The wild grape genome sequence provides insights into the transition from dioecy to hermaphroditism during grape domestication

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Grapevine has a major economical and cultural importance since antiquity. A key step in domestication was the transition from separate sexes (dioecy) in wild *Vitis vinifera* ssp. *sylvestris* (*V. sylvestris*) to hermaphroditism in cultivated *Vitis vinifera* ssp. *vinifera*. While the grapevine sex locus is known to be small, its precise boundaries, gene content and the sex-determining genes are unknown. Here we obtained a high-quality *de novo* reference genome for *V. sylvestris* and whole-genome resequencing data of a cross. Studying SNP segregation patterns, gene content and expression in wild and cultivated accessions allowed us to build a model for sex determination in grapevine. In this model, up- and down-regulation of a cytokinin regulator is sufficient to cause female sterility and reversal to hermaphroditism, respectively. This study highlights the importance of neo-functionalization of Y alleles in sex determination and provides a resource for studying genetic diversity in *V. sylvestris* and the genomic processes of grapevine domestication.

Dioecy is rare in flowering plants (~6%) but over-represented among crops (~20%)[1]. In some cases, both wild and cultivated plants are dioecious (*e.g.* date palm, asparagus, persimmons). Other crops, such as grapevine, papaya, and strawberry, derive from dioecious progenitors and switched to hermaphroditism during domestication. The genes underlying this switch are currently not known in any crop. In *Vitis sylvestris*, wild females produce morphologically bisexual flowers, with retracted anthers that produce few and infertile pollen[2], while male flowers undergo early ovule abortion[3]. Genetic analyses identified a 143-kb haplotype responsible for sex-determination in the grapevine reference genome[4,5]. Two candidate genes for female sterility have been proposed, in particular *APRT3*[6], a putative cytokinin regulator.

However, the sequence of the sex locus was not available in *V. sylvestris* and no causative mutations have been identified. A genetic and evolutionary model to explain sex determination and switch to hermaphroditism in grapevine is currently lacking.

We sequenced a female individual of *V. sylvestris* using SMRT-sequencing (120X). Contigs were assembled with falcon-unzip[7] and the grapevine reference genome[8] (PN40024 – version 12X.2) was used to build pseudomolecules. We obtained a high-quality diploid assembly of 469 Mb with a contig N50 of 1.7 Mb, 98% of the gene content anchored on chromosomes and a BUSCO evaluation of 95% (Supplementary Table 1, Supplementary Figure 1), comparing favorably to other recently published plant genomes[9,10]. We annotated 39,031 protein-coding genes on primary contigs.

To study sex determination, we localized the sex locus, which was fully included in the 5th largest contig of the assembly. We also sequenced and assembled bacterial artificial chromosomes (BACs) covering the sex locus in a male of another *V. sylvestris* population and in *Vitis vinifera* cv. Cabernet-Sauvignon. To identify sex-linked polymorphisms in *V. sylvestris,* we produced a cross and resequenced in paired-end Illumina short reads the whole genomes of the parents and 10 offspring, yielding 43-74 millions of read pairs per individual (Supplementary Table 2). To overcome issues due to X-Y divergence, we used an iterative SNP-tolerant mapping procedure[11], eventually mapping 98.2% of reads in average (mapping coverage by individual 14x-28x, Supplementary Table 3-6). Single nucleotide polymorphisms (SNPs) that segregate with sex in our cross were identified with an empirical approach and with SEX-DETector^{++}, a new version of the probabilistic method SEX-DETector that identifies sex-linked genes from patterns of segregation in a cross from RNA-seq data[12], which we developed here to analyse genomic data. As SNPs close to the sex locus might be genetically linked to the locus in a particular cross, we used public whole-genome resequencing data[13] (Figure 1a-b, Supplementary Tables 7-8) to determine the boundaries of

75 the sex locus. We searched for SNPs that were sex-linked in our cross and that were always
76 heterozygous in males and homozygous in females in the validation dataset. We found that
77 the X haplotype of the sex-locus spanned 111 kb on chromosome 2 (4,810,929 to 4,921,949
78 bp, Figure 1b-c, Supplementary Figures 2-5) and we obtained a final dataset of 1,865 XY
79 SNPs (Supplementary Data File 1). To investigate changes associated with transition to
80 hermaphroditism, we investigated the genotypes at the sex locus in publicly available DNA-
81 seq data of 13 grapevine cultivars[14] (Supplementary Table 9). Out of 13 cultivars, six
82 harbored recombinant genotypes. The 5' part of the sex locus was always either XH or HH
83 and spanned 93 kb (4.810-4.903 Mb), H indicating the hermaphrodite haplotype, derived from
84 the Y haplotype[5]. The remaining part of the sex locus (4.903-4.922Mb) was either XX or XH,
85 but never HH (Figure 1b, Supplementary Table 10). The genes essential for the male
86 phenotype should therefore be located in the 93 kb region of the sex locus.

87 Next, we investigated the age of the sex locus, and the presence of deletions and insertions of
88 transposable elements in the Y haplotype that may indicate degeneration. We calculated the
89 synonymous divergence (dS) between X and Y coding DNA sequences (CDS) of XY gene
90 pairs (Figure 1c) as a proxy of the age of the system (Figure 1d). The two genes at the
91 opposite limits of the non-recombining region had a dS of 0.03, suggesting that suppression of
92 recombination occurred very recently and in a single step in *V. sylvestris*. The maximum dS
93 was 0.0424, which is to our knowledge lower than any of the other systems dated in plants
94 (*Mercurialis annuua* has a maximum dS of 0.05[15]). To identify deletions in the Y haplotype,
95 we compared the X haplotype and two BAC contigs covering the Y haplotype
96 (Supplementary Figures 2-5). We also measured the mean mapping coverage in our
97 resequencing dataset, searching for two-fold reductions in males. This revealed eight regions
98 spanning 500 to 5,500 bp and affecting one predicted gene (Supplementary Figure 5). We also
99 detected insertions of transposable elements in the Y haplotype (Supplementary Figures 2-5,

100 Supplementary Data File 2). This suggests that the Y haplotype of *V. sylvestris* is already
101 degenerating despite recombination suppression being very recent.

102 Theoretical work predicts that XY sex chromosomes should combine a recessive male-
103 sterility mutation (X) and a dominant female-sterility mutation[16] (Y). To identify candidates,
104 we compared the gene content of X, Y and H haplotypes (Figure 2a) and searched for
105 presence/absence patterns and loss-of-function mutations (Figure 2b). We also mapped a
106 public RNA-seq dataset of males, females and hermaphrodites flower buds of *V. sylvestris* at
107 four developmental stages[13] (Supplementary Table 11), measuring the total (Figure 3a) and
108 the allele-specific (X, Y and H, Figure 3b) expression of transcripts.

109 First, we investigated the presence of X recessive mutations possibly to causing male sterility.
110 We found that the gene at one limit of the sex locus, annotated as *INAPERTURATE* (*INP1)*[17],
111 showed a 8-bp deletion in exon2 in all female haplotypes, resulting in a premature stop codon
112 and a truncated protein (Figure 2b). *INP1* loss-of-function mutant in *A. thaliana* lacks pollen
113 apertures[18], similarly to the pollen of female *V. sylvestris*[2]. We found that *INP1* was highly
114 specific of mature flower buds of *V. vinifera*, consistent with a role in late pollen
115 development[19]. It remains to be shown that the absence of apertures in grapevine female
116 pollen is sufficient to cause sterility, as inaperturate sterile pollen evolved independently at
117 least six times in Eudicots in association with dioecy[20], but the *A. thaliania INP1* knock-out
118 mutant is fertile[19]. We also found that four genes were absent from the X haplotype (Figures
119 1a-2b Supplementary Figure 2). They included a gene previously annotated[5] as a short
120 homologue of Ethylene-overproducer-like-1 (*ETOL1)*, but also showing homology to *NPG1*.
121 *NPG1* is essential to pollen germination in *A. thaliana*[21], and the ethylene pathway has been
122 shown to be determinant in floral morph determination in *Cucumis melo*[22]. Three other genes
123 were also absent from the X haplotype, namely a gene encoding a short peptide of unknown
124 function, *NAP1* and *UAP56A*. *NAP1* encodes a nucleosome assembly protein in *A. thaliana*.

125 Two homologues of *UAP56A,* a DEAD-box ATP-dependent RNA helicase, have been shown
126 to regulate programmed cell death during tapetum development in *Oryza sativa*[23], and
127 disrupting them led to male sterility. None of these three genes were significantly expressed in
128 the early developmental stages of the RNA-seq dataset that were produced to study ovule
129 development (Supplementary Figure 6), likely because male sterility occurs later than female
130 sterility in *V. sylvestris* flowers[13]. *INP1* and the four X-deleted genes were all present in at
131 least one haplotype in cultivars. These results suggest that several recessive deletion
132 mutations affecting genes involved in tapetum and pollen development may cause the male
133 sterility syndrome (Figure 4).

134 Second, we searched for dominant mutations causing female sterility in the Y haplotype and
135 for possible mechanisms of reversion to hermaphroditism. So far, sex-determining genes that
136 have been identified in plants were Y-specific genes that arose through duplication[24–26]. The
137 Y-specific genes described above (*INP1*, *ETOL1*, *UNK* and *UAP56*) are not expressed at the
138 stage where ovule abortion occurs and are unlikely candidates for female sterility
139 (Supplementary Figure 6). Therefore, we searched for differential expression of X and Y
140 alleles in XY gene pairs. Three genes showed higher total expression in males than in females
141 in the sex locus: *VviPLATZ*, *VviFSEX* and *APRT3* (Figure 3a). *VviPLATZ* and *APRT3* showed
142 a similar expression pattern in males, with a two-fold induction during bud development.
143 However, *APRT3* was also expressed in female while *VviPLATZ* expression was male-
144 specific. Interestingly, only the Y alleles of *VviPLATZ* and *APRT3* (*VviPLATZy* and *APRT3y*)
145 were induced in male buds (Figure 3b, Supplementary Figure 7). *VviFSEX* showed a
146 decreasing versus stable expression in female and males, respectively, with equal expression
147 of its X and Y alleles. The promoters of *APRT3x* and *APRT3y* were structurally similar, but a
148 Y-specific transposable element may be present upstream of *VviPLATZy* (Supplementary
149 Text), which could explain its up-regulation. *APRT3* is the ortholog of a gene encoding an

150 enzyme of cytokinin elimination. Exogenous application of cytokinin is sufficient to restore
151 female fertility in male *V. sylvestris*[27]. Previous *In situ* hybridisation work suggested that
152 APRT3 was induced during male bud development and absent in female buds[6]. Here we
153 found that *APRT3x* is expressed both in female and male buds, and is an ubiquitously
154 expressed gene in *V. vinifera* (Supplementary Figure 8, Supplementary Table 12). Since all
155 analysed cultivars possessed at least one *APRT3x* allele (recombinant *APRT3x/APRT3x*
156 genotypes were observed but no *APRT3y/APRT3y*, Figure 1b), we hypothesize that *APRT3x*
157 performs essential functions in *V. sylvestris* and in grapevine. *VviPLATZ* is a transcription
158 factor of the PLATZ family. Interestingly, in the RNA-seq dataset, we found that *VviPLATZy*
159 was expressed at a lower level in hermaphrodites than in males and that *APRT3* was not
160 induced as in males (since it is RNA-seq data, the hermaphrodite genotype at *APRT3* is
161 however unknown, Figure 3a). A tentative scenario for female sterility would be that
162 *VviPLATZy* specifically activates *APRT3y*, leading to a decrease in cytokinin concentration in
163 ovule and causing its abortion (Figure 4). Interestingly, a 10 kb repeated element is present
164 1.2 kb upstream of *APRT3* in all H haplotypes but absent from X haplotypes (Figure 2a), and
165 might influence the expression of *APRT3*. From an evolutionary perspective, female sterility
166 in grapevine would originate from neo-functionalization of the Y allele of an ubiquitous gene,
167 the X allele conserving the ancestral function. Down-regulation of *VviPLATZy* and/or
168 *APRT3y* may be sufficient to cause reversal to hermaphroditism (Figure 4, Supplementary
169 Text). In addition (but not in a mutually exclusive way), transition to hermaphroditism may be
170 caused by recombination upstream of the *APRT3* promoter leading to the *APRT3x/APRT3x*
171 genotype that is observed in several cultivars (Figure 1b).

172 Following an extensive analysis of the *V. sylvestris* sex locus, we have proposed a model for
173 sex determination in grapevine, based on a combination of deletion of X alleles and neo-
174 functionalisation of Y alleles. In our model, the switch from dioecy to hermaphroditism was

175 easy, as a single genomic change (possibly two) was needed. This is in agreement with recent
176 studies[28] suggesting that dioecy is rare in flowering plants because it has a high reversion rate
177 to hermaphroditism. Future genetic and functional studies will allow dissecting the role of the
178 pinpointed genes in different aspects of the male sterility syndrome, female sterility and
179 reversal to female fertility in hermaphrodites.

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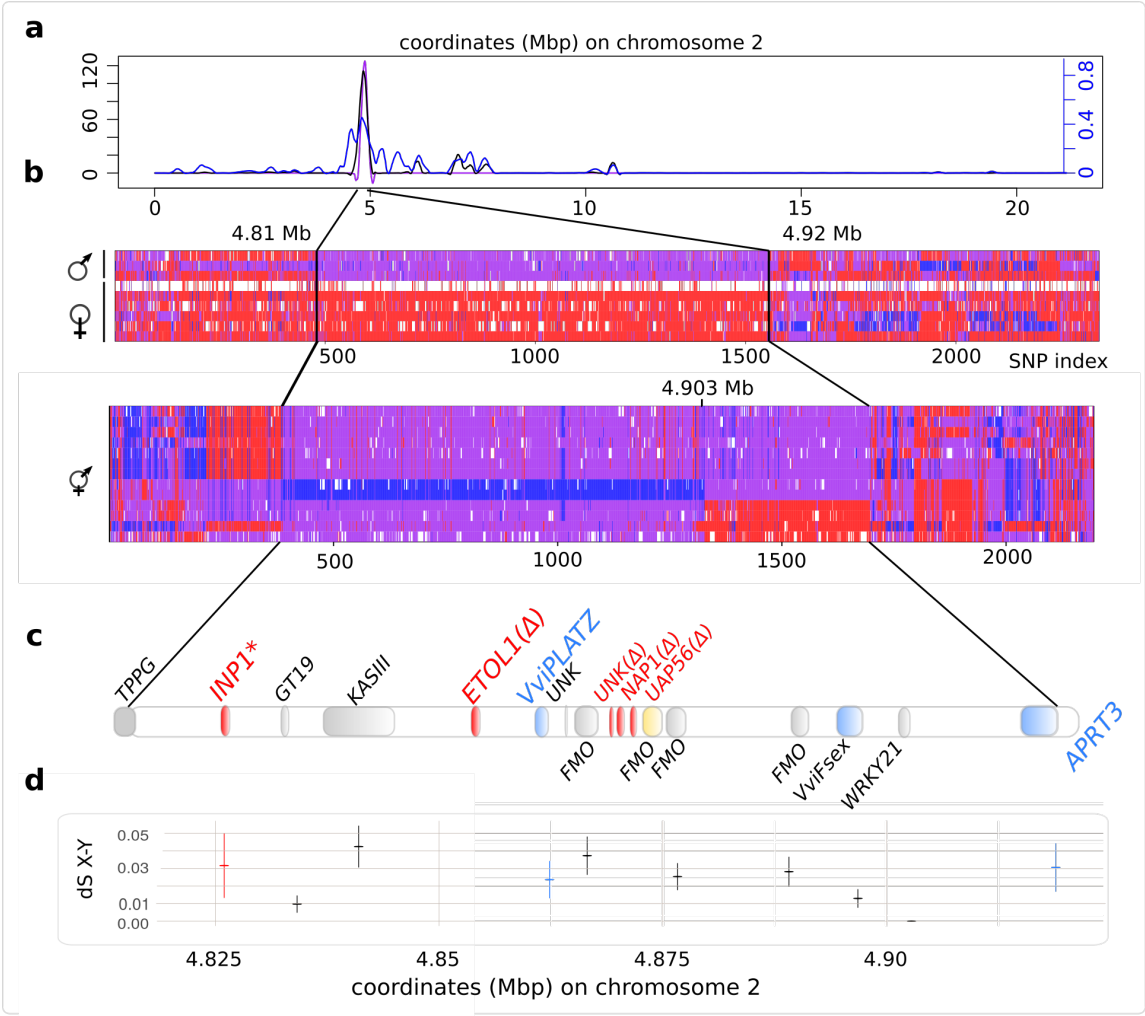
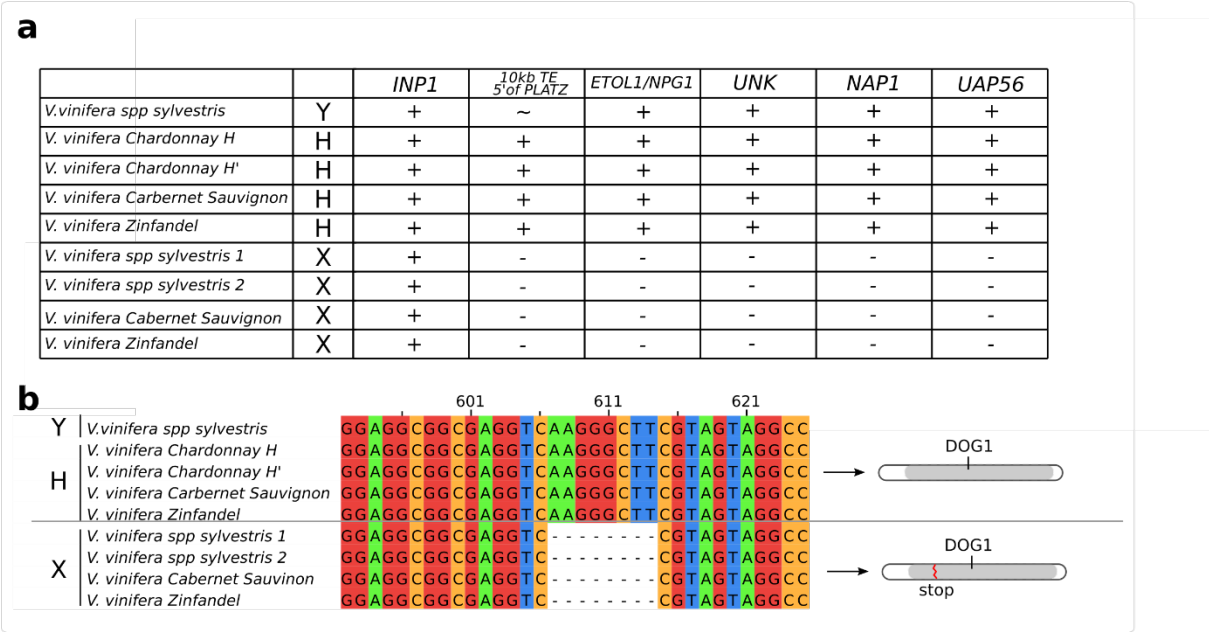


Figure 1: Limit, gene content and synonymous divergence in the sex locus of *V. sylvestris* and *V. vinifera*. a, Detection of sex-linked SNPs on chromosome 2. left y axis: adjusted SNP number in 10kb windows, black curve: XY single nucleotide polymorphisms detected by SEX-DETector++ in a cross of *V. sylvestris*. Purple curve: candidate XY SNPs that show heterozygosity in males and homozygosity in females in a validation dataset of public whole-genome resequencing is drawn in purple; right y-axis (blue curve): adjusted mean posterior probability of being XY for SNPs in 10kb windows. **b,** Genotype of nine *V. sylvestris* individuals (top) and thirteen grapevine cultivars (bottom) in a validation dataset of public whole-genome resequencing, at locations of candidates XY SNPs. Red, purple and blue, and white traits represent XX, XY, YY and missing genotypes, respectively. The black lines highlight the limits of shared XY SNPs between the cross resequenced in the present study and the validation dataset. **c,** Gene content and annotation and in the sex locus (approximate position). Genes highlighted in red are absent (Δ) or possess a frameshift deletion in the X haplotype. Genes highlighted in blue are induced in males. The yellow gene is absent in the Y haplotype. **d,** synonymous divergence between X and Y allele in the sex locus (+/- standart error), reflecting the age of recombination suppression. dS was only computed for genes present in both haplotypes.



a

		INP1	10kb TE 5'of PLATZ	ETOL1/NPG1	UNK	NAP1	UAP56
V.vinifera spp sylvestris	Y	+	~	+	+	+	+
V. vinifera Chardonnay H	H	+	+	+	+	+	+
V. vinifera Chardonnay H'	H	+	+	+	+	+	+
V. vinifera Carbernet Sauvignon	H	+	+	+	+	+	+
V. vinifera Zinfandel	H	+	+	+	+	+	+
V. vinifera spp sylvestris 1	X	+	-	-	-	-	-
V. vinifera spp sylvestris 2	X	+	-	-	-	-	-
V. vinifera Cabernet Sauvignon	X	+	-	-	-	-	-
V. vinifera Zinfandel	X	+	-	-	-	-	-

Figure 2: Presence-absence patterns and frameshifts mutations in X, Y and H haplotypes. a, Presence-absence patterns of five genes absent or truncated in the X haplotype of *V. sylvestris*, and of a 10kb repeated element 1.2 kb uptream of a gene of the PLATZ family of transcription factors. The tild indicates that the estimated length of the gap in the Y haplotype inferred is consitent with the presence of the element, but is has not been sequenced yet. **b,** An 8-bp deletion in exon 2 of the *INP1* gene is shared by X haplotypes and absent in Y or H haplotypes. It results in a premature stop codon, leading to a shorter protein. The DOG protein domain, involved in DNA binding, is truncated.

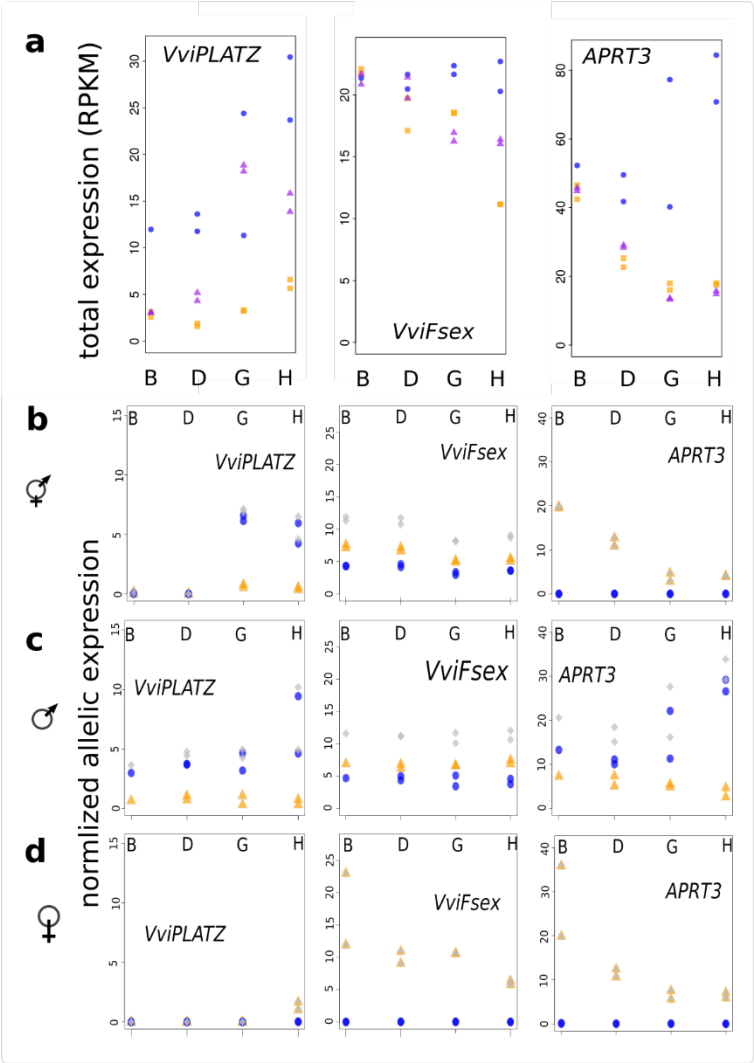


Figure 3: Total and allele-specific expression of female-sterility candidates during flower bud development. B, D, G and H represent early development stages of flower development sequenced in RNA-seq in Ramos et al[13]. **a,** total normalized expression (RPKM) of *VviPLATZ*, *VviFsex* and *APRT3* in males (blue circles), females (orange squares) and spontaneous hermaphrodites (purple triangles) of *V. sylvestris*. Each point represents a biological replicate. **b,** allele-specific normalized expression. Blue dots, orange triangles, and grey diamonds represent Y-specific, X-specific and summed expression, respectively. RNA-seq data were genotyped and the coverage of X and Y variants was extracted from vcf file and averaged by gene. Read counts were normalized by library.

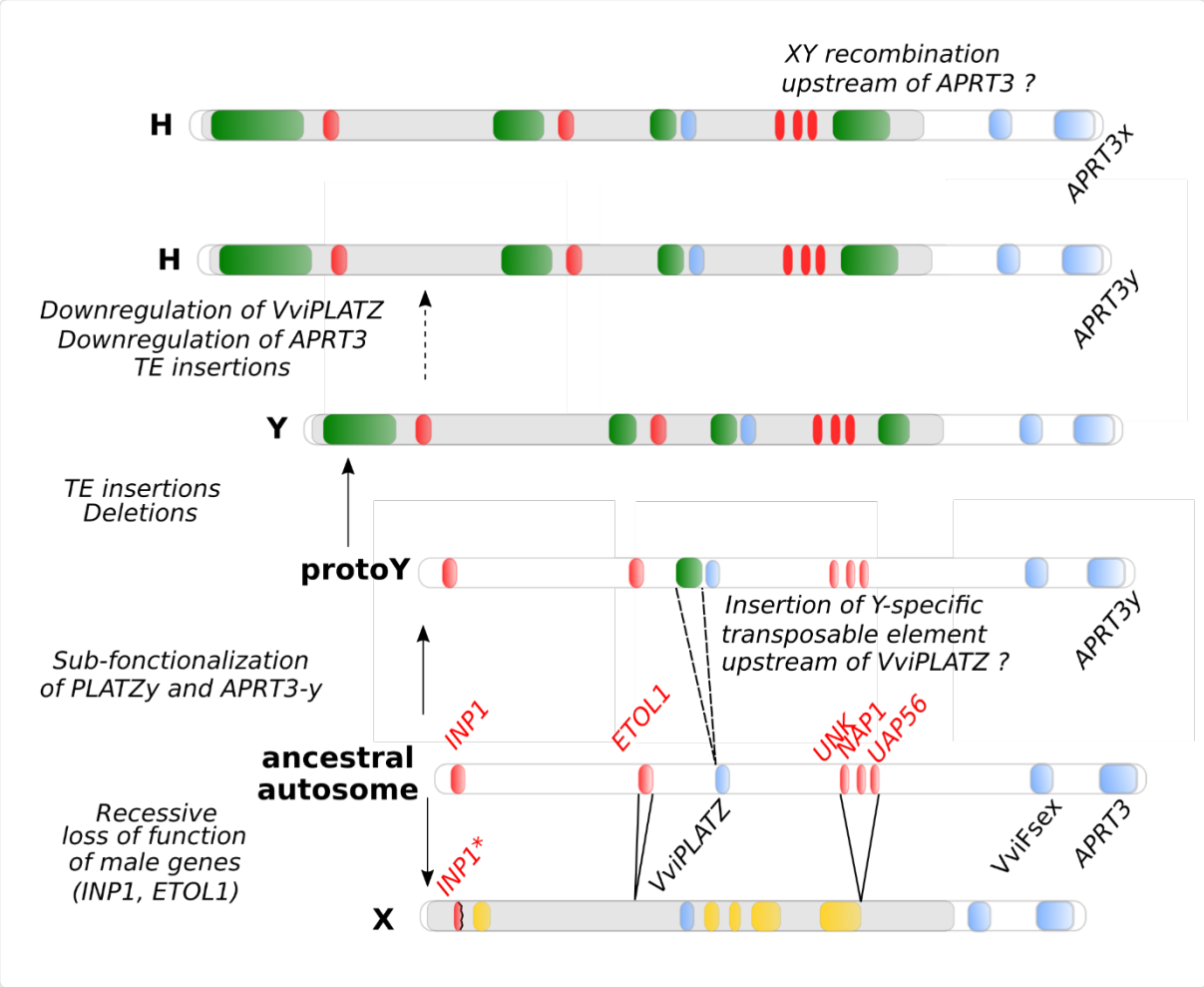


Figure 4: Evolutionary genomic scenario of the formation of the X and Y haplotypes and reversal to hermaphroditism. Red: genes partially or completely deleted in the X haplotype. Blue: genes differentially expressed between male, female and hermaphrodite during early flower bud development. On the schematic sex locus, yellow highlights represent X-hemizygote regions (*i.e.*, absent from the Y), green highlights represent transposable elements, red highlights male-sterility candidates and blue highlights female-sterility candidates. Grey highlight represents the part of the sex locus where homology between X and Y is reduced due to deletions or insertions.

Data Availability

All raw reads, genome assembly and gene annotation are currently under submission.

Code Availability

The source code of SEX-DETector++ is available at the url *https://gitlab.in2p3.fr/sex-det-family/sex-detector-plusplus.*

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Contributions

GABM, PH, CR, ED, RT and RB conceived the research. VD and ED obtained plant material and DNA for cross resequencing and genome sequencing. WM obtained DNA for whole genome PacBio sequencing. TF and RB obtained plant material and DNA for BAC sequencing, and with SS defined probes for BAC screening. SV and HBe performed BAC library production and screening, BAC sequencing and assembly. AV, CR and JC assembled the genome. RB and TF analysed BAC sequences and assigned them to sex haplotyes. SS sequenced the gaps between BAC contigs. WM, HBe, ER, KJ and CR coordinated the sequencing of the genome. ED and CR coordinated the sequencing of the transcriptome. GABM coordinated the sequencing of the cross. AV, CR and JC assembled the genome. JG and RT assembled the transcriptome. AV, CR and ES annotated the genome and the BAC sequences. FG, FP, DG, JK and GABM developed SEX-DETector++. HBa, AV and CR analysed RNA-seq data. HBa analysed WGS data and performed structural and molecular evolution analysis of the sex locus. HBa, CR and RB performed integrative analysis of sex determination. PH, RT, GABM and RB funded the study. CR, GABM and RB coordinated the project. HBa and GABM wrote the manuscript, with contributions from all authors.

Guide to Supplementary Material

Material and Methods:

- Statistical analyses and data visualisation.
- Sequencing data
- Genome assembly, cleaning, polishing, anchoring and ordering.
- BAC generation and sequencing.
- Genome and BAC annotation.
- Development of SEX-DETector++.
- Characterization of the sex-linked region in the *V. sylvestris* genome.
- Integrative search for sex-determining genes in the sex locus.
- Supplementary References

Supplementary figures:

-Supplementary Figure 1: BUSCO analysis of the genome annotation.

-Supplementary Figure 2-4: Structural comparisons of X and Y, X and H and Y and H haplotypes, respectively.

-Supplementary Figure 5: Density of sex-linked SNPs in a cross between two *V. sylvestris* parents.

-Supplementary Figure 6: Total normalized gene expression in males, females and hermaphrodites of *V. sylvestris* for genes in the sex locus during flower bud development.

-Supplementary Figure 7: Allele-specific expression of X and Y alleles of XY genes pairs of *Vitis sylvestris* during the development of flower buds.

-Supplementary Figure 8: Distribution of organ-specificity expression of genes in *Vitis sylvestris*.

Supplementary tables:

-Supplementary Table 1: Genome assembly and anchoring statistics

-Supplementary Table 2: Summary statistics of resequencing data of a cross between two *V. sylvestris* parents

-Supplementary Table 3: Mode of the gaussian distribution of mapping coverage for each sample for the dataset of whole genome resequencing of a cross in *V. sylvestris*.

-Supplementary Table 4: Summary statistics of iterative SNP-tolerant mapping for the resequencing dataset of a cross in *V. sylvestris*.

-Supplementary Table 5: Summary statistics of missingness per sample for each iteration of SNP-calling in the dataset of whole-genome resequencing of a cross in *V. sylvestris*.

-Supplementary Table 6: Number of homozygous and heterozygous SNPs called at each iteration in the dataset of whole-genome resequencing of a cross in *V. sylvestris*.

-Supplementary Table 7: Whole-genome resequencing dataset of the ncbi short read archive that were mapped to the *Vitis sylvestris* genome.

-Supplementary Table 8: Statistics of mapping and SNP-calling of a public whole-genome resequencing dataset of the ncbi that was mapped to the *Vitis sylvestris* genome.

-Supplementary Table 9: Summary of the genotype of 13 cultivars in the sex locus on chromosome 2 inferred from whole-genome resequencing data.

-Supplementary Table 10: Predicted genes in the sex locus of *V. sylvestris*. Coordinates and geneID are indicated for the *V. sylvestris* reference genome.

-Supplementary Table 11: Mapping statistics of public RNAseq libraries of flower buds of female, male and hermaphrodite *V. sylvestris* against the *V. sylvestris* genome.

-Supplementary Table 12: Public RNAseq dataset of *V. vinifera* and *V. sylvestris* mapped to the *Vitis sylvestris* genome.

Supplementary text:

This text describes the results of BAC assembly and the attemps to characterize and to cover the 13kb gap on the male haplotypes.

Supplementary data files:

-Supplementary_DataFile1.xlsx: genomic location of sex-linked single nucleotide polymorphisms in the genome of *Vitis sylvestris*.

-Supplementary_DataFile2.xlsx: genomic location of repeated elements in the genome of *Vitis sylvestris*.

-egnep.Vvi.for_Sylvestris.cfg: configuration file of Eugene annotation.

Supplementary Information

Table of contents

Material and Methods 2
Supplementary Figures 10
Supplementary Tables 19
Supplementary Text 33

Material and Methods

Statistical analyses and data visualisation. Unless stated otherwise, statistical analyses were carried out in R v3.4.4 (2018-03-15)[29]. Data visualisation were performed with R or with circos v0.69-6[30]. Adeneget[31] was used to visualize SNP data in R.

Sequencing data

Whole genome sequencing (PacBio – long reads) of Sylvestris C1-2. The accession of Sylvestris C1-2 with plant code 8500.Col.C1-2, origin of the geographic location Sainte-Croix-en-Plaine, Haut-Rhin (68), France, was used for extraction of high-molecular-weight genomic DNA. The extraction was performed by the CNRGV (Centre National de Ressources Génomiques Végétales) – INRA Toulouse. They started with 1G of frozen material (-80°C), crushed it with liquid nitrogen and used the Genomic-tip 100/G kit (Qiagen) for the extraction. The SMRT library preparation and sequencing on PacBio RSII platform (P6-C4 chemistry) was done by the IGM Genomics Center at the University of California, San Diego, following the standard PacBio protocols. A total of 129X coverage was obtained in order to perform *de novo* genome assembly.

Whole genome sequencing (Illumina – short reads) of Sylvestris C1-2. DNA libraries with two different library size from leaves of Sylvestris C1-2 were prepared in order to perform 2*151 paired-end short reads sequencing (Illumina). One library had a library size of 740bp (860 bp with adapters) and the other library had a library size of 392bp (512bp with adapters). The two libraries were sequenced on the same flowcell lane. The libraries preparation and Illumina sequencing was performed by the EPGV (Etude du Polymorphisme des Génomes Végétaux) – INRA. A total of 338,109,086 reads was obtained (~100X coverage) for all samples together, used to polish the Sylvestris C1-2 PacBio assembly.

RNA-sequencing of Sylvestris C1-2. RNA-sequencing was performed on six samples of the Sylvestris C1-2 accession. Three biological replicates of whole green berries and three biological replicates of whole mid-ripening berries were sequenced. The RNA-seq library preparation was performed with the TruSeq Stranded mRNA Library Prep Kit (Illumina) and was sequenced in paired-end (2*100b) on HiSeq4000 platform (Illumina technology). Library preparation and sequencing was performed by the GenomEast platform – Strasbourg. A total of 1,113,531,260 reads was obtained for all samples together, used to assemble a Sylvestris transcriptome and perform genes annotation.

Whole genome resequencing of a cross between C1-5 (Female Vitis sylvestris) and Martigny_2 (Male Vitis sylvestris). These two accessions were crossed in the Inra Colmar ampelographic collection. The accession C1-5 female *Vitis sylvestris* (plant accession number at Inra Colmar 8500.Col.C1-5) originated from the geographic location Sainte-Croix-en-Plaine, Haut-Rhin (68), France, and the male accession Martigny_2 (plant accession number at Inra Colmar 8500.Col.1), was an accession originating from the geographic location Martigny, Swiss. The progeny was grown at Inra Colmar greenhouses. The DNA samples of both parents, of five male descendants (44613.Col.5026T, 44613.Col.5028T, 44613.Col.5029T, 44613.Col.5033T and 44613.Col.5053T) and five female descendants (44613.Col.5035T, 44613.Col.5040T, 44613.Col.5046T, 44613.Col.5050T and 44613.Col.5057T) were sequenced. The quantity and quality of the DNA extracted from

leaves (DNeasy plant mini kit, Qiagen) of 12 individuals from the cross (2 parents and 10 offspring) was checked prior to sequencing. Nanodrop analysis indicated that concentrations were always above 110 ng.µl^{-1}, and that more than 5 µg DNA was available for all samples. Quality (fragment size) was checked using capillary electrophoresis (Fragment Analyzer) and was satisfactory. Based on quality check, we adapted sonication (using Covaris E220) in order to get mostly fragments of 250 bp. Twelve Illumina libraries were constructed and were pooled for two lanes of sequencing on an Illumina Hiseq 4000 machine in 2x100bp paired-end mode. This yielded between 85 and 149 millions reads per individual (Supplementary Table 2), which roughly corresponded to 17X to 30X coverage of re-seq data per individual.

Genome assembly, cleaning, polishing, anchoring and ordering.

Shortly, the Sylvestris C1-2 *de novo* genome assembly was performed with Falcon-integrate (Falcon + Falcon-unzip), in order to obtain a diploid assembly (the two haplotypes). With Falcon, PacBio reads were self-corrected, assembly was performed, haplotypes were generated, assembly was polished with Arrow and finally we obtained a phased diploid assembly of Sylvestris C1-2.

Genome assembly and phasing. The FALCON-integrate 1.8.4 tool used is available on github: https://github.com/PacificBiosciences/FALCON-integrate/tree/1.8.4. The FALCON parameters used for the *Vitis sylvestris* genome assembly are taken from the genome assembly of *Vitis vinifera* cv Cabernet Sauvignon paper[7] (pa_HPCdaligner_option = -v -dal128 -e0.75 -M60 -l2500 -k18 -h1250 -s100 -w8; ovlp_HPCdaligner_option = -v -dal128 -M60 -e.96 -l1500 -s100 -k24 -h1250; pa_DBsplit_option = -a -x500 -s200; ovlp_DBsplit_option = -s200; falcon_sense_option = --output_multi --min_idt 0.70 --min_cov 4 --max_n_read 400 --n_core 8; falcon_sense_skip_contained = False; overlap_filtering_setting = --max_diff 120 --max_cov 120 --min_cov 4 --bestn 10 --n_core 8). The phasing and haplotypes creation was performed with Falcon-unzip, with default parameters. The assembly was polished with FALCON and PacBio reads using Arrow (available in Falcon-integrate).

Assembly polishing and finishing. After the FALCON run, we performed additional polishing with Illumina reads. First, illumina reads were aligned with bwa[32] mem and -M option on FALCON's genome assembly. Then, alignments were filtered with samtools to keep only primary alignments and concordant pairs. Finally, alignments were filtered with bamtools to keep alignments with an edit distance <= 5. These filtered aligned reads are used to polish the assembly with the pacbio-util (version 0.2) from pacbio-utilities tool (https://github.com/douglasgscofield/PacBio-utilities). Then, the same alignment was performed on the genome polished with pacbio-utils and this genome was polished with Illumina reads and PILON (v1.22 - https://github.com/broadinstitute/pilon). A few haplotigs may have remained in the primary contigs file. A tool is available, purge_haplotigs, to find these false primary contigs in order to move them to the haplotigs file. We used this tool to correct this and to finish our genome asssembly (v1.0.4 - commit 6414f68 - https://bitbucket.org/mroachawri/purge_haplotigs/src/master/).

Quality control of the assembly. Assembly statistics such as number of contigs, N50 and L50 were calculated with a home-made script. Genome assembly completeness was assessed with BUSCO[33] with the genome mode, the embryophyta_odb9 lineage and the *Arabidopsis* species

options (version 2.0 - https://gitlab.com/ezlab/busco). Nucmer tool (from MUMmer tool: https://sourceforge.net/projects/mummer/ - nucmer (version 3.1) parameters: -maxmatch -l 100 -c 500) and the grapevine reference genome[8] (PN40024, version 12X.2 - https://urgi.versailles.inra.fr/Species/Vitis/Data-Sequences/Genome-sequences) were used to align the Sylvestris assembly to the PN40024 reference assembly in order to see completeness of this Sylvestris assembly. Nucmer and grapevine reference genome were also used to anchor and order Sylvestris contigs into chromosomes (with additional parameters –r –q), as it was done in the Chardonnay genome assembly paper[34].

BAC generation and sequencing.

Generation of BAC library of a Vitis sylvestris male sequence. To obtain the DNA sequence of the Y allele in the sex region, we proceeded as follows. We chose a *Vitis vinifera* ssp. *sylvestris* male from a wild population spontaneously growing on a hill forest near Montpellier (France), on the Northern slope of the Pic Saint Loup mountain. Its male phenotype was confirmed over five years of observation of the flowers, both on the forest plant and on 5 of its clones planted in the INRA Vassal Grape Collection in Marseillan, France (introduction name: Lambrusque PSL10; introduction number: 8500Mtp107). High Molecular Weight DNA was isolated from 40 g of PSL10 young leaves following the cell nuclei extraction method described in Peterson *et al.*[35] (2000) with slight modifications[36]. The long DNA fragments were partially digested with EcoRI restriction enzyme, and fragments from 100 to 250 kb were selected. Sized and eluted DNA was then ligated into a pAGIBAC-EcoRI cloning vector and cloned into DH10B T1R *Escherichia coli* strain (Invitrogen). The resulting BAC clones were plated on a solid selective medium and organised in barcoded microplates using a robotic workstation QPix2 XT (Molecular Devices). The BAC library was named Vsy-B-Lamb. It consists of 27,648 clones of 113kb size in average, representing a 6.4x genome equivalent coverage. The library was replicated for security reason and the two copies were stored in separate freezers at -80°C (resource available at https://cnrgv.toulouse.inra.fr/fr).

BAC library screening. The bacterial clones were deposited on a macroarray nylon membrane (22x22 cm), following a 6x6 grid pattern. Three copies of this gridded macroarray were created. To select BAC clones carrying the DNA fragments from the sex locus, we used specifically designed radio-labelled probes. These probes were defined using the sequences published in Picq et al (2014)[5]: VSVV006, VSVV007, VSVV009, VSVV010, VSVV011 (GeneID GSVIVT01001275001, GSVIVT01001277001, GSVIVT01001286001, GSVIVT00007310001, GSVIVT00007312001 respectively). The hybridisation of 3 separate pools of probes allowed to spot around 200 putatively positive BAC clones in total; the 30 clones showing the most intense spots were individually tested via Real-Time PCR using the same sequences used for probe design and 9 clones were validated. The same clustering of the 9 positive clones into two groups of alleles was obtained using two approaches: the first assignation based on their melting temperature curves similarities; and the second based on Sanger sequencing of internal and BAC-end sequences. These two groups correspond to the two alternate alleles expected in a "XY-like" sex region. Internal and BAC-ends sequences were also used to map the BACs on each other, for each allele, with the objective to sequence

138 the minimal number of clones with an optimised overlapping to cover the whole region
139 (minimum tilling paths composed of 3 and 4 clones respectively).

140 *BAC sequencing and assembly of X and Y haplotypes*. The sequences of the 7 sex-region
141 BACs of *V. sylvestris* were obtained via a PacBio RSII sequencer (P6C4 chemistry).
142 Sequencing was done in a pool of 20 individually tagged BAC clones. We used 2 µg of each
143 individual BAC clone DNA to prepare the PacBio SMRT® 10kb library. GeT-PlaGe
144 Genomic Platform (INRA-Toulouse, France) handled the sample loading on the RSII device
145 and the data retrieval. We performed the detection and removal of residual *E. coli* sequences
146 on raw reads. After a second cleaning step consisting in detecting and removing the vector
147 sequences, individually-tagged BAC sequences were assembled with the HGAP workflow
148 (https://github.com/PacificBiosciences/Bioinformatics-Training/wiki/HGAP).
149 For each of the two groups of BACs identified as above, the BAC sequences were joined
150 using their overlapping, so to form a long "haplotig". One haplotig was then assigned to the
151 "male haplotype" and the other to the "female haplotype" using the sex-discriminating
152 polymorphisms described by Picq et al[5] (2014), namely 10 SNPs for VSVV006, 7 SNPs for
153 VSVV007 and 6 SNPs for VSVV009. These SNPs were found to be 100% associated with
154 sex, in a worldwide collection of 22 males, 23 hermaphrodites and 91 females (Picq et al[5]
155 2014), and we confirmed that the two haplotigs had either all male SNPs or all female SNPs.

156

157 **Genome and BAC annotation.**

158 *Genome annotation*. RNA Illumina paired-end reads obtained from grape berries of Sylvestris
159 C1-2 (see above) were used to assemble a transcriptome in order to annotate the genome.
160 Prinseq-lite[37], Ribopicker[38] and STAR[39] were used to clean the data, and the assembly was
161 made using Trinity[40]. We assembled 398,189 putative transcripts (422,652,106 bp), with a
162 N50 of 2,217 bp, which roughly corresponds to the average size of Pinot noir transcripts
163 (2,056 bp). TransDecoder[40] detected 187,734 CDS with an average size of 832 bp (of which
164 60,985 CDS are larger than 832 bp). A BUSCO analysis detected 1,237 genes, of which 1,166
165 (94%) are complete. EuGene-EP[41] (Eukaryote Pipeline - v1.4 - http://eugene.toulouse.inra.fr/)
166 was used to perform genes annotation on the Sylvestris assembly. The protein databases used
167 for the genes annotation were the TAIR10, swissprot and uniprot plants databases. The
168 transcriptomes used for the genes annotation were our Sylvestris transcriptome, 812 manual
169 annotated genes, and transcript sequences of *Vitis vinifera* from NCBI - 2017-11-08. The
170 configuration file with all the parameters used is available as supplementary data
171 (egnep.Vvi.for_Sylvestris.cfg). Primary contigs and haplotigs were annotated separately, with
172 the same parameters. Finally, we obtained two genes annotations, one for primary contigs and
173 one for haplotigs, with different files: genes annotation in a gff3 file,
174 gene/mrna/cds/ncrna/protein sequences in separate fasta files and some statistics per gene.

175 *BAC annotation*. The BAC sequences (*V. sylvestris* Male haplotype P2, *V. sylvestris* Male
176 haplotype P1, *V. sylvestris* Female haplotype, *V. vinifera* Cabernet Sauvignon Hermaphrodite
177 genome sequences and the annotation with EuGene[41] (Eukaryote Pipeline - v1.4 -
178 http://eugene.toulouse.inra.fr/) was started, with same parameters and reference files than for
179 the genome annotation. The protein databases used for the genes annotation were the TAIR10,
180 swissprot and uniprot plants databases. The transcriptomes used for the genes annotation were
181 our Sylvestris transcriptome, 812 manual annotated genes, and transcript sequences of *Vitis*

182 *vinifera* from NCBI - 2017-11-08. The configuration file with all the parameters used is
183 available as supplementary data (egnep.Vvi.for_Sylvestris.cfg). Then the annotations specific
184 to BACs were extracted and used as final BACs annotation. We did not run Eugene on the 5
185 BAC sequences alone because EuGene learns from data and we did not want to introduce a
186 bias due to the low number of BAC sequences.

187

188 **Development of SEX-DETector++.**
189 We developed a new version of the SEX-DETector software, that implements a probabilistic
190 method to study SNPs segregation in a family, taking into account genotyping errors. SEX-
191 DETector++ is coded in C++ and uses additional algorithmic optimisation to reduce the
192 running time and memory usage by about two orders of magnitude compared to the original
193 code. SEX-DETector++ thus allows convenient usage on large, genome-wide genotyping
194 datasets for which the original code would require prohibitively large running times and
195 memory. The underlying model is the same as in the original code, but new functionalities
196 were added to deal with genomic data as an input of the method (in the vcf format). The code
197 is publicly available at https://gitlab.in2p3.fr/sex-det-family/sex-detector-plusplus, where
198 technical and installation details can also be found.

199

200 **Characterization of the sex-linked region in the *V. sylvestris* genome.**
201 *SNP-tolerant mapping and SNP discovery from whole-genome resequencing data.*
202 Divergence between X and Y alleles can prevent SNP discovery. To avoid this issue, we
203 carried out an iterative SNP-tolerant mapping similarly to the procedure described in Prentout
204 *et al.*[11]. Raw reads of twelve individuals of the same family (2 parents and 5 descendants of
205 each sex) were first mapped against the *V. sylvestris* genome with gsnap[42] v2018-07-04 (-m
206 0.1) in standard mode. At each iteration step, SNP calling was performed: variants were called
207 with samtools mpileup v1.3.1[43] followed with Varscan v2.4.3[44] mpileup2snp (Min coverage
208 8, Min reads2 2, Min var freq 0.2, Min avg qual 15, P-value thresh 0.01). Additional filters
209 were applied: minimum frequency of variants reads between 0.25 and 0.75 for heterozygote
210 genotypes in individual samples; maximum coverage of twice mode of the gaussian
211 distribution for each sample, only bi-allelic SNPs, minor frequency variant higher than 0.05,
212 maximum rate of missing data 0.2. A SNP database was build with gsnap utilities to perform
213 a SNP-tolerant mapping at the next iteration. We carried out two iterations of SNP-tolerant
214 mapping until the rate of discovery of new XY SNPs (see below) became low. Finally, a 4th
215 step of SNP-tolerant mapping with more stringent parameters (-m 0.05, primary alignments
216 only) was performed in order to reduce the rate of false-positive SNPs.

217 *Detection and validation of sex-linked single nucleotide polymorphisms.* In order to detect
218 SNPs linked to the sex-determining region, we run SEX-DETector++. This yielded 4,113 sex-
219 linked SNPs (with a posterior probability high than 0.6), 90.5% of them on chromosome 2. As
220 the sex-determining regions is assumed to be small[5] and the low number of offspring in our
221 cross gives us access to few recombination events, the sex-linked region in our analysis may
222 be larger than the actual sex-determining region (SNP close to the sex locus tend to be linked
223 to sex in a particular cross). To determine the boundaries of the sex-determining region,
224 independent public data were analyzed in order to identify shared XY SNPs between different

populations. WG-reseq and RNA-seq data from wild and cultivated grapevines were mapped against the *V. sylvestris* genome with gsnap v2018-07-04, SNPs were called with varscan v2.4.3. We searched for candidate XY-SNPs that were heterozygote in all male samples and homozygote in all female samples of the validation dataset. Validated XY-SNPs spanned 111-kb on chromosome 2 (from 4,810,929 to 4,921,949 bp), which indicated the limits of the sex-determining region. In order to complement SEX-DETector++ analysis, we also performed an empirical one. We searched for SNPs that overlapped the sex-determining region, were homozygote in female individuals of the cross, and heterozygote in male individuals, allowing two missing alleles. This empirical analysis retrieved all 1,406 XY SNPs identified by SEX-DETector++ plus 459 additional ones (+ 32.6%). This final dataset of 1,865 XY SNPs was used for downstream analyses.

Determination of the synonymous divergence between X and Y alleles in the sex-linked region. The dataset of XY SNPs was used to build X and Y allelic pseudosequences in coding regions, using a custom python script to substitute reference positions by X or Y SNPs respectively in the genome in respect with strand, and to extract and concatenate coding DNA sequences for each gene of the sex locus. The yn00 program of the PAML suite[45] was used to estimate synonymous divergence (dS) with standard error estimation.

Structural characterization of the sex-linked region in the V. sylvestris genome.
Structural gene annotations in the sex-linked regions were extracted from Eugene (See annotation Section). Predicted proteins were mapped against the ncbi nr database with blastp and functional annotations were manually analyzed. The best hit in *Arabidopsis thaliana* was used to name the genes. Transposable elements were detected with Red[46].

Integrative search for sex-determining genes in the sex locus.
Comparison of gene and TE content in X, Y and H haplotypes. In addition to the female whole genome of *V. sylvestris*, we obtained assemblies for BACs of X and Y haplotypes of *V. sylvestris* and H and F haplotypes of Cabernet-Sauvignon (cf. BAC sequencing and assembly Section), H designating modified Y haplotypes found in hermaphrodites. Blastn (ncbi-blast v2.2.3) were carried out between pairs of haplotypes (X-Y, X-H, Y-H, X from whole-genome and X from BACs). Whole-haplotype comparisons were visualized with circos to identify presence/absence patterns. Genic sequences were extracted in all haplotypes using Eugene annotations and blastn, and aligned with mafft v7 online service[47] to identify structural differences and mutations. The sequences of the putative sex-determining candidates were blasted against assemblies of other cultivated grapevine genomes (Chardonnay, Cabernet-Sauvignon and Zinfandel) to assess their presence or absence.

Total and allele-specific expression of sex-linked genes. We retrieved raw reads from 23 public libraries of female, male and hermaphrodite bud flowers samples from *V. sylvestris* at four developmental stages[13]. Reads were mapped on the *V. sylvestris* genome with gsnap v2018-07-04 (-m 0.1), with a mode tolerant to XY SNPs. Read counts were obtained with htseq-count v0.10.0[48] and normalized by computing the RPKM (reads per count per millions of reads mapped). To specifically measure the expression of X and Y alleles, we performed SNP calling (gsnap –m 0.05, varscan max missing data 0.4 and minor allele frequency 0.05),

267 and extracted the positions corresponding to XY SNPs located in CDS from the vcf file. For
268 each gene and library, we read the number of reads mapping on reference and variant alleles
269 (corresponding to X and Y alleles respectively) with bedtools interesect[49]. Reads numbers
270 were summed by gene and normalized by the total number of reads in the variant file for the
271 library and the length of transcripts.

272 *Organ-specific expression of sex-linked genes in V. vinifera.* We retrieved and mapped
273 transcriptomic data of *V. sylvestris* and *V. vinifera* in several organs and conditions against the
274 genome of *V. sylvestris* (berries, developing seeds, leaves under normal, drought and
275 pathogenic conditions, stem, early and mature flower buds, Supplementary Table 12). We
276 measured the gene expression levels in the different conditions, and computed an index of
277 organ-specificity (Tau[50]) on normalized expression levels (log(read count per kilobase per
278 millions of mapped reads)). The Tau specificity index ranges between 0 and 1 and typically
279 display two modes near 0 and 1 indicating ubiquitous and organ-specific genes respectively.

280

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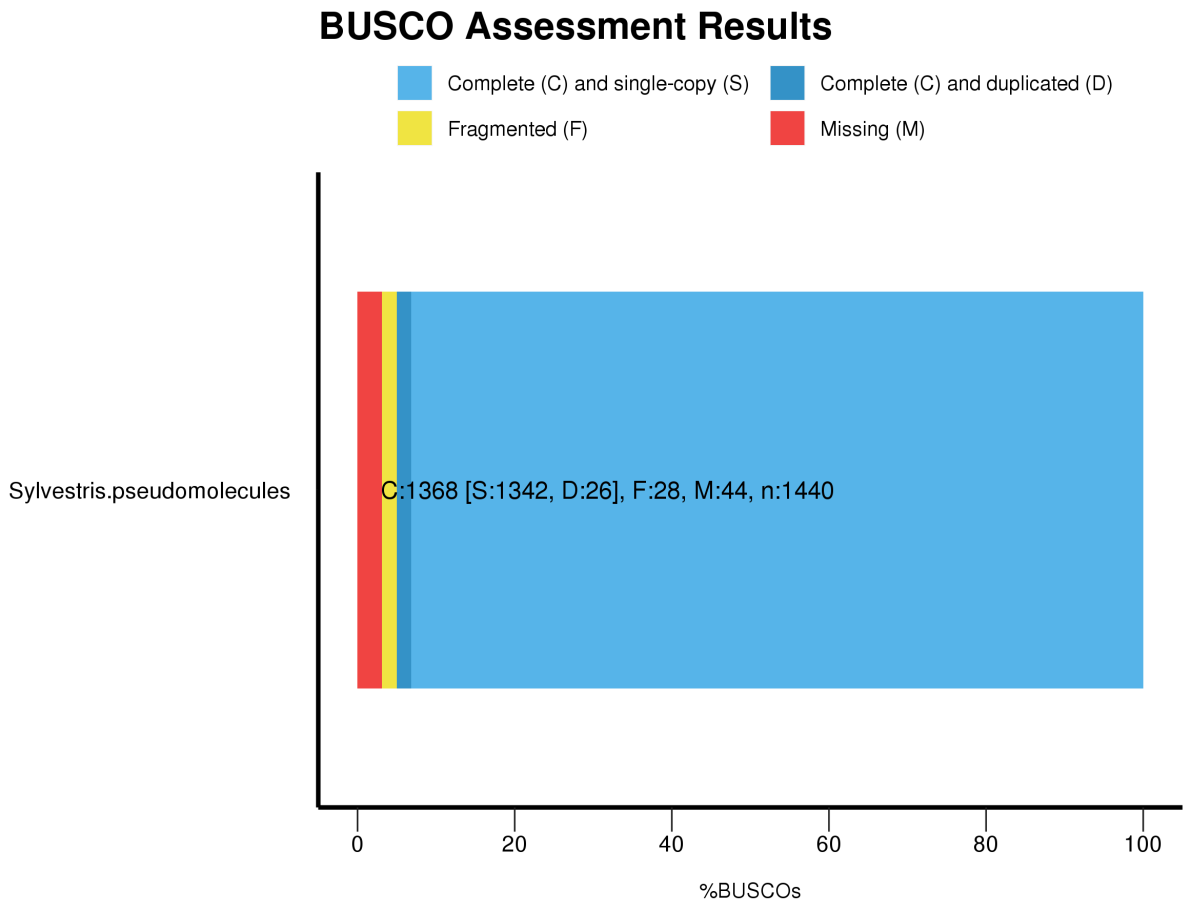
330

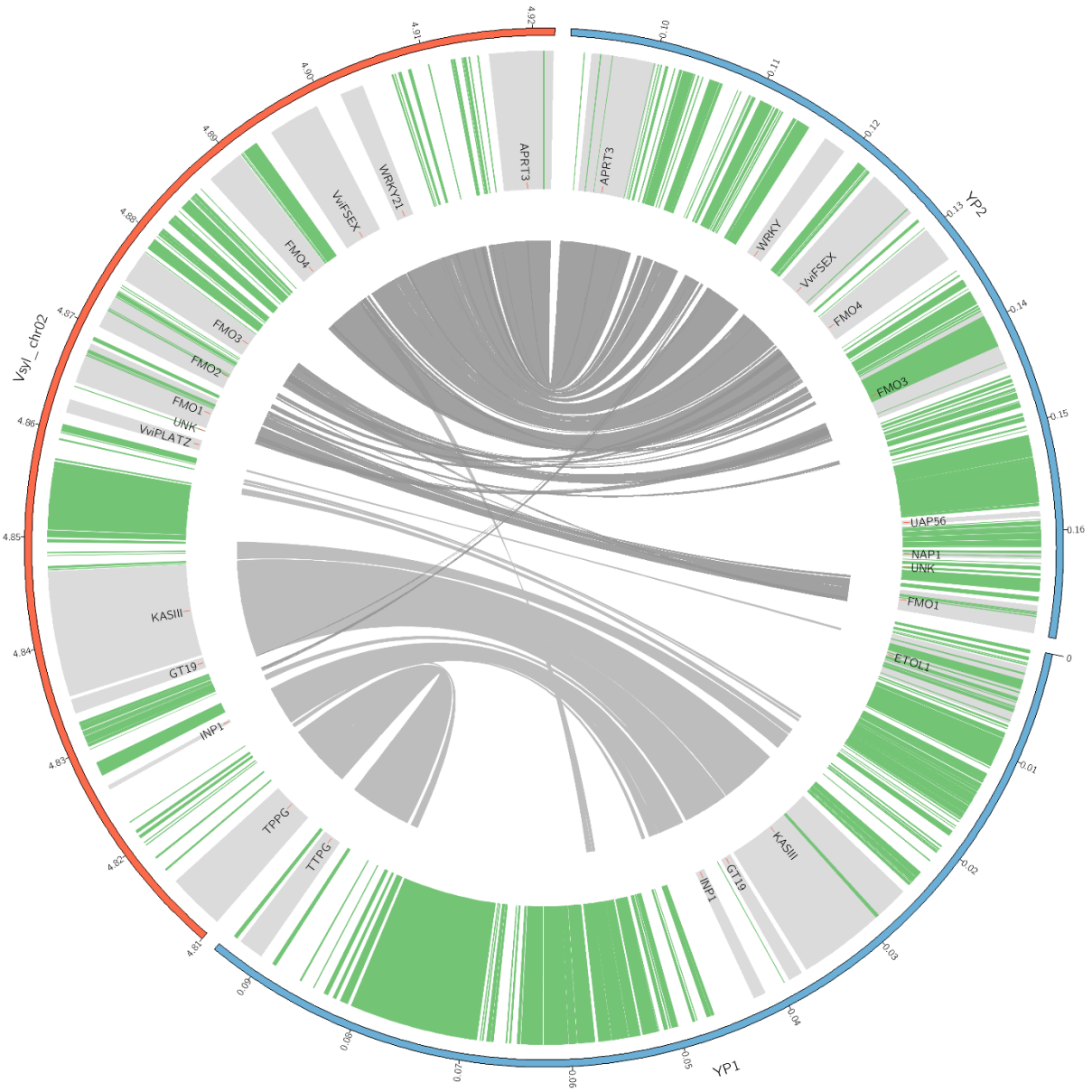
331

Supplementary Figures

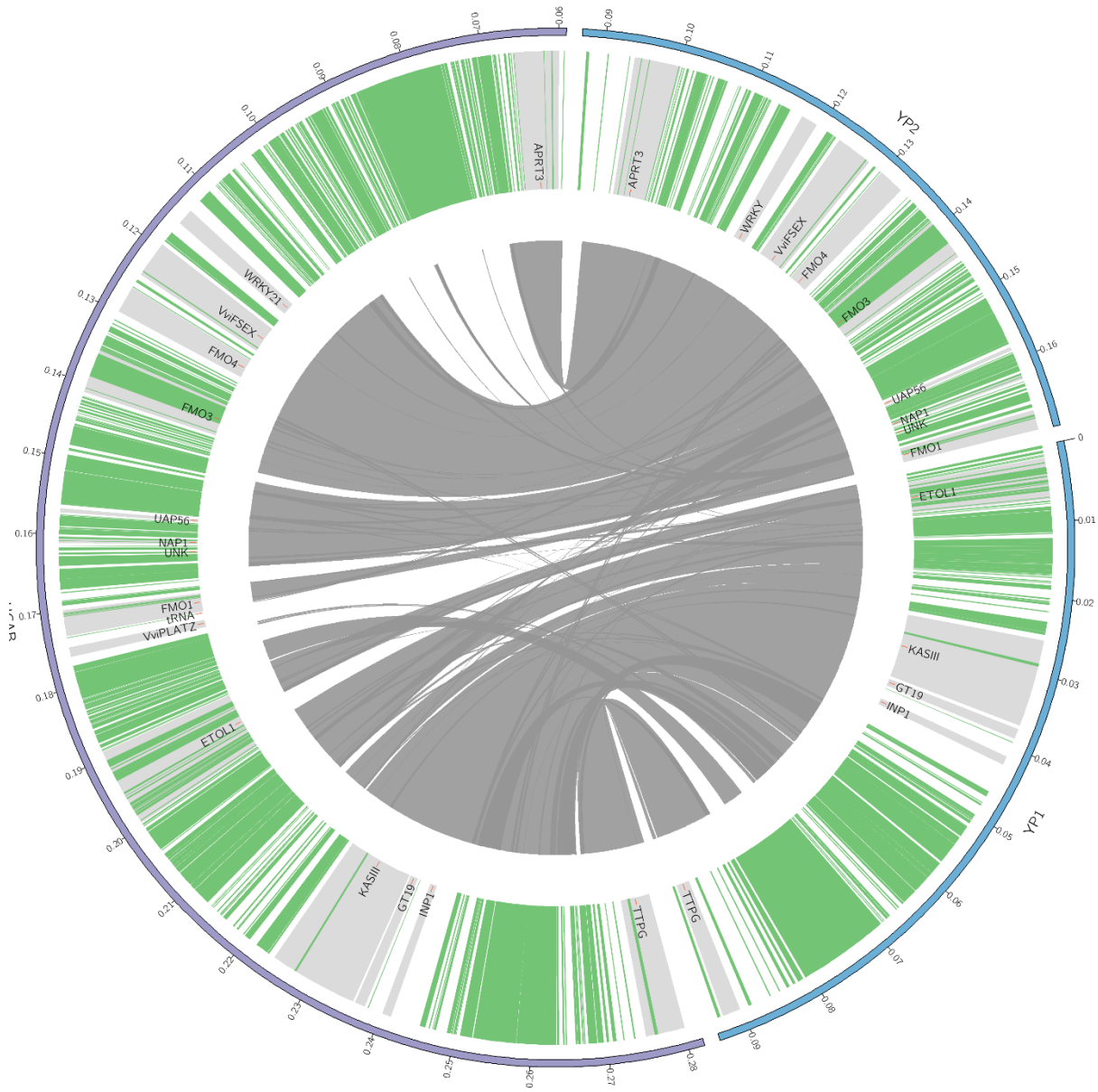
Supplementary Figure 1: BUSCO results on *V. sylvestris* pseudomolecules. Out of 1440 genes in the BUSCO dataset, 95% are complete.



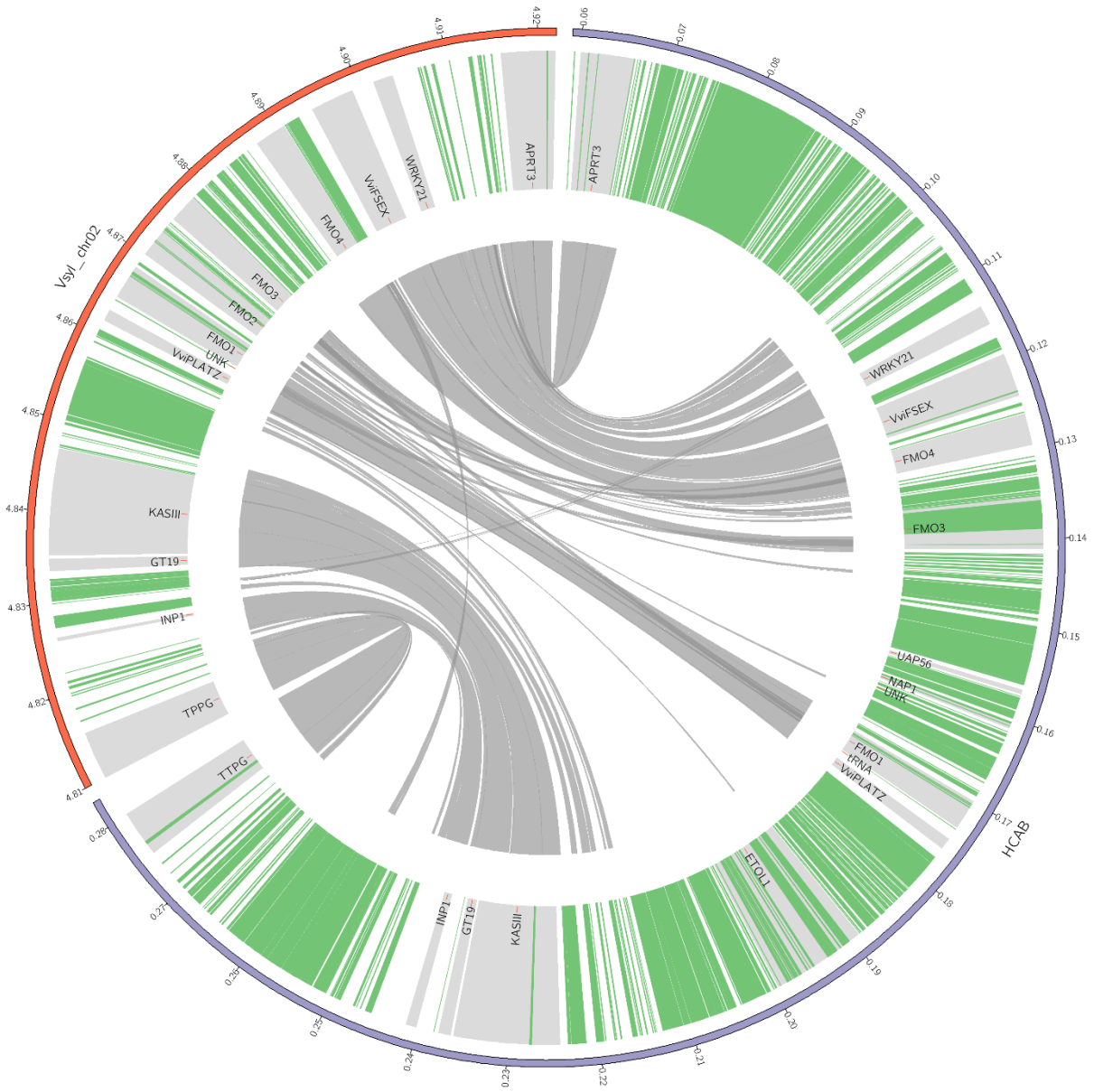
Supplementary Figure 2: Structural comparison of X and Y haplotypes of the sex locus of *V. sylvestris*. Outer to inner track: circular representation of pseudomolecules; limits of genes (obtained from Eugene and verified with blastn) in grey and repeats in green; synteny relationships (blastn hits with an e-value lower than 0.01. YP1 and YP2 are two BAC contigs covering the Y locus, with a gap of an estimated size of 13kb, in which presence of the *PLATZy* allele has been confirmed by PCR (see Supplementary Text). Coordinates are indicated in Mbp.



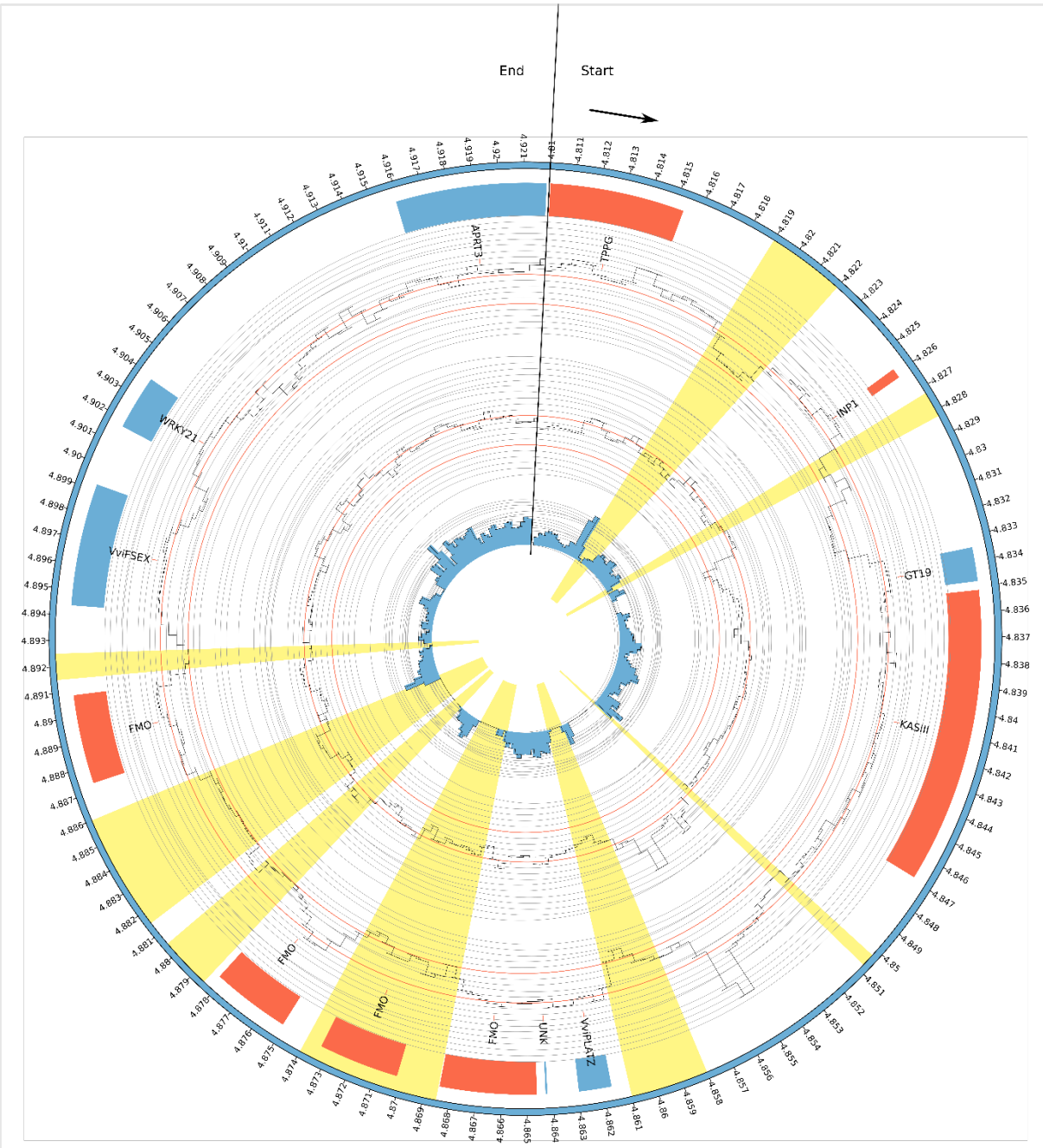
Supplementary Figure 3: Structural comparison of a Y haplotype of *V. sylvestris* and a H haplotype of *V. vinifera* cv. Cabernet Sauvignon, H referring to the modified Y haplotype found in hermaphrodites. Outer to inner track: circular representation of pseudomolecules; limits of genes (obtained from Eugene and verified with blastn) in grey and repeats in green; synteny relationships (blastn hits with e-values lower than 0.001). YP1 and YP2 are two BAC contigs covering the Y locus, with a gap of an estimated size of 13kb, in which presence of the *PLATZy* allele has been confirmed by PCR (see Supplementary Text). The purple ideogram represents the H haplotype. A large insertion between *WRKY* and *APRT3* is present in the H haplotype of Cabernet Sauvignon, but is not shared by all H haplotypes (*e.g.* only of the two H haplotypes of Chardonnay, not shown). Coordinates are indicated in Mbp.



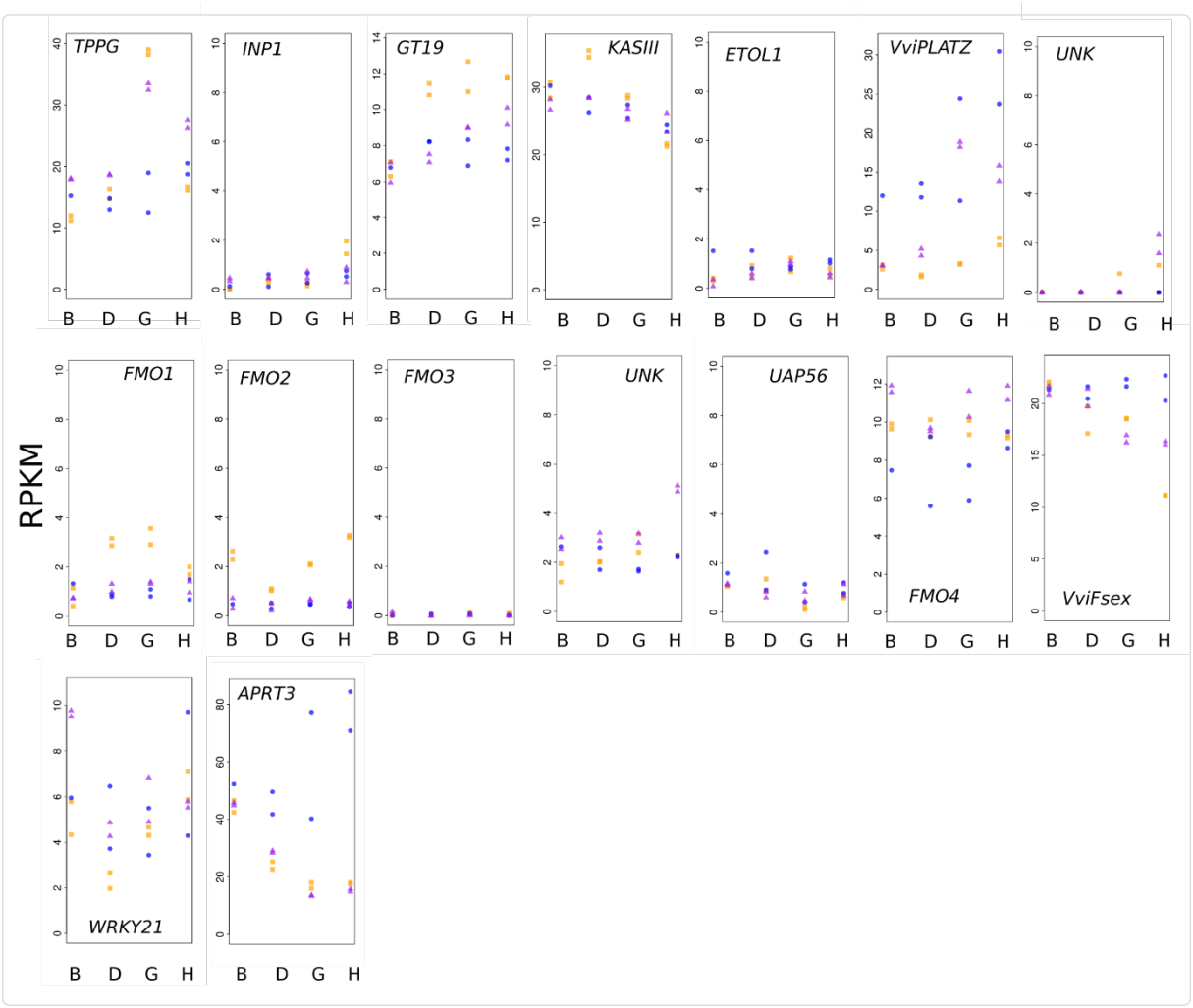
Supplementary Figure 4: Structural comparison of X haplotype of *V. sylvestris* and a H haplotype of *V. vinifera* cv. Cabernet Sauvignon. Outer to inner track: circular representation of pseudomolecules; Outer to inner track: circular representation of pseudomolecules; limits of genes (obtained from Eugene and verified with blastn) in grey and repeats in green; synteny relationships; synteny relationships (blastn hits with e-values lower than 0.001). Coordinates are indicated in Mbp.



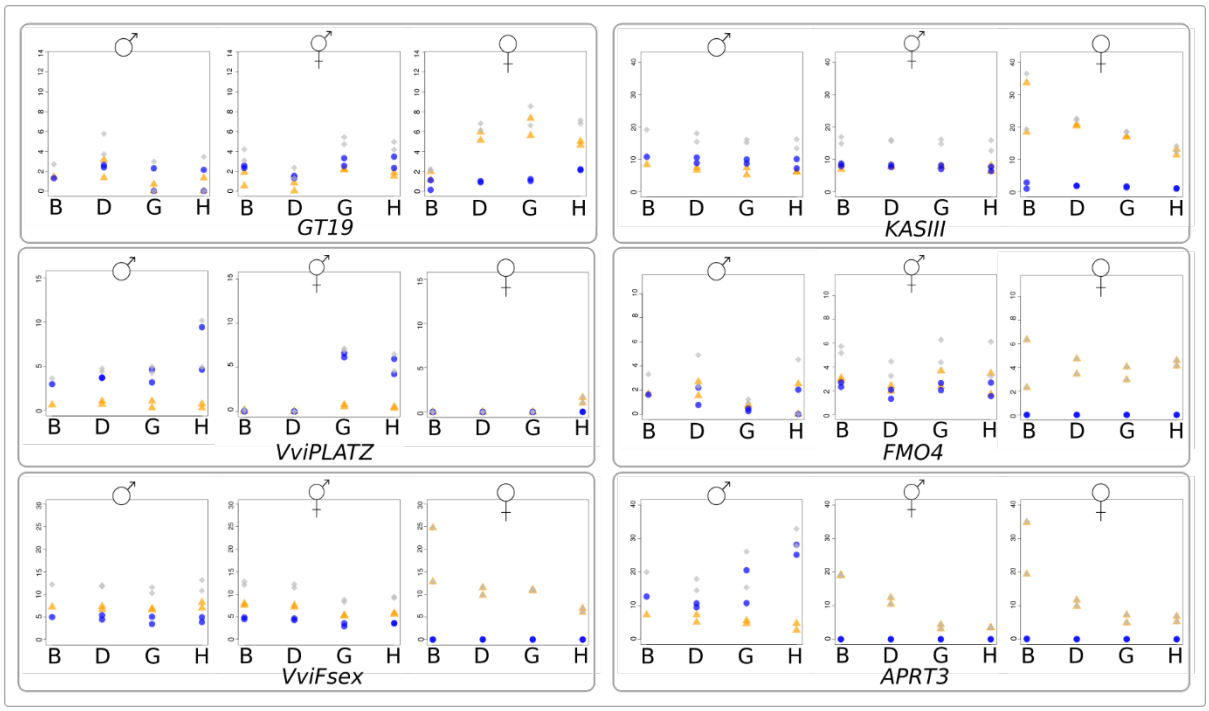
Supplementary Figure 5: Density of XY-SNPs and location of X-hemizygote regions in a cross in *V. sylvestris*. Track, form outter to inner track: genomic coordinates on chromosome 2 (from 4.810 to 4.922 Mb), location and name of genes (blue = direct strand, red = reverse strand), Depth of mapping coverage in a male individual (RCDN9), from 0 to 50, Depth of mapping coverage in a female individual (RCDN16), number of XY SNPs (from 0 to 64). All values were computed by 1-kb overlapping windows. The number of XY SNP was not normalized in order to reflect the proportion of informative sites in each window. Yellow highlights represent the approximate limites of X-hemizygote regions inferred from a two-fold reduction of mapping coverage in males.



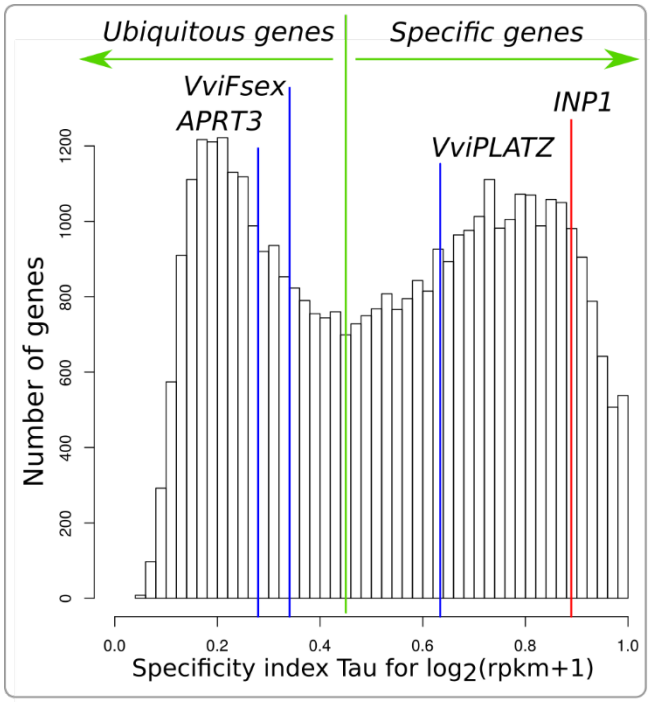
Supplementary Figure 6: Total normalized gene expression in males, females and hermaphrodites of *V. sylvestris* for genes in the sex locus during flower bud development. Letters B to H reffer to successive developmental stages. Expression in females, males and hermaphrodites are represented by orange, purple and blue colors, respectively.



Supplementary Figure 7: Allele-specific expression of X and Y alleles of XY genes pairs of *Vitis sylvestris* during the development of flower buds. The X and Y allelic expression from B to H-stages flower buds is shown for females, males and hermaphrodites. Orange triangles, blue dots and grey losanges represent X, Y and total allelic expression respectively. Allele-specific expression was computed only for genes with sufficient mapping coverage on at least 5 XY SNPs. The y scale is different for each gene but shared for a given gene between females, males and hermaphrodites. Two replicates are represented for each condition, except for the male B stage for which there was no replicate.



Supplementary Figure 8: Distribution of organ-specificity expression in *Vitis sylvestris* genes. The value of the specificity index Tau for four genes of the sex locus in indicated by vertical lines.



Supplementary Tables

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403

404 **Supplementary Table 1:** Assembly and anchoring statistics of the *V. sylvestris* genome.

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	Primary contigs	Anchored primary contigs	Haplotigs
Number of contigs	591	476	3,781
Mean length (b)	792,724	947,992	83,879
Median length (b)	421,056	584,494.5	36,553
Max length (b)	6,865,695	6,865,695	2,541,772
L50	82	77	483
N50 (b)	1,711,677	1,773,898	173,606
Assembly size (b)	468,500,071	451,244,463	317,149,633

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408 **Supplementary Table 2**: Summary statistics of resequencing data of a cross between two *V. sylvestris*
409 parents.

Sample ID	Genotype	Type	Sex	Concentraton ADN ng/µl	Number of Reads	Number of Pairs
RCDN8	C1-5	mother	Female	158	91,689,312	45,844,656
RCDN9	Martigny 2	father	Male	113	140,875,256	70,437,628
RCDN10	5026T	offspring	Male	150	149,542,818	74,771,409
RCDN11	5028T	offspring	Male	145	128,590,900	64,295,450
RCDN12	5029T	offspring	Male	145	85,976,170	42,988,085
RCDN13	5033T	offspring	Male	145	130,071,908	65,035,954
RCDN14	5053T	offspring	Male	155	123,960,284	61,980,142
RCDN15	5035T	offspring	female	110	109,564,824	54,782,412
RCDN16	5040T	offspring	female	110	132,609,212	66,304,606
RCDN17	5046T	offspring	female	130	111,932,706	55,966,353
RCDN18	5050T	offspring	female	110	106,023,554	53,011,777
RCDN19	5057T	offspring	female	120	125,195,484	62,597,742

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412 **Supplementary Table 3:** Mode of the gaussian distribution of mapping coverage for each sample for
413 the dataset of whole genome resequencing of a cross in *V. sylvestris*. The values are identical for each
414 iteration of mapping and therefore indicated only once. The maximum coverage allowed corresponds
415 to the maximum coverage that was allowed when filtering SNP, in order to remove repeated positions.

416

Sample ID	Mode of gaussian distribution	Maximum coverage allowed
RCDN8	16	32
RCDN9	25	50
RCDN10	26	52
RCDN11	22	44
RCDN12	14	28
RCDN13	22	44
RCDN14	21	42
RCDN15	19	38
RCDN16	23	46
RCDN17	19	38
RCDN18	18	36
RCDN19	22	44

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419 **Supplementary Table 4:** Summary statistics of iterative SNP-tolerant mapping for the resequencing
420 dataset of a cross in *V. sylvestris*.

Sample ID	Sex	Iteration 1			Iteration 2		
		Number of mapped reads	fraction of mapped reads	fraction of paired reads	Number of mapped reads	fraction of mapped reads	fraction of paired reads
RCDN8	F	89,951,765	0.981	0.969	90,089,021	0.983	0.971
RCDN9	M	138,338,630	0.982	0.971	138,544,634	0.983	0.973
RCDN10	M	146,671,291	0.981	0.968	146,904,980	0.982	0.971
RCDN11	M	125,913,524	0.979	0.966	126,122,927	0.981	0.969
RCDN12	M	84,214,280	0.98	0.967	84,352,679	0.981	0.969
RCDN13	M	127,437,731	0.98	0.967	127,645,009	0.981	0.969
RCDN14	M	121,369,732	0.979	0.966	121,578,081	0.981	0.968
RCDN15	F	107,360,998	0.98	0.967	107,538,009	0.982	0.97
RCDN16	F	130,211,584	0.982	0.971	130,408,313	0.983	0.973
RCDN17	F	109,719,948	0.98	0.968	109,900,170	0.982	0.97
RCDN18	F	104,161,079	0.982	0.971	104,315,801	0.984	0.973
RCDN19	F	122,729,069	0.98	0.967	122,927,332	0.982	0.97

421

Sample ID	Iteration 3			Iteration 4		
	Number of mapped reads	fraction of mapped reads	fraction of paired reads	Number of mapped reads	fraction of mapped reads	fraction of paired reads
RCDN8	90,095,720	0.983	0.972	90,029,816	0.982	0.97
RCDN9	138,552,746	0.984	0.973	138,452,568	0.983	0.972
RCDN10	146,916,364	0.982	0.971	146,801,740	0.982	0.969
RCDN11	126,131,254	0.981	0.969	126,036,403	0.98	0.968
RCDN12	84,358,053	0.981	0.969	84,294,600	0.98	0.968
RCDN13	127,653,125	0.981	0.969	127,558,702	0.981	0.968
RCDN14	121,585,814	0.981	0.968	121,490,591	0.98	0.967
RCDN15	107,545,495	0.982	0.97	107,458,696	0.981	0.968
RCDN16	130,416,024	0.983	0.973	130,319,644	0.983	0.972
RCDN17	109,907,858	0.982	0.97	109,819,091	0.981	0.969
RCDN18	104,322,423	0.984	0.973	104,244,590	0.983	0.972
RCDN19	122,935,210	0.982	0.97	122,840,655	0.981	0.969

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423 **Supplementary Table 5:** Summary statistics of missingness per sample for each iteration of SNP-
424 calling in the dataset of whole-genome resequencing of a cross in *V. sylvestris*. Statistics were
425 obtained with vcftools version 0.1.15. N_MISS is the number of missing sites, F_MISS is the
426 frequency of missing sites.

	Iteration 1		Iteration 2		Iteration 3		Iteration 4	
Sample ID	N_MISS	F_MISS	N_MISS	F_MISS	N_MISS	F_MISS	N_MISS	F_MISS
RCDN8	280,469	0.0685	257,255	0.0576	260,910	0.0557	259,983	0.0556
RCDN9	80,415	0.0196	78,141	0.0175	82,728	0.0177	82,577	0.0177
RCDN10	34,051	0.00831	45,378	0.0102	55,432	0.0118	57,368	0.0123
RCDN11	72,953	0.0178	79,836	0.0179	91,745	0.0196	90,777	0.0194
RCDN12	418,876	0.102	385,349	0.0863	397,437	0.0848	395,665	0.0847
RCDN13	59,795	0.0146	67,214	0.0151	77,745	0.0166	78,511	0.0168
RCDN14	81,071	0.0198	75,386	0.0169	80,678	0.0172	79,763	0.0171
RCDN15	112,434	0.0274	103,972	0.0233	110,945	0.0237	110,066	0.0236
RCDN16	51,669	0.0126	58,007	0.0130	66,040	0.0141	66,795	0.0143
RCDN17	112,887	0.0276	106,827	0.0239	114,224	0.0244	112,998	0.0242
RCDN18	138,484	0.0338	133,571	0.0299	143,660	0.0307	142,622	0.0305
RCDN19	54,255	0.0132	53,412	0.0120	58,227	0.0124	57,799	0.0124

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429 **Supplementary Table 6:** Number of homozygous and heterozygous SNPs called at each iteration in
430 the dataset of whole-genome resequencing of a cross in *V. sylvestris*. "% increase het step4/step1"
431 indicates the percentage of increase in the number heterozygous SNPs detected between the first and
432 fourth step of iterative mapping.

		Iteration 1		Iteration 2	
Sample ID	Category	Number of homozygous SNPs	Number of heterozygous SNPs	Number of homozygous SNPs	Number of heterozygous SNPs
RCDN8	M	380,285	1,674,962	428,741	1,915,267
RCDN9	F	763,168	1,868,596	813,700	2,079,174
RCDN10	S	419,471	2,336,345	449,943	2,581,917
RCDN11	S	447,082	2,129,876	481,153	2,359,784
RCDN12	S	376,336	1,784,861	429,508	2,032,012
RCDN13	S	411,888	2,172,007	443,658	2,407,092
RCDN14	S	446,637	2,141,363	486,468	2,390,231
RCDN15	D	411,587	2,004,887	451,048	2,242,332
RCDN16	D	441,713	2,240,436	475,968	2,482,939
RCDN17	D	388,490	2,099,888	426,892	2,352,568
RCDN18	D	394,402	2,152,081	434,417	2,406,320
RCDN19	D	397,321	2,055,753	429,151	2,293,760

433

	Iteration 3		Iteration 4			
Sample ID	Number of homozygous SNPs	Number of heterozygous SNPs	Number of homozygous SNPs	Number of heterozygous SNPs	% increase hom step4/step1	% increase het step4/step1
RCDN8	442,840	2,061,120	442,698	2,062,008	16.41	23.11
RCDN9	832,308	2,241,277	830,372	2,242,601	8.81	20.02
RCDN10	460,069	2,756,124	459,710	2,757,158	9.59	18.01
RCDN11	494,110	2,522,773	491,744	2,524,116	9.99	18.51
RCDN12	443,293	2,175,229	442,599	2,174,875	17.61	21.85
RCDN13	454,834	2,569,706	453,889	2,569,619	10.20	18.31
RCDN14	499,287	2,557,644	498,176	2,559,111	11.54	19.51
RCDN15	463,449	2,400,577	462,964	2,400,871	12.48	19.75
RCDN16	487,714	2,654,078	486,224	2,655,479	10.08	18.53

RCDN17	438,941	2,512,766	439,365	2,516,241	13.10	19.83
RCDN18	447,979	2,565,013	447,227	2,567,305	13.39	19.29
RCDN19	438,548	2,459,524	438,145	2,460,816	10.27	19.70

434

435 **Supplementary Table 7:** Whole-genome resequencing dataset of the ncbi short read archive (SRA)
436 that were mapped to the *Vitis sylvestris* genome. All samples were part of study SRP108271. Sample
437 SRS2238702 had a lower mapping coverage than other samples, resulting in a high rate of missing
438 data.

SRA run ID	Sample Accession	Domestication status	Organism Name	Origin / cultivar	Sex
SRR5627780	SRS2238707	cultivated	*Vitis vinifera*	Thompson RLK	Hermaphrodite
SRR5627781	SRS2238705	cultivated	*Vitis vinifera*	Muscat of Alexandria	Hermaphrodite
SRR5627782	SRS2238704	cultivated	*Vitis vinifera*	Thopson 2A	Hermaphrodite
SRR5627783	SRS2238703	wild	*Vitis vinifera subsp. sylvestris*	Turkmenistan1	Malqe
SRR5627784	SRS2238702	wild	*Vitis vinifera subsp. sylvestris*	Armenia	Female
SRR5627785	SRS2238701	wild	*Vitis vinifera subsp. sylvestris*	Pakistan1	Female
SRR5627786	SRS2238700	wild	*Vitis vinifera subsp. sylvestris*	Turkmenistan2	Male
SRR5627787	SRS2238699	wild	*Vitis vinifera subsp. sylvestris*	Azerbaijan1	Female
SRR5627789	SRS2238698	wild	*Vitis vinifera subsp. sylvestris*	Georgia	Female
SRR5627790	SRS2238696	wild	*Vitis vinifera subsp. sylvestris*	Azerbaijan2	Female
SRR5627791	SRS2238695	wild	*Vitis vinifera subsp. sylvestris*	Pakistan3	Female
SRR5627792	SRS2238694	wild	*Vitis vinifera subsp. sylvestris*	Pakistan2	Male
SRR5627793	SRS2238693	cultivated	*Vitis vinifera*	Semillion	Hermaphrodite
SRR5627794	SRS2238692	cultivated	*Vitis vinifera*	Riesling	Hermaphrodite
SRR5627795	SRS2238691	cultivated	*Vitis vinifera*	Cabernet Sauvignon	Hermaphrodite
SRR5627796	SRS2238690	cultivated	*Vitis vinifera*	Primitivo	Hermaphrodite
SRR5627797	SRS2238689	cultivated	*Vitis vinifera*	Pinot Noir	Hermaphrodite
SRR5627798	SRS2238688	cultivated	*Vitis vinifera*	Gamay Noir	Hermaphrodite
SRR5627799	SRS2238687	cultivated	*Vitis vinifera*	Chardonnay	Hermaphrodite
SRR5627800	SRS2238686	cultivated	*Vitis vinifera*	Aramon	Hermaphrodite
SRR5627801	SRS2238684	cultivated	*Vitis vinifera*	Zinfindel	Hermaphrodite
SRR5627802	SRS2238685	cultivated	*Vitis vinifera*	Traminer	Hermaphrodite

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441 **Supplementary Table 8:** Statistics of mapping and SNP-calling of a public whole-genome
442 resequencing dataset of the ncbi that was mapped to the *Vitis sylvestris* genome.

443

SRA run ID	Status	Number of reads	Proportion of mapped reads	Proportion of paired reads
SRR5627780	cultivated	70,284,983	0.989	0.975
SRR5627781	cultivated	76,803,804	0.991	0.98
SRR5627782	cultivated	80,937,324	0.992	0.981
SRR5627783	wild	80,232,477	0.986	0.974
SRR5627784	wild	35,879,048	0.954	0.92
SRR5627785	wild	105,896,803	0.991	0.978
SRR5627786	wild	80,948,588	0.99	0.979
SRR5627787	wild	76,824,067	0.985	0.97
SRR5627789	wild	80,107,055	0.989	0.977
SRR5627790	wild	79,079,895	0.965	0.938
SRR5627791	wild	105,783,610	0.991	0.979
SRR5627792	wild	115,956,970	0.991	0.976
SRR5627793	cultivated	77,221,117	0.992	0.98
SRR5627794	cultivated	77,644,996	0.992	0.98
SRR5627795	cultivated	71,775,105	0.992	0.98
SRR5627796	cultivated	169,763,362	0.972	0.942
SRR5627797	cultivated	90,757,557	0.993	0.981
SRR5627798	cultivated	66,283,833	0.992	0.983
SRR5627799	cultivated	454,053,065	0.982	0.956
SRR5627800	cultivated	96,530,135	0.992	0.98
SRR5627801	cultivated	223,282,216	0.992	0.978
SRR5627802	cultivated	60,767,595	0.99	0.974

444

445 **Supplementary Table 9:** Summary of the genotype of 13 cultivars in the sex locus on chromosome 2
446 inferred from whole-genome resequencing data. Cultivars were genotyped at XY SNPs. H reffers to
447 the modified Y haplotypes in hermaphrodites. Raw SNP data are shown in Figure 1a.

SRA run ID	Origin / cultivar	Genotype 4.810-4.903 Mb	Genotype 4.903-4.21Mb
SRR5627780	Thompson RLK	XH	XH
SRR5627781	Muscat of Alexandria	XH	XH
SRR5627782	Thopson 2A	XH	XH
SRR5627793	Semillion	XH	XH
SRR5627794	Riesling	HH	XH
SRR5627795	Cabernet Sauvignon	XH	XH
SRR5627796	Primitivo	XH	XH
SRR5627797	Pinot Noir	XH	XX
SRR5627798	Gamay Noir	XH	XX
SRR5627799	Chardonnay	HH	XH
SRR5627800	Aramon	XH	XX
SRR5627801	Zinfindel	XH	XH
SRR5627802	Traminer	XH	XX

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451 **Supplementary Table 10:** Predicted genes in the sex locus of *V. sylvestris*. Coordinates and geneID
452 are indicated for the *V. sylvestris* reference genome, therefore the absolute position of X-deleted genes
453 is not shown.

Gene ID	Start	End	Strand	Functional annotation	Genotype in hermaphrodites
000003Fg0028711	4,810,035	4,815,364	-	trehalose phosphate phosphatase	from exon 9, XH or HH
000003Fg0028721	4,825,791	4,826,183	-	Protein INAPERTURATE POLLEN1 (INP1)	XH or HH
000003Fg0028731	4,833,463	4,834,804	+	xyloglucan galactosyltransferase (GT19)	XH or HH
000003Fg0028741	4,835,150	4,846,883	-	3-oxoacyl-[acyl-carrier-protein] synthase (KASIII)	XH or HH
NA				Ethylene-overproducer-like1 / No Pollen Germination 1 (ETOL1)	XH or HH
000003Fg0028751	4,861,687	4,862,981	+	transcription of the PLATZ family (VviPLATZ)	XH or HH
000003Fg0028761	4,864,219	4,864,291	+	tRNA-gly	XH or HH
000003Fg0028771	4,864,636	4,868,462	-	flavin mono-oxygenase (FMO)	XH or HH
000003Fg0028781	4,870,175	4,873,359	-	flavin mono-oxygenase (FMO)	XH or HH
NA				ATP-dependent DEAD BOX helicase (UAP56)	XH or HH
NA				NUCLEOSOME ASSEMBLY PROTEIN 1 (NAP1)	XH or HH
NA				Uncharacterized protein (UNK)	
000003Fg0028791	4,875,086	4,878,202	-	flavin mono-oxygenase (FMO)	XH or HH
000003Fg0028801	4,887,323	4,890,854	-	flavin mono-oxygenase (FMO)	XH or HH
000003Fg0028811	4,894,375	4,899,225	+	Uncharacterized protein (VviFSEX)	XH or HH
000003Fg0028821	4,901,701	4,903,864	+	WRKY transcription factor 21 (WRKY21)	XH or HH
000003Fg0028831	4,915,923	4,921,889	+	adenine phospho-ribosyltransferase (VviAPRT3)	XH or HH

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468 **Supplementary Table 11:** Mapping statistics of public RNA-seq libraries of flower buds of female,
469 male and hermaphrodite *V. sylvestris* (Ramos et al. 2013) against the *V. sylvestris* genome. B to D
470 represent successive developmental stages.

SRA ID	Sex	Stage	Number of reads	% of reads mapped	% of reads assigned to a gene
SRR1239522	Female	B	13,571,346	95	84.2
SRR1239524	Female	D	14,596,473	95.5	84.9
SRR1239526	Female	G	14,111,102	95.4	84.3
SRR1239528	Female	H	18,464,325	94.7	82.9
SRR1239530	Male	B	17,247,932	95	84.1
SRR1239532	Male	D	17,244,273	94.7	83.7
SRR1239534	Male	G	7,711,531	95.3	84.1
SRR1239536	Male	H	17,830,620	94.5	83.6
SRR1239537	Hermaphrodite	B	19,832,905	94.2	83.4
SRR1239539	Hermaphrodite	D	13,814,366	94.9	83.7
SRR1239541	Hermaphrodite	G	14,239,068	95	83.1
SRR1239543	Hermaphrodite	H	15,564,147	96	83.9

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474 **Supplementary Table 12:** Public RNA-seq dataset of *V. vinifera* and *V. sylvestris* mapped to the *Vitis*
475 *sylvestris* genome.

SRA ID	Description	Cultivar	Data
SRR1709065	BERRY2.WD1	Pinot Noir	Illumina Single
SRR1709064	BERRY2.WD2	Pinot Noir	Illumina Single
SRR1709063	BERRY2.WD3	Pinot Noir	Illumina Single
SRR1709068	BERRY2.WW1	Pinot Noir	Illumina Single
SRR1709067	BERRY2.WW2	Pinot Noir	Illumina Single
SRR1709066	BERRY2.WW3	Pinot Noir	Illumina Single
SRR5989171	BERRY3.EL35.S1	Riesling	Illumina Single
SRR5989170	BERRY3.EL35.S2	Riesling	Illumina Single
SRR5989174	BERRY3.EL36.S1	Riesling	Illumina Single
SRR5989175	BERRY3.EL36.S2	Riesling	Illumina Single
SRR5989178	BERRY3.EL38.S1	Riesling	Illumina Single
SRR5989177	BERRY3.EL38.S2	Riesling	Illumina Single
SRR3056887	BUD2.Efs1	Prieto Picudo	Illumina Paired
SRR3056888	BUD2.Efs2	Prieto Picudo	Illumina Paired
SRR3056889	BUD2.Efs3	Prieto Picudo	Illumina Paired
SRR3056884	BUD2.Nfs1	Prieto Picudo	Illumina Paired
SRR3056885	BUD2.Nfs1	Prieto Picudo	Illumina Paired
SRR3056886	BUD2.Nfs1	Prieto Picudo	Illumina Paired
SRR2043187	BUD3.PC1	Corinto Bianco	Illumina Paired
SRR2043218	BUD3.PC2	Corinto Bianco	Illumina Paired
SRR2043221	BUD3.PC3	Corinto Bianco	Illumina Paired
SRR2043222	BUD3.SD1	Pedro Ximenes	Illumina Paired
SRR2043223	BUD3.SD2	Pedro Ximenes	Illumina Paired
SRR2043224	BUD3.SD3	Pedro Ximenes	Illumina Paired
SRR1553992	FLO.CK.1h.12DBB	Labruscan grape	Illumina Single
SRR1553993	FLO.CK.1h.12DBB	Labruscan grape	Illumina Single
SRR1554086	FLO.CK.24h.11DBB1	Labruscan grape	Illumina Single
SRR1554085	FLO.CK.24h.11DBB2	Labruscan grape	Illumina Single
SRR1553990	FLO.GA3.1h.12DBB	Labruscan grape	Illumina Single
SRR1553991	FLO.GA3.1h.12DBB	Labruscan grape	Illumina Single
SRR1553996	FLO.GA3.24h.11DBB1	Labruscan grape	Illumina Single
SRR1554080	FLO.GA3.24h.11DBB2	Labruscan grape	Illumina Single
SRR1302044	FLO2.SDL	Not specified	Illumina Paired

SRR1302041	FLO2.WT	Not specified	Illumina Paired
SRR519455	LEAF1.1DPI	Pinot Noir	Illumina Paired
SRR519456	LEAF1.2DPI	Pinot Noir	Illumina Paired
SRR519449	LEAF1.ctl1	Pinot Noir	Illumina Paired
SRR519450	LEAF1.ctl2	Pinot Noir	Illumina Paired
SRR519452	LEAF1.ctl3	Pinot Noir	Illumina Paired
SRR6706487	LEAF2.4DD1	Pinot Noir	Illumina Paired
SRR6706486	LEAF2.4DD2	Pinot Noir	Illumina Paired
SRR3056876	LEAF4.MtLS	Chasselat Cioutat	Illumina Paired
SRR3056875	LEAF4.MtLS	Chasselat Cioutat	Illumina Paired
SRR1926309	Mix.Wild.Ctl.1DPI1	Wild	Illumina Single
SRR1926307	Mix.Wild.Ctl.1DPI2	Wild	Illumina Single
SRR1926305	Mix.Wild.Ctl.1DPI3	Wild	Illumina Single
SRR1926310	Mix.Wild.Ctl.5DPI1	Wild	Illumina Single
SRR1926308	Mix.Wild.Ctl.5DPI2	Wild	Illumina Single
SRR1926306	Mix.Wild.Ctl.5DPI3	Wild	Illumina Single
SRR1926303	Mix.Wild.In.1DPI1	Wild	Illumina Single
SRR1926300	Mix.Wild.In.2DPI1	Wild	Illumina Single
SRR1926299	Mix.Wild.In.3DPI1	Wild	Illumina Single
SRR1926304	Mix.Wild.In.5DPI1	Wild	Illumina Single
SRR1926302	Mix.Wild.In.5DPI2	Wild	Illumina Single
SRR1926301	Mix.Wild.In.5DPI3	Wild	Illumina Single
SRR6300212	SEED.noSD.1	Red Globe X Crimson Seedless F1 hybrid	Illumina Paired
SRR6300211	SEED.noSD.2	Red Globe X Crimson Seedless F1 hybrid	Illumina Paired
SRR6300209	SEED.SD.1	Red Globe X Crimson Seedless F1 hybrid	Illumina Paired
SRR6300208	SEED.SD.2	Red Globe X Crimson Seedless F1 hybrid	Illumina Paired
DRR093297	STEM1.1	Pinot Noir	Illumina Single
DRR093298	STEM1.2	Pinot Noir	Illumina Single

Supplementary Text

Results of BAC sequencing and assembly

The final results were: 1) a female haplotype composed of the overlapping of 08J18 and 20H04 BAC sequences, of a total 204,082 bp length. This haplotype maps correctly between position 4,866,319 and 5,119,669 of the chromosome 2 of the *Vitis vinifera* reference genome (cultivar PN40024, 12X.V0, NCBI), which is known to correspond to the female haplotype of the Pinot Noir cultivar (Fechter et al 2012, Picq et al 2014); and 2) a male haplotype composed of two non-overlapping contigs, contig1: BACs 27H5 and 65K18 (171,096bp) and contig2: BACs 9D16, 15F19 and 71N3 (169,751bp). The male haplotype does not map well on the grape genome reference, as the hermaphrodite haplotype of the PN40024 was not assembled on the chromosome 2 but its differing sequences were left unassigned (chromosome unknown). On the reference genome 12X.V0, the borders of the contig1 map at positions chr2:4,856,490 and chrUn:16,079,507; the borders of contig2 map at chr2:4,953,113 and chr:5,142,793. Based on the closest blast hits of the male contigs on the female and hermaphrodite haplotypes of the reference genome, as well as on the other available long-reads PacBio genomes (the Chardonnay sequences of Zhou et al 2019[19]; the Cabernet Sauvignon sequences of Chin et al 2016[9]; as well as the *Vitis sylvestris* female sequences of this work), we estimated the length of the gap between contig1 and contig2 at around 13kb.

Covering the 13kb gap on the male haplotype

Further efforts were attempted to cover the gap between the two non-overlapping male contigs. As a gap of 13kb is too long to be covered by long-range PCR even when using high-quality TAQ, we made the hypothesis that the *PLATZ* gene, for which we have positional evidence in several female and hermaphrodite genotypes, is positioned within this gap in the male haplotype too, and can be used to split the 13kb in two shorter parts, easier to amplify by long-range PCR. The positions of *PLATZ* on H and F haplotypes were obtained from: the reference genome of PN40024, on which the *PLATZ* female allele maps at chr2:4949289..4950582 and its hermaphrodite allele at chrUn16091133..16092418; the Chardonnay PacBio sequences (Zhou et al 2019); the Cabernet Sauvignon PacBio sequences (Chin et al 2016); as well as the *Vitis sylvestris* female PacBio sequences (this work). In addition, the *PLATZ* male allele was also found by microassembly of reads carrying Y alleles detecting in this work from the males of the *Vitis sylvestris* cross (this work); for facility we call this male version *PLATZy*.

Based on this hypothetical construct (contig1-gap1-PLATZy-gap2-contig2), we defined several pairs of primers based on the contig1- and contig2 ends and on *PLATZy*. The primers were defined exploiting polymorphisms between the F and M haplotype, so to be sure to amplify only the M haplotype. Starting from primers based on *PLATZy* and contig2 (forward: ACTCCCCTGTTTCTCTCCGA and reverse: TCATGTTGCGTCTAGATCGGT), we were able to obtain a single band of 3.2kb, corresponding to gap2, by long-range PCR on the *Vitis sylvestris* PSL10 male. DNAs from one female *V. sylvestris* and the hermaphrodite Pinot noir

519 (respectively with introduction codes 8500Mtp110 and 193Mtp81, INRA Vassal Grape
520 Collection) did not provide any amplification, as expected. The forward and reverse Sanger
521 sequences of this PCR product were obtained through an AB1 sequencer, and have 93-99%
522 identity with the same region on 3' of *PLATZ*, of the reference genome PN40024, and the
523 Cabernet Sauvignon, the Chardonnay and the *Vitis sylvestris* female PacBio sequences. We
524 can thus confirm that in the male haplotype, *PLATZy* is located in the expected region
525 between *ETOL1* and the first *FMO*.

526 On the other hand, we did not succeed to sequence gap1, as PCR amplifications using several
527 combinations of primers defined in contig1 and *PLATZ* always provided multiple bands,
528 confirming that gap1 is highly likely to correspond to a repeated element, as observed in the
529 reference genome PN40024, Cabernet Sauvignon, Chardonnay and *Vitis sylvestris* female.

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