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1 **A derived ZW chromosome system in *Amborella***
2 ***trichopoda*, representing the sister lineage to all other**
3 **extant flowering plants**

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29 Abstract

- 30 • The genetic basis and evolution of sex determination in dioecious plants is emerging as an active area
31 of research with exciting advances in genome sequencing and analysis technologies. As the sole species
32 within the sister lineage to all other extant flowering plants, *Amborella trichopoda* is an important model
33 for understanding the evolution and development of flowers. Plants typically produce only male or female
34 flowers, but sex determination mechanisms are unknown for the species.
- 35 • Sequence data derived from plants of natural origin and an F1 mapping population were used to identify
36 sex-linked genes and the non-recombining region.
- 37 • *A. trichopoda* has a ZW sex determination system. Analysis of genes in a 4 Mb non-recombining sex-
38 determination region reveals recent divergence of Z and W gametologs, and few Z- and W-specific genes.
- 39 • The sex chromosomes of *A. trichopoda* evolved less than 16.5 My ago, long after the divergence of the
40 extant angiosperms.

41 Introduction

42 Dioecy (separate male and female plants) is one of the many sexual systems exhibited by flowering plant species.
43 Dioecy has evolved repeatedly in angiosperms and *c.* 6% of all species (i.e. more than 15,000 species) are dioe-
44 cious (Renner, 2014). Though detailed knowledge of the molecular mechanisms of sex determination in dioecious
45 plants is rather scarce, purely environmental sex determination seems to be rare (Renner, 2014; Charlesworth,
46 2016), and most dioecious species are therefore thought to have genetic sex determination. However, only *c.* 50
47 species with homomorphic or heteromorphic sex chromosomes have been reported, either using light microscopy
48 or classical genetic inheritance studies, and more recently with genomic approaches (Ming et al., 2011; Muyle
49 et al., 2017; Renner and Müller, 2021), and the exact sex-determining genes have been identified in even fewer
50 species (Akagi et al., 2014; Harkess et al., 2017; Akagi et al., 2019; Harkess et al., 2020; Müller et al., 2020).
51 Because dioecy evolved many times independently, different dioecious clades could have different mechanisms
52 of sex determination, so these need to be identified in each clade separately. Very often, the sex chromosomes
53 are only mildly diverged and homomorphic (with some notable exceptions; cf Renner and Müller, 2021).

54 *Amborella trichopoda* is an understory shrub endemic to New Caledonia and the sole extant species on the
55 sister lineage to the clade including all other angiosperms, which form the most diverse division of plants. It
56 seems clearly established that the most recent common ancestor of all living angiosperms had bisexual flowers,
57 and that the transition to dioecy in the *A. trichopoda* lineage occurred after it diverged from the last common
58 ancestor of all other flowering plants (Endress and Doyle, 2009; Sauquet et al., 2017). Despite the independent
59 origin of dioecy within the *A. trichopoda* lineage, unraveling the mechanism of sex determination in this species
60 could help to shed light on the evolution and development of unisexual flowers in other angiosperms and the
61 evolution of sex chromosomes more generally.

62 *A. trichopoda* plants grown from seeds collected in the wild exhibit a sex ratio of nearly 50:50 (Anger et al.,
63 2017) making genetic sex determination in the species plausible. However, there are also reports of individual
64 plants exhibiting shifts sex over time (Buzgo et al., 2004; Anger et al., 2017). In fact, the reference genome
65 sequence (Amborella Genome Project, 2013) was derived from a genotype (Santa Cruz Arboretum accession
66 no. 75.3) that was first documented as male but cuttings produced female flowers (Buzgo et al., 2004). Here we

67 test for a genetic basis for sex determination in *A. trichopoda*, and go on to characterize the sex determination
68 region in the *Amborella* genome.

69 **Materials and methods**

70 For the initial inference of sex-linkage, we obtained RNA-seq data of 20 individuals (10 females, 10 males;
71 see Supporting Information Table S1 and Supporting Information, Materials and Methods S1) grown from
72 seeds collected in the wild (hereafter referred to as the “wild population”. Sequence reads were aligned with
73 STAR (Dobin et al., 2013) to the draft chromosomal assembly of the *A. trichopoda* genome (V6.1, available on
74 CoGe, genome ID 50948), consisting of 13 pseudomolecules containing $\sim 95\%$ of the genomic scaffolds. Single
75 Nucleotide Polymorphisms (SNPs) were inferred with reads2snp (Gayral et al., 2013) and analyzed with SDpop,
76 a probabilistic framework based on the modeling of allele and genotype frequencies in populations (Käfer et al.,
77 2021). These RNAseq data from flower buds (between stages 5 and 6 according to Buzgo et al. 2004) were also
78 used to infer sex-biased expression with edgeR (Robinson et al., 2010).

79 We furthermore used shotgun DNA seq data from an F1 mapping population: parents along with 16 female
80 and 17 male F1 offspring; these data will be referred to as the “F1 mapping population”, see Supporting
81 Information Table S2. These data were also aligned to the V6.1 draft chromosomal assembly (using BWA; Li
82 and Durbin 2009). Genotypes were inferred with freebayes (Garrison and Marth, 2012). Putative W- and Z-
83 specific regions in the assembly were identified using coverage differences in genomic windows of 20 Kpb between
84 females and males. These were used to construct masks of the W- and Z-specific sequences for remapping the
85 sequencing data on the W- and Z-masked genomes (see Supporting Information, Materials and Methods S1).

86 Using the mappings of both datasets on the masked genome assemblies, we used SDpop and SEX-DETECTOR++
87 (Muyle et al., 2016; Badouin et al., 2020) to infer the Z- and W-haplotypes of the detected gametologous genes.
88 Synonymous divergence (dS) between these haplotypes was calculated with codeML (Yang, 2007). Because
89 of high sampling variance in gene-wise divergence values (Takahata and Nei, 1985), average dS values per
90 genes using both datasets were calculated. To estimate the time since recombination suppression, we used the
91 maximum average dS value in two ways. First, based on population genetics results, the divergence between
92 two sequences that do not recombine is $2\mu t + \pi$ (Nei and Li, 1979; Takahata and Nei, 1985), where π is the
93 nucleotide diversity, t the time since separation and μ the per-base mutation rate, which is again linked to π
94 and the effective population size N_e by $\pi = 4N_e\mu$. We used the estimates of population genetic parameters and
95 generation time obtained by the Amborella Genome Project (2013). Second, we compared the obtained dS value
96 to the estimated divergence of orthologous genes between *Amborella* and *Nuphar* by the One Thousand Plant
97 Transcriptomes Initiative (2019), and scaled this difference using estimates of the crown age of the angiosperms
98 (Salomo et al., 2017; Sauquet and Magallon, 2018).

99 **Results**

100 We applied SDpop (Käfer et al., 2021) to the wild population RNA-seq data, and found Z/W segregation
101 of sex-linked SNPs. SDpop inferred 49 ZW gametologs and nine putatively Z-hemizygous genes (Supporting
102 Information, Results S1 and Supporting Information Table S3), which represents 0.28% of all transcribed genes.
103 Of the ZW gametologs, 33 (67%) were located in a single region of *c.* 4 Mb on chromosome 9 of the reference
104 genome assembly (Fig. 1). Twelve of the genes inferred as ZW occurred on scaffolds that were not assigned

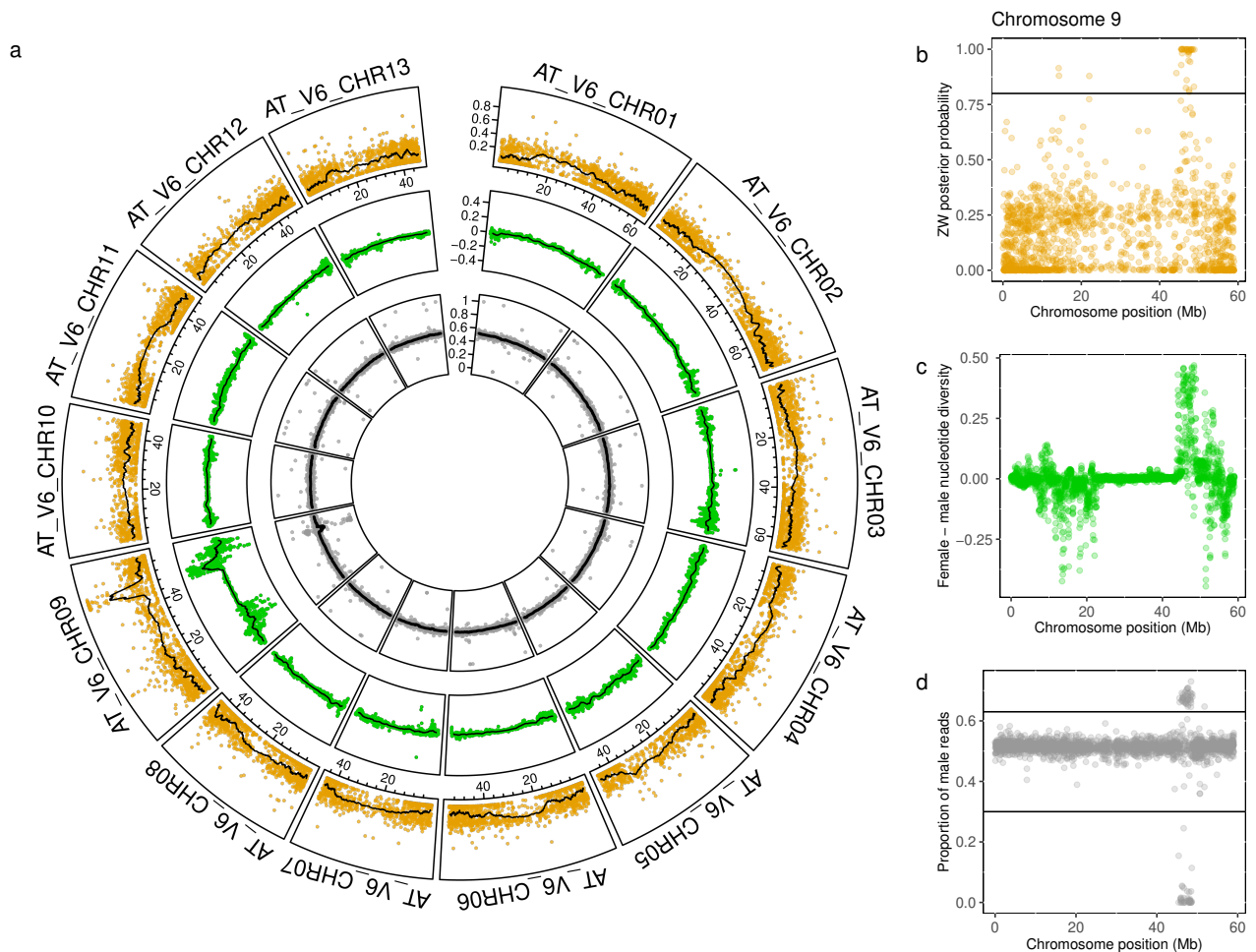


Fig. 1 Sex-linkage inferred from sequencing data in the whole *Amborella trichopoda* genome (a) and on chromosome 9 (b, c, d). Orange (panel a, outer graphs; panel b): SDpop posterior scores for the ZW segregation type per gene. The black line in (b) indicates the posterior score threshold (0.8) for classification as sex-linked. Green: (panel a, middle graphs; panel c): difference in average nucleotide diversity per gene between females and males. Grey (panel a, inner graphs; panel d): proportion of reads from males for each gene. The black lines in (d) indicate the thresholds (0.30 and 0.63) beyond which the genes were considered putatively sex-linked. In (a), the black lines represent the average in a sliding window of 50 genes.

105 to 13 chromosomal pseudomolecules, suggesting that they may reside on alternative haplotypes for the non-
 106 recombining region of chromosome 9 in the primary chromosomal assembly.

107 Using the 33 genotyped and phenotyped progeny and their parents from the F1 mapping population, we
 108 first assessed whether 671 female-specific SNP variants identified in the wild population transcripts were only
 109 present in the seed parent and female progeny. Indeed, males in the mapping population were found to have no
 110 more than five of the putatively female-specific variants, whereas females contained at least 465 of the female-
 111 specific SNP variants identified in the wild-collected population (Supporting Information Table S2). Thus, the
 112 divergence between Z and W genes is conserved among *A. trichopoda* populations.

113 We then used the mapping population to estimate the frequency of the sites that were heterozygous in
 114 females and homozygous in males, and found a clear excess of female-heterozygous SNPs in the sex-determining
 115 region identified by SDpop (Fig. 1). Furthermore, the male-versus-female coverage ratio showed a remarkable
 116 pattern in this region, with some genes having equal coverage for both sexes, others no male coverage, and

117 yet others for which two thirds of the reads were from males. As the existence of sex chromosomes (or any
118 structural heterozygosity) was not considered in the haploid assembly process, the assembled sex determination
119 region placed on chromosome 9 is a chimeric mix of fragments derived from the W and Z chromosomes likely
120 together with regions of consensus sequence for the W and Z haplotypes. Accordingly, divergent regions of the
121 W and Z haplotypes not included in the chromosomal pseudomolecules were assembled in unassigned scaffolds.
122 For the inference of divergence and sex-biased gene expression, we thus obtained two masks for the assembly
123 based on the coverage, one discarding W-specific regions, and the other Z-specific regions of the chromosome 9
124 assembly and scaffolds that were not included in the chromosomal pseudomolecules (see Supporting Information,
125 Results S1; Supporting Information Fig. S1 and Supporting Information Fig. S2; and Data Files S1 and S2).

126 Realigning the wild population RNAseq data on these masked assemblies allowed detection of slightly more
127 sex-linked genes using SDpop (Supporting Information Table S3). As these RNAseq data only contain expressed
128 genes (*c.* 50% of all genes), we used the DNA-reseq data from the mapping population to expand our inference
129 of putatively sex-linked genes. First, we analysed the data with SEX-DETECTOR (Muyle et al., 2016; Badouin
130 et al., 2020) that assigns genes as sex-linked or not based on the transmission of alleles from parents to progeny.
131 When the non-recombining region is small, as is the case here, many genes are in linkage disequilibrium with this
132 region due to the limited number of crossovers in one generation, and SEX-DETECTOR overestimates the size of
133 the non-recombining sex-determination region (Supporting Information Fig. S3). We thus used the divergence
134 between the predicted Z and W haplotypes as a second criterion for inference of sex-linkage: genes with *dS*
135 values higher than 0.005 (i.e., 2.5 times higher than the genome-wide heterozygosity) were considered as potential
136 ZW gametologs. Second, we used the coverage data from the mapping-population sequencing: we considered
137 as potentially sex-linked the genes that Fig. 1d and Supporting Information Fig. S2). Using these criteria,
138 we identified 211 potentially sex-linked genes, of which 140 are on chromosome 9 and 69 on the unassigned
139 scaffolds (Supplementary Data File 3). Among these, stringent analyses of the wild-population and F1 mapping
140 populations data implicated four gene models as W-specific (AmTr_v6.0_c9.20670.1, AmTr_v6.0_c9.20870.1,
141 and AmTr_v6.0_s3897.A.10.1) and three as Z-specific (AmTr_v6.0_c9.20390.1, AmTr_v6.0_c9.20460.1, and
142 AmTr_v6.0_s2752.A.10.1; Data File S3). These assignments are conservative and it is likely that more W-
143 and Z-specific genes may be identified in the future as high-fidelity long read data are generated and used to
144 construct a fully phased diploid assembly.

145 We calculated the synonymous divergence (*dS*) using the mapping on the two masked assemblies, and using
146 the RNAseq as well as the DNA-reseq data (Fig. 2). As the sampling variance of these values is predictably
147 high, we used the average of the two sequence datasets as our best estimate for the *dS* value of each gene
148 (cf Takahata and Nei, 1985). The maximum *dS* value is between 0.05 and 0.06, depending on the masking
149 of the reference. We calculated the time since recombination suppression based on these values in two ways,
150 either using a population genetics-based (i.e., comparing gametolog divergence to the average level of nucleotide
151 polymorphism) or phylogenetically based (i.e. comparing gametolog divergence to divergence estimates with
152 other species; see Supporting Information, Results S1). The phylogeny-based estimate yields a wider range of
153 divergence-time estimates (3.2 to 16.5 million years ago) than the population genetics-based estimate (7.2 to 8.7
154 million years ago), but both estimates rely heavily on their respective underlying assumptions (e.g. molecular
155 clock, generation time, past population size) which cannot be directly inferred nor tested.

156 Using our RNAseq data (from the wild population), we quantified sex-related expression biases. Whereas the
157 number of female-biased genes is approximately equal to the number of male-biased genes genome-wide, male-

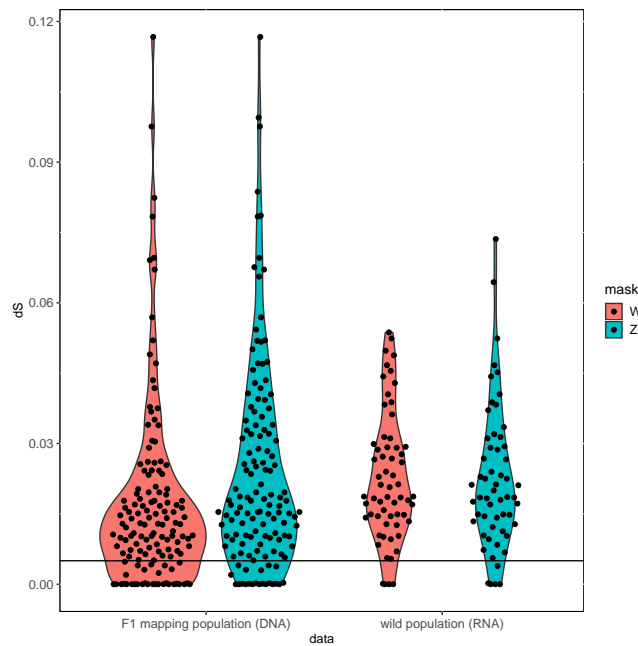


Fig. 2 Distribution of synonymous divergence (dS) between the Z and W copies of gametologous genes of *Amborella trichopoda*. For each dataset (mapping population DNA-reseq or wild population RNAseq), Z and W haplotypes were obtained after mapping on the “W masked” (red) and “Z masked” (blue) genomes. The areas of the “violins” are scaled according to the number of gametolog pairs (points). The horizontal black line indicates the threshold (0.005) above which genes were considered as sex-linked in the mapping population data.

158 biased genes seem to be over-represented in the non-recombining region (Supporting Information Table S4;
 159 Supporting Information Fig. S4). Of the sex-linked female-biased genes, only two almost completely lacked
 160 male expression (AmTr_v6.0_c9.19430.1 and AmTr_v6.0_s1359.A.20.1; Supporting Information, Results S1).
 161 These genes could have a role in female sex determination, but their function is currently not known.

162 The mapping population data showed another intriguing pattern on chromosome 9: male offspring were more
 163 often heterozygous than female offspring in a region from *c.* 10 to *c.* 20 Mb (Fig. 1). Upon closer inspection, it
 164 appears that this occurs particularly at SNP positions for which the mother, but not the father, was heterozygous
 165 (Supporting Information Fig. S5). This pattern is not typical of pseudoautosomal transmission in regions closely
 166 linked to the non-recombining region, nor can it be explained by allele loss (e.g. through lethality) or by increased
 167 heterozygosity in the mother (Supporting Information, Results S1; Supporting Information Fig. S6). An obligate
 168 recombination event between the left and the right arms of chromosome 9 during meiosis in the mother would
 169 be able to cause such a pattern, although this also would imply that the less frequent (minor) alleles all occur on
 170 the same copy of chromosome 9. Although a weak deviation from purely autosomal allele-genotype equilibria
 171 was also detected in the wild population (Fig. 1), more data (e.g. using different parents for an F1) are needed
 172 before a precise mechanistic hypothesis for this pattern can be formulated and tested.

173 Discussion

174 We here show that *A. trichopoda* has sex chromosomes of the ZW type, with female heterogamy. Based on the
 175 current haploid genome assembly, the non-recombining region is estimated to comprise only *c.* 4 Mb long of
 176 the 59 Mb sex chromosome. The maximum synonymous divergence of *c.* 5 to 6% indicates that recombination

177 suppression occurred rather recently, at least much more recently than the divergence of *Amborella* and other
178 living angiosperms. Although it might be possible that the first genes for which recombination was suppressed
179 are not expressed or have been lost, the fact that Z and W haplotype sequences were collapsed in the current
180 assembly and that we were not able to unambiguously identify more than a handful of Z- or W- specific genes
181 also suggests that the *A. trichopoda* sex chromosomes are rather young.

182 Sex change is well documented in *A. trichopoda*, in wild as well as in cultivated plants (Buzgo et al., 2004;
183 Grosse-Veldmann et al., 2011; Anger et al., 2017). Our results show that the individual used for reference genome
184 sequencing (Amborella Genome Project, 2013) is genetically female with a Z and W gametologs, although it had
185 produced male flowers in the past. Sex changes are not uncommon in dioecious angiosperms, even in those with
186 clearly heteromorphic sex chromosomes (Ainsworth, 2000). The identification of the non-recombining region
187 should allow researchers to determine the genetic sex for comparison with the phenotypic sex, and help gain
188 insight in the genes involved in sex determination.

189 Most plant sex chromosomes described so far are of the XY type (Ming et al., 2011; Renner and Müller,
190 2021), and this observation is considered by some as support for the hypothesis that dioecy evolves from gyn-
191 odioecy through the occurrence of a recessive female-determining gene and a dominant male-determining gene
192 (Charlesworth and Charlesworth, 1978). However, few sex determination mechanisms have been described at
193 the gene level (cf Renner and Müller, 2021), and there seem to be many different developmental and evolutionary
194 pathways to the production of unisexual flowers (Diggle et al., 2011; Golenberg and West, 2013).

195 Our inference that the ZW sex determination system is a recently derived trait in *A. trichopoda* underscores
196 the fact that the species is not ancestral nor “basal”. The *Amborella* lineage and the ancestral lineage of
197 all other flowering plants diverged from their most recent common ancestor (MRCA) in the early Cretaceous
198 or even late Jurassic (Magallón et al., 2015; Silvestro et al., 2021). Thus, the biology of extant *Amborella*
199 populations has been molded by more than 130 million years of evolution, since the early diversification of the
200 extant flowering plants. However, *A. trichopoda* has retained some characteristics that are hypothesized to be
201 shared with the MCRA of living angiosperms (e.g. free floral organs, including an undifferentiated perianth of
202 tepals, and carpels that contain an apical, secretion-filled canal for pollen-tube growth; Endress and Igersheim
203 2000; Sauquet et al. 2017), and no genome duplication has occurred in the *Amborella* lineage since diverged
204 from the other angiosperms (Amborella Genome Project, 2013). Thus, further study of the genes involved in
205 sex determination in *A. trichopoda* might shed light on the regulatory pathways that underlie development of
206 unisexual flowers in other angiosperms.

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216 Author contributions

217 Conceptualization: GABM, CS, JK, JLM, AB Methodology: JK, FP, GABM, JLM, AB Software: JK, FP,
218 Formal analysis: JK, AB, GL, AB, JB, AH Investigation: JK, AB, AAR, GL, JC, CS, GABM, JLM Resources:
219 BF, GG, PR, CWdP Writing - Original Draft: JK, GABM, JLM, CS Writing - Review & Editing: all authors,
220 Visualization: JK, AB, Supervision: GABM, JLM, CS, JK Project administration: GABM, JLM, CS, Funding
221 acquisition: GABM, CS, JLM, CWdP

222 Data availability

223 The data that support the findings of this study are openly available in SRA, bioprojects PRJNA748676 and
224 PRJNA212863. We used the annotated V6.1 draft genome of *Amborella trichopoda* available on CoGe, genome
225 ID 50948 (genomevolution.org/coge/GenomeInfo.pl?gid=50948).

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309 **Supporting Information**

- 310 Additional supporting information may be found in the online version of this article.
- 311 **Materials and Methods S1** Additional information about the data and analyses of sex linkage.
- 312 **Table S1** RNAseq statistics for the twenty individuals grown from seeds collected in the wild.
- 313 **Table S2** Read depths for the individuals from the F1 family sequenced using DNA-reseq data.
- 314 **Results S1** Detailed description of the results of the analyses.
- 315 **Table S3** Summary of sex-linkage inference using the RNAseq data from a wild population.
- 316 **Fig. S1** Masking of putative W- and Z-specific regions in the non-recombining region of chromosome 9.
- 317 **Fig. S2** Re-seq coverage per gene using mapping on the unmasked and masked genome assemblies.
- 318 **Fig. S3** Results of the sex-linkage analysis in the mapping population data.
- 319 **Table S4** Sex-biased gene expression analysis.
- 320 **Fig. S4** Sex-biased gene expression inferred from RNAseq data from flower buds.
- 321 **Fig. S5** Distribution of heterozygosity between parents and offspring in the mapping population.
- 322 **Fig. S6** Allele transmission on chromosome 9.
- 323 **Data File S1** bed file indicating the putative W-specific regions for masking.
- 324 **Data File S2** bed file indicating the putative Z-specific regions for masking.
- 325 **Data File S3** Table (xlsx format) with all putatively sex-linked gene models and gene-level statistics.