at 55 °C, 90 sec extension at 72 °C and a final extension of 10 min at 72 °C.

P. tremula and P. tremuloides

For the previously described *TOZ19* marker (Pakull et al., 2015), the PCR program followed a 3 min denaturation step at 94 °C, 42 cycles of 30 sec denaturation at 94 °C, then annealing at 50 °C for 45 sec, extension for 1 min at 72 °C and a final extension at 72 °C for 10 min.

P. trichocarpa, P. deltoides and P. nigra

The PCR-RFLP assay (Geraldes et al., 2015), here named HEMA1_*Tsp*RI, was performed using the following program: denaturation at 98 °C for 30 sec followed by 35 cycles of 10 sec denaturation at 98 °C, annealing at 58 °C for 30 sec, extension

Table 1
PCR primer sequences designed and used to determine sex in different *Populus* species. The marker TOZ13-1 was used as a PCR control for *P. alba*, *P. adenopoda*, *P.* × *tomentosa*, *P. grandidentata*, *P. tremula* and *P. tremuloides*.

Primer name	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Size of PCR product (bp)	*Populus* species
ARR17	TCAGTGTACATGTCTAATGACAAGC	AGCCCTAGAATTACGCCTCC	826	*P. alba*, *P. adenopoda*, *P.* × *tomentosa* (W chromosome)
ARR17_IR	AGAGAGCATTGGAGTATTTGGG	GTTGAGGTGGTTAGACATTGTGG	400	*P. grandidentata* (Y chromosome)
TOZ19-2	GACGCCATCAAGATTGTGGATTCACCA	GTATCAGGATGGAACATGAGAGTAGTTACG	500	P. *tremula*, *P. tremuloides* (Y chromosome)
HEMA1_*Tsp*RI	TGATCATGGGCATTATAACCAA	TGGACAATGGTCAAACAGTCC	559	*P. trichocarpa*, P. *deltoides*, *P. nigra* (Y chromosome)
TOZ13-1	TTAGGTGCTGATGGTTTGGTAAAGCTA	CTTGCATGTGGATCATCAACTCAAGATCA	260	PCR control (autosome)

at 72 °C for 30 sec and then a final extension for 5 min at 72 °C. Amplicons were digested with the enzyme *Tsp*RI (New England Biolabs, Ipswich, MA), using 5 µL of PCR product, 0.5 µL of *Tsp*RI, 1 µL of buffer and water to reach a final volume of 10 µL. The reaction was incubated at 37 °C overnight. On an agarose gel, females (XX) are expected to present PCR products at 382 bp and 178 bp, while males (XY) additionally have Y-specific fragments at 279 bp and 281 bp.

All PCR products were checked by gel electrophoresis at 100 volts for 60 minutes using a 1 % or 1.5 % agarose gel with 0.1 x TAE as a running buffer. The DNA was stained with RotiSafe from Roth (Karlsruhe, Germany).

Results

P. adenopoda and P. qiongdaoensis appear to feature a ZW system of sex determination

To date, the only described poplar species that exhibits female heterogamety is white poplar (*P. alba*) (Müller et al., 2020; Paolucci et al., 2010; Yang et al., 2021). Sex determination in *P. alba* relies on a presence/absence mutation of the *ARR17* gene. In females (ZW), three complete copies of *ARR17* can be found in the SDR of the W chromosome, while *ARR17* is absent from the Z chromosome. Males (ZZ) thus do not carry any *ARR17* sequence (Müller et al., 2020; Yang et al., 2021).

To test whether the same ZW system could be present in sister species of *P. alba* we downloaded publicly available next generation sequencing (NGS) data of *P. alba, P. adenopoda* and *P. qiongdaoensis* and mapped it to the *P. tremula* v2.2 genome (Schiffthaler et al., 2019), which represents a high-quality reference genome assembly for the section Populus. This allowed us to specifically analyze the region where the master regulator of sex determination, *ARR17*, is located. The normalized read coverage of several *P. alba* samples demonstrated female-specific coverage in the *ARR17* region as reported before (Figure 1a) (Müller et al., 2020). Strikingly, a near identical pattern can be observed in *P. adenopoda* (Figure 1b) suggesting that the origin of the sex-determining systems of *P. adenopoda* and *P. alba* may be shared. Interestingly, the outgroup species of *P. alba* and P. *adenopoda*, *P. qiongdaoensis* also appears to feature a ZW system of sex determination (Figure 1c). Putative males only carry a small part of the *ARR17* gene. Additional female-specific hemizygous regions around the *ARR17* gene are shared with *P. alba* and *P. adenopoda*.

To provide further evidence for the putative female heterogametic system in *P. adenopoda*, we tested a fragment of the *ARR17* gene as a female sex-marker (ARR17), while employing a fragment of the *TOZ13* gene (TOZ13-1) as PCR control (Pakull et al., 2015). All samples are expected to show a PCR product for TOZ13-1 and only females should present a PCR product for ARR17. We tested the marker on individuals of *P. alba* (Figure 2a) and *P. adenopoda* (Figure 2b), and on individuals of the Chinese white poplar *P.* × *tomentosa* (Figure 2b), which is considered a hybrid between *P. alba* and *P. adenopoda* (Wang et al., 2019). While the marker perfectly distinguishes between males and females based on presence/absence of *ARR17* in *P. alba* and *P.* × *tomentosa* (Figure 2), all of the *P. adenopoda* individuals, which were of unknown sex, failed to show an *ARR17* PCR product. Since the genomic region amplified by the ARR17 marker shows no indication of structural variation and no SNPs or indels in the primer binding sites in the *P. adenopoda* resequencing data, our samples are probably all males. Taken together, our results indicate that the ZW systems of sex determination of *P. alba*, *P. adenopoda* and *P. qiongdaoensis* might have a shared origin. Nevertheless, further phylogenetic and synteny analyses should be performed to precisely characterize the SDRs of *P. adenopoda* and *P. qiongdaoensis*. In any case, our results demonstrate that ARR17 can be used as a sex marker in *P. alba*, *P. adenopoda* and their hybrids.

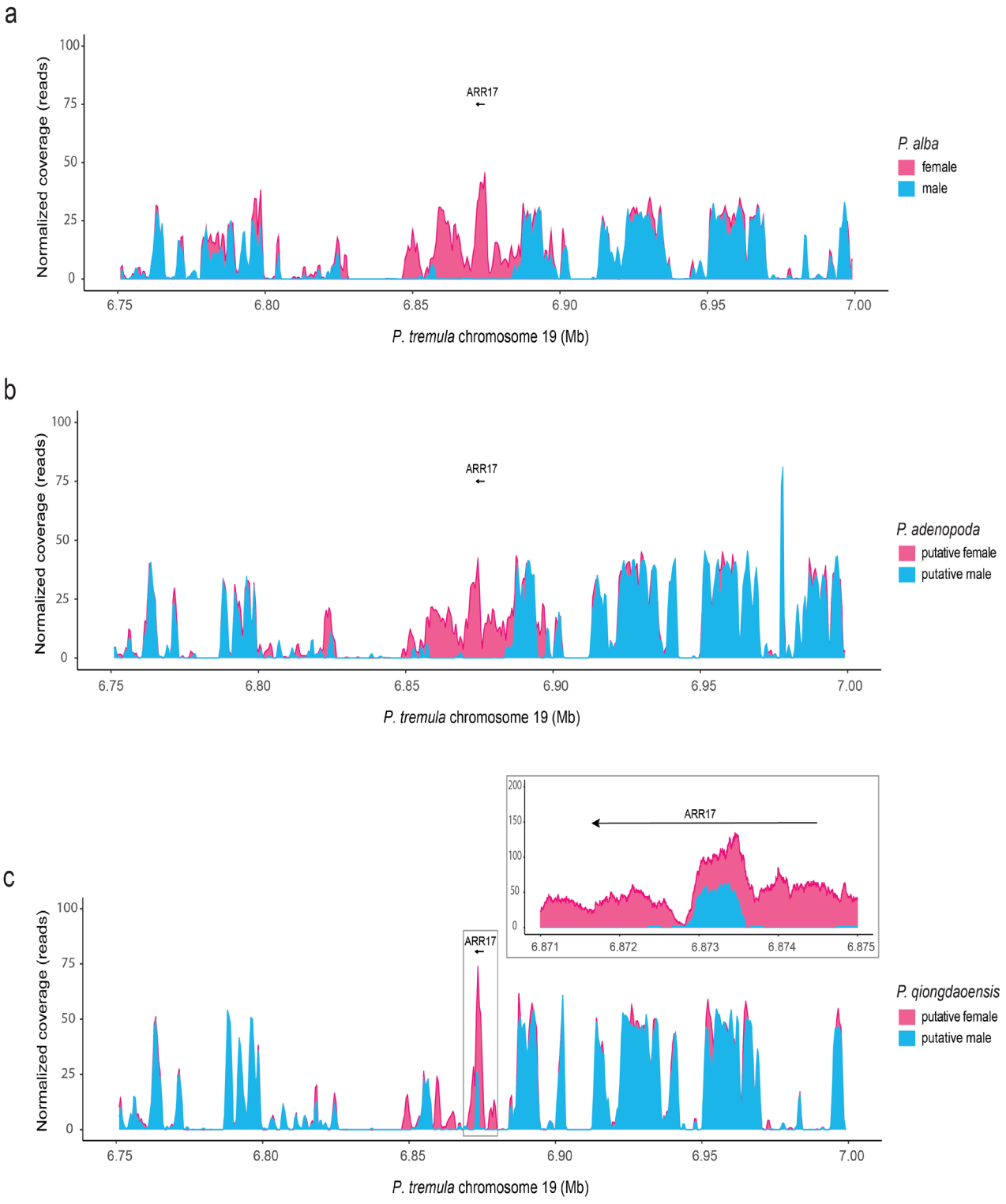

Figure 1
Mean normalized DNA-seq coverage in 1000 bp windows (with a step size of 500 bp) along the *ARR17* genomic region of the *P. tremula* reference genome (Schiffthaler et al., 2019). Sequencing reads were filtered for a mapping quality >10. *ARR17* is represented by a black arrow. (a) White poplar (*P. alba*) (12 female samples and 12 male samples). (b) Chinese aspen (*P. adenopoda*) (3 putative females and 5 putative males, grouped by the presence or absence of *ARR17*). (c) *P. qiongdaoensis* (4 putative female samples and 2 putative male samples, grouped by the presence or absence of *ARR17*). Zoom of *ARR17* region in panel (c) presents the raw sequencing coverage instead of an average in sliding windows.

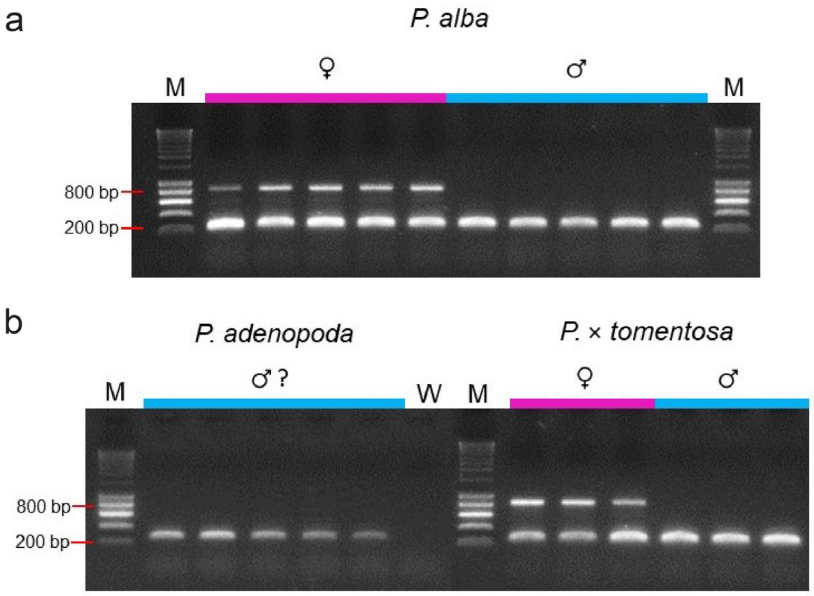

Figure 2
PCR amplifications of the gene *ARR17* in *Populus* species with a ZW system of sex determination. (a) *P. alba*, (b) *P. adenopoda* and *P.* × *tomentosa*. Samples were run on a 1 % agarose gel. The upper PCR product represents ARR17 and the lower PCR product TOZ13-1. M = Smart Ladder (Eurogentech, Cologne, Germany), W = water control. Fragment sizes of the DNA ladder are given on the left side. The *P. adenopoda* were of unknown sex, while the sex of all other individuals is documented.

The Y chromosome-specific ARR17 inverted repeat as a sex marker in P. grandidentata

Few studies have been carried out in the bigtooth aspen *Populus grandidentata*. The described male-specific aspen sex-linked marker TOZ19 (Pakull et al., 2015) did not yield successful PCR amplification in this species. Therefore, to develop an alternative molecular sex marker for *P. grandidentata*, we decided to test the *ARR17* inverted repeat sequences (ARR17_IR), which are located in the male-specific region of the Y chromosome in the aspen species *P. tremula* and *P. tremuloides*. Sanger sequencing of a part of the *P. grandidentata* ortholog of Potri.019G047600, a gene located close to *TOZ19*, identified a SNP (at position 6,721,236 bp of chromosome 19 of the poplar reference genome Potri v3.0 (Tuskan et al., 2006)) that was heterozygous (A/G) in all 19 male samples tested but homozygous (G/G) in all 48 females. These results indicated an XY system of sex determination. Since the male-specific heterozygous variant is located within the aspen SDR based on the aspen reference genome, *P. grandidentata* may share the same genetic basis of sex determination with *P. tremula* and P. *tremuloides*. We therefore tested several *ARR17* primer pairs comprising two forward primers, that can only work on an inversely repeated sequence. One of these primer pairs, here termed ARR17_IR, successfully amplified part of the male-specific *ARR17* inverted repeat sequence in male *P. grandidentata* individuals but did not yield any PCR product in *P. tremula* or *P. tremuloides*. This male-specific amplification can be utilized to discriminate female and male *P. grandidentata* individuals (Figure 3).

Previously described sex markers in different Populus species

The sex of non-flowering aspens can be determined using the male-specific sex-linked gene *TOZ19*, which has been validated in over 80 samples in previous work (Pakull et al., 2015). Through a PCR amplification, sex in *P. tremula* and *P. tremuloides* can be determined. Here we reproduced these results, where all samples presented a control PCR product (TOZ13-1) and only males presented a PCR product for the *TOZ19* sequence (T0Z19-2) (Figure 4).

Another sex-linked marker has been developed for genotyping of a male-specific single nucleotide polymorphisms (SNPs), which is located in a Y-chromosomal partial duplicate of the *HEMA1* gene, in *P. trichocarpa*, *P. deltoides* and *P. nigra* (Geraldes et al., 2015). We validated this PCR-RFLP marker in independent samples (Figure 5). The fragments match to the *in silico* prediction and the overall pattern is completely reproducible.

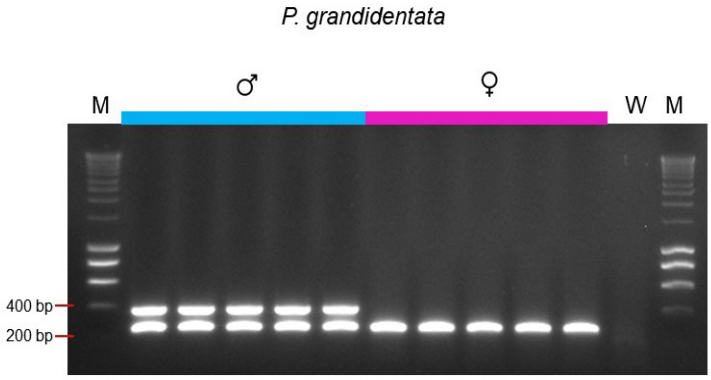

Figure 3
PCR amplification of Y chromosome-specific *ARR17* inverted repeat sequences as a molecular marker to determine sex in *P. grandidentata*. Samples were run on a 1 % agarose gel. The upper PCR product represents the *ARR17* inverted repeat (ARR17_IR) and the lower PCR product the TOZ13-1 control. M = Smart Ladder (Eurogentech, Cologne, Germany), W = water control. Fragment sizes of the DNA ladder are given on the left side.

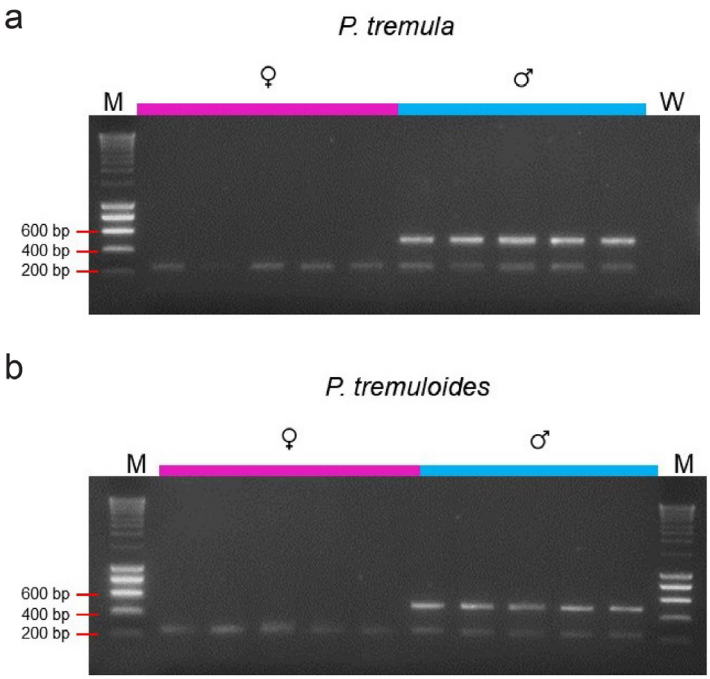

Figure 4
PCR amplification of *TOZ19* in the aspens (a) *P. tremula* and (b) *P. tremuloides*. Samples were run on a 1 % agarose gel. The upper PCR product represents the TOZ19-2 and the lower PCR product the TOZ13-1 control. M = Smart Ladder (Eurogentech, Cologne, Germany), W = water control. Fragment sizes of the DNA ladder are given on the left side.

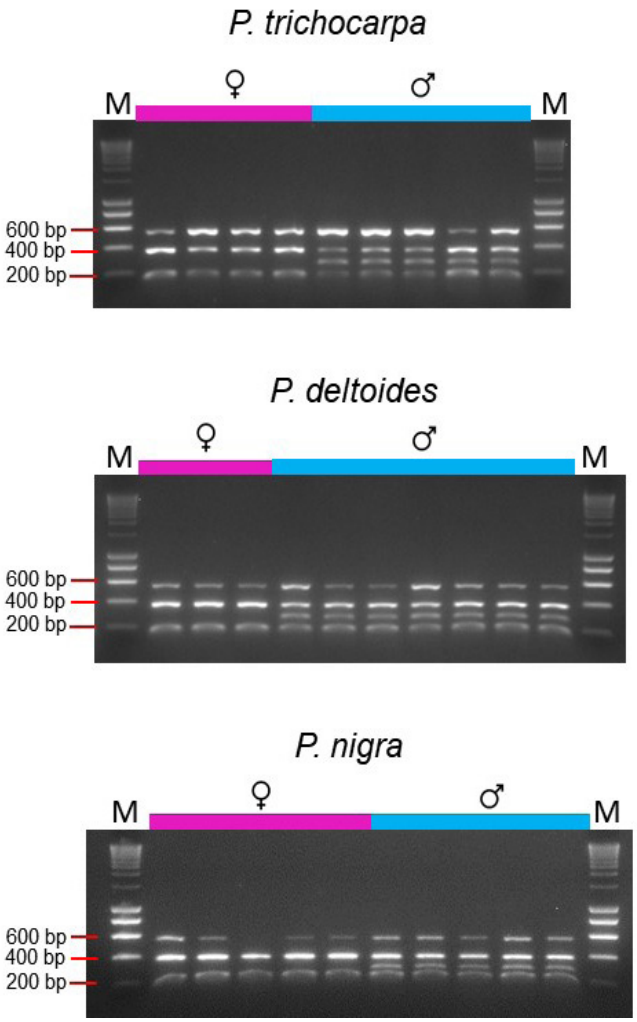

Figure 5
PCR-RFLP assays of (a) *P. trichocarpa*, (b) *P. deltoides* and (c) *P. nigra*. The fragment at 600 bp represents the undigested PCR product (HEMA_*Tsp*RI). Females present PCR products at 400 bp, and 200 bp, while males present Y-specific PCR products at 300bp in addition to the ones at 400 bp and 200 bp. Samples were run on a 1.5 % agarose gel. M = Smart Ladder (Eurogentech, Cologne, Germany). Fragment sizes of the DNA ladder are given on the left side.

Discussion

Tree species can take a long time before producing flowers for the first time. In the genus *Populus*, time to flowering is often more than a decade. To assess the sex of a plant at an earlier stage or outside the flowering season, molecular markers can be employed. Such markers have been described for different dioecious species including several poplar species (Geraldes et al., 2015; Pakull et al., 2015). Here, we used the *ARR17* gene, which functions as a sex switch in poplars, to differentiate female and male individuals in *P. alba*, a species with female heterogamety (ZW system). A nearly identical sex-specific coverage pattern of re-sequencing data of *P. alba* and *P. adenopoda* along the *ARR17* genomic region suggests that these two ZW systems of sex determination may have a shared origin. Additionally, the outgroup species of *P. alba* and *P. adenopoda*, *P. qiongdaoensis* exhibits partially shared female-specific coverage along the *ARR17* locus. While we cannot exclude three independent evolutionary origins without more detailed analyses including high-quality genome assemblies, the phylogenetic relationship of the three species is consistent with a single origin early in the evolution of the section Populus (Figure 6). The described ARR17 sex marker may thus work in *P. alba*, *P. adenopoda* and their respective hybrids and potentially also in *P. qiongdaoensis*. In line with this, sex identification using our PCR-based ARR17 marker was successful in the Chinese white poplar (*P.* × *tomentosa*). Phylogenetic analyses (An et al., 2020; Gao et al., 2019) support the assumption that *P.* × *tomentosa*, a closely related species of both *P. alba* and *P. adenopoda*, is the

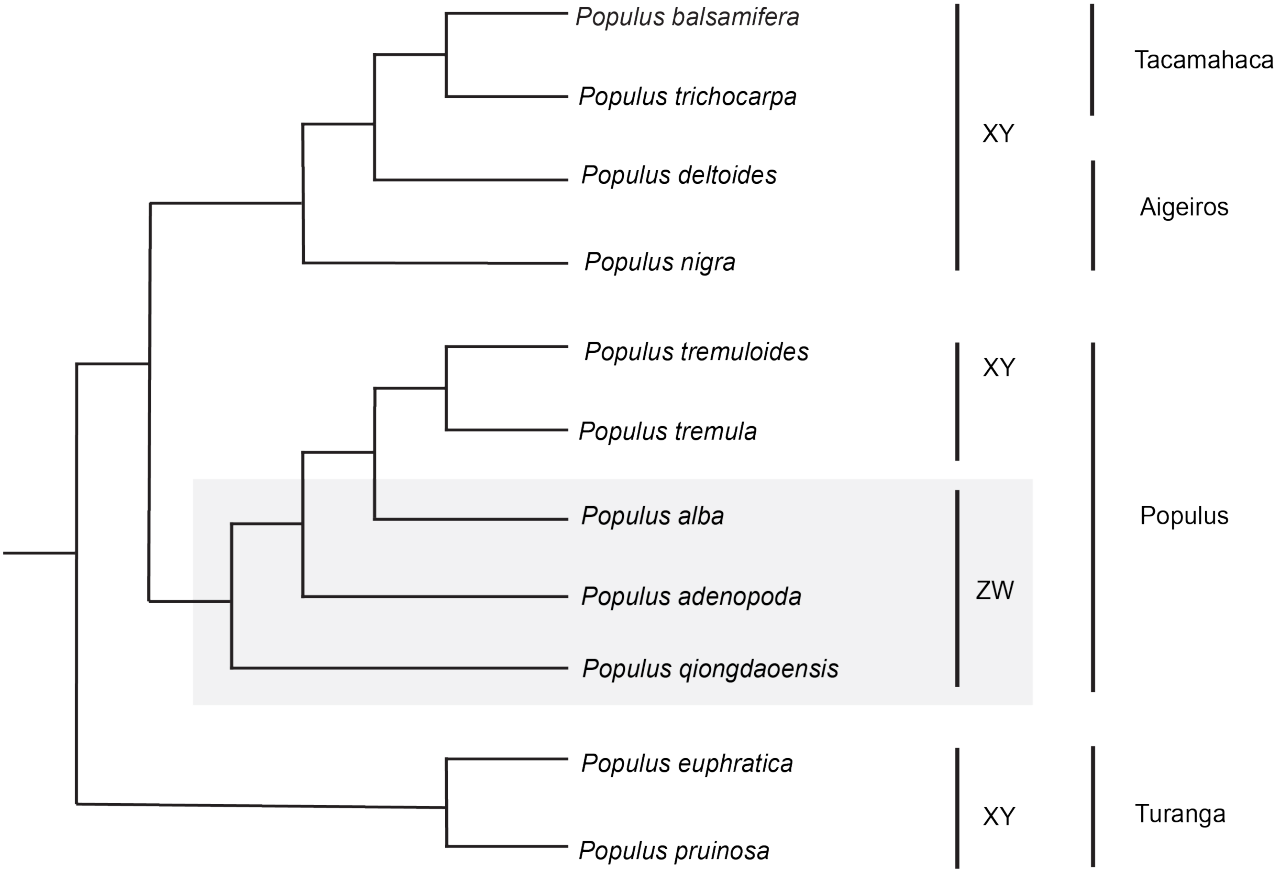

Figure 6
Phylogenetic relationships among *Populus* species based on Wang et al. (2020) with respective sections (Tacamahaca, Aigeiros, Turanga and Populus) and sex determination systems XY or ZW, in the case where *P. alba*, *P. adenopoda* and *P. qiongdaoensis* feature a ZW system.

result of hybridization between these two species, where *P. adenopoda* is the female parent and *P. alba* the male parent.

The identification of the genetic basis and the molecular mechanisms of sex determination in several species of the genus *Populus* provides insights into sex chromosome evolution. The genus *Populus* is characterized by a diversity of different sex chromosomal systems demonstrating several sex chromosome turnover events. Sex-determining regions are located at different genomic positions including different chromosomes (Geraldes et al., 2015; Kersten et al., 2014; Müller et al., 2020; Yang et al., 2021). Additionally, *P. alba* exhibits a ZW system as opposed to the XY systems of most other species. The analyses of *P. adenopoda* and *P. qiongdaoensis* reported here indicating ZW systems in those species as well, suggest that the ZW system of *P. alba* may not have evolved specifically in the *P. alba* lineage. Shared female-specific hemizygous regions argue for a common origin and would phylogenetically place the evolution of the ZW system to an early ancestor of the section *Populus* (Figure 6) (Wang et al., 2020). While the derived nature of the XY system found in the aspens *P. tremula* and *P. tremuloides* has been reported before (Müller et al., 2020, Zhou et al., 2020) our results indicate that it re-evolved from the ZW system found in *P. alba*, *P. adenopoda* and *P. qiongdaoensis*. Strikingly, this new XY system is mechanistically identical to the much older system found in several species of the *Populus* sections Tacamahaca, Aigeiros and Turanga, demonstrating a remarkable example of convergent evolution (Figure 6).

To summarize all poplar sex markers in one paper (Figure 7), we included results from PCR amplification utilizing previously described sex markers. The male-specific aspen homologue of the *P. trichocarpa* gene Potri.019G047300 (named *T0Z19*, (Pakull et al., 2015)) is located within the SDR of aspens. The *TOZ19* gene is only present in males, while it is missing completely (*P. tremuloides*) or partially (*P. tremula*) in females (Pakull et al., 2015). The paralog Potri.013G079600 (named *TOZ13*) is not sex-linked and can be amplified in all male and female aspen and white poplar species tested. It can therefore serve as a PCR control.

There are few studies involving the bigtooth aspen (*P. grandidentata*) making it difficult to determine its exact phylogenetic relationships although it appears to be an outgroup to the other aspen species (An et al., 2020). However, it seems to behave differently from the other aspens, since PCR amplification of the *TOZ19* gene does not work in this species. As an

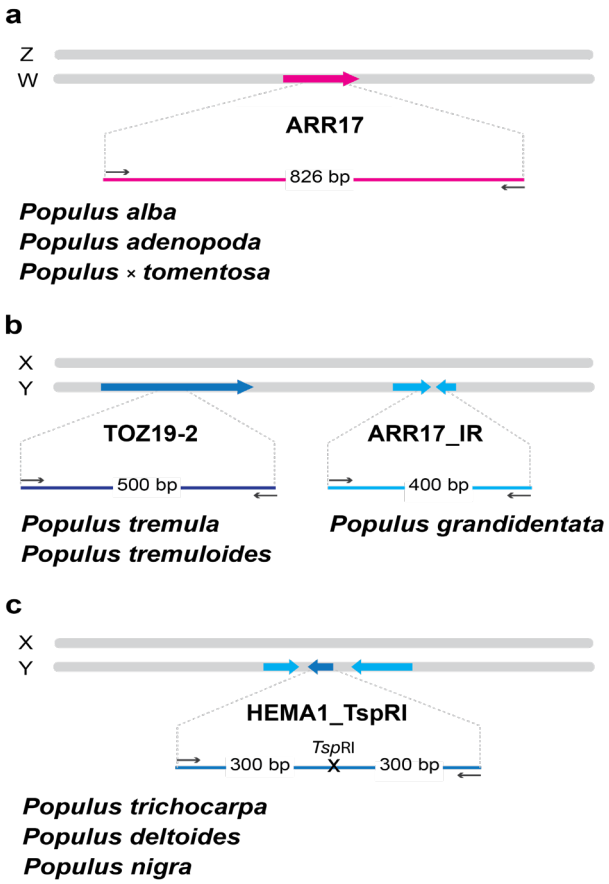

Figure 7
Summary of poplar sex markers. (a) Marker ARR17 in *P. alba*, *P. adenopoda* and *P.* × *tomentosa*. (b) Marker TOZ19-2 in aspens (*P. tremula* and *P. tremuloides*); ARR17_IR in *P. grandidentata* (c) Marker HEMA1_*Tsp*RI in *P. trichocarpa*, *P. deltoides* and *P. nigra*. In XY systems, ARR17 is present but not sex-linked.

alternative, the *ARR17* inverted repeat was used as a male-specific marker (ARR17_IR) to differentiate males from females. The results demonstrate a male heterogametic system in *P. grandidentata*. Nevertheless, both the genomic location of the SDR and the mechanism of action remain unknown. It would be interesting to assemble the genome of a male *P. grandidentata* individual to resolve the male-specific sequence of the Y chromosome (MSY) of that species.

Just as the TOZ19 marker that only works for the aspens *P. tremula* and *P. tremuloides*, the *ARR17* inverted repeat (ARR17_IR) marker only works for *P. grandidentata*. The *ARR17* inverted repeat is non-coding. While there must be some sequence conservation for the siRNAs to specifically target the *ARR17* locus for RNA-directed DNA methylation, the siRNAs are rather generic (Müller et al., 2020). They span a relatively wide genomic region of approximately 1,000 bp and mutations within the *ARR17* inverted repeat may thus not affect its overall function. Again, an assembly of the MSY of *P. grandidentata* would be interesting for a comparison between the different aspen species.

In conclusion, our work reveals new details on the evolution of sex determination in the *Populus* genus. This allowed the development of additional molecular markers to determine the sex of non-flowering poplar individuals in different species. Additional sex-chromosomal systems will be likely uncovered in the genus, which is characterized by frequent sex chromosome turnover. It will be interesting to elucidate potential biological reasons for these evolutionary dynamics in future studies.

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