nature portfolio

Peer Review File

Allopolyploidization from two dioecious ancestors leads to recurrent evolution of sex chromosomes

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In this manuscript, the authors assembled the genomes of a female allotetraploid weeping willow and a male diploid Salix dunnii and characterized the sex determination system in weeping willows. Based on genomic features of sex chromosomes and the locations of partial and intact ARR17s. The results provide insights into the sex chromosome revolution after allopolyploidization.

I have a question about the determination of the ZW system in weeping willow. As described in the method section, the k-mers were generated from high-quality reads and mapped onto the genome (76 chromosomes) of a weeping willow. Because the genome sequences were from a female weeping willow. Male-specific k-mers couldn't be mapped. Further assembly of these male-specific k-mers could help to identify Z/Y-specific genomic regions. Another suggestion is to sequence one male weeping willow with low coverage using HiFi. The genome sequences of male individuals would provide more detail about the reorganization of sex chromosomes.

Reviewer #2:
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The manuscript entitled "Allopolyploidization from two dioecious ancestors leads to recurrent evolution of sex chromosomes" by He et al. investigated the evolution of sex chromosome in allopolyploid weeping willow, S. babylonica, through whole genome comparative analysis involving various species from the parental Salix and Vetrix clades. Their research explored the transition from XY system to ZW system following allopolyploidization, specifically focusing on chromosomes 7 and 15. While their comparative genome analysis is comprehensive, certain aspects of the discussion in the main text are challenging to follow. I have some questions regarding their results and conclusions, which are detailed in an attached file.

Peer Review for Allopolyploidization from two dioecious ancestors leads to recurrent evolution of sex chromosomes

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Major comments:

1. **Line 153-156:** I suggest renaming the A and B subgenomes to S and V subgenomes, respectively, named after the *Salix* and *Vetrix* clades. This would alleviate the confusion between subgenome and allele labels (Aa, Ab, Ba, Bb, making it clearer and easier to understand. Using Sa, Sb, Va, Vb would be more intuitive if there is no problem.

2. **Supplementary Figure 9:** Could you provide a collinearity analysis between all four haplotypes (Aa, Ab, Ba, Bb) in S. babylonica and ab haplotypes in S. dunni on chr15? I am interested in whether the genome structures Aa and Ab from the Salix clade in S. babylonica and S. dunni are more similar compared to the Ba and Bb genomes from the Vetrix clade. Is there a notable structural difference in the Ba genome (W)? Additionally, the gene density on Chr15a in S. dunnii appears inconsistent between figures (a) and (b), even though they cover nearly the same regions. Could you please check this?

3. **Line 191 and Figure 2b:** You identified three inversion events between the X and Y chromosomes in *S. dunnii*. Could you add an additional arrow in Figure 2b to indicate one of these inversions?

4. **Lines 202-204:** I find it challenging to follow your explanation here. Could you provide any references concerning gamete production in the Vetrix clade? When referring to "ancestor," do you mean S. artutifolia? Additionally, it seems you are suggesting that ancestral sex determination in male S. babylonica is governed by XY system on chr15 from Vetrix genome, instead of ZW system. Is that correct? Could you rephrase these

sentences for easy understanding?

5. **Supplementary Figure 11:** This phylogeny figure is difficult to interpret. For example, adding markers to differentiate between Salix and Vetrix clades and highlighting genes from Y and Z could enhance clarity.

6. **Lines 209-214:** The discussion here is crucial for understanding your findings but is currently hard to follow. Adding a simplified diagram in Supplementary Figure 11, focusing solely on Chr15, could be beneficial. Probably, it will be more simplified figure than Figure 4.

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8. **Lines 233-234:** Please consider rephrasing this sentence for clarity: "Gene counts are

similar between W-SLR (323 genes) and Z-SLR (306 genes), though their total lengths and repeat lengths differ."

9. **Lines 239-240 and Figure 3f:** This section is somewhat confusing. Could you elaborate on the divergence and expansion of sex-linked regions in the main text? According to Supplementary Table 12, are these specific to SLR? Adding sentences and modifying table are helpful for understanding the characteristics of sex chromosomes.

10. **Figure 4:** This is a useful summary, but it's unclear why S. purpurea is shown with only one chromosome 7. Should it depict two chromosome 7s? In S. babylonica, shouldn't the differing lengths of 7Aa and 7Ab be similar, if they are derived from chromosomes 7X7X in the Salix clade.

11. **Figure 5:** Could you verify the accumulation of small RNA on chromosomes 19Ba, Bb, Aa, Ab in males, in comparison to their gene expression levels?

12. **Lines 276-279:** You discuss the dosage effect from the Z chromosome. Is it possible that the W chromosome also acquires a significant role for being female from X chromosome,

perhaps in inhibiting small RNA production of partial ARR14?

13. **Lines 332-338 and Supplementary Figure 13:** The current description of your scenario is somewhat challenging to follow. To enhance clarity, could you update the chromosome notation in the main text from 7X7X15X15Y to 7Sx7Sx15Vx15Vy? Similarly, in Supplementary Figure 13, please consider renaming subgenome A to S and subgenome B to V to maintain consistency with earlier suggestions. If the acquisition of the W chromosome is a crucial event in sex determination, 7S7S7V7V-15S15S15Vx15Vy represents male, and subsequently, 7S7S7V7V-15S15S15Vw15Vz transitions to female. How do you think about this possibility?

Minor comments:

1. Reference 20 have been already published in New Phytol.
2. L327: 15Z (Ba) -> 15Z (Bb)?

Reviewer #3:
Remarks to the Author:
Major comments:

Please engage the reader in a discussion of the biological significance of your findings. Currently, the paper seems most appropriate as a genome sequencing report. The Salix babylonica system is an excellent model for the study of sex chromosome turnover and sexual antagonism. Unfortunately, this broader evolutionary context is barely mentioned in the manuscript.

The presentation of polyploidy is also incomplete. The introduction and discussion focus on sex chromosome duplication but the downstream targets of the sex determining gene also are impacted by the change in gene dosage.

In the abstract, the authors mention that their results point to rapid evolution and reversion of sex chromosomes. Where is the relative speed of evolution assessed?

How does the S. dunnii genome assembly published here differ from the chromosome-scale assembly published by the authors in 2021?

Nearly 400 Salix species have nrDNA ITS and/or plastid loci sequenced. These could be analyzed to better resolve the diploid ancestry of S. babylonica.

There are a number of missing words and typos through the ms that need to be fixed. Some of them change the meaning of the sentences from what I think is meant, others obfuscate the results.

Minor Comments:

Using hyphenated “Salix-clade” vs. “Vetrix¬-clade” is helpful to the reader in the beginning but then is lost in later half of the ms. Please use it consistently throughout the paper.

line 40 one pair of
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which showed that dmrt1 homeologs, autosomal gene which interacts with the sex determining gene dmw, have been pseudogenized multiple times independently in Xenopus after polyploidization.
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line 78 replace interestingly - convince your reader that it is interesting, don't tell them it is.
line 83 delete exciting
line 87 in Salicaceae is well known
line 97 the Vertix clade
lines 99-102 Please make changes to this very long sentence to clarify meaning. I suggest removing “In order” and “which have not previously been sequenced”.
I suggest changing the wording of line 101-102 to:
. . . a haplotype-resolved chromosome-level genome of a female allotetraploid weeping willow and a haplotype-resolved genome of a male diploid Salix dunnii of the Salix-clade with an XY system on chromosome 7.
line 102 Salix dunnii previous genome sequence published in 2021 - reference 24.
line 106 Be specific about what kind of polyploidization event is expected to have occurred: auto- or allo-?
line 107 Add -clade to Vetrix.
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line 148-149 Change “phylogenetic position” to “ancestry”.
line 153 Change “separated” to “resolves” or a similar word.
line 157 Change “built” to “estimated”.
line 163 Therefore, S. babylonica may have emerged during 6.2–2.91 Mya. is redundant - delete
line 164-5 Nearly 400 species of Salix have plastid and/or nrDNA ITS sequence in Genbank, analysis of these should answer this question.
line 174 Spell out MF and CQ
line 187 check Fst sampling in methods
line 197 replace “imply” with “suggest” and remove first use of “most likely”
line 199-200 I think this should be plural, “ ancient sex-linked genes”.
line 212 delete "were"
line 249 delete first "the"
lines 249-250 This concluding sentence lacks a definitive statement on the results of this study. Be explicit about your findings and in what way your system differs from other sexual systems in angiosperms.

line 263 reword to describe what was sequenced (RNA, small RNA) and then the number of reads
line 265 the 3 stages are not described in the methods
line 271 19 not 19s
line 282 are catkins the same as flower buds?
line 294 delete interestingly
line 307 Remove “However,”
line 310 Some suggestions for these sentences to improve clarity: Previous studies do not provide direct evidence that dioecious plants can break through the polyploidy limit. Diospyros kaki and Mercurialis annua, both male heterogametic systems, reverted to non-dioecious sex determination systems after genome doubling, while the diploid and polyploid ancestors of the octoploid dioecious Fragaria chiloensis were likely hermaphroditic.
line 324 pair of sex chromosomes was retained

Reviewer #4:
Remarks to the Author:
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Point-by-point response

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors assembled the genomes of a female allotetraploid weeping willow and a male diploid Salix dunnii and characterized the sex determination system in weeping willows. Based on genomic features of sex chromosomes and the locations of partial and intact ARR17s. The results provide insights into the sex chromosome revolution after allopolyploidization.

I have a question about the determination of the ZW system in weeping willow. As described in the method section, the k-mers were generated from high-quality reads and mapped onto the genome (76 chromosomes) of a weeping willow. Because the genome sequences were from a female weeping willow. Male-specific k-mers couldn't be mapped. Further assembly of these male-specific k-mers could help to identify Z/Y-specific genomic regions. Another suggestion is to sequence one male weeping willow with low coverage using HiFi. The genome sequences of male individuals would provide more detail about the reorganization of sex chromosomes.

Response: Many thanks to the reviewer for this suggestion, however, and we do apologize if we have misunderstood something, the weeping willow has ZW system on chromosome 15. Because both sexes carry at least one Z chromosome, we do not expect any male-specific *k*-mers other than a low number of false positives. In contrast, if a species carries a XY system, it should have male-specific *k*-mers related to the Y chromosome. We apologize if our writing was unclear, and have revised the relevant sentence to reduce confusion - "We expect male (male heterogamety, male carrying XY and female carrying XX) or female (female heterogamety, female carrying ZW and male carrying ZZ) specific k-mers on sex-liked region of sex chromosome(s)."

Furthermore, because we obtained high-quality 15W and 15Z sex chromosome assemblies from the female, further sequencing of 15Z from a male are not necessary. We have added the distribution of male-specific *k*-mers, which represent a low incidence of false positives, in the revised Supplementary Fig. 7, which reveals a non-significant male-specific *k*-mer signal throughout the genome.

Reviewer #2 (Remarks to the Author):

The manuscript entitled "Allopolyploidization from two dioecious ancestors leads to recurrent evolution of sex chromosomes" by He et al. investigated the evolution of sex chromosome in allopolyploid weeping willow, S. babylonica, through whole genome comparative analysis involving various species from the parental Salix and Vetrix clades. Their research explored the transition from XY system to ZW system following allopolyploidization, specifically focusing on chromosomes 7 and 15. While their comparative genome analysis is comprehensive, certain aspects of the discussion in the main text are challenging to follow. I have some questions regarding their results and conclusions, which are detailed in an attached file.

Response: Many thanks for your helpful comments. We have revised the manuscript throughout as you suggest, and we hope that the improved version meets with your expectations.

Major comments:

1. **Line 153-156:** I suggest renaming the A and B subgenomes to S and V subgenomes, respectively, named after the Salix and Vetrix clades. This would alleviate the confusion between subgenome and allele labels (Aa, Ab, Ba, Bb, making it clearer and easier to understand. Using Sa, Sb, Va, Vb would be more intuitive if there is no problem.

Response: This is a great suggestion! We have replaced **A** and **B** with **S** and **V** in the MS.

2. **Supplementary Figure 9:** Could you provide a collinearity analysis between all four haplotypes (Aa, Ab, Ba, Bb) in S. babylonica and ab haplotypes in S. dunni on chr15? I am interested in whether the genome structures Aa and Ab from the Salix clade in S. babylonica and S. dunni are more similar compared to the Ba and Bb genomes from the Vetrix clade. Is there a notable structural difference in the Ba genome (W)? Additionally, the gene density on Chr15a in S. dunnii appears inconsistent between figures (a) and (b), even though they cover nearly the same regions. Could you please check this?

Response: We used chr15 a and b of *S. dunnii* and 15Sa, 15Sb, 15Va, and 15Vb of the *S. babylonica* to conduct collinearity analysis (Supplementary Fig. 9a). As you guessed, the gene structure of 15a and 15b in *S. dunnii* are more similar to 15Sa and 15Sb in *S. babylonica* than to 15Vw and 15Vz. There are obvious inversions between 15Vw and other chromosomes.

Thank you for your suggestion to check the gene density plots. We discovered some issues in the presentation and have fixed them.

3. **Line 191 and Figure 2b:** You identified three inversion events between the X and Y chromosomes in S. dunnii. Could you add an additional arrow in Figure 2b to indicate one of these inversions?

Response: Added.

4. **Lines 202-204:** I find it challenging to follow your explanation here. Could you provide any references concerning gamete production in the Vetrix clade? When referring to "ancestor," do you mean S. artutifolia? Additionally, it seems you are suggesting that ancestral sex determination in male S. babylonica is governed by XY system on chr15 from Vetrix genome, instead of ZW system. Is that correct? Could you rephrase these sentences for easy understanding?

Response: We apologize for the confusion; however, this is a complex situation to explain. The “ancestor” means a male from the *Vetrix* clade. A male (15Z15Z) in female heterogamety can only produce unreduced gametes with 15Z, while 15W gametes can only come from a female. In contrast, a male (15X15Y) in a male heterogametic species can produce both 15X and 15Y gametes. We have revised the relevant sentences “A diploid male (15Z15Z) in a female heterogametic species can only produce 15Z gametes, and cannot produce 15W, which must come from a female, while a diploid male (15X15Y) in male heterogametic species can produce unreduced gametes with 15X and 15Y.”

5. **Supplementary Figure 11:** This phylogeny figure is difficult to interpret. For example, adding markers to differentiate between Salix and Vetrix clades and highlighting genes from Y and Z could enhance clarity.
Response: Thank you for this suggestion. We have revised the tree accordingly.

6. **Lines 209-214:** The discussion here is crucial for understanding your findings but is currently hard to follow. Adding a simplified diagram in Supplementary Figure 11, focusing solely on Chr15, could be beneficial. Probably, it will be more simplified figure than Figure 4.
Response: We added a simplified diagram in Supplementary Figure 11 as you suggest. We revised the **Evolutionary origin of *Salix babylonica*** to clarify our hypothesis and relevant evidence.

7. **Lines 221-223:** I am uncertain if the variation in gene numbers and repeat sequences necessarily indicates that autosome 7Aa originated from a sex chromosome. In Figure 3b and Supplementary Figure 12, could you compare 4 haplotypes (7Ba-7Bb-7Aa-7Ab) in S. babylonica with 2 haplotypes (7XY) in S. dunnii? If their genomic structures are similar to 7X in S. dunnii, it could support your hypothesis.
Response: We have conducted collinearity analysis for 7Va-7Vb-7Sa-7Sb of *S. babylonica* and 7X and 7Y of *S. dunnii* (Fig. 3d). The 7Sa and 7Sb genomic structures are similar to 7X in *S. dunnii*.

8. **Lines 233-234:** Please consider rephrasing this sentence for clarity: "Gene counts are similar between W-SLR (323 genes) and Z-SLR (306 genes), though their total lengths and repeat lengths differ."
Response: Revised.

9. **Lines 239-240 and Figure 3f:** This section is somewhat confusing. Could you elaborate on the divergence and expansion of sex-linked regions in the main text? According to Supplementary Table 12, are these specific to SLR? Adding sentences and modifying table are helpful for understanding the characteristics of sex chromosomes.
Response: We deleted Figure 3f and revised the sentences. We stated the regions in the second column of Supplementary Table 12, and revised the table to make it clearer.

10. **Figure 4:** This is a useful summary, but it's unclear why S. purpurea is shown with only one chromosome 7. Should it depict two chromosome 7s? In S. babylonica, shouldn't the differing lengths of 7Aa and 7Ab be similar, if they are derived from chromosomes 7X7X in the Salix clade.
Response: The genome of *S. purpurea* was published by Zhou et al. (2020, https://doi.org/10.1186/s13059-020-1952-4). They only assembled one chromosome 7. Hence, we only presented one. The 7Aa/Sa and 7Ab/Sb were derived from 7X more than 2.91 million years ago. The differing lengths likely reveal the rediploidization following allopolyploidization. More haplotype-resolved genome assemblies could help us to explore the mechanisms of the differing lengths of homologous pairs. Although we agree that this is an interesting direction for future work, we respectfully suggest that it is beyond the scope of the current paper.

We added the lengths of all the assembled chromosomes in Supplementary Table 5 and 6 for future discussion.

11. **Figure 5:** Could you verify the accumulation of small RNA on chromosomes 19Ba, Bb, Aa, Ab in males, in comparison to their gene expression levels?

Response: As you hypothesized, we detected the accumulation of small RNA in intact *ARR17*-like gene regions of the chromosomes 19Va, 19Vb, 19Sa, and 19Sb in males (Supplementary Figure 16). In male flower buds, there is an accumulation of small RNAs near the intact *ARR17*-like duplicates, while in female flower buds, there is almost none.

12. **Lines 276-279:** You discuss the dosage effect from the Z chromosome. Is it possible that the W chromosome also acquires a significant role for being female from X chromosome, perhaps in inhibiting small RNA production of partial ARR14?

Response: Your hypothesis is very insightful, and we agree that this is indeed possible. Unfortunately, we did not find such a factor. More sequenced allotetraploid willows (e.g., *Salix alba*, *S. pentandra*, *S. chienii*) could facilitate the future detection of such factors. We have added a sentence to state that is possible. However, the duplicated intact *ARR17*-like genes on chromosome 19 could play such a role too. Please see the next response.

13. **Lines 332-338 and Supplementary Figure 13:** The current description of your scenario is somewhat challenging to follow. To enhance clarity, could you update the chromosome notation in the main text from 7X7X15X15Y to 7Sx7Sx15Vx15Vy? Similarly, in Supplementary Figure 13, please consider renaming subgenome A to S and subgenome B to V to maintain consistency with earlier suggestions. If the acquisition of the W chromosome is a crucial event in sex determination, 7S7S7V7V-15S15S15Vx15Vy represents male, and subsequently, 7S7S7V7V-15S15S15Vw15Vz transitions to female. How do you think about this possibility?

Response: We have replaced all A and B with S and V as you suggested. In the section "**Sex determination and *ARR17*-like genes in *S. babylonica***", we explain how the 7S7S7V7V-15S15S15Vx15Vy and 7S7S7V7V-15S15S15Vw15Vz determine femaleness. To clarify this, we added "Duplicated intact *ARR17*-like genes on chromosome 19s made new females" in the revised **Supplementary Figure 12**.

Minor comments:

1. Reference 20 have been already published in New Phytol.

Response: Yes, revised.

2. L327: 15Z (Ba) -> 15Z (Bb)?

Response: Yes, revised.

Reviewer #3 (Remarks to the Author):

Major comments:

Please engage the reader in a discussion of the biological significance of your findings. Currently, the paper seems most appropriate as a genome sequencing report. The Salix babylonica system is an excellent model for the study of sex chromosome turnover and sexual antagonism. Unfortunately, this broader evolutionary context is barely mentioned in the manuscript.

Response: Many thanks for this suggestion, in discussion and abstract, we wrote "how these polyploid lineages overcome the complex and unbalanced allelic combinations of sex determination alleles to establish stable dioecy is not yet known" and "Polyploidization presents an unusual challenge for species with sex chromosomes, as it can lead to complex combinations of sex chromosomes that disrupt reproductive development." As such, we present *Salix babylonica* as a model for allopolyploidization between lineages with different sex chromosomes. We have added relevant sentences to highlight the importance of the key results: "Our results also suggested that sex chromosome turnover (15XY to 15ZW) happened during allopolyploidization."…"The origination and turnover of *S. babylonica* sex chromosomes"…"These results suggest that XY to ZW transitions occurred independently in different willow species."

The presentation of polyploidy is also incomplete. The introduction and discussion focus on sex chromosome duplication but the downstream targets of the sex determining gene also are impacted by the change in gene dosage.

Response: Many thanks for this suggestion. According to Leite Montalvão et al. (2022, https://doi.org/10.1098/rstb.2021.0217), the expressed intact *ARR17*-like gene eventually inhibits *PI* to suppress stamen and determine femaleness. Hence, we added the expression data of the *PI*-like genes of female and male buds (see the new **Supplementary Figure 17**). As we expected, the *PI*-like genes were expressed in males not in females.

In the abstract, the authors mention that their results point to rapid evolution and reversion of sex chromosomes. Where is the relative speed of evolution assessed?

Response: We deleted "rapid" from this sentence. The timing of allopolyploidization is in the results.

How does the S. dunnii genome assembly published here differ from the chromosome-scale assembly published by the authors in 2021?

Response: He et al., (2021) published a female genome of *S. dunnii*, which only includes the 7X chromosome. In current paper, we assembled both 7X and 7Y from a male individual. The 7Y of the *S. dunnii* is the first Y chromosome of the *Salix*-clade. We have stated this in the introduction in order to make the advance clearer.

Nearly 400 Salix species have nrDNA ITS and/or plastid loci sequenced. These could be analyzed to better resolve the diploid ancestry of S. babylonica.

Response: We thank the reviewer for this suggestion, but respectfully suggest that others have already done this analysis. *S. babylonica* is clustered in a tetraploid group in the plastome tree (Wagner et al., 2021, 10.3389/fpls.2021.662715). Similarly, Gulyaev et al., (2022) also revealed the same tetraploid group, and suggested the tetraploid group likely arose from allopolyploid offspring of crosses between species from clades *Salix* and *Vetrix*. ETS and ITS sequences failed to reveal the nuclear origination (Wu et al., 2015, 10.1186/s12862-015-0311-7). We suggest that it is not necessary to repeat this recent work, and do agree that more future genomes from the tetraploid group will help us to understand the origin of the clade.

There are a number of missing words and typos through the ms that need to be fixed. Some of them change the meaning of the sentences from what I think is meant, others obfuscate the results.

Response: Thanks for your comments. We have revised the ms accordingly.

Minor Comments:

Using hyphenated “Salix-clade” vs. “Vetrix¬-clade” is helpful to the reader in the beginning but then is lost in later half of the ms. Please use it consistently throughout the paper.

Response: Revised.

line 40 one pair of

Response: Corrected.

line 44 19s ?

Response: Four homologous chromosomes of 19. Revised.

lines 52-53 This is fairly general statement could use more citations to back it up and could use explicit examples of the “several interesting questions” presented.

Response: Added. We have also clarified our interesting questions, how does the polyploid lineage overcome unbalanced sex chromosome dose effects and how does the duplicated sex chromosome revert to autosomal inheritance.

line 56 citation needed

Response: We deleted this sentence, because it is hard to estimate relative speed of evolution in diploid vs. polyploid.

lines 63-64 “complex combinations of chromosomes” seems like an over-statement. I could see how two chromosome systems being added together through allopolyploidy may lead to complex interactions between sex chromosomes.

Response: We replaced the complex with “diverse”.

lines 64-65 Is this loss of dioecy a reversion to hermaphroditism or a change to sterility?

Response: We added the non-dioecious system “monoecy” as an example.

line 67 "sometimes instantaneously" citation needed

Response: Added.

lines 69-70 Are there instances where duplicate sex chromosomes are retained? If so, these sorts of exception seem relevant to share with the reader.

Response: Added.

line 73 Of relevance here is the study by Bewick et al. 2011 (Evolution 65:698–712)
which showed that dmrt1 homeologs, autosomal gene which interacts with the sex determining gene dmw, have been pseudogenized multiple times independently in Xenopus after polyploidization.

Response: Thanks, this is an excellent paper. We have now cited it in the introduction.

line 74 Why is the "retention and loss of sex chromosomes in allopolyploidization" and "its effects on gene content and sex determination" remain unexplored? I can guess that it is genomic sequencing technology that can now allow us to do this kind of thing. It is best to be explicit about why. This would help motivate why readers might care about S. babylonica sex chromosomes in general.

Response: Yes, haplotype-resolved genome assemblies give us the opportunity to explore questions like these. We have revised the sentence.

lines 76-78 Evidence needed for the claim that the weeping willow (Salix babylonica) is one of the best-known trees in the world? On what grounds was it expected to be an allotetraploid? And why was it suspected it to have arisen from a cross between species from the Salix-clade and Vetrix-clades within the genus Salix? These hypotheses about the origin of Salix babylonica need to be addressed explicitly.

Response: We added information on native occurrence and the widespread use as ornamental tree**,** specifically in the Northern hemisphere. "the best-known trees" is reported by Isebrands, & Richardson (2014) in *Poplars and willows: trees for society and the environment*. We added also the book by Newsholme (1992) who gives information on the worldwide natural and cultivated distribution. The *Salix babylonica* is known to be tetraploid, clustered in a tetraploid group, and likely arose from crosses between species from the *Salix*- and *Vetrix*-clade within the genus *Salix* according to phylogenetic incongruence between the chloroplast and nuclear trees of *Salix*. See Fig 1. of our study. Thus, *S. babylonica* will become a model system for study of allopolyploidy. We have revised the relevant sentences.

line 78 replace interestingly - convince your reader that it is interesting, don't tell them it is.

Response: Deleted.

line 83 delete exciting

Response: Deleted.

line 87 in Salicaceae is well known

Response: Corrected.

line 97 the Vertix clade

Response: Added.

lines 99-102 Please make changes to this very long sentence to clarify meaning. I suggest removing “In order” and “which have not previously been sequenced”.

Response: Deleted and revised.

I suggest changing the wording of line 101-102 to:
. . . a haplotype-resolved chromosome-level genome of a female allotetraploid weeping willow and a haplotype-resolved genome of a male diploid Salix dunnii of the Salix-clade with an XY system on chromosome 7.

Response: Thanks. Revised as you suggest.

line 102 Salix dunnii previous genome sequence published in 2021 - reference 24.

Response: Yes, a female genome (only includes 7X) of *S. dunnii* was assembled based on Oxford Nanopore Technologies (ONT) long reads. We stated it in the introduction.

line 106 Be specific about what kind of polyploidization event is expected to have occurred: auto- or allo-?

Response: allo-. Revised.

line 107 Add -clade to Vetrix.

Response: Added.

line 147 “We confirmed that S. babylonica is a tetraploid based on flow cytometry and an allotetraploid according to our . . . “

Response: Revised.

line 148-149 Change “phylogenetic position” to “ancestry”.

Response: Done.

line 153 Change “separated” to “resolves” or a similar word.

Response: Done.

line 157 Change “built” to “estimated”.

Response: Done.

line 163 Therefore, S. babylonica may have emerged during 6.2–2.91 Mya. is redundant – delete

Response: Deleted.

line 164-5 Nearly 400 species of Salix have plastid and/or nrDNA ITS sequence in Genbank, analysis of these should answer this question.

Response: Please see the answer to the fifth of Major comments.

line 174 Spell out MF and CQ

Response: Done.

line 187 check Fst sampling in methods

Response: Checked and added.

line 197 replace “imply” with “suggest” and remove first use of “most likely”

Response: Done.

line 199-200 I think this should be plural, “ ancient sex-linked genes”.

Response: Added.

line 212 delete "were"

Response: Deleted.

line 249 delete first "the"

Response: Deleted.

lines 249-250 This concluding sentence lacks a definitive statement on the results of this study. Be explicit about your findings and in what way your system differs from other sexual systems in angiosperms.

Response: Added. We have discussed this question in another paper (Wang, Y., Gong, G. N., Wang, Y., Zhang, R. G., Hörandl, E., Zhang, Z. X., ... & He, L. (2024). Gap-free X and Y chromosome assemblies of *Salix arbutifolia* reveal an evolutionary change from male to female heterogamety in willows, without a change in the position of the sex-determining locus. *New Phytologist*, meanwhile published)

line 263 reword to describe what was sequenced (RNA, small RNA) and then the number of reads

Response: Done.

line 265 the 3 stages are not described in the methods

Response: We wrote it “In October 2023 (stage 1), December 2023 (stage 2), and February 2024 (stage 3)” in **Plant material.** We added “three stages” in **RNA extraction and library preparation.**

line 271 19 not 19s

Response: Corrected.

line 282 are catkins the same as flower buds?

Response: We used both flower buds and catkins in the current paper. To facilitate comparisons among the three species and considering available RNA datasets, we only detect the dosage compensation based on catkins.

line 294 delete interestingly

Response: Deleted.

line 307 Remove “However,”

Response: Done.

line 310 Some suggestions for these sentences to improve clarity: Previous studies do not provide direct evidence that dioecious plants can break through the polyploidy limit. Diospyros kaki and Mercurialis annua, both male heterogametic systems, reverted to non-dioecious sex determination systems after genome doubling, while the diploid and polyploid ancestors of the octoploid dioecious Fragaria chiloensis were likely hermaphroditic.

Response: Replaced the sentences as you suggested.

line 324 pair of sex chromosomes was retained

Response: Revised.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: Thank you for reviewing our manuscript.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors have addressed most of my concerns. I would recommend the publication of this manuscript.

Reviewer #2:
Remarks to the Author:
Thank you for revising your manuscript. Your revisions have been addressed satisfactorily and have improved the clarity and quality of the manuscript.

Reviewer #4:
Remarks to the Author:
The authors have provided satisfactory changes in response to my reviews.