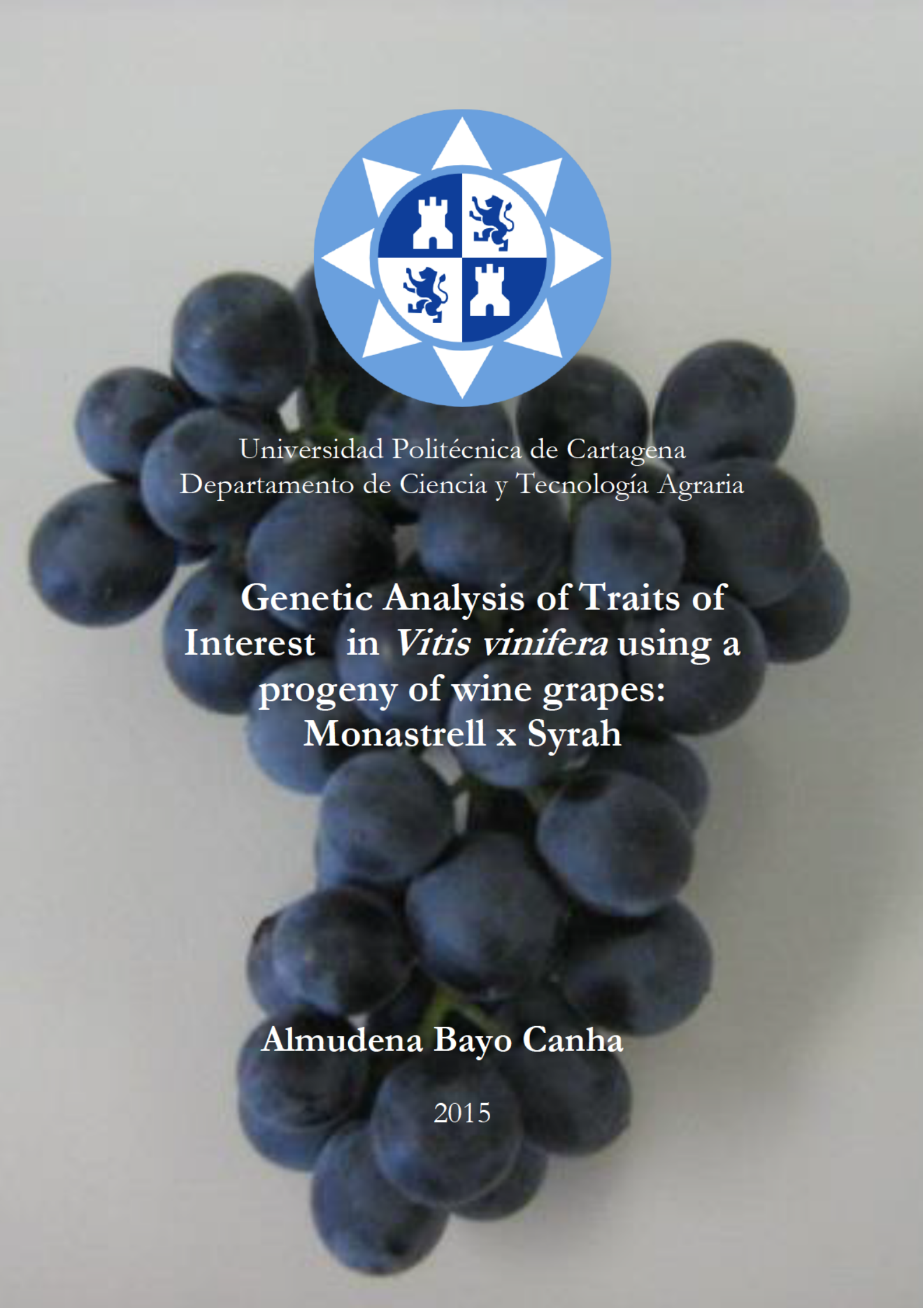

Universidad Politécnica de Cartagena
Departamento de Ciencia y Tecnología Agraria

Genetic Analysis of Traits of Interest in *Vitis vinifera* using a progeny of wine grapes: Monastrell x Syrah

Almudena Bayo Canha

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Dra. Leonor Ruiz García

2015

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DT-16

CONFORMIDAD DE SOLICITUD DEAUTORIZACIÓN DE DEPÓSITO DE TESIS DOCTORAL POR EL/LA DIRECTOR/A DE LA TESIS

D./Dª. Leonor Ruiz García Director/a de la Tesis doctoral "Genetic analysis of traits of interest in *Vitis vinifera* using a progeny of wine grapes: Monastrell x Syrah"

INFORMA:

Que la referida Tesis Doctoral, ha sido realizada por D/Dª. Almudena Bayo Canha, dentro del programa de doctorado "Técnicas Avanzadas en Investigación y Desarrollo Agrario y Alimentario", dando mi conformidad para que sea presentada ante la Comisión de Doctorado para ser autorizado su depósito.

La rama de conocimiento en la que esta tesis ha sido desarrollada es:

X Ciencias
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En Cartagena, a 17 de Septiembre de 2015

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Fdo.: Leonor Ruiz García

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Universidad Politécnica de Cartagena

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D./Dª. Marcos Egea Gutiérrez-Cortines Tutor/a de la Tesis doctoral "Genetic analysis of traits of interest in *Vitis vinifera* using a progeny of wine grapes: Monastrell x Syrah"

INFORMA:

Que la referida Tesis Doctoral, ha sido realizada por D/Dª. Almudena Bayo Canha, dentro del programa de doctorado "Técnicas Avanzadas en Investigación y Desarrollo Agrario y Alimentario", dando mi conformidad para que sea presentada ante la Comisión de Doctorado para ser autorizado su depósito.

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Universidad Politécnica de Cartagena

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D/Dª. Francisco Artés Hernández, Presidente/a de la Comisión Académica del Programa "Técnicas Avanzadas en Investigación y Desarrollo Agrario y Alimentario".

INFORMA:

Que la Tesis Doctoral titulada, "Genetic analysis of traits of interest in *Vitis vinifera* using a progeny of wine grapes: Monastrell x Syrah", ha sido realizada, dentro del mencionado programa de doctorado, por D/Dª. Almudena Bayo Canha, bajo la dirección y supervisión del Dr/ Dra. Leonor Ruiz García.

En reunión de la Comisión Académica de fecha 16/9/2015 , visto que en la misma se acreditan los indicios de calidad correspondientes y la autorización del Director de la misma, se acordó dar la conformidad, con la finalidad de que sea autorizado su depósito por la Comisión de Doctorado.

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En Cartagena, a 16 de septiembre de 2015

EL PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA

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Fdo: Francisco Artés Hernández

COMISIÓN DE DOCTORADO

FONDAZIONE EDMUND MACH

San Michele all'Adige, September 10 2015

To whom it concerns

Based on my experience, I confirm that the work done by Almudena Bayo Cahna "Genetic analysis of traits of interest in *Vitis vinifera* using a progeny of wine grape: Monastrell x Syrah" is suitable for evaluation to earn the doctoral degree.

In particular, the topics described in the thesis are actual and relevant to viticulture and oenology. The adopted approach is correct and consistent with that reported by other authors working in a similar field. The phenotypic traits have been evaluated in multiple seasons, which is important to minimize environmental effects and make results as robust as possible. The F_1 progeny is adequate in size for the plant species under study and segregates for the examined traits. The linkage maps are dense enough for QTL analysis. Several QTLs confirm the results reported by other authors and additional QTLs are new findings. Candidate genes have been proposed, which represent a valuable resource for further validation.

Sincerely,

Dr. Laura Costantini Laura Costantini

Fondazione Edmund Mach- Research and Innovation Center

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Grapevine Applied Genomics Group

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Institut National de la Recherche Agronomique
Centre de Montpellier

Unité Mixte de Recherche
Amélioration Génétique et Adaptation des Plantes
Méditerranéennes et Tropicales

Montpellier, August 14th, 2015

Dear Madam, Dear Sir,

After carefully reading the summary of the PhD work carried out by Almudena Bayo Canha at IMIDA in Murcia (Spain), I can attest that the quality of this work seems to be suitable to be submitted to an appropriate jury to postulate to the Doctor degree. A large amount of data has been generated (genotyping and phenotyping of 22 traits on a quite large population in several years) and these data have been adequately analysed with classical methods to detect QTLs, for which candidate genes have been proposed. These results have been compared to previously published ones. QTL stability among years has been discussed. After further discussion on trait correlations and heritability, as well as QTL colocalizations, the results obtained can be submitted for publication in an international peer-reviewed journal.

Sincerely,

Dr Agnès Doligez
Research Ingeneer

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Specially thanks to Stella Grando team: Laura Costantini, Juri Batillana, Francessco Emanuelli, Madda Sordo, Silvia Lorenzi and Valentina Catalano. Thanks for your help and friendship during my stay in Italy.

A mi compañero de viaje en la vida, gracias por compartir todo conmigo. Por ser mi ancla, mi brújula cuando me pierdo, por creer en mi siempre, por tener sueños e ilusiones compartidos. La mitad de esta tesis es tuya, al igual que la mitad de tu tesis es mía, eso es así.

A los chicos de la seda, por nuestras conversaciones frikis que nos hacían evadirnos de la realidad por un rato. A Mercedes y María por su amistad y apoyo en los comienzos. A Luis y Lola por esas risas sanas. A Lola especialmente por la amistad que se inició en ese pasillo y que hoy en día sigue creciendo, pese a un océano de por medio. A Marga por su apoyo

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A mi tutor en la universidad, el Dr. Marcos Egea por acompañarme durante esta andanza y ayudarme con tanto papeleo. Este agradecimiento se hace extensivo a la Dra. Julia Weiss por ser ellos quienes me mostraron este apasionante mundo de la genética, gracias por todo lo que me enseñasteis en clase y después por los años de laboratorio donde tuve la suerte de aprender mucho a vuestro lado. Gracias por ser mis maestros.

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A todos vosotros que habéis compartido conmigo este trozo de camino, gracias por hacer mas llevadero este viaje. Gracias a vuestro apoyo uno de mis sueños se ha hecho realidad.

Abbreviations

Ac	Acidity
Ant	Anthocyanins
AFLP	Amplified Fragment Length Polymorphism
BAC	Bacterial Artificial Chromosome
BW	Berry weight
CAPS	Cleaved Amplified Polymorphic Sequence
CC	Cluster compactness
CG	Candidate Genes
CeGen	Centro Nacional de Genotipado
cDNA	complementary Deoxyribonucleic acid
cM	Centimorgan
CN	Cluster number
CW	Cluster weight
DHPLC	Denaturing High-Performance Liquid Chromatography
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides triphosphate
D.O.	Denominations of Origin
EST	Expressed Sequence Tag
FAOSTAT	Food and Agriculture Organization of the United Nations
FEM	Fondazione Edmund Mach
Fi	Fertility index
Fw	Flowering
Ge	Estimated Genome size
LG	Linkage group
LGW	Linkage-group-wide thresholds
Gob	Observed Genome length
GW	Genome-wide thresholds
IGGP	International Grape Genome Program
IMIDA	Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario
Kb	Kilobase
KW	Kruskal-Wallis
LOD	Likelihood Ratio or Logarithm of odds
Ma	Malic acid
MAS	Marker-Assisted Selection
MQM	Multiple QTL Mapping
MLE	Maximum Likelihood Estimators
Mn	Monastrell
MYB	Myeloblastosis family of transcription factors
NCBI	National Center for Biotechnology Information
OIV	International Organization of Vine and Wine
P	Production

PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RGA	Resistance gene analogs
Rp	Ripening
SAMPL	Selective Amplification of Microsatellite Polymorphic Loci
SCAR	Sequence Characterized Amplified Regions
SIM	Simple Interval Mapping
SNP	Single Nucleotide Polymorphism
Sp	Sprouting
SSCP	Single Stranded Conformational Polymorphism
SSR	Simple Sequence Repeat
STR	Short Tandem Repeats
S-SAR	Sequence-Specific Amplification Polymorphism
Sy	Syrah
Ta	Annealing Temperature
Taq	*Thermus aqualticus*
Tar	Tartaric acid
TCA	Tricarboxylic acid
TSS	Total soluble solids
VMC	Vitis Microsatellite Consortium
Vr	Veraison

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Chapter 1. Phenotypic segregation and relationships of agronomic traits in Monastrell x Syrah wine grape progeny

Chapter 2. Genetic linkage maps construction

Chapter 3. QTLs Analysis

I. SUMMARY

I SUMMARY

1. Introduction

The wine industry has a high value throughout the world; it is of economic, cultural and historical importance in each productive area. Currently, the competition in the wine markets makes it necessary to seek and provide alternatives. On the one hand, climatic changes cause problems, so that varieties well adapted to the new conditions are required. On the other hand, the high market competitiveness drives the development of new varieties with high berry quality, for transforming into innovative and balanced wines. Vine breeders are working to provide new options for growers to adapt to these new scenarios.

In wine grapes there are some traits that are important for viticulture and the winery, such as phenological period, productivity, and the quality of the berries. Most of these traits are controlled by a large number of genes of minor effect, together with influences of the environment. Early identification of individuals carrying the desired allele combinations allows breeders to grow larger effective populations, which results in decreased maintenance and evaluation costs. The identification of genes and molecular markers underlying quality traits will help accelerate the breeding process, creating new opportunities for crop improvement.

One approach to the improvement of conventional breeding is the identification of chromosomal regions or QTL (Quantitative Trait Loci) involved in the genetic control of quality traits, based on genetic maps and phenotypic evaluation of a segregating progeny. The purpose of QTL mapping is to identify the number and location of the genetic determinants responsible for the variation of the quantitative traits under study and their stability among different years. The linkage of markers with heritable traits is used to associate the genotype of an individual with the expressed phenotype, and would make marker-assisted selection (MAS) very efficient.

2. Objectives

The aim of the IMIDA's wine grape breeding program is to obtain new varieties well adapted to Murcia's climate conditions and with better genetic composition. The goal of this work is the identification of the major genetic determinants for a given phenotypic trait in genetic maps and their co-localization with the position of candidate gene sequences related to the relevant phenotype. For this purpose, three specific objectives were considered:

1. The phenotypic evaluation of a progeny derived from a controlled cross between the wine grape cultivars Monastrell and Syrah.

2. The construction of grapevine genetic maps, using this progeny and molecular markers.

3. The use of these maps and the phenotypic data of the progeny for QTL analyses, in order to develop helpful markers for breeding programs.

3. Material and Methods

Plant Material

This study is based on an F_1 progeny of 229 hybrids obtained from controlled crosses between the wine grape varieties Monastrell (female progenitor) and Syrah (male progenitor). Monastrell is a thick-skinned black grape, high in tannins and late maturing. It is a variety of great hardiness and high resistance to drought and it needs a good deal of insolation. It is very well adapted to the dry conditions of the Mediterranean climate, and is spread across the whole Mediterranean basin. Monastrell is the most planted wine variety in Murcia (Spain), and is the predominant variety included in the Denominations of Origin (DOs) of Jumilla, Yecla and Bullas, Alicante and Almansa, being grown in the Spanish regions of Murcia, Castilla-La Mancha and Comunidad Valenciana. Syrah is a dark-skinned grape, high in flavors and soft tannins and early maturing. Wines made from Syrah are often powerfully

flavored and full-bodied. In Murcia, Syrah is also used for blending with Monastrell due to the acidity, color, and tannin levels of Syrah that provide a favorable ageing potential.

This progeny segregates for some of the most important traits in wine grapes, like fertility, ripening time, bunch compactness, berry size, color, and acidity. The vines of this population were grown on their own roots under standard conditions of irrigation, fertilization, and pest and disease control, in an experimental field of the IMIDA in Murcia (South-east Spain).

Marker analysis

Total DNA extraction from young leaves was performed using the commercial kit "DNeasy Plant Mini Kit" (Qiagen, IZASA, Spain), following the manufacturer's protocol. The parent and mapping populations were genotyped using microsatellites (SSR, Simple Sequence Repeat), SNPs (Single Nucleotide Polymorphism) and CAPS (Cleaved Amplified Polymorphic Sequence) markers. Three hundred and thirty-five SNP markers (Lijavetzky et al. 2007; Cabezas et al. 2011) were analysed in the Spanish National Genotyping Centre (CeGen) with the SNPlex technology, 138 of them resulting informative for linkage mapping. In addition, eight new SNP-based markers were mapped, after their identification and development by applying the candidate genes (CG) approach (Pflieger et al. 2001) at the Fondazione Edmund Mach (San Michele all'Adige, Italy), in collaboration with the research team of Dr. Stella Grando. These candidate genes were selected based on different QTLs intervals. For suitable polymorphisms primers a mini-sequencing protocol was applied, employing the SNaPshot Multiplex Kit protocol. For SSR, 177 were first analyzed with the genitors and six progeny individuals (NCBI UniSTS GeneBank database) to select the most informative ones. Of these, 104 were selected for linkage mapping according to their segregation type. The SSR markers were analyzed by PCR, and the

products were separated by capillary electrophoresis using an ABI Prism 3730 Genetic Analyzer sequencer; the fragments were sized using GeneMapper software (Applied Biosystems). Finally, the mapping population was also genotyped with the CAPS marker 20D18CB9, linked to berry color (Walker et al. 2007).

Linkage maps

Genetic maps were developed with JoinMap 3.0 software (Van Ooijen and Voorrips 2001), applying the Kosambi function for the estimation of map distances (Kosambi 1944). Linkage groups (LGs) and marker order were determined using threshold values of 4.0 for LOD (logarithm of odds) and 0.4 for recombination rate. The LGs were numbered from LG1 to LG19 according to the international agreement achieved within the IGGP (International Grape Genome Program; www.vitaceae.org). Most markers showing distorted segregation were originally included in the map calculation unless they significantly affected the order of neighbouring markers. The markers order obtained was kept in round 2, but in some cases the markers order was fixed according to the position of the markers in the database of the grape genome sequences (www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/). Female, male and consensus genetic maps were aligned using MapChart v2.2 software (Voorrips 2002).

Phenotypic evaluation

Twenty-two segregating agronomic traits were evaluated for each genotype in three or six seasons (2008-2013). All statistical analyses were performed using SPSS 18.0 for Windows. Differences between years for each trait were analysed by the Kruskal-Wallis test, and the correlation between traits was calculated by the Spearman test.

Phenology-related traits

Budbreak was measured as the date when 50% of the buds were in Baggiolini stage C (Baggiolini 1952), and flowering as the date when 50% of the flowers were opened and the anthers were visible. Veraison was considered as the date when 50% of the berries were coloring and/or softening. Ripening time was considered when colored grapes reached 13.5 ºBaumé and white ones reached 12.5 ºBaumé.

Productive and morphological traits

Productive and morphological traits were evaluated at harvest (ripeness) in the laboratory, except the fertility index - which was scored before flowering as the number of inflorescences per young shoot. The average cluster weight was calculated using all the clusters per genotype, and the average berry weight was calculated using 300 berries taken randomly per genotype. For berry shape and the number of seeds per berry, 30 berries taken randomly were sampled per genotype. Berry skin color was determined visually as uncolored or colored. Cluster density, cluster shape, and berry shape were classified following the OIV codes.

Enology-related traits

These analyses of each sample were performed in triplicate; each one with approximately 100 g of berries taken randomly. Total soluble solids were determined as ºBaumé, using an Atago RX-5000 digital refractometer. The juice pH and titratable acidity were determined by titration with 0.1 N NaOH, using a Metrohm 686 automatic titrator. The titratable acidity was expressed as g/L tartaric acid equivalent. Tartaric and malic acids were measured using enzymatic kits. Potassium content was determined by atomic absorption spectrometry, using a Unicam 969 spectrophotometer, and expressed as g/L. The phenolic potential of the grapes was determined based on the method

described by Saint-Cricq et al. (1998). Total and extractable anthocyanins contents were assayed by measuring the absorbance at 520 nm at pH 1.0 and pH 3.6, respectively, and were expressed as mg/L. The extractability index was calculated as described by Romero-Cascales et al. (2005).

QTL analysis

The QTL analysis was done using MapQTL® 4.0 software (Van Ooijen et al. 2002). It was based on three different methods: the Kruskal-Wallis non-parametric test (KW; Lehmann 1975), simple interval mapping (SIM; Lander and Botstein 1989) and multiple QTL mapping (MQM; Jansen and Stam 1994). Initially, possible QTLs were identified by the KW and SIM methods. Both genome-wide and linkage-group-wide LOD thresholds corresponding to a significance level of α=0.05 were established from 1,000 permutations (Churchill and Doerge 1994). The significant and/or suggestive QTLs detected with SIM were considered. Then, scored markers in those regions were used as cofactors in MQM analysis. The QTL position was estimated from the location of the maximum LOD value with a 1-LOD support interval. The additive effect and percentage of phenotypic variance explained by each QTL were estimated from the MQM model.
The normality of each trait distribution was evaluated by the Kolmogorov–Smirnov test.

4. Results and Discussion

Phenotypic evaluation

Continuous variation and transgressive segregation were observed for most of the evaluated characters. The phenotypic data distributions were very similar in the different years analyzed.

Phenological traits

The mean values of sprouting, flowering, and ripening (days since 1st January) and the mean length of the veraison-ripening interval showed significant differences among the years of the study. Syrah was the earlier parent for all the phenology-related traits. Most of the hybrids were later than Monastrell for flowering and veraison, and most of them showed sprouting and ripening times between those of the two progenitors.

Productive and morphological traits

The mean fertility index (0.6) of the progeny was lower than the values of both progenitors. Over 74% of the hybrids showed lower fertility than both progenitors. The mean cluster weight (84 g) of the progeny was lower than that of both progenitors, and 86% of the hybrids were distributed in the low-cluster-weight range (below 161 g). For cluster compactness, 5% of the hybrids showed loose clusters and 29% medium clusters. The berries of Monastrell and Syrah are colored and, in agreement with the expected Mendelian segregation for a monogenic, dominant trait (3:1), 74% and 26% of the progeny showed colored and uncolored berries, respectively.

Enological traits

The total acidity ranged between 2.6 and 10.6 g/L, the average value of the progeny being higher than the values of both progenitors. However, the mean tartaric acid content of the progeny was lower than that of both progenitors. The malic acid content in the progeny ranged between 0.8 and 6.5 g/L and was less than that of tartaric acid. The total anthocyanins ranged between 235 and 2969 mg/L and only 17% of the hybrids had values higher than that of Syrah.

Correlation between traits

Several associations between traits were revealed within each year, with Spearman coefficients higher than 0.5. Many of them were significant in all years analyzed and concerned the component variables of the same character: a positive, high correlation between veraison-ripening interval and ripening date; a positive, high correlation between visual color, total anthocyanins, extractable anthocyanins, and extractability index; a negative, moderate correlation between total acidity and pH. Cluster weight correlated positively with berry weight, fertility index, and cluster shape, but negatively with tartaric acid. Total acidity correlated negatively with ripening, veraison-ripening interval, and visual color.

Linkage maps

The total number of molecular markers useful for linkage analysis in the MnxSy mapping progeny was 251 (104 SSRs, 146 SNPs, and 1 CAPS), of which 84% allowed discrimination between maternal and paternal inherited alleles. The complete linkage map of Monastrell consisted of 160 molecular markers distributed in 19 linkage groups (LGs) covering 1035 cM, with an observed coverage of 61% and an average distance between loci of 7.02 cM. The complete linkage map of Syrah consisted of 186 molecular markers distributed in 19 LGs covering 1038 cM, with an observed coverage of 60% and an average distance between loci of 6.22 cM. The integrated map covered 1174 cM with 238 markers, with an average distance between loci of 5.23 cM and an observed coverage of 76%.

QTLs detection

Phenotypic data and the three maps developed (Monastrell, Syrah, and integrated) were used to perform QTL analysis. These analyses were carried out using a multiple QTL model (MQM) based on QTLs detected previously

via interval mapping (SIM). The QTL analysis was made separately at the genome-wide-level (GW) and linkage-group-level (LGW), at 95% significance. Five significant QTLs were detected for sprouting at the GW level - on LGs 1, 7, 8, 13, and 14 - but only the QTL on LG 7 was stable over the years studied (consistent). They explained between 6% and 12% of total variance. The QTLs on LGs 7 and 14 were previously reported by other authors. For flowering time, three QTLs were found - on LGs 5, 7, and 14 - but only the QTL on LG 7 was consistent, explaining up to 19% of total phenotypic variance. The *VvFT* (*Flowering locus T*) gene was found within the confidence interval of the QTL detected on LG 7. Four significant QTLs were detected for veraison, but only the QTL on LG 2 was consistent, explaining up to 22% of total phenotypic variance. The QTLs on LGs 5, 8, and 11 were detected only in one year (2008), but were reported by other authors (except the QTL on LG 5). Ripening date and the veraison-ripening period were found to be under the control of two genomic regions (LGs 2 and 17), explaining between 7% and 18% of total phenotypic variance. *VvMybA*, a transcription factor that control the presence or absence of color in grapes, was found within the confidence interval of the QTL detected on LG 2.

Two significant QTLs for fertility were detected on LGs 3 and 5, explaining between 6% and 26% of total variance. Although the region on LG 3 was detected only in one year, it was reported previously by other authors. The QTL on LG 5 was consistent and also was reported by other authors. Berry weight was under the control of six genomic regions (LGs 1, 5, 7, 14, 17, and 19), which explained between 6% and 12% of total variance. The QTLs located on LGs 5, 14, and 17 were consistent. A cytochrome P450 78A-like protein linked with tomato weight was found within the confidence interval of the QTL detected on LG 17. For cluster compactness, two QTLs, localized on LGs 2 and 5 explained up to 28% of phenotypic variance.

Total acidity was shown to be under the control of two genomic regions (LGs 1 and 2), which explained between 7% and 18% of total phenotypic variance. For the ratio of total soluble solids to total acidity, three QTLs were found - on LGs 1, 2, and 4 - but only the QTL on LG 2 was consistent, explaining up to 19% of total phenotypic variance. The *CBL01* (*Calcineurin B-like protein 01*) gene was found within the confidence interval of the QTL detected on LG 2. This protein acts as a complex, together with a CBL-interacting protein kinase (CIPK), in the activation of a K^+ channel of the Shaker family VvK1.2. Although for tartaric acid only one consistent QTL was found, on LG 16 at the LGW level (suggestive), an important gene was located within this region. *L*-idonate dehydrogenase is the key enzyme involved in the conversion of *L*-idonate to 5-keto D-gluconic acid, that produces oxalic acid, which is the only pathway known in the synthesis of tartaric acid in grapes. Malic acid was under the control of seven genomic regions (LG 4, 5, 8, 9, 15, 17, and 18), which explained between 11% and 29% of total phenotypic variance. For the ratio of tartaric acid to malic acid, three QTLs were found, on LGs 5, 8, and 11; these explained between 11% and 21% of variance. The *PDC1* (*Pyruvate Decarboxylase 1*) gene was found within the confidence interval of the QTL detected on LG 8. The increase in ethanol is linked with pyruvate production, derived from malic decarboxylation. *PDC* is the key enzyme in the fermentative metabolism and in the production of ethanol. One significant and consistent QTL was detected for total anthocyanin, on LG 2, and explained up to 80% of total phenotypic variance. The transcription factors involved in anthocyanin synthesis, *VvMybA1*, *VvMybA2*, and *VvMybA3,* were located within this region.

5. Conclusions

The study of this progeny during several seasons revealed that crossing Monastrell and Syrah can generate a large phenotypic variability that may be useful in the development of new cultivars with improved attributes. The joint analysis of these phenotypic data and of genetic variations at SSR and SNP loci allowed us to identify several QTLs for phenological stages, productivity, and morphological and enological traits in wine grapes. These results open new perspectives for future studies on the genetic determinism of quality traits in grapevine.

II. GENERAL INTRODUCTION

II. GENERAL INTRODUCTION

The **grapevine** is one of the oldest crops in the world and still one of the most important in economic terms. Originating in Asia, it is grown throughout the temperate regions. In addition to making wine and other alcoholic beverages, the berries are consumed fresh (table grapes) or after drying and are used to obtain juice.

Figure 1. Botanic draw of *Vitis vinifera*. Font: www.lavid.eu

The grapevine is a perennial, climbing plant having a twisted trunk with very long and flexible stems with jointed nodes. It attaches itself to natural supports using specialized organs called tendrils borne opposite the leaves. The leaves are alternate, petiolate, large, and consist of five pointed lobes. Its

flowers are greenish, small, and regular; the stamens occur opposite the petals and the pistil has two carpels (Mullins et al. 1992; Hidalgo 2002). The fused petals, called calyptra or cap, remain connected at the apex, while splitting along the base from the receptacle. The sepals degenerate early in flower development. Cultivated varieties generally have hermaphrodite flowers, while the wild forms are dioecious, with plants having only female (containing a functional pistil and either producing recurved stamens and sterile pollen or lacking anthers) or only male flowers (possessing erect functional anthers and lacking a fully developed pistil) (Srinivasan and Mullins 1976). The fruit of the grapevine is the grape, a berry that is more or less round in shape, juicy, and forms clusters. This berry is constituted by an outer film called the skin (epicarp), the pulp (mesocarp), and the endocarp, which generally contains a maximum of four seeds.

II.I ORIGIN

The history of the grapevine is closely related to the development of human culture, and has been recently reviewed by This et al. (2006). While the oldest known fossils of the Vitaceae family date from the lower Eocene, about 56 million years ago, the first wild vines appeared in Europe in the Pliocene (2-2.5 million years ago). Sheltered during the Quaternary in the Mediterranean basin and to the south of the Caspian Sea, at the end of this cold period they migrated to the east-west Mediterranean basin. Currently, the grapevine is found from the Atlantic coasts of Europe to Tajikistan and the western Himalayas, and from the slopes of the Rhine to the northern forests of Tunisia (Zohary and Hopf 2000; Hidalgo 2002; McGovern 2003).

The hunter-gatherers of the Paleolithic (2.8 million - 8500 years BC) used the berries of wild vine (*V. vinifera* ssp. *silvestris*) as a food source (Zohary 1996). From that moment, man began to select hermaphrodite vine plants since their clusters bore more berries. Also selected were those plants whose berries were

larger and with a higher sugar content (Zohary and Hopf 2000). The selection was carried out both to obtain food and for wine making, a fact confirmed by the discovery of Neolithic pottery vessels (8500-4000 BC) containing the remains of seeds and tartaric acid (McGovern et al. 1996; Hidalgo 2002). This process of domestication resulted in the cultivated vine (*V. vinifera* ssp. *sativa*), which differs from the wild vine at both the phenotypic and genetic levels (reviewed by This et al. 2006). In addition to the archaeological and historical evidence suggesting that the first domestication of the vine occurred in the Middle East (McGovern et al. 1996), other genetic studies around the Mediterranean basin support the existence of secondary domestication events, independent of the initial domestication process in the center of diversity of the species (Arroyo-García et al. 2006; Lopes et al. 2009; Myles et al. 2011). Recent analysis of the genetic relationships between wild Spanish vines and cultivated vines suggests a genetic contribution of the wild accessions in Spain to contemporary Western varieties (De Andrés et al. 2011).

The expansion of the domesticated grapevine took place from the center of diversity of the vine, the Middle East, to regions such as the Jordan Valley (4000 BC), Egypt (3000 BC), the southern and central mountains of Zagros (Iran), and Lower Mesopotamia (around 3000 BC). The expansion towards the west is documented in Crete (2200 BC) and the coasts of the Italian and Iberian peninsulas (800 BC) (McGovern 2003). The cultivation of the grapevine reached China and Japan about 1200 BC. The expansion of wine consumption was made possible initially by the Phoenicians, who transported wine around the Mediterranean Sea. The Romans spread it throughout Europe and gave names to the different varieties. For them, wine was associated with the high classes of society and usually was drunk mixed with water, and always in company and at social events. More details about the production and producers of wine were found in Egyptian pictures dating from around 1500 B.C. In Spain, the first findings were in a Phoenician

settlement in Almuñecar, where some vessels had remains of high quality wine from Egypt, dating from 717 B.C. (Hidalgo 2002). During the Imperial Roman Empire it reached many temperate regions of Europe, even reaching Germany. At the end of the Roman influence, the cultivation of grapevines was common in most of the parts of Europe where it is cultivated today.

In the Middle Ages, the Catholic Church – by means of the crusades - spread the cultivation of the grapevine and facilitated the exchange of germplasm. The spread of Islam to North Africa, Spain, and the Middle East also had an important role in the expansion of grape cultivation, especially table grapes (Royer 1988). During the Middle Ages varietal names appeared that are still used today. Later, missionaries introduced the cultivated vine into America, first as seeds (since they are easy to carry) and then as cuttings brought from France, Germany, Spain, Italy and Eastern Europe. In the nineteenth century, cuttings were also taken to South Africa, Australia, and New Zealand, and later to North Africa. The connexions between table grape varieties confirm that there is a closer relationship among them than for wine varieties. This shows that there has been more inter-breeding than for wine varieties, which are more ancient. Nonetheless, grapes have high heterozygosity compared with other domesticated crops and it is worth highlighting that relatively few varieties are used in the wine industry around the world. This poor variability among the cultivars helps to explain the phylloxera impact at the end of the 19th century in Europe, which necessitated the use of American vitis rootstocks and attempts to preserve the varieties through cuttings. However, for the winemaking industry cultivar homogeneity is important and it is achieved with the use of vegetative propagation. The southward movement of grapevines in Europe and western Asia would have been largely restricted by the east-west mountain ranges. This may explain the existence of only one *Vitis* sp. (*V. vinifera*) from the Atlantic coast of Europe to the western Himalayas, whereas China possesses

about 30 species, and North and Central America about 34 species (Jackson 2008).

II.II TAXONOMY

In taxonomic terms, the grapevine occurs within the division Magnoliophyta, class Magnoliatae, subclass Rosidae, superorder Celastranae, order Rhamnales, family Vitaceae, and genus *Vitis* – agronomically, *Vitis* is the most important genus of the Vitaceae. Originating from warm or temperate zones of the northern hemisphere, in America, Europe, and Asia, *Vitis* consists of approximately 60 inter-fertile species (This et al. 2006). Grapevines are distinguished from related genera primarily by floral characteristics. According to Galet (1967), in the genus *Vitis* two sections or subgenera can be distinguished: *Muscadinia* and *Euvitis*.

The subgenus *Muscadinia* includes three species (*V. rotundifolia* Michx, *V. munsoniana* Simpson, and *V. popenoeii* Fen) from the warm and temperate regions of southeastern North America. These species are characterized by the presence of simple tendrils, adherent bark with lenticels, nodes without a diaphragm, berries that are not very sweet and whose maturation is staggered, and a diploid chromosome number (2n) of 40.

The subgenus *Euvitis* includes more than 50 species characterized by the presence of bifurcated tendrils, bark that peels off, nodes with a diaphragm, and a diploid chromosome number (2n) of 38. Grapevines are thought to be ancestrally hexaploids. Its species can be classified by their natural geographical range, as American and European-Asian. Among the latter, the species *Vitis vinifera* is the only one with a significant economic importance, and the only one employed in wine production. Currently, two forms of this species co-exist in Eurasia and North Africa: the cultivated form *V. vinifera* ssp. *vinifera* (or *sativa*) and the wild form *V. vinifera* ssp. *silvestris* (or *sylvestris*). This separation into subspecies was based mainly on morphological

differences due to the selection or process of domestication of grapevine by man over time.

II.III GENOME

Although the cultivated grapevine has hermaphrodite flowers that can be self-pollinated, cross-fertilization by insects and wind is normal; thus, it has a highly heterozygous genome with many lethal recessive mutations (Olmo 1979; Thomas and Scott 1993). Recently the genome of Pinot Noir was sequenced (Velasco et al. 2007), as well as a highly homozygous line of the same variety, obtained by successive backcrosses (Jaillon et al. 2007). This sequencing work has yielded more detailed knowledge of specific characteristics of the grapevine genome that had previously been inferred using methods such as flow cytometry (Lodhi and Reisch 1995) or genetic maps (Lodhi et al. 1995; Dalbó et al. 2000; Doligez et al. 2002; Adam-Blondon et al. 2004; Fischer et al. 2004; Riaz et al. 2004; Fanizza et al. 2005; Cabezas et al. 2006; Doligez et al. 2006 a and b; Di Gaspero et al. 2007; Mejía et al. 2007; Troggio et al. 2007; Welter et al. 2007; Costantini et al. 2008; Salmaso et al. 2008; Vezzulli et al. 2008; Doligez et al. 2010).

The grapevine genome is distributed in 19 chromosomes (2n = 38), with a size of between 487.1 Mb (Jaillon et al. 2007) and 504.6 Mb (Velasco et al. 2007). It is, therefore, of a size similar to that estimated for the balsam poplar of California [*Populus trichocarpa*, 485 Mb (Tuskan et al. 2006)] and rice [*Oryza sativa*, 420 Mb (Goff et al. 2002)], almost four times that of the Arabidopsis genome [*Arabidopsis thaliana*, 125 Mb (Kaul et al. 2000)], half that of tomato [*Solanum lycopersicum*, 1,000 Mb (Lodhi and Reisch 1995)], a sixth of that of corn (*Zea mays*, 3,000 Mb), and 33 times less than that of wheat [(*Triticum spp.*, 16.000 Mb) (Goff et al. 2002)]. The estimated number of genes is 29,585 to 30,434, with an average of 372 codons and five exons per gene (Jaillon et al. 2007; Velasco et al. 2007).

Analysis of the elements with repetitive sequences revealed that 24.7 to 41.4% of the total genome are transposable elements, more abundant in grapevine than in the poplar, rice, or Arabidopsis. The distribution of these repetitive elements is not very uniform throughout the genome, being very abundant in introns (Jaillon et al. 2007). In addition, 88,909 microsatellite sequences (repetitions of very short and simple motifs) have been identified, which occupy 2.1 Mb, representing 0.42% of the total genome (Velasco et al. 2007).

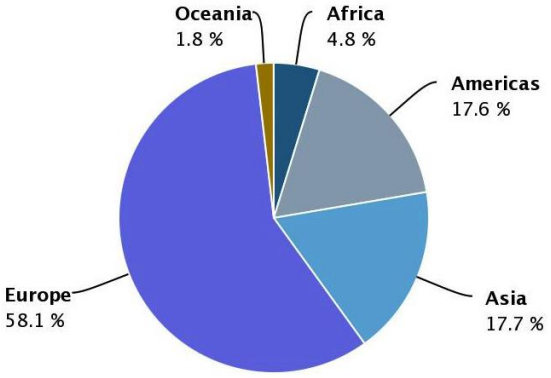
Velasco et al. (2007) identified and assessed two million polymorphisms arising from the substitution of one nucleotide in a sequence by another or SNPs (single nucleotide polymorphisms), with a frequency of 4.0 SNPs per kb, being less frequent in coding regions than in non-coding regions. Of the genes, 86.7% contain one or more SNPs, which is very interesting when it comes to using them as genetic markers, because the SNPs can cause natural phenotypic variation.

II.IV ECONOMIC IMPORTANCE

The cultivation of the grapevine is an activity of great economic importance worldwide. In 2007, more than 7.7 million hectares of grapevine were cultivated, producing 66.5 million tonnes of grapes (OIV 2011), which makes grapes the fourth-most-produced fruit, after bananas, oranges, and apples (FAO 2007). Europe is the largest producer of grapes, with 44% of world production. The list of European grape-growing countries is headed by Italy, France, and Spain, the latter being the fifth-greatest producer on a global basis. Of the global grape production, 30.2% is consumed fresh (table grapes), with Asia the largest producer - led by China, Iran, Turkey, and India. Spain lies in twelfth place, and is the second-largest producer of table grapes in Europe, behind Italy. Of the 1,023 million ha of vine farmed in Spain in 2013, 97.4% are dedicated to wine production, 2% to table grapes, 0.3% to the

production of raisins, and 0.3% to vineyard nurseries (www.mapa.es). The European Union is the largest wine producer in the world, accounting for about two-thirds of global production. Of the estimated 25.2 million tonnes of grapes produced in the EU in 2013, the vast majority (91%) were destined to wine production.

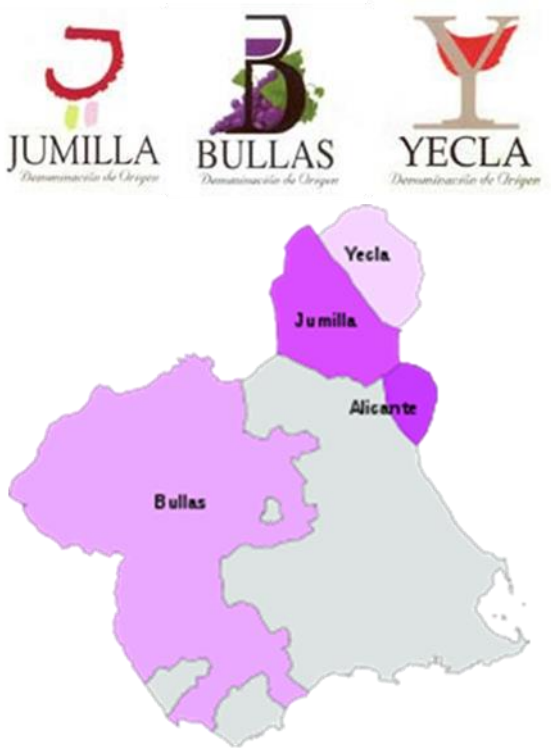
Figure 2. World production of grapes by continent (FAOSTAT)



World wine production in 2013 was 218.6 million hectolitres (hl), representing an increase of about 24 million hl with respect to 2012. The largest producers of wine were Italy with 44.9 million hl (16.1% of world production), Spain with 42.7 million hl (15.3% of world production), and France with 42 million hl (15.1% of world production). Outside of Europe the production was 114.4 million hl, being highest in the USA (22 million hl), Argentina (15 million hl), Chile (12.8 million hl), and Australia (12.5 million hl). Spain is the country with the greatest cultivated area for this crop in both Europe (30% of the total area) and the World (13.4%). Here, wine production was stable during a number of years at about 40 million hl, but in 2013/2014 it increased to 52 million hl (FEGA; Federación Española de Garantía Agraria). In 2014 Spain took the lead in wine sales by volume but the average price was lower. By contrast, Italy weakly decreased its exports by volume but increased the profit. The cultivated areas inside Spain are shared out thus: 48.8% Castilla-La Mancha, 8.7% Extremadura, Valencia, Castilla and

León. In Spain there are 90 Geographic Denominations of Origin (D.O.), three of being located in Murcia (Bullas, Jumilla, and Yecla).

Figure 3. D.O. areas in the Region of Murcia



The Jumilla D.O., created in 1966, has 44 wineries and 1993 viticulturists. This D.O. also comprises different areas of the Castilla-La Mancha region; Ontur, Fuente Álamo, Montealegre del Castillo, Tobarra, Albatana, and Hellín. The Yecla D.O. was formed in 1975 with 5824 ha, 493 viticulturists, and eight wineries. The D.O. Bullas was created in 1994 with 1036 ha and 496 winegrowers. This D.O. comprises several towns such as Bullas, Cehegín, Caravaca, Moratalla, and Lorca. Murcia has 30,916 ha of vineyards with a production of 187,814 tonnes in 2013. In this year the exports were 123,000 tonnes, with a value of 159 million Euros; this supposed 6.24% of total national exports. In Spain this activity generated (in 2013/2014) 73,816 jobs, 9% more than in previous years, due to the higher production than in previous seasons.

II.V GRAPE AND WINE: QUALITY

Although wine consists of two primary ingredients, water and ethanol, its quality depends on the quality of the grapes. The subtle differences that distinguish one varietal wine from another depend on a large number of compounds. The taste and mouth-feel sensations of a wine are due primarily to the few compounds that occur individually at concentrations above 0.1g/liter. These include water, alcohol (ethanol), fixed acids (primarily tartaric and malic or lactic acids), sugars (glucose and fructose), and glycerol. Other than alcohol, wine generally contains about 0.8-1.2 g of aromatic compounds/liter. This constitutes about 1% relative to the wine's ethanol content. The basic flavor of wine depends on additional 20 or more compounds. The most common aromatic compounds are fusel alcohols, volatile acids, and fatty acid esters. Of these, fusel alcohols often constitute 50% of all volatile substances other than ethanol. The vast majority of chemicals found in wine are the metabolic by-products of yeast activity during fermentation. By comparison, the number of aromatic compounds derived from grapes is comparatively small. Nevertheless, these often constitute the compounds that make varietal wines distinctive. Even the mysteries of the benefits of ageing and barrel maturation are now yielding their secrets. This knowledge is beginning to guide vineyard and winery practise toward the production of more consistent and better-quality wine. Plant breeders are also using this information to streamline the development of new grape varieties.

Given the great importance of the cultivation of wine grapes in the agriculture of the region of Murcia, and ultimately in its economy, it is vital that genetics-based breeding projects aimed at yielding new varieties are developed, to maintain - and even improve - the competitiveness that characterizes this region. Such new varieties, adapted to the agro-climatic conditions of our region, would provide a higher profit margin for the farmer

and keep this ancient tradition, one of the foundations of our culture and economy, alive.

II.VI BREEDING

II.VI.I Traits of interest

Phenological traits

The phenology of plants consists of complex events that depend on the combination of different factors; external, such as the environment, and internal, such as the expression of different genes and hormonal levels. Phenology correlates specific phases of growth with climatic conditions (Mullins et al. 1992) and for farmers it is important to know these stages in order to optimize treatments or agricultural practices. For breeders it is crucial to find new cultivars with good adaptation to specific climatic conditions, for the optimal development of the vineyard. In the last decade has been an increase in temperatures and this change affects the phenological processes in grapevine areas (Jones and Davis 2000; Duchêne and Schneider 2005).

Classically, there are four phenological phases in grape: sprouting, flowering, veraison, and ripening. The optimal achievement of all of them allows a satisfactory yield. Bud break is an important event that occurs after dormancy, it being essential that plants sprout homogeneously in order to have an optimal development. Bud break, in a temperate region, is expressed by the number of chilling hours that are required for this event to begin. In these latitudes, vines have a dormant period; this is induced by short days, decreasing temperatures and a reduction of growth. In the same way, the initiation of growth depends on the external temperature and light as well as the internal status of the plant that stimulates growth. Three phases form this stage: paradormancy, endodormancy, and ecodormancy (Lang et al. 1987). In paradormancy buds are latent because of internal conditions but could sprout if the shoot tip or leaves are removed. In endodormacy buds are blocked by

physiological factors. Then, the plant transition to ecodormancy occurs when a range of chilling hours are accumulated, and buds break when environmental conditions became favorable (Horvath et al. 2003). Dormancy is crucial in temperate areas to survive in winter conditions. Bud break has been studied by several authors (Wake and Fennell 2000; Keilin et al. 2007; Halaly et al. 2008; Ophir et al. 2009; Díaz-Riquelme et al. 2012). All the studies concluded that bud burst has similarities with oxidative stress and hypoxia, is associate with catalase activity, and that some hormones as ethylene or ABA are involved in this phase. Bud break has a high influence in the rest of the phenological events, so its delay affects the normal development of the vine. In the Northern hemisphere, this stage occurs at the end of March or beginning of April, when temperatures rise after winter.

Flowering is the next step after bud break, and the onset of it depends on the previous stage. This event is absolutely linked with the production; therefore, flowering is a crucial step on the path to an optimal yield, one of the main objectives in the vineyard. It is well known that the initiation of the flowering process begins with the formation of the anlagen, in the previous year, followed by the floral induction in the subsequent season. The 'anlagen' is an "uncommitted primordial" and could develop in a tendril, shoot, or inflorescence, depending on external and internal cues. At bud break time flowering re-commences its development; the numbers of branches and flowers are not fixed and depend on the endo and exo conditions that prevail at this moment. Cytokinins and gibberellins are the main hormones that control the flowering event. Gibberellins promote the lateral meristem formation and tendril development. In contrast, cytokinins promote inflorescence development. Carmona et al. (2008) reviewed the genetic control of this phase and how the cultural practices affect the final production. The genes involve in this pathways are extensively studied in Arabidopsis (reviewed by Roux et al. 2006). In *Vitis* many genes involved in

floral induction (Joly et al. 2004; Boss et al. 2006; Sreekantan and Thomas 2006; Carmona et al. 2007; Almada et al. 2009; Díaz-Riquelme et al. 2009), identity of floral meristem (Carmona et al. 2002; Calonje et al. 2004; Joly et al. 2004; Boss et al. 2006; Díaz-Riquelme et al. 2009) and identity of floral organs (Boss et al. 2001; 2002; 2003; Sreekantan et al. 2006; Poupin et al. 2007; Díaz-Riqueleme et al. 2009) has been studied. Of the environmental factors, high temperature and the light intensity are the factors that most influence in this event. Bunches in vines are formed by racemes with branches that produce the typical conical form of the inflorescence. The number of flowers per inflorescence and the whole production is almost constant between varieties and years if there are no environmental stresses that alter the flowering event. Grapevine cultivars are hermaphrodites and so the pollination is ensured, but only 20-30% of the flowers proceed to fruit set. This is favorable, to have a balanced yield in the field and to produce an optimal quality of berries.

The berry development is characterized by two sigmoidal growing cycles (Coombe 1992). The first phase begins with fruit set and finishes with veraison, the principal process being cell division. In this stage, the berry is an organ with chlorophyll, green and hard, which primarily accumulates acids. This phase includes two separate stages; in the first, the embryo develops but there is little cell division in seeds and pericarp; in the second stage the embryo grows, the length of this stage determining the ripening date of the variety. The second phase begins with the onset of veraison and includes different processes, such as softening (cell-wall degradation), cell expansion, and sugar, color, and aromatic compounds accumulation. These processes still carry on during the ripening phase and conclude at the harvest date. So, the beginning of veraison determines the harvest date. The transcriptomic analysis of this phase showed that a strong cellular reprogramming occur in the transition between pre- and post- veraison (Terrier et al. 2005; Deluc et al. 2007, Pilati et al. 2007; Lijavetzky et al. 2012).

The hormonal balance through berry development changes; in the first phase there are an accumulation of auxins, cytokinins and gibberellins that promote cell division. After veraison the main hormones are ethylene, abscisic acid and brassinosteroids (Conde et al. 2007). Although grapes are no-climateric fruits, different works with ethylene show its connections with maturation process (Chervin et al. 2004; 2008). Moreover the accumulation of abscisic acid is faster in the post-veraison stage. Some works showed that ABA enhances sugar and anthocyanins accumulation and decreases acid concentration (Çakir et al. 2003; Sun et al. 2010). The final size of the berry depends on environmental and genetic factors, such as temperature and water availability; as well, the number of seeds is closely correlated with berry size. Also, it is limited by the capacity of cellular expansion and the numbers of cells that are fixed since stage I of development. The skin becomes thin in the ripening process, as the capacity of expansion of the mesocarp cells is higher than that of the skin cells. Degradation events of the cell wall are triggered, as the action of polygalacturonases and cellulases allows cell expansion (Barnavon et al. 2001; Nunan et al. 2001; Glissant et al. 2008), because the vacuoles need to increase in volume to store different metabolites.

Harvest date is closely related with the grape quality. In this sense, grape quality is the result of a set of many characteristics that hopefully mean a good product for consumers. For wine grapes it depends on the sugar/acidity ratio, the phenols concentration, and the volatile aromas formed. Acidity is generated in the first stage of growth, and at the onset of veraison it starts to decrease through several actions: dilution, synthesis inhibition, gluconeogenesis, and the use of acids as a source of energy. Sugar is accumulated during ripening as the berries act as sinks. In the same way, anthocyanins and phenols begin to be synthesized during veraison. Temperature affects all of these processes: in cold regions grapes have less sugar and more acids, and vice versa in warm places. In wine production the

fermentative process transforms sugar into alcohol, and the acid level contributes to wine stability. There are a lot of factors and processes that control ripening, depending on the variety, year, and region, as well as the kind of wine to be produced, so the optimal time for harvest is a complex decision for growers.

Productivity and morphological traits

There are different factors influencing the productivity, such as the variety, environmental conditions, or cultural practices. The cultivar production is mainly controlled by fruit set; although the cultivated vines are hermaphrodites only the 20-30% of total flowers develop into fruits (Mullins et al. 1992). In vine cultivars, the berry is the essential unit of the yield but the fertility index, measured as the number of clusters per shoot, is the major indicator of fruitfulness in the vineyard (Fanizza et al. 2005). The number of clusters is more important than cluster weight for final production. In the same way, the cluster weight depends more on the number of berries that the weight thereof. Other crucial component for final production is the compactness. It is associated with the healthy of the cluster and the final quality of the berries (Vail and Marois 1991). Dense clusters are more susceptible to fungal attack. Besides, greater compactness of the cluster implies a heterogeneous cluster. The different ripeness of the berries within the cluster depend on the position and sun exposure of each berry, and affect the final juice composition. Yield in grapes is mainly controlled by winter pruning but it could be modified in the productive season. Thinning clusters is a common practice to modify the final production. Also this technique is applied to transform the cluster shape. Nevertheless this technique could induce undesirable responses on the quality, and berry damages (Tardaguila et al. 2012).

Wine industry prefers a small berry size, because it increases the skin-to-flesh ratio and enhances the pigments and other secondary metabolites in the wine. Berry development has three different phases in a double sigmoid curve: the first involves cell division; the second is correlated with veraison; the third is related with cell enlargement (Coombe 1973; 1992). The final size depends on the plasticity of the cell wall that allows the cell enlargement by water and sugar accumulation. For growers, it is very important to have a variety with equilibrated production, which assures homogeneous and healthy maturity in all the berries. For these reasons the cluster size and the compactness become important parameters on breeding programs.

Enologycal traits

Acidity is one of the main enological characters for both table and wine grapes, because the ratio sugar/acidity makes the taste pleasant or unpleasant for consumers. In particular, the level and composition of the acid fraction in the must influences the development of balanced and stable wines, since acidity affects the growth of the microorganisms that are needed in the fermentation process of wine. Although the acid composition depends on the cultivar, there are two main acids, tartaric and malic. They make up 70-90% of the total acidity in berries. Their synthesis starts in the first phase of berry growth, when the fruit is green and herbaceous, and decreases in the final phase, when sugars start to accumulate. Acids decrease due to degradation processes and their dilution by the increase of water and sugar accumulation in the vacuoles. Tartaric acid shows a peak of accumulation in the pre-veraison stage and then the level keeps constant. Then a dilution occurs due to the water accumulation in the vacuoles in the final step of grape maturity. For malic acid the accumulation occurs in the first phase of fruit growth, as tartaric acid, but it is synthetized through sugar metabolism. Contrary to tartaric acid it shows a degradation process post-veraison. Malic acid degrades

during the ripening process because it is involved in other processes such as energy provision. Several genes, related with the production and degradation of this acid, have been studied and cloned (reviewed by Sweetman et al. 2009).

Another main trait in wine grapes is the color, which is crucial for the final quality of the wine. Anthocyanins are responsible for this character and their accumulation in grapes starts in the veraison stage and continues through all the ripening process. Color has been extensively studied in some species, such as petunia (*Petunia hybrids*), snapdragon (*Antirrhinum majus*), or maize (*Zea mays*). Anthocyanins belong to a big family of flavonoids which have six important groups: chalcones, flavones, flavonols, flavandiols, anthocyanins, and condensed tannins. Their function is linked with protection against pathogens and UV and with insect attraction for pollination. In grapes, anthocyanins are stored in the skin vacuoles, although a few cultivars can accumulate them in the flesh too. Tannins occur in the skin and, mainly, in the seeds of the grapes, contributing to wine stability. In general, the flavonoids have beneficial effects on human health.

Anthocyanins differ according to: i) the number and position of the hydroxyl groups; ii) the degree and position of the methylation of the hydroxyl groups; iii) the nature and number of the sugars linked and their position; iv) the nature and number of the aromatic acids linked with the sugars (acylation). All of these different combinations result in five basic anthocyanins: cyanidin, peonidin, delphinidin, petunidin, and malvidin. In *Vitis vinifera* the most usual forms of these anthocyanins are 3-monoglucoside, 3-acetylglucoside, and 3-p-coumaroylglucoside. Malvidin-3-O-glucoside is the most abundant anthocyanin in grapes.

These secondary metabolites derive from the shikimate pathway. The principal genes involved in this pathway were cloned in *Vitis* by Sparvoli et al (1994), using the previously described genes from petunia and snapdragon.

Further work by Boss et al (1996) studied the expression pattern of these genes in flowers and berries of grapes.

The presence of color in *Vitis vinifera* and other *Vitis* genus depends on a MYB transcription factors family located on chromosome 2 (Kobayashi et al. 2004; Walker et al. 2007; Azuma et al. 2008). The insertion of a retrotransposon in a promoter region and the presence of one SNP mutation in a coding sequence are responsible of the absence of color. A more comprehensive study of this family (Fournier-Level et al. 2009) revealed 32 polymorphisms in a core collection of natural source. The 84% of total anthocyanin variance could be explained by the combination of one retrotransposon, three SNPs and one insertion/deletion.

II.VI.II Molecular Markers

Genetic markers represent genetic differences between individual organisms or species. Generally, they do not represent the target genes themselves but act as 'signs' or 'flags'. Genetic markers that are located in close proximity to genes (tightly linked) may be referred to as gene 'tags'. Such markers themselves do not affect the phenotype of the trait of interest because they are located only near or 'linked' to genes controlling the trait. All genetic markers occupy specific genomic positions within chromosomes called 'loci' (singular 'locus'). There are three major types of genetic markers: morphological or visible markers which themselves are phenotypic traits; biochemical markers, which include allelic variants of enzymes called isozymes; and DNA or molecular markers (Jones et al. 1997; Winter and Kahl 1995). Morphological markers are usually visually characterized phenotypic characters such as fruit color, growth habits or berry shape. Isozymes markers are differences in enzymes that are detected by electrophoresis and specific staining. The major disadvantages of morphological and biochemical markers

are that they may be limited in number and are influenced by environmental factors or the developmental stage of the plant (Winter and Kahl 1995).

Since the 1970s, molecular techniques have permitted the detection and study of individual variations or polymorphisms at the level of the DNA sequence, and therefore the development of molecular genetic markers. Such polymorphisms can be detected by a diverse range of techniques which allow the genetic differentiation of organisms of the same or distinct species (De Vienne et al. 2003; Collard et al. 2005). Normally, a small fragment of DNA, that may or may not contain genes, is analyzed. DNA markers are the most widely used type of marker predominantly due to their abundance.

These techniques can be classified into two main groups: those based on the polymerase chain reaction (PCR) and the rest. The first, in turn, can be classified according to whether they amplify known sequences or random sequences, although there are markers that share both characteristics. The amplification of DNA fragments and the direct evaluation of the differences in length of the amplified products, without the need for transfer and hybridization, represented a qualitative change in the possibilities of using DNA markers in genetics-based plant breeding.

All molecular markers can be visualized, so that the alleles carried by an individual for a given marker can be identified. The display can be carried out by electrophoretic techniques on agarose or acrylamide gel, or by using sequencers. Depending on the information that molecular markers may provide, they are classified as dominant or codominant. A marker is dominant when homozygous individuals are indistinguishable from heterozygous ones, so that they are described by presence/absence of a fragment. If a dominant marker is visualized on an agarose gel as a band, it is impossible to know whether the individual carries the marker on both copies of the chromosome (homozygous) or on only one (heterozygote). The co-dominant markers do allow one to distinguish between homozygous and heterozygous individuals.

Such markers always amplify two alleles, so that two bands will be displayed if the individual is heterozygous, but only a single band if it is homozygous (Nuez et al. 2000; De Vienne et al. 2003). Below are described some of the markers used most widely for grapevine.

The first molecular markers used, the RFLP (Restriction Fragment Length Polymorphisms), were developed in the early 1980s (Botstein et al. 1980; Wyman and White 1980). With these markers, the polymorphism will be detected as a difference in the length of the restriction fragments generated. They are co-dominant in character and possess major drawbacks: the requirement for information prior to their evaluation and their high economic and labor costs (García-Mas et al. 2000; De Vienne et al. 2003). The RFLPs were followed by a long list of markers that appeared in tandem with the evolution of the techniques of molecular analysis; here are described the ones used most in grapevine. The polymorphism of the RAPD (Random Amplified Polymorphic DNA) is generated by amplification of genomic DNA by PCR, using a single primer with a short (8-10 bp) and random sequence. They are markers that are simple and cheap to obtain, but have drawbacks: they are dominant by nature, have low reproducibility between laboratories, and are not transferable between crossings and, therefore, even less so between species (Williams et al. 1990; Welsh et al. 1991; García-Mas et al. 2000). The AFLP (Amplified Fragment Length Polymorphisms) are based on the combination of digestion with restriction enzymes and selective PCR (Vos et al. 1995). They are dominant markers, more reproducible than the RAPDs, and do not require prior information, but do need more complex and expensive technology (García-Mas et al. 2000; De Vienne et al. 2003). Some markers, such as SAMPLs (Selective Amplification of Microsatellite Polymorphic Loci) (Vogel and Scolnick 1998) and the S-SAPS (Sequence-Specific Amplification Polymorphisms) (Waugh et al. 1997), are derived from a modification of the AFLPs technique. The markers of type RFLP, RAPD,

or AFLP can be converted into specific PCR markers that are easier to assess, such as the SCARs (Sequence Characterized Amplified Regions). These markers, which can be codominant, allow the detection of a single locus, but require prior information on the sequence to be studied. The conversion to SCARs-type markers can cause loss of polymorphism (Konieczny and Ausubel 1993). To solve this drawback, the fragments amplified by PCR can be digested with restriction enzymes to generate polymorphism. This results in markers of the CAPS type (Cleaved Amplified Polymorphism Sequence) and their variant dCAPS; both are codominant in character. The SSCPs (Single Strand Conformation Polymorphisms) are developed by taking advantage of changes in the three-dimensional configuration caused by variations in the DNA sequence, which result in differences in mobility on the acrylamide gel, giving rise to polymorphism (Orita et al. 1989). This technique, which requires specific amplification by PCR of a region, is useful to detect polymorphisms which are due to only one base. In this work we have mainly used two types of molecular markers that are detailed below: microsatellites and SNPs.

Microsatellites (SSRs, Simple Sequence Repeats)

Microsatellites, SSRs (Simple Sequence Repeats), and STRs (Short Tandem Repeats) are hypervariable genomic regions constituted by a small repeating unit of di-tri or tetranucleotides in tandem, although some more complex combinations are also possible. The design of primers specific for the unique sequences that flank the redundant region allows PCR amplification of the repeat region. The genetic basis of the polymorphism detected is the variability of the number of tandem repeats between individuals and, consequently, the size of the amplified fragment. When the differences in length of the amplified fragments are great, they can be assessed on agarose gels, although their evaluation on acrylamide gels or in DNA

sequencers is more common. The use of these markers has been limited by the difficulty in locating them in the genome and the need to predetermine the sequences flanking the repeat. They are expensive to develop and their use is limited to species where this information exists or where it can be transferred from related species. Once this information is obtained, they are markers of low cost have easily interpretable genetic profiles, allowing the exchange of information between laboratories (García-Mas et al. 2000; De Vienne et al. 2003).

A microsatellite may have many alleles, depending on the number of repetitions present in different individuals. This is because these sequences have a higher mutation rate than any other type of sequence. Although the reason for this is not fully elucidated, it may be because, during DNA replication, the newly formed strand pairs with the wrong repetition, so that the DNA polymerase can add or delete one or more copies of the repeat in the new DNA strand (Moxon and Wills 1999). It can also be due to errors in recombination (Oliveira et al. 2006).

Besides being highly polymorphic and abundant markers distributed throughout the genome, they are codominant, highly reproducible, and transferable between crossings, since the sequences flanking the repeats are conserved between individuals of the same species and partly between related species. Therefore, they have been the molecular markers used most commonly in genetic studies (Thomas et al. 1994; Bowers and Meredith 1997; Oliveira et al. 2006).

Currently, all available information about the microsatellites identified in the grapevine genome is in the NCBI database UniSTS (http://www.ncbi.nlm.nih.gov/) data. Many of them have been described in research journals such as the VMC (Di Gaspero et al. 2000, Pellerone et al. 2001, Arroyo-García and Martínez-Zapater 2004), developed by the private consortium Vitis Microsatellite Consortium (Agrogene SA, Moissy, Cramayel,

France), as well as the VrZAG (Sefc et al. 1999), VVS (Thomas and Scott 1993, Thomas et al. 1994), VVMD (Bowers et al. 1996, 1999), SCU (Scott et al. 2000), UDV (Di Gaspero et al. 2005), VVI (Merdinoglu et al. 2005), and VCHR (Cipriani et al. 2008).

SNPs (Single Nucleotide Polymorphisms)

The SNPs are markers whose polymorphism is caused by variations in a single nucleotide in the DNA sequence. By their nature, SNPs can be present in any genomic region; so, they can mark any gene of interest for breeding and offer the possibility to exploit a huge variation. The level of polymorphism is very similar between coding and non-coding regions, although it is slightly higher in the latter. It is the most abundant polymorphism in most organisms (Rafalski 2002 a, b), but requires prior knowledge of the sequence of the alleles under investigation. In grapevine the presence of an SNP has been observed every 64 or 250 bp, according to studies (Lijavetzky et al. 2007; Velasco et al. 2007). Although they are biallelic markers, they have the advantages of being codominant and highly reproducible between laboratories and detection techniques, as the alleles differ by one nucleotide in a given position, rather than by their sizes (as with the rest of the molecular markers). For the above reasons, they are considered interesting and useful markers, both in the construction of genetic maps and in the identification of varieties (García-Mas et al. 2000; De Vienne et al. 2003).

The detection of SNPs necessarily involves the amplification of genomic sequences, which may come from cDNAs, BACs (Bacterial Artificial Chromosomes), or databases published with ESTs (Expressed Sequence Tags). There are several methods for detecting SNPs in these sequences: the development of SSCPs (Orita et al. 1989) and CAPS (Konieczny and Ausubel 1993), the re-sequencing of ESTs (Lijavetzky et al. 2007) and terminal

sequences of BACs (Salmaso et al. 2008), or the sequencing of complete genomes – such as the recently fully-sequenced genome of grapevine (Jaillon et al. 2007; Velasco et al. 2007). Due to recent advances in DNA sequencing, progress is being made in large-scale production of this type of polymorphism in plants (Margulies et al. 2005; Shen et al. 2005; Streemers and Gunderson 2005; Tobler et al. 2005).

The assessment of the polymorphism can be accomplished on gels, which separate the amplification products generated using specific primers, one of which matches the 3' end with the nucleotide originating from the polymorphism. Polymorphisms can also be resolved by denaturing high-performance liquid chromatography (DHPLC), provided the melting points of the PCR products of the alleles under study are known, based on their sequences. The usefulness of SNPs lies in their widespread use; due to this, more sophisticated methods have been proposed, which involve the use of microchips.

II.VI.III Maps

Genetic maps in animal and plants are based on the concept of linkage. A genetic or linkage map is a representation of the relative positions of markers or genes within a chromosome or linkage group. It is said that two markers are linked when they exist on the same chromosome and tend to be inherited together in the recombination event of meiosis. The population employed for their construction can derive from a cross (F1), one or more backcrosses (F1+n), or a group of individuals that are not directly related (germplasm).

The construction of a genetic map involves the estimation of the genetic distance or mapping distance between two loci, estimated two by two. This distance is defined as the value of the recombination fraction between loci; that is, the proportion of the new associations formed between two pairs

of markers with respect to the total number of associations. Its minimum value is 0 and the maximum 0.5, the latter value corresponding to the situation of independent genetic loci. The distance between markers is a relative value for each cross as it depends on the markers being considered (Kearsey and Pooni 1998; Nuez 2000; De Vienne 2003b).

After checking the linkage using a chi-squared test, one can estimate the recombination value by different methods. The methods based on regression and maximum likelihood give smaller standard deviations of the estimates, and therefore are the ones used most in the different software packages available. Once the pairs of values for the recombination between loci have been calculated, it is necessary to define their significance; that is, the probability that two loci are linked with the calculated value versus the probability that they are independent with the same segregation. This is known as the LOD (Likelihood Ratio, or Logarithm of Odds) value, the logarithm of the ratio of inequality (Morton 1955). A LOD >3 is equivalent to saying that the alternative hypothesis (linkage) is 1,000 times more likely than the null hypothesis (independence). The level of significance or LOD value is chosen by the researcher. Increasing its value will avoid the false inclusion of markers in the different linkage groups (LGs), and the number of groups established will increase. When a good set of data is available, and the appropriate markers for the available population are used, the number of LGs will be equal to the haploid complement of the species in question. It is not advisable to lower the LOD value in order to impose this situation; values greater than 3 are recommended (Kearsey and Pooni 1998; Nuez 2000; De Vienne 2003b). To organize the linked markers, different algorithms that calculate the goodness of a particular map (among all the possible ones) have been described (Lander and Green 1987). The JoinMap program automatically calculates the arrangements that minimize the squares of the differences between the observed values of recombination (obtained directly

from all pairs of values) and the ones estimated on the map. Thus, commencing from the two closest markers in the group to be ordered, new loci are added and ultimately the goodness of the map is obtained as a chi-squared value. Provided common markers are available, this program also allows the maps obtained to be integrated with different or related populations, based on the distances between pairs of values (Stam 1993). The integration of maps allows establishment of the order of the markers genotyped initially in other populations, and the transfer of markers to the varieties used for the construction of the integrated map. The robustness of a map will depend on both the size of the population used and on the presence of markers showing biased or non-Mendelian segregation, as well as genotyping errors.

The formula relating the distance on a linkage map with the recombination fraction is known as a map function. The two map functions used most were developed by Haldane (Haldane 1919) and Kosambi (Kosambi 1944). That of Haldane assumes no interference while that of Kosambi considers the presence of positive interference. Thus, the Kosambi distance considers that the probability of a crossover in an interval decreases with the existence of a crossover in the adjacent interval. This distance is expressed in centimorgan (cM): one cM is the distance separating two loci between which the expected number of crossovers is 0.01. Values between 40 and 50 cM indicate that the loci segregate independently (Kosambi 1944).

Genetic maps in grapevine: Due to the highly heterozygous nature of the grapevine, the strategy for the construction of genetic maps is the 2-way pseudo-testcross test strategy (Grattapaglia and Sederoff 1994), using F_1 populations derived from intra or interspecific crosses, or self-crosses. It involves the construction of two maps of the same cross, one with the segregation due to the meiosis of the mother and one from the father. The

first genetic maps of grapevine were constructed by Lodhi et al. (1995) from a population of 60 F1 individuals, the result of crossing interspecific hybrids, using RFLP, RAPD, and isozymes markers. Since then, there have been numerous projects to develop genetic maps from intra- and interspecific crosses of grapevine, and the type of molecular markers used has varied over time. The first maps were constructed primarily with AFLPs, RAPDs, CAPS, and SSCPs (Dalbó et al. 2000; Doligez et al. 2002; Grando et al. 2003; Doucleff et al. 2004; Fischer et al. 2004; Cabezas et al. 2006).

Microsatellite markers (SSRs) are very useful for comparing homologous LGs of different maps, because they are highly transferable between laboratories and also due to their co-dominant character (Grando et al. 2003). Due to this, in 2004 they began to be used more frequently (Adam-Blondon et al. 2004; Riaz et al. 2004; Fanizza et al. 2005; Doligez et al. 2006 a, b;. Lowe and Walker 2006; Riaz et al. 2006; Di Gaspero et al. 2007; Mejía et al. 2007; Welter et al. 2007; Costantini et al. 2008; Salmaso et al. 2008; Vezzulli et al. 2008; Battilana et al. 2009; Duchêne et al. 2009; Fournier-Level et al. 2009; Doligez et al. 2010; Riaz et al. 2011). The map built by Adam-Blondon et al. (2004) from 245 SSRs in a progeny of an intraspecific cross of *V. vinifera* (Shiraz x Grenache) has been used as a reference by the IGGP (International Grape Genome Program) for the establishment of the nomenclature of the LGs of *V. vinifera.*

From 2007 onwards, improvements in the technology that facilitates the development of SNP-type markers, and the huge abundance of these polymorphisms in the DNA of grapevine, have led to the gradual incorporation of these markers into genetic maps. This has enabled the mapping of a large number of molecular markers developed from candidate genes involved in controlling the traits of interest. Thus, one can detect whether a particular gene co-localizes with a QTL, suggesting a possible role of the gene in the character (Di Gaspero et al. 2007; Troggio et al. 2007;

Welter et al. 2007; Costantini et al. 2008; Salmaso et al. 2008; Vezzulli et al. 2008; Battilana et al. 2009; Duchêne et al. 2009). Although SNPs have allowed the mapping of more genes, we have also found polymorphisms of the SSR or AFLPs type, starting from gene sequences derived from ESTs or cDNAs (Scott et al. 2000; Decroocq et al. 2003; Riaz et al. 2006).

The first integrated map was built by Doligez et al. (2006a), using five progenies developed by different research groups: one from the selfing of Riesling, and others derived from the crosses Shiraz x Grenache, Chardonnay x Bianca, Riesling x Cabernet Sauvignon, and of table grape varieties MTP2223-27 (Dattier de Beyrouth x 75 Pirovano) and MTP2121-30 (Alphonse Lavallée x Sultanina). In this reference map the order of 502 SSR markers was established; these are highly transferable and very useful for the construction of other genetic maps.

Di Gaspero et al. (2007) constructed an integrated map based on the search for QTLs associated with resistance to diseases. This map was constructed from four parental maps, derived from the interspecific crosses Chardonnay x Bianca and Cabernet Sauvignon x 20/3, using SSR markers and derivatives analogous to resistance genes (RGAs). Bianca and 20/3 are hybrids obtained by crossing, for which 80% of the genetic background corresponds to different species of the genus Vitis. This map was obtained in order to establish the chromosomal location of the markers derived from RGAs, using SSRs whose order was previously established by Doligez et al. (2006a). Vezulli et al. (2008) constructed an integrated map from three intraspecific crosses (Pinot Noir x Shiraz, Shiraz x Grenache, Cabernet Sauvignon x Riesling), which includes 283 SSRs and 501 SNPs. Cabezas et al. (2011) published an integrated map based on populations obtained from the crosses between eight varieties of grapevine (Dominga, Autumn Seedless, Monastrell, Cabernet Sauvignon, Ruby Seedless, Moscatuel, Muscat Hamburg, and Sugraone), which included 168 SSRs and 202 SNPs.

For a map to be effective in the study of a character, it must be built with progeny which segregate for the desired character, so it is appropriate that the parents be phenotypically distinct for it. Following this approach, a range of useful maps has been generated for the investigation of a large number of fruit quality traits such as seedlessness (Doligez et al. 2002; Cabezas et al. 2006; Mejía et al. 2007; Costantini et al. 2008), size and weight of berries (Doligez et al. 2002; Fanizza et al. 2005; Cabezas et al. 2006; Mejía et al. 2007; Costantini et al. 2008; Doligez et al. 2013), berry color (Doligez et al. 2002; Fischer et al. 2004; Fournier-Level et al. 2009), and muscat flavor (Sevini et al. 2004; Doligez et al. 2006 b; Battilana et al. 2009; Duchêne et al. 2009). This tool has also been used to study productivity - evaluated as the total yield per plant, number of bunches per plant, number of berries per cluster, and cluster weight (Fanizza et al. 2005), or the number of inflorescences per shoot (Doligez et al. 2010). Other maps have allowed the study of phenological characters such as the times of bud break, flowering, veraison, and ripening (Mejía et al. 2007; Costantini et al. 2008; Duchêne et al. 2012; Grzeskowiak et al. 2013).

In the study of resistance to diseases and pests, maps from interspecific crosses have been used, in which at least one parent belonged to a species of the genus Vitis of American origin, which showed some resistance. Thus, there are maps on which analysis has been carried out of the QTLs for resistance to diseases such as mildew, powdery mildew, and black or gray rot (Lodhi et al. 1995; Dalbó et al. 2000; Fischer et al. 2004; Di Gaspero et al. 2007; Welter et al. 2007; Salmaso et al. 2008), the bacterium *Xylella fastidiosa*, responsible for Pierce's disease (Doucleff et al. 2004; Riaz et al. 2006), or pests like the nematode Xypinema index (Doucleff et al. 2004; Xu et al. 2008). Grafts and rootstocks have also been studied by using genetic maps, since there are many characters of the species of the Vitaceae that can contribute to cultivation of grafted varieties of *V. vinifera*. Among these characters are

resistance to pests such as phylloxera and nematodes, tolerance of saline soils, vigorous growth, induced chlorosis, and the success of grafting (Lowe and Walker 2006).

After determining the involvement of a region (or regions) of the genome in a character, after a previous analysis of QTLs, some authors have employed a strategy of partial mapping, saturating this/these area(s) with additional markers in order to obtain more information about it/them (Adam-Blondon et al. 2001; Duchêne et al. 2009; Riaz et al. 2011).

II.VI.IV Mapping QTLs

A QTL is a region of the genome responsible for the quantitative variation in the expression of a character. The first step in the genetic mapping of a locus that controls a quantitative character or QTL is the determination of the existence of a significant statistical association between the segregation of the character and the segregation of a genetic marker in a given population (Jensen 1989). This association indicates that the genetic marker is linked to a locus that controls all or part of the quantitative character. Once the linkage map for a population that shows continuous variation for a character has been obtained, the basic and simplified procedure for the analysis of QTLs for this character is similar to that used to construct the map. In the intervals between adjacent pairs of markers, the LOD is calculated for the presence of a locus influencing the trait under study (the probability of the presence of a QTL versus the probability of its absence). If the LOD score exceeds the critical threshold value, there is evidence of a QTL linked to the marker. The result is an assessment of the chromosomal regions affecting this character (Kearsey and Pooni 1998; De Vienne and Causse 2003).

There are several computer programs for the analysis of QTLs that employ very similar approaches (De Vienne and Causse 2003). Most of them

begin with the simple mapping interval (SIM) proposed by Lander and Botstein (1989). The programs calculate, by regression, the association between the values of the characters under study, for each individual, and their genotype, for each marker and at regular intervals between markers. This regression depends on some values, called maximum likelihood estimators (MLEs), which maximize the probability that the phenotypic data are linked to a possible QTL. The most effective estimator is the one that reflects the phenotypic effect caused by the substitution of one allele in this QTL (the effect would be zero if there were no linkage of the QTL). These MLEs are compared with the MLEs obtained under the assumption that there is no linked QTL, with the following equation:

LOD = Log10 (MLE QTL linked/MLE QTL not linked)

where LOD (Logarithm of Odds) reflects the estimated fit; that is, the probability of the presence of a QTL versus the probability of its absence. If the LOD exceeds a certain threshold value, the presence of a QTL is signaled. The statistical significance of the QTLs is obtained by calculating a threshold LOD value by different methods (Doerge and Rebaï 1996). Most computer programs use the empirical method developed by Churchill and Doerge (1994), based on the theory of permutation (Fischer 1935), which consists of randomly changing or permuting the quantitative data of the individuals of the mapped population, in order to destroy any relationship between the phenotypic values and the markers or genotypic values. This method, being an empirical method (which studies the data directly) and non-parametric (not applied to a model with a particular distribution), is valid for any experimental design, is undemanding in terms of the population size or mapping density, and can be applied to data that does not follow a normal distribution. Although not dependent on the mapping densities, Krugliak and Lander

(1995) recommended the use of the threshold obtained with a dense map, in order to minimize false positives. The accuracy with which the threshold values are calculated will be determined by the number of permutations. A number of 1,000 permutations is considered appropriate for a significance level of $\alpha = 0.05$ (Doerge and Churchill 1996).

The SIM analyzes pairs of markers throughout the genome, and provides information on all the chromosomes in a single analysis, so that it can detect several QTLs simultaneously. However, with this method QTLs are mapped one at a time, regardless of the presence of other QTLs (mapped or not). Therefore, this method by itself has little value since a quantitative characteristic - by definition - is controlled by several genes. The effect of other unlinked QTLs is equivalent to the environmental effect and reduces the LOD value. As a result, the power of detection can be compromised, and the estimates of the location and effects of the QTLs may be biased (Jansen 1993). The linkage between two QTLs involved in the control of the character of interest leads to shifts in the peaks of maximum LOD.

However, the SIM allows detection of those markers that may be involved in the control of the character. These markers are used as cofactors in the models developed for the analysis of multiple QTLs, using a multiple regression model. The result is the reduction of background noise and better definition of the LOD values. Jansen (1992, 1993, 1994a) put forward a method very similar to that proposed by Zeng (1994), which combines multiple regression with the SIM. This method involves setting up models for a single QTL (one for each interval) and the use of markers selected as cofactors for the regression of the phenotype on the genotype. In this way it is possible to eliminate the effects of possible QTLs elsewhere in the genome. This framework for the unification of models used to detect and map multiple QTLs is called MQM (Jansen 1994a), which stands for Multiple-QTL Models and also for Marker-QTL-Marker (which reflects the insertion of QTLs

between markers in the linkage map). Jansen (1994b) showed that the probability of a Type II error (QTL present but not detected) in the case of unlinked QTLs (on different chromosomes) is lower with MQM than with the SIM. Also, the detection of different linked QTLs (on the same chromosome) is much easier with MQM than with the SIM, both for QTLs with (mutually neutralizing) opposite effects, which often go undetected, and for QTLs with the same sign (Martínez and Curnow 1992).

Currently, the procedure proposed by Van Ooijen (2009) for the detection and mapping of QTLs involves an initial search for possible QTLs using a multiple linear regression or SIM. Then, those markers closest to the detected QTLs are designated as cofactors, which will play the role of close QTLs in the subsequent MQM analysis. This MQM analysis will search for QTLs along the genome, like the SIM, but, due to the presence of the selected cofactors, the residual variance is reduced and - therefore - the power of detection of other QTLs increases. After the first MQM analysis the position of some QTLs detected earlier may vary, so the nearest cofactors must be marked again and the MQM analysis repeated. This process is repeated as often as necessary until a set of cofactors that maximizes the probability profile of the QTLs detected is obtained.

Computer programs generally produce, as part of their outputs, together with the estimated fit, a LOD value, the percentage of explained variability, and the additive component. The program used in this work, MapQTL® v 4.0 (Van Ooijen et al. 2002), incorporates the MQM and SIM methods, along with the permutations test. These methods are widely used by the scientific community.

QTLs in grapevine: In grapes QTL detection has mostly been used to investigate the genes related to resistance to diseases such as powdery and downy mildew and Pierce's disease (Doucleff et al. 2004; Fischer et al. 2004;

Barker et al. 2005; Krivanek et al. 2006; Riaz et al. 2006; Riaz et al. 2008; Xu et al. 2008; Coleman et al. 2009; Marguerit et al. 2009). It has also been used to examine the genes related to a range of agronomic traits: berry size, seed number, seed fresh and dry weights, berry weight, inflorescence and flower morphology, number of inflorescences per shoot, flowering date, timing and duration of flowering and of veraison, veraison-ripening interval, architecture of the inflorescence, aroma profile, firmness, acidity, anthocyanin content, number of clusters per vine, sexual traits, and fertility (Doligez et al. 2002; Fischer et al. 2004; Fanizza et al. 2005; Cabezas et al. 2006; Mejía et al. 2007; Costantini et al. 2008; Marguerit et al. 2009; Doligez et al. 2010; Mejía et al. 2011; Duchêne et al. 2012; Fechter et al. 2012 Grzeskowiak et al. 2013; Viana et al. 2013; Correa et al. 2014; Fechter et al. 2014; Azuma et al. 2015; Carreño et al. 2015; Chen et al. 2015; Costantini et al. 2015; Houel et al. 2015). Although the QTLs have been detected using maps constructed by means of interspecific crosses, as in the case of disease resistance, they contribute to our knowledge of the chromosomal regions and genes involved in the traits of interest in *V. vinifera.*

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III. OBJECTIVES

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The cultivation of wine grapes in the Region of Murcia is of great importance both economically and socially. The variety Monastrell is of great economic value - not only in the Region of Murcia, where it constitutes the main variety in the Jumilla, Yecla, and Bullas Denomination of Origin (D.O.) areas, but also in other areas of the Mediterranean.

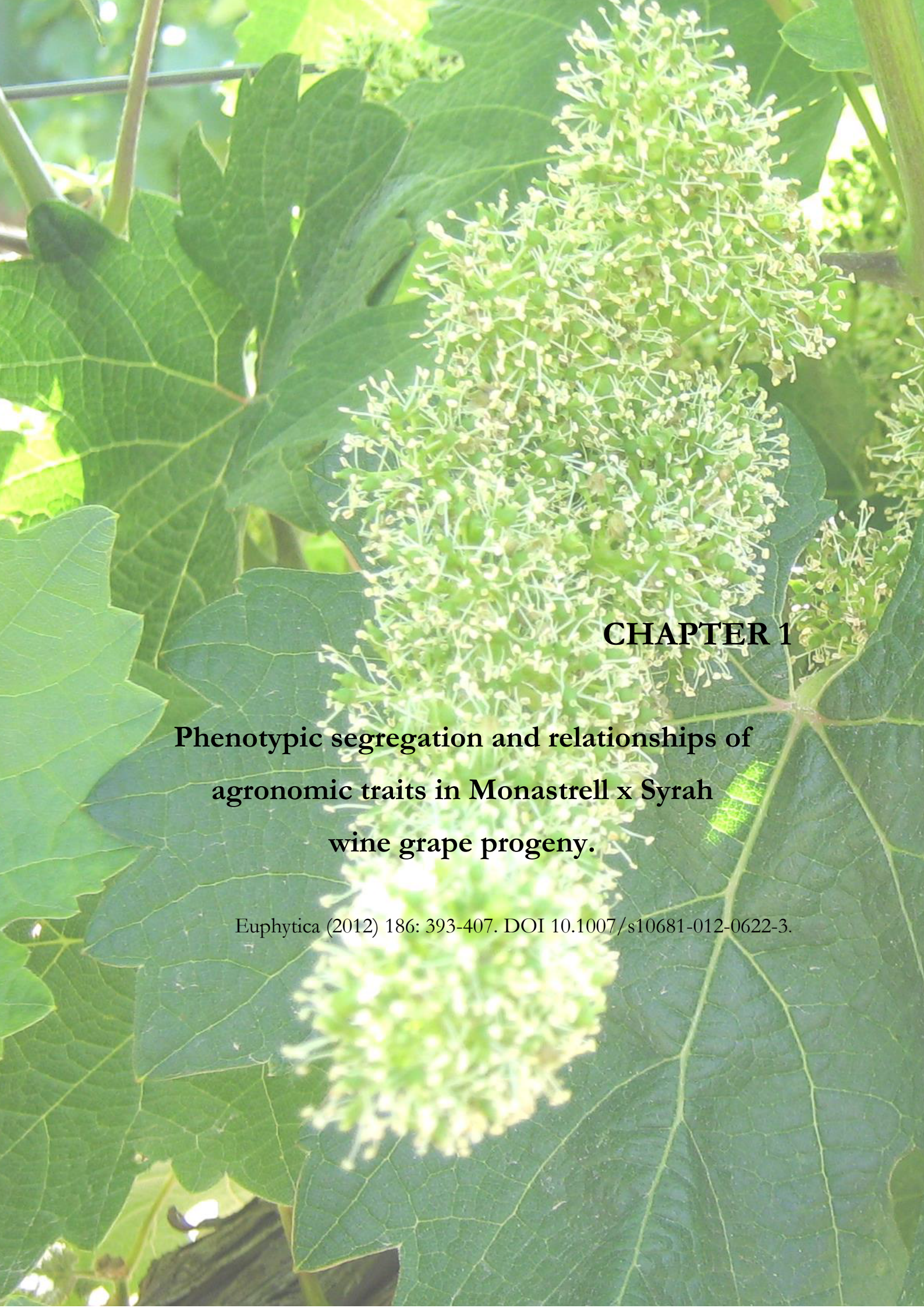
This variety has a great potential regarding grape and wine quality. Nevertheless, the IMIDA's wine grape breeding program aims to obtain new varieties well adapted to Murcia's climate conditions and with better genetic composition. These results will have a big socio-economic impact, helping to boost the profitability of the crop and increase the yield and quality of the wines of the area, by providing new varieties that are better adapted to the edaphoclimatic conditions of the area. These varieties will respond to the new social challenges and to the consumers who demand better-quality and healthy products. All this, undoubtedly, will consolidate and promote the DOs of the wines of Murcia, and will increase the competitiveness, production, and exportation of these wines, contributing significantly to the increased economic growth of this sector in the Region of Murcia.

The global goal of this work is the identification of the major genetic determinants for a given phenotypic trait in genetic maps and their co-localization with the position of candidate gene sequences related to the relevant phenotype. For this purpose, we considered three specific objectives:

1. The phenotypic evaluation of a progeny derived from a controlled cross between the wine grape cultivars Monastrell and Syrah.

2. The construction of grapevine genetic maps using this progeny and molecular markers.

3. The use of these maps and the phenotypic data of the progeny for QTL analyses, in order to develop helpful markers for breeding programs.



CHAPTER 1

Phenotypic segregation and relationships of agronomic traits in Monastrell x Syrah wine grape progeny.

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1.1 Introduction

Vitis vinifera L. is a temperate-climate species cultivated widely in the countries of the Mediterranean basin, principally for wine and fresh fruit. Wine production, historically the most-important use for grape berries, is based primarily on traditional cultivars of *Vitis vinifera* which have been perpetuated for centuries by vegetative propagation. Different studies have shown that the growth rate and quality of the grape can be affected by climate change, representing a risk to present and future grape production (Schultz 2000; Brunet et al. 2007; Duchêne et al. 2005; 2010; Jones et al. 2005; Keller 2010). Given this context, the development of new cultivars with new or improved attributes, caused by better combinations of alleles at multiple loci, is important.

Berry quality is a determinant factor of wine quality, and it is related to certain productive, morphological, and physicochemical parameters (Conde et al. 2007; Jackson 2008). Lower numbers of clusters per plant and smaller clusters have a positive effect on wine quality and reduce the production costs by decreasing the pruning of clusters (Howell 2001; Morris et al. 2004). Cluster structure and compactness affects berry illumination, ripeness homogeneity, disease susceptibility, and, therefore, fruit quality (Mullins et al. 1992; Vail and Marois 1991). Fruit quality is also determined by different physicochemical parameters such as sugar content, acidity level and their ratio, and phenolic compounds. During fermentation, the sugar is converted to alcohol and this determines whether the wine tastes thin and watery or alcoholic and hot. Excessive acidity produces wines that are too tart, but grapes that are deficient in acid produce wines that have a flat and uninteresting taste. Acidity is also involved in the inhibition of oxidation and spoilage, and it is an important factor in wine stability. In this respect, a high content of potassium in grape berries may have a negative impact on wine quality, mainly because it decreases free tartrate during winemaking and

therefore reduces the tartrate:malate ratio, which is undesirable for high-quality wines (Conde et al. 2007; Jackson 2008).

Grape berry skin color, one of the qualities used for selection in breeding programs for wine grapes, results from the vacuolar accumulation of anthocyanins in berry skin cells. In grapevine, most of the structural genes encoding the enzymes of the anthocyanins biosynthetic pathway have been identified (Sparvoli et al. 1994; Boss et al. 1996). Two adjacent transcription factors isolated from *V. vinifera*, *VvmybA1* and *VvmybA2*, are involved in the control of the anthocyanins pathway (Kobayashi et al. 2002; 2004; Walker et al. 2007; Azuma et al. 2008). The presence or absence of anthocyanins in grape berries segregates as a monogenic trait determined by a locus in linkage group 2 (Doligez et al. 2002; Fischer et al. 2004; Salmaso et al. 2008). Recently, it was suggested that the color locus is a cluster of *MYB* genes, including *VvmybA1* and *VvmybA2*, located on chromosome 2 (Matus et al. 2008; Azuma et al. 2009; Fournier-Level et al. 2009).

In addition, grapevine phenology determines the production window of cultivars and their adaptation to local environmental conditions (Coombe 1988; Jackson 2008). The most-important grapevine developmental stages are sprouting, flowering, veraison, and ripening. The time between these phenological stages varies greatly with grapevine variety, climate, and geographical location (Coombe 1988; Jackson 2008). Climatic changes can alter the environmental conditions, and cultivars adapted to specific regions may become less productive, necessitating the development of new viticulture techniques for the new conditions or a change of cultivar.

Grapevine breeding programs involve the crossing of heterozygous cultivars and selection in the F_1, or later, for one or a few of the best hybrids. Few traits of viticultural importance are controlled by single genes or genes of major effect, including berry color (Doligez et al. 2002), flesh development (Fernandez et al. 2006), flower hermaphroditism (Dalbò et al. 2000; Riaz et al.

2006), and seedlessness (Lahogue et al. 1998; Doligez et al. 2002; Cabezas et al. 2006; Mejía et al. 2007; Costantini et al. 2008). Many traits of agricultural significance exhibit quantitative inheritance, which is often the result of multiple genes of minor effect (reviewed by Costantini et al. 2009; Martínez-Zapater et al. 2009; Welter et al. 2011). The analysis of segregating progenies allows the efficiency of cross-breeding programs to be improved, increasing our knowledge of the inheritance and genetic architecture of quantitative traits (Mackay 2001). The establishment of correlations between traits might reduce the number of characters that need to be evaluated in future genotypes or progenies. Furthermore, it is important to know these correlations given that the improvement of one trait could have unfavorable impacts on others. Previous studies obtained relationships between some of the quality parameters in grapevine (Gawel et al. 2000; Jones and Davis 2000; Wei et al. 2002; Mpelasoka et al. 2003; Liu et al. 2007; Costantini et al. 2008; Liang et al. 2009; Leão et al. 2010).

Monastrell is the major red wine grape variety grown in Murcia and sometimes it is blended with other cultivars like Syrah or Cabernet Sauvignon. The main objective of the wine grape breeding program at the IMIDA is to obtain new cultivars well adapted to Murcia's climate conditions and with better genetic composition, derived from crosses involving Monastrell as a parental genotype. With this purpose, a Monastrell x Syrah F_1 progeny was generated. The goal of this work was to study the transmission of different agronomic and fruit quality traits in this progeny, as well as the correlations between these traits, in order to improve the selection of hybrids of interest for future studies. Four types of data have been evaluated and discussed: phenological, productive, morphological, and enological.

1.2 Materials and Methods

1.2.1 Plant material

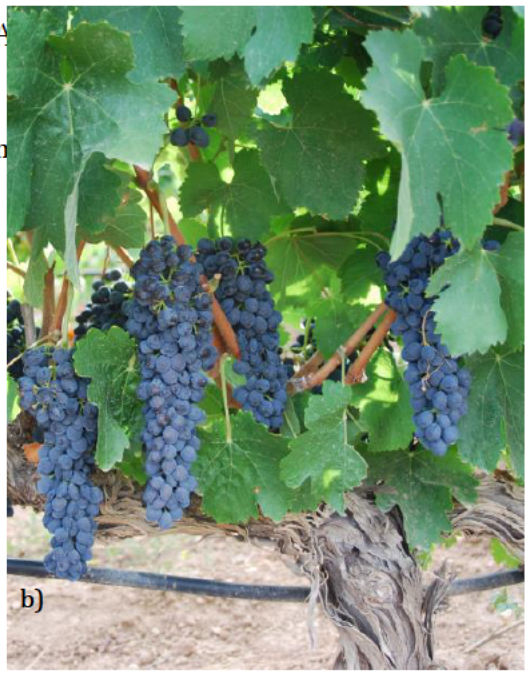
A segregating progeny of 229 plants resulting from hybridization between the wine grape cultivars Monastrell (female progenitor) and Syrah (male progenitor) was used in this work. A plant of each hybrid and representative plants of both progenitors were grown on their own roots under standard conditions of irrigation, fertilization, and pest and disease control, in the same experimental field of the IMIDA in Murcia (Southeast Spain) from the year 2000. The vine and row spacings were 1.25 and 2.5 m, respectively. The vines were pruned to two two-bud spurs (four nodes). Monastrell (unknown progenitors), a traditional Spanish variety adapted to the dry conditions of the Mediterranean climate, is cultivated widely in Murcia and contributes to the characteristic features of wines from this region. Syrah (Mondeuse Blanche x Dureza) (http://www.vivc.de) is a foreign variety, very well adapted to the warm climate of Murcia, which blends very well with Monastrell, contributing quality characters. This progeny segregates for several agricultural traits (phenology, yield, morphology, and enology) and has been used also to produce an integrated genetic map of *Vitis vinifera* (Bayo-Canha et al. in preparation). Therefore, the plants derived from Monastrell self-pollination and from pollen-donors other than Syrah were identified genetically and discarded using the microsatellite (SSR, Simple Sequence Repeat) loci segregating <1:1:1:1>. The genetic identity of Monastrell and Syrah was verified by genotyping 6 SSR loci: VVMD5, VVMD7, VVMD27, VrZAG62, VrZAG79, and VVS2 (This et al. 2004).

The total DNA was extracted from 50 mg of frozen young leaves using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA), following the manufacturer's protocol. The SSR analyses were performed according to a previously described method (Ibáñez et al. 2003). The PCR products were separated by capillary electrophoresis performed on an ABI Prism 3730

genetic analyzer and the fragments were sized using GeneMapper® software (Applied Biosystems, Carlsbad, CA, USA

Figure 1.1. a) Mon

a)



b)

1.2.2 Phenotypic evaluation

Twenty-two segregating agronomic traits were evaluated for each genotype in three consecutive seasons (2008-2010). One hundred and fifty-two of the 229 progeny individuals bore fruit in 2008, 153 in 2009, and 176 in 2010. The numbers of individuals scored for phenotypic evaluation varied depending on the year and trait because of environmental conditions and disease incidence. In addition, the numbers of individuals analyzed for enological parameters were lower due to the minimal quantities of material necessary for these analyses.

Phenology-related traits

The numbers of hybrids evaluated for phenological traits varied from 141 to 229 (see Table 1.1). Sprouting was measured as the date when 50% of the buds were in Baggiolini stage C, and the flowering as the date when 50% of the flowers had opened and the anthers were visible (Baggiolini stage I) (Baggiolini 1952). Veraison was considered as the date when 50% of the berries were coloring and/or softening, and ripening as the date when three berries picked randomly from the top, medium, and bottom regions of several

clusters reached approximately 13.5 ºBaumé (ºBé) (colored grapes) or 12.5 ºBé (uncolored grapes) using a hand refractometer. The veraison-ripening interval was calculated as the number of days from veraison to ripening.

Productive and morphological traits

Productive and morphological traits were evaluated at harvest (ripeness), except the fertility index which was scored before flowering. The numbers of hybrids evaluated for productive traits varied from 132 to 174 (Table 1.1). The fertility index was scored as the number of inflorescences per young shoot. The average weight of the cluster was calculated using all the clusters per genotype, and the berry weight was calculated using about 300 berries taken randomly per genotype.

The numbers of individuals evaluated for morphological traits varied from 117 to 174; from 141 to 173 for number of seeds per berry (Table 1.1); from 122 to 160 for cluster density; from 117 to 120 for cluster shape; from 142 to 169 for berry shape; from 142 to 174 for berry skin color. For berry shape and the number of seeds per berry, 30 berries taken randomly were sampled per genotype. Berry skin color was determined visually as uncolored or colored. The cluster density was coded into five phenotypic groups, following the code OIV 204 (O.I.V. 2009): 1, very loose; 3, loose; 5, medium; 7, dense; 9, very dense. The cluster shape was divided into six phenotypic groups: conical, conical with wings, funnel shaped, cylindrical, cylindrical with wings, and double branched. Finally, the berry shape was classified into three phenotypic groups, following the code OIV 223 (O.I.V. 2009): 2, globose; 3, broad ellipsoid; 4, narrow ellipsoid.

Enology-related traits

The numbers of hybrids evaluated for enological parameters varied from 74 to 160 (Table 1.1). The physicochemical analyses of each sample were performed in triplicate at harvest. About 100 g of berries from different

positions within each cluster, considered as one replication, were mixed and squeezed to determine the physicochemical parameters of the juice. Total soluble solids were determined as ºBaumé, using an Atago RX-5000 digital refractometer (Atago, Tokyo, Japan). The juice pH and titratable acidity were determined by titration with 0.1 N NaOH, using a Metrohm 686 automatic titrator (Metrohm, Herisau, Switzerland). The titratable acidity was expressed as g/L tartaric acid equivalent. Tartaric and malic acids were measured using enzymatic kits from Boehringer Mannheim GmbH (Mannheim, Germany). The potassium content was determined by atomic absorption spectrometry, using a Unicam 969 spectrophotometer (Thermo Elemental, UK), and expressed as g/L. The phenolic potential of the grapes was determined based on the method described by Saint-Cricq et al. (1998), macerating the grapes for 4 hr at two pH values (3.6 and 1.0). The original pH 3.2 solution was exchanged for one of pH 3.6, which is better suited to the musts from Murcia (Romero-Cascales et al. 2005; Romero et al. 2010). The total and extractable anthocyanins contents of the two solutions were then assayed by measuring the absorbance at 520 nm at pH 1.0 and pH 3.6, respectively, and expressed as mg/L. The extractability index was calculated as described by Romero-Cascales et al. (2005).

1.2.3 Genotypic evaluation of the color

To establish whether the visual berry skin color phenotype and the total anthocyanins content were correlated with the *VvmybA* genotype, the CAPS (Cleaved Amplified Polymorphic Sequence) marker 20D18CB9 (Walker et al. 2007) was tested against the progeny and parental plants, using a PCR assay. The amplification product obtained using the primers 20D18CB9f (5´-GATGACCAAACTGCCACTGA-3´) and 20D18CB9r (5´-ATGACCTTGTCCCACCAAAA-3´) was then restricted with *Dde*I and separated by gel electrophoresis on 2.5% agarose gels, using 1x TBE buffer. The separated DNA fragments were visualized under UV light, after staining

with ethidium bromide, and documented with Gel Doc XR software (Bio-Rad).

1.2.4 Statistical analysis

The normality of each trait distribution was evaluated by the Kolmogorov-Smirnov test. Differences between years for each trait were analyzed by the Kruskal-Wallis test. The correlation between traits was calculated by the Spearman test at $P < 0.01$. The cluster analysis of the quantitative phenotypic data was carried out using the squared Euclidian distance combined with the average linkage clustering method. All statistical analyses were performed using SPSS 18.0 for Windows.

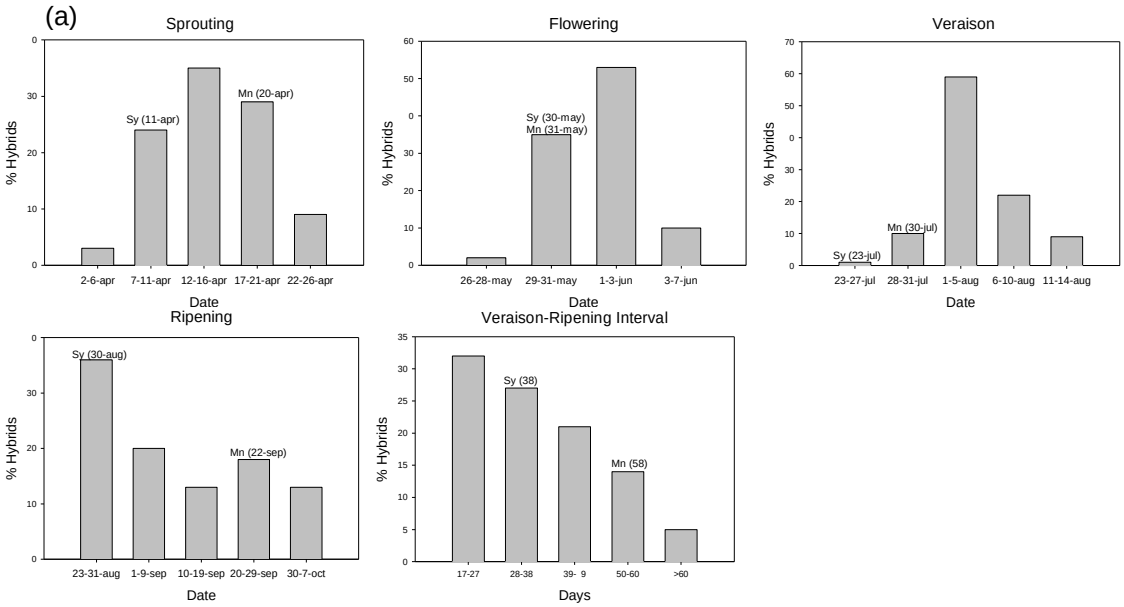
1.3 Results

1.3.1 Phenotypic evaluation

The phenotypic data distributions, which are shown in Fig. 1.2 for season 2010, were very similar in the three years analyzed. Continuous variation and transgressive segregation were observed for the characters evaluated, except for the shape and visual color of the berries (Fig. 1.2b).

The Kolmogorov-Smirnov test indicated that only malic acid and potassium showed a normal distribution in the three years studied. Berry weight distribution was normal in 2008 and 2009, number of seeds per berry and total soluble solids (ºBé) in 2009, and total acidity in 2008. The Kruskal-Wallis test revealed a significant year effect ($P < 0.05$) for all the traits except for the fertility index, cluster shape, visual color of the berries, total acidity, and total and extractable anthocyanins.

Figure 1.2. Distribution of the progeny for different agronomic traits in 2010.



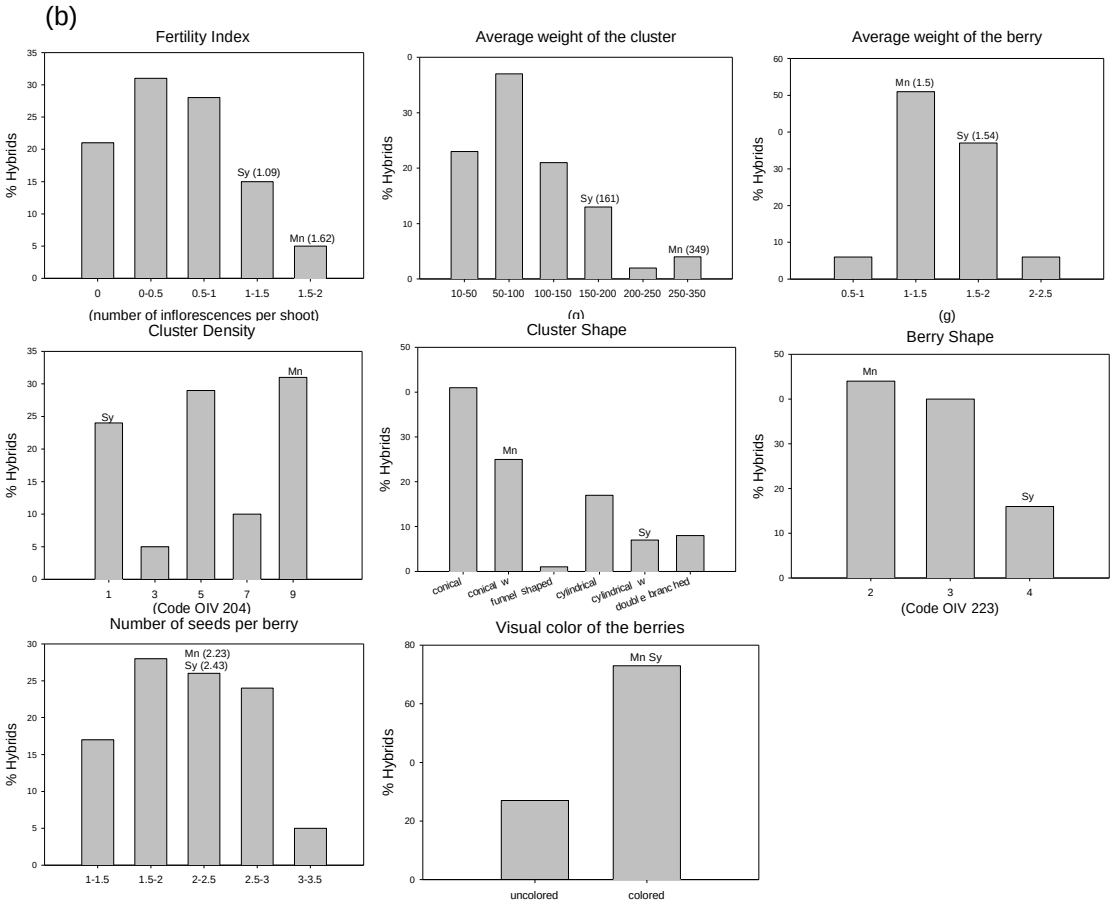
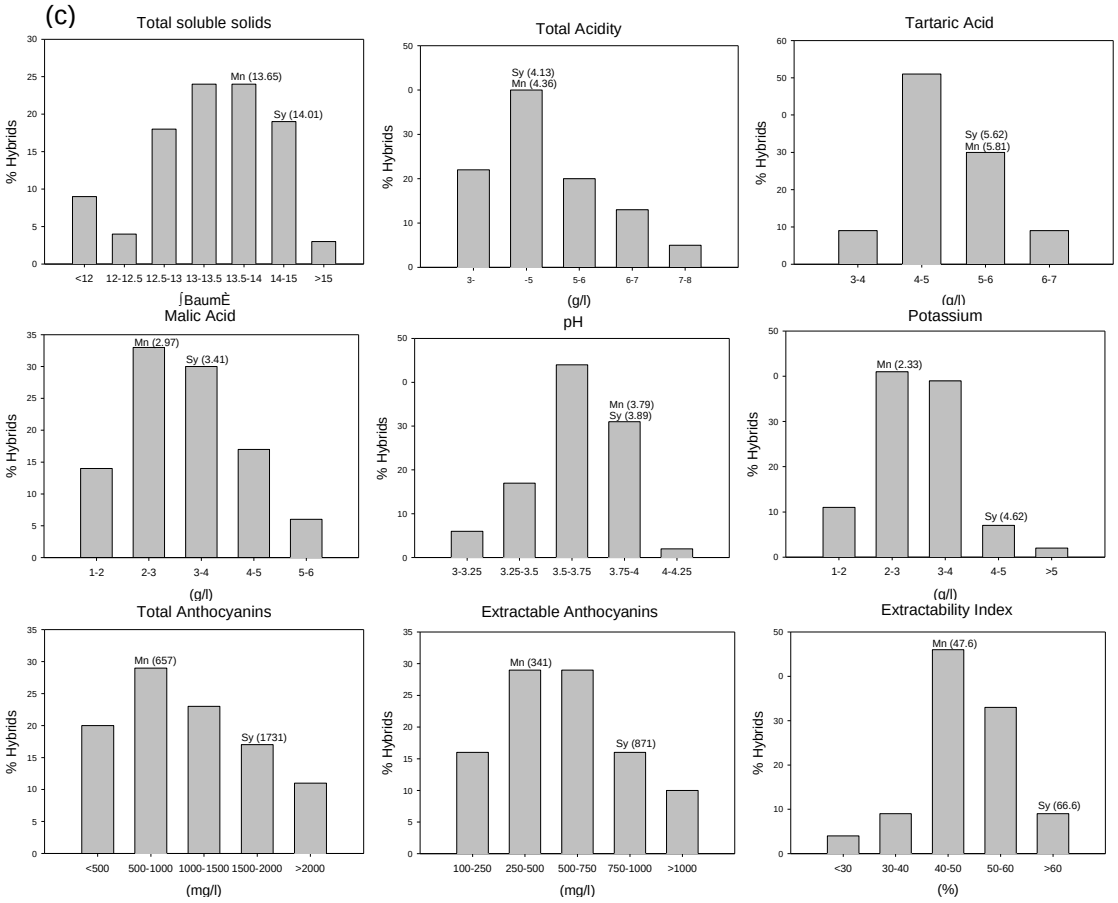


Figure 1.2. Continued.



The positions of the parents are indicated: Monastrell (Mn) and Syrah (Sy). (a) Histograms for phenological traits. (b) Histograms for productive and morphological traits. (c) Histograms for enological traits.

Phenological traits

The average length of the growing season (from sprouting to ripening) was 141 days. The mean values of sprouting, flowering, and ripening (days since 1st January) and the mean length of the veraison-ripening interval showed significant differences among the three years of the study (Table 1.1). These differences show the influence of the environmental conditions on these traits, although the different numbers of plants analyzed each year could also have a partial effect on the differences. Syrah was the earlier parent for all the phenology-related traits (Fig. 1.2a). Most of the hybrids were later than Monastrell for flowering and veraison (63% and 94%, respectively). The mean dates of flowering and veraison of the progeny were delayed as the hybrids aged. The same happened for ripening, except in 2009 (Table 1.1). Since the

colored hybrids require higher contents of sugar for ripening, the greater number of colored plants analyzed in 2010 (127), compared to 2008 (101) and 2009 (109), could have delayed the ripening in this year. However, this does not hold when comparing 2009 to 2008. The veraison-ripening interval was between 15 and 67 days, and 9% of the hybrids showed a veraison-ripening interval greater than that of Monastrell (Table 1.1, Fig. 1.2a).

Table 1.1. Mean values of 18 agronomic traits evaluated in the F_1 Monastrell x Syrah progeny.

	Year						Total		
	n	2008	n	2009	n	2010	*x*	*m*	*M*
Sprouting (days since 1st January)	229	99 a	228	106 b	228	103 c	103	90	118
Flowering (days since 1st January)	150	146 a	149	149 b	176	151 c	149	138	167
Veraison (days since 1st January)	147	211 a	150	210 a	173	215 b	213	201	223
Ripening (days since 1st January)	142	241 a	151	236 b	174	252 c	244	229	275
Veraison-ripening interval (days)	141	30 a	149	26 b	170	37 c	31	15	67
Fertility index	152	0.6 a	153	0.6 a	174	0.6 a	0.6	0.0	2.2
Cluster weight (g)	138	77.2 a	149	77.2 a	167	98.1 b	84.9	6.0	334.9
Berry weight (g)	132	1.15 a	149	1.2 a	171	1.5 b	1.3	0.5	2.4
Number of seeds per berry	141	2.4 a	149	2.2 a	173	2.2 a	2.3	1.0	3.8
Total soluble solids (ºBé)	119	13.7 a	141	14.8 b	160	13.3 c	13.9	10.8	17.3
Total acidity (g/L)	119	5.1 a	141	5.1 a	154	4.9 b	5.03	2.6	10.6
Tartaric acid (g/L)	96	5.4 a	97	5.2 a	119	4.9 b	5.2	2.8	7.9
Malic acid (g/L)	96	3.7 b	97	3.1 a	119	3.1 a	3.3	0.8	6.5
pH	119	3.8 a	141	4.1 b	157	3.7 a	3.8	2.9	5.7
Potassium (g/L)	96	2.8 a	97	2.3 a	119	3.0 b	2.6	1.3	5.0
Total anthocyanins (mg/L)	-	-	74	1108 a	82	1125 a	1117	235	2969
Extractable anthocyanins (mg/L)	-	-	74	688 a	82	554 b	618	108	1569
Extractability index (%)	-	-	74	34.9 a	82	48.1 b	41.9	13.2	82.4

Values with different letters show significant differences between years at the 5% level, according to de LSD test. n, number of plants evaluated each year, and mean values for each year. Mean (*x*), minimum (*m*), and maximum (*M*) values for the three years.

Productive and morphological traits

The distribution of productive and morphological traits for the progeny in 2010 is shown in Fig. 1.2b. The mean fertility index (0.6) and the mean cluster weight (84.9 g) of the progeny were lower than the values of both progenitors (Table 1.1, Fig. 1.2b). Seventy-four percent of the hybrids were distributed in the low fertility index range (below 1.1) and 86% in the low cluster weight range (below 161 g). In relation to bunch compactness, 5% and

29% of the hybrids showed loose and medium clusters, respectively (Fig. 1.2b), which are less sensitive to diseases than compact clusters. The berries of Monastrell and Syrah are colored and in agreement with the expected Mendelian segregation for a monogenic dominant trait (3:1): 74% and 26% of the progeny showed colored and uncolored berries, respectively (Fig. 1.2b).

Enological traits

The total acidity ranged between 2.6 and 10.6 g/L with an average value in the progeny higher than the values of both progenitors. However, the mean tartaric acid content of the progeny was lower than that of both progenitors (Table 1.1, Fig 1.2c). Eighty-one percent of the hybrids were distributed in the low tartaric acid range and 71% in the low pH range (Fig 1.2c). The malic acid content in the progeny ranged between 0.8 and 6.5 g/L and was less than that of tartaric acid (Table 1.1).

Colored plants were analyzed in the winery for total anthocyanins, extractable anthocyanins, and extractability index in 2009 and 2010 (Table 1.1). The total anthocyanins ranged between 235 and 2969 mg/L and only 17% of the hybrids had values higher than Syrah (above 1731 mg/L) (Fig. 1.2c). The values of extractable anthocyanins ranged between 108 and 1569 mg/L and only 15% of the hybrids had values higher than Syrah (above 871 mg/L). Finally, 46% of the hybrids showed an optimal extractability index, between 40 and 50%.

1.3.2 Correlations between traits

Table 1.2 shows the correlations between the traits detected by the Spearman correlation test ($P < 0.01$), excluding the traits that show none (berry shape), one weak correlation (fertility index, cluster shape, cluster density, and tartaric acid), or two weak correlations to other traits (number of seeds per berry). The values of the coefficients correspond to the year 2010.

Several associations between traits were revealed within each year, although few correlations were significant, with Spearman coefficients higher than 0.5. Many of them were significant in all years analyzed and concerned the component variables of the same character: a positive high correlation between veraison-ripening interval and ripening date; a positive high correlation between visual color, total anthocyanins, extractable anthocyanins, and extractability index; a negative moderate correlation between total acidity and pH. Also, a positive moderate correlation between total acidity and malic acid was found in 2008 and 2010 (Table 1.2).

Nevertheless, correlations between different traits were also detected in the three years analyzed or in two of the years (Table 1.2). With regard to the correlations between the different phenological characters, veraison correlated weakly with ripening and flowering, and flowering correlated moderately with sprouting. Cluster weight correlated positively with berry weight, fertility index ($r = 0.49$), and cluster shape ($r = 0.37$), and negatively with tartaric acid ($r = -0.27$). The number of seeds per berry only correlated weakly ($r = 0.31$) with cluster density in 2009 and 2010, and with potassium (in 2008 and 2009; $r = 0.36$). Total soluble solids (ºBé) correlated negatively with veraison ($r = -0.40$ in 2008 and $r = -0.38$ in 2009) and positively with visual color, pH, and total and extractable anthocyanins. Visual color, total anthocyanins, and extractability index correlated positively with ripening and veraison-ripening interval. Total acidity correlated negatively with ripening, veraison-ripening interval, and visual color. Furthermore, malic acid correlated positively with sprouting and potassium, and negatively with veraison-ripening interval. Finally, pH correlated positively with veraison-ripening interval ($r = 0.44$ in 2008 and $r = 0.43$ in 2009), visual color, extractable anthocyanins, and potassium. Correlations observed in only one year, as well as discordant correlations over different years, were not considered.

Table 1.2. Phenotypic correlations among 16 traits (Spearman correlation coefficient) during the three years. The values of the coefficients correspond to the year 2010.

	Sp	Fl	Vr	Rp	Vr_Rp	VC	CW	BW	TSS	TA	Mal	pH	K	T_Ant	E_Ant	EI
Sprouting	1	**.64**	ns	ns	ns	ns	ns	ns	ns	ns	.27	ns	ns	ns	ns	ns
Flowering		1	**.27**	ns	ns	ns	ns	**.24**	ns	ns	ns	ns	ns	ns	ns	ns
Veraison			1	**.32**	ns	ns	ns	ns	-ns	ns	ns	ns	ns	ns	ns	ns
Ripening				1	**.95**	.48	ns	ns	ns	**-.50**	ns	ns	ns	ns	ns	.35
Veraison-Ripening Interval					1	**.48**	ns	ns	ns	**-.50**	-.60	ns	ns	.27	ns	.31
Visual Color of the berries						1	ns	ns	.56	-.31	i	**.27**	ns	.81	.81	.81
Cluster Weight							1	**.41**	ns	ns	ns	ns	ns	ns	ns	ns
Berry Weight								1	ns	ns	ns	ns	ns	ns	ns	ns
Total Soluble Solids									1	ns	ns	.40	ns	.55	.56	ns
Total Acidity										1	.66	**-.50**	ns	ns	ns	ns
Malic acid											1	ns	.64	ns	ns	ns
pH												1	**.46**	ns	.24	ns
Potassium													1	ns	ns	ns
Total Anthocyanins[a]														1	.99	.84
Extractable Anthocyanins[a]															1	.77
Extractability Index[a]																1

Abbreviations: Sp, Sprouting; Fl, Flowering; Vr, Veraison; Rp, Ripening; Vr_Rp, Veraison-ripening interval; VC, Visual color of the berries; CW, Cluster weight; BW, Berry weight; TSS, Total soluble solids; TA, Total acidity; Mal, Malic acid; K, Potassium; T_Ant, Total anthocyanins; E_Ant, Extractable anthocyanins; EI, Extractability index.
Boldface font indicates correlations which are significant at the 0.01 level in the three years evaluated, and underlined font indicates correlations which are significant in two of the years.
ns, not significant; i, inconsistent; -, negative correlation.
[a] based only on years 2009 and 2010.

1.3.3 Cluster analysis of quantitative phenotypic data

Progeny classification based on quantitative phenotypic data for 18 traits (all the phenotypic data evaluated except that of the visual color, cluster density, and cluster and berry shape) was carried out using only hybrids that had no missing values for these traits within each year. The uncolored hybrids were analyzed using the data of the three years, and the colored hybrids using only the data of 2009 and 2010. The hybrids were grouped on the basis of the same criteria with high significance for wine grape breeding in all the years analyzed: malic acid for uncolored grapes, and the content and extractability of anthocyanins for colored grapes. Fig. 1.3 shows the groups obtained, based only on the evaluation of 2010.

The 37 uncolored hybrids were grouped into two main clusters (Fig. 1.3a), in general accordance with their malic acid and potassium contents. Cluster one included the hybrids (from '93' to '65') with the highest average contents of malic acid and potassium (4.8 g/L and 3.6 g/L, respectively). Cluster two included the hybrids (from '30' to '183') with average malic acid and potassium contents of 3.2 g/L and 2.3 g/L, respectively. The hybrid '59' showed the lowest malic acid and potassium contents (1 g/L and 1.6 g/L, respectively) and was placed outside of the others.

The 82 colored hybrids were grouped into two main clusters (Fig. 1.3b), in general accordance with their content and extractability of anthocyanins and their cluster and berry weights. Group one included the hybrids (from '196' to '46') with the lowest content (average of 952 mg/L) and extractability (average of 479 mg/L) of anthocyanins, and the highest cluster and berry weights (averages of 121 g and 1.45 g, respectively). Group two includes the hybrids (from '104' to '222') with the highest content (average of 2292 mg/L) and extractability (average of 1061 mg/L) of anthocyanins, and the lowest cluster and berry weights (averages of 76 g and 1.31 g, respectively).

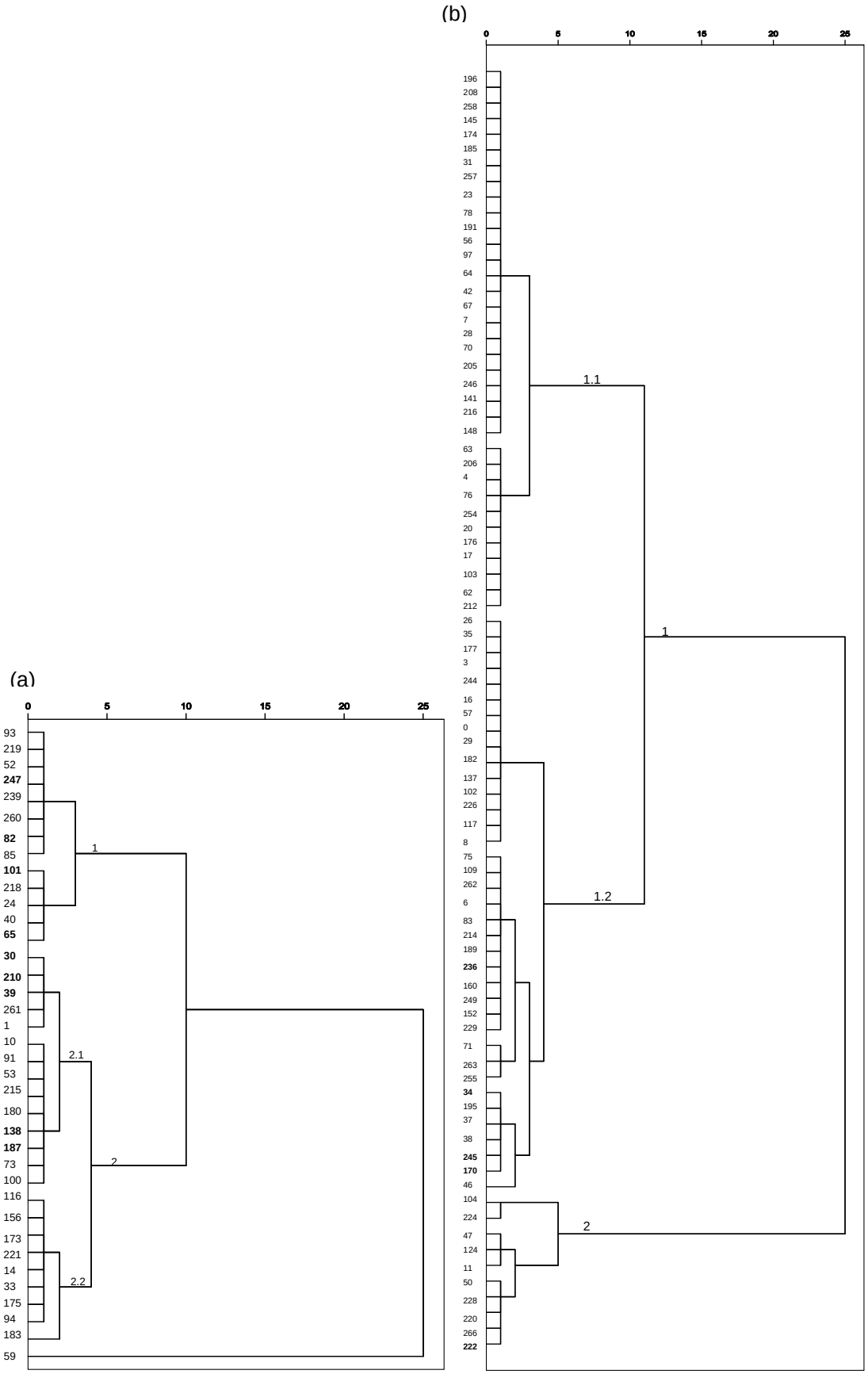


Figure1.3. Dendrogram of 37 uncolored (a) and 82 colored (b) Monastrell x Syrah hybrids, based on 18 quantitative agronomic traits. Boldface font indicates pre-selected hybrids.

In the experimental winery of the IMIDA, a desirable new white variety should have pH < 3.7, malic acid > 3 g/L, and a berry weight of 1-1.6 g. Three uncolored hybrids ('247', '65', and '30') showed these values in the three years analyzed, and six ('82', '101', '210', '39', '138', and '187') in two of the years (Fig. 1.3a, bold font). Likewise, a desirable new red variety should have pH < 3.8, total anthocyanins > 1400 mg/L, extractable anthocyanins > 700 mg/L, and a berry weight < 1.5 g. Five colored hybrids ('236', '34', '245', '170', and '222') showed these values in the two years analyzed (Fig. 1.3b, bold font). These white and red hybrids could be pre-selected from the initial breeding population.

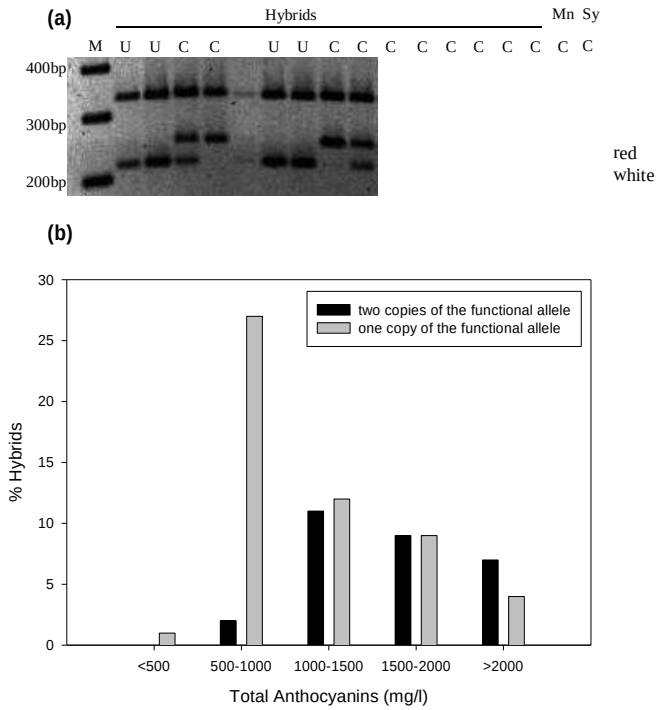
1.3.4 Association of visual berry color and total anthocyanins content with allelic composition for *VvmybA*

The 186 F1 plants phenotyped for visual berry color, together with the Monastrell and Syrah progenitor cultivars, were tested also for color genotype with the CAPS marker 20D18CB9, which flanks the *VvmybA* genes (Walker et al. 2007). An example of a gel used to score the marker is presented in Fig.1.4a. All 137 plants bearing colored-skinned berries carried one (51%) or two copies (23%) of the functional allele, while all 49 uncolored-skinned berries were homozygous for the non-functional allele (26%). Thus, 100% of the berry color phenotype can be explained on the basis of the PCR-established *VvmybA* genotype.

Eighty-two of the 186 F_1 plants analyzed for visual color were analyzed also for total anthocyanins content in 2010. The LSD test for the average anthocyanins content of the corresponding genotypic classes showed significant differences ($P < 0.05$) between homozygous (two copies) and heterozygous plants (one copy). Although total anthocyanins differed even within the same genotypic classes (Fig. 1.4b), hybrids with two copies of the

functional allele had significantly higher anthocyanins contents (average value of 1637 mg/L) than hybrids with only one copy (average of 913 mg/L). Ninety-two percent of the hybrids homozygous for the functional allele were distributed in the high-anthocyanins-content range (above 1000 mg/L), in comparison with the 34% of the heterozygous hybrids (Fig. 1.4b).

Figure 1.4. Association of visual berry color and total anthocyanins content with the *VvmybA* genotype.



(a): PCR analysis of *VvmybA* alleles for uncolored (U) and colored (C) Monastrell (Mn) x Syrah (Sy) hybrids, with the CAPS marker 20D18CB9. M, molecular weight marker.

(b): Average distribution of 82 colored hybrids for total anthocyanins. The number of copies of the functional *VvmybA* allele is indicated.

1.4 Discussion

This study shows the high phenotypic variability found in a cross between two different wine grape cultivars that may be useful in the development of new cultivars with improved attributes. Most of the phenotypic parameters evaluated showed continuous variation within the

progeny, suggesting a polygenic inheritance in agreement with previous studies (Sato et al. 2000; Wei et al. 2002; Liu et al. 2007; Costantini et al. 2008; Shiraishi et al. 2008; 2010; Liang et al. 2009; Duchêne et al. 2010; 2011).

In recent decades, the shortening of the intervals between phenological events has been reported in several areas of cultivation of the grapevine (Duchêne and Schneider 2005; Ramos et al. 2008; Soar et al. 2008) and it is accepted that elevated temperatures can impair the quality of grapes and wines (Jones and Davis 2000; Jones et al. 2005; Keller 2010). High temperatures are associated with elevated sugar content and tend to make the winemaking process more expensive because low-acid grape juice requires addition of tartaric acid. Likewise, high temperatures have been correlated with elevated synthesis of anthocyanins, but above 35 ºC anthocyanins stop accumulating and may even be degraded (Spayd et al. 2002; Mori et al. 2007). A possible adaptation to this climate change scenario in warm areas, like that of Murcia, is to grow late-ripening varieties with longer veraison-ripening intervals. These varieties might avoid the highest temperatures and have enough time to achieve full maturation in this area. Our results show that the possibility of obtaining offspring with a ripening date later than Monastrell (13-19%) and with a longer veraison-ripening interval (4-15%) is low. These late hybrids should be used as new progenitors to obtain offspring with a phenology adapted to this area in the future.

Also important is the selection of plants with better quality attributes, with regard to their adaptation to hot conditions. In this sense, the results of this work suggest that in order to get a high tartaric/malic acid ratio in the progeny, which improves the stability of wine, the mean tartaric acid content of the parents should be high, consistent with the results obtained by Liu et al. (2007). Moreover, the distribution of total content and extractability of anthocyanins in our progeny exhibited an additive model, and allowed the

identification of hybrids that showed total levels of anthocyanins above 1400 mg/L (24-32%) and those with levels of extractable anthocyanins above 700 mg/L (25-59%), the values preferred for the IMIDA's breeding program. As expected, the mean total soluble solids of the progeny were lower than the values of both progenitors, due in part to the presence of white hybrids that require lower contents of sugar for their ripening. Inheritance patterns for table grape sugars were investigated previously in progenies by Liu et al. (2007). They concluded that parental sugar content has no significant effect on progeny sugar content. Finally, a low number of clusters per plant, together with small clusters, has a positive effect on wine quality and reduces the production costs by decreasing the cluster pruning (Morris et al. 2004). Fertility and cluster weight distribution exhibited a dominant model towards low values while berry weight exhibited an additive model, consistent with previous studies (Eibach 1990; Fanizza et al. 2005; Doligez et al. 2010). Nevertheless, these low values in our work could be a consequence of the mean value being calculated for the first years of production - when productivity is usually low - and of the narrow planting.

The selection of a particular phenotype can be a long process, due to the juvenile period (3-5 years) of grapevine plants and the additional time necessary for evaluation of important traits for wine production. On the one hand, phenotypic correlations between the traits of interest might be used to reduce the number of these to be evaluated in future studies. In this sense, we found that hybrids with high contents of anthocyanins also showed high anthocyanins extractability ($r = 0.99$). In addition, our results suggest that selection in breeding programs for hybrids homozygous for the functional color allele would be more likely to produce hybrids with high contents of anthocyanins. Similar results were reported also by Lijavetzky et al. (2006) and Azuma et al. (2008). So, the genotypic evaluation of color could be an

important tool in the pre-selection of hybrids with high contents and extractability of anthocyanins in the IMIDA wine breeding progeny. Although we also found a high correlation between ripening and the veraison-ripening interval (r = 0.95), the low correlation of these phenological events with veraison denotes the importance of assessing both veraison and ripening.

On the other hand, phenotypic correlations between traits may also restrict breeding progress given that the improvement of one trait could have unfavorable impacts on other traits. Thus, it is important to know the relationships between the traits of interest and to define the breeding objectives. In this work, only a few correlations were significant, with Spearman coefficients higher than 0.5. Weak or no correlations among traits indicate that each trait is genetically independent, such as fertility index, berry shape, cluster shape, cluster density, and tartaric acid. The intermediate correlation between sprouting and flowering (r = 0.64) could imply certain tendency of the late-sprouting hybrids to bloom later. To obtain balanced wines, it is necessary to have veraison-ripening periods that allow sugars to accumulate to favorable levels, maintain acid structure, and produce the optimum profile for phenols and flavor and aroma compounds. The data reported here indicate that selection for long veraison-ripening intervals would reduce the total acidity (r = -0.50) and malic acid (r = -0.60) and might also result in a weak increase of anthocyanins (r = 0.27). The negative correlation between ripening and acidity (r = -0.50) is in agreement with the data published by Wei et al. (2002) and in contrast to the positive correlation reported by Jones and Davis (2000). Excessive potassium in grape berries may have a negative impact on wine quality, mainly because it reduces the tartrate:malate ratio, which is undesirable for high-quality wines. The correlation of potassium with pH (r = 0.46) and malic acid (r = 0.64) is in agreement with previous results (Hale 1977; Boulton 1980; Gawel et al. 2000;

Mpelasoka et al. 2003). High potassium concentrations in a berry may impede malate transfer from the vacuolar storage pools to the cytoplasm and therefore may decrease the rate of malate degradation through malate respiration (Hale 1977). Other useful relationships were the moderate correlations between total soluble solids and total and extractable anthocyanins.

Seeds produce and act as sinks for hormones, which induce rapid growth of the developing ovary by increasing cell division and cell expansion (Bohner and Bangerth 1988). The relationship between seed number and berry size was reviewed by Ollat et al. (2002). In table grape, as in other fruit species, there is a positive correlation of berry weight or size with seed number (Coombe 1973) and a negative correlation with seedlessness (Wei et al. 2002). However, in our study with wine grapes, no correlation was found for berry weight and the number of seeds per berry, as Costantini et al. (2008) found in table grape. In accordance with these results, some QTLs (Quantitative Trait Loci) for seed number have been reported on linkage groups independent of QTLs for berry weight (Doligez et al. 2002; Cabezas et al. 2006; Mejía et al. 2007; Costantini et al. 2008).

1.5 Conclusions

From the above results we deduce that crossing Monastrell and Syrah can generate a large phenotypic variability that may be useful in the development of new cultivars with improved attributes. Breeding new, late-ripening varieties is a way to adapt the grapevine phenology to climate change in hot areas, and from this progeny we can select hybrids with a ripening date later than Monastrell. The selection of new parents should be based on high tartaric acid content, to improve the wine structure. We also suggest that the genotypic evaluation of the color could be an important tool in the pre-

selection of hybrids with high content and extractability of anthocyanins. This progeny is maintained under experimental vineyard conditions, so pre-selected hybrids must be grafted and cultivated in commercial wine grape conditions in order to carry out new analyses of quality and micro-vinification procedures. Besides the common quality parameters, combinations of aromas, flavors, and tannins - which contribute to the unique and typical 'varietal character' - will be selected in these new cultivation conditions, depending on the type or style of wine that will be made.

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CHAPTER 2

Genetic linkage maps construction

2.1 Introduction

Molecular markers are differences at the chromosomal level made visible by molecular techniques. They enable us to follow the chromosomal segments which are passed on from one generation to the next. Molecular markers can be used to verify the identity of cultivars, to distinguish and compare cultivars, and also in the construction of genetic linkage maps and to study segregation of genes and the origin and inheritance of heritable traits. A linkage map is a representation of the genome of a biological species. In a diploid species every individual carries two genomes, one inherited from the mother and one from the father. Corresponding chromosomes from these two genomes are called homologous chromosomes. Molecular markers correspond to physical locations of DNA on these chromosomes (marker loci) and they allow the detection of differences between the homologs at the marker loci. These differences are called the marker alleles or the allelic variant of the marker.

The construction of genetic linkage maps is nowadays a powerful tool to understand the genetic determination of many relevant grapevine traits that are generally controlled by multiple genes. The number of markers used to construct these maps varies dramatically and ranges from as few as 84 to more than 500. Lodhi et al. (1995) developed the first maps in grapevine using markers such as RAPD (Random Amplified Polymorphic DNA), RFLP (Restriction Fragment Length Polymorphisms), and isoenzymes. In the subsequent years several maps were generated, including different types of markers: AFLP, RAPD, CAPS, and SSCP (Dalbó et al. 2000; Doligez et al. 2002; Grando et al. 2003; Doucleff et al. 2004; Fischer et al. 2004; Cabezas et al. 2006). These maps were improved with the addition of microsatellite (SSR) markers, which enabled the comparison of different genetic maps because they are highly transferable between laboratories and due to their co-dominant

character (Adam-Blondon et al. 2004; Riaz et al. 2004; 2006; Fanizza et al. 2005; Doligez et al. 2006a,b; Lowe and Walker 2006; Di Gaspero et al. 2007; Mejía et al. 2007; Welter et al. 2007; Costantini et al. 2008; Salmaso et al. 2008; Vezzulli et al. 2008; Battilana et al. 2009; Duchêne et al. 2009; 2012; Fournier-Level et al. 2009; Doligez et al. 2010; Riaz et al. 2011). The numbering of linkage groups has been performed according to Adam-Blondon et al. (2004). The reference map of Doligez et al. (2006a) was constructed using 502 loci SSR as well as five individual mapping populations.

The development of SNP-type markers favored the gradual incorporation of these markers into genetic maps, together with markers developed from candidate genes (Di Gaspero et al. 2007; Troggio et al. 2007; Welter et al. 2007; Costantini et al. 2008; Salmaso et al. 2008; Vezzulli et al. 2008; Battilana et al. 2009; Duchêne et al. 2009; Cabezas et al. 2011; Emanuelli et al. 2013; Grzeskowiak et al. 2013).

Low and Walker (2006) generated the first interspecific rootstocks linkage map. While most maps to date have been constructed for *V. vinifera*, Blanc et al. (2012) recently published an SSR-based map for *M. rotundifolia* that showed a high degree of similarity to the *V. vinifera* reference map of Doligez et al. (2006a).

These maps were used in QTL mapping of many relevant grapevine traits such as phenology, fertility, berry weight and composition, seedlessness, berry firmness, muscat flavor, cluster architecture, and tolerance to abiotic stresses or resistance to diseases and infestations (see, in particular, Costantini et al. 2009, as well as Battilana et al. 2009; Doligez et al. 2010; 2013; Riaz et al. 2011; Huang et al. 2012; Duchêne et al. 2012; Emanuelli et al. 2013; Grzeskowiak et al. 2013; Barba et al. 2014; Correa et al. 2014; Coupel-Ledru et al. 2014; van Heerden et al. 2014; Carreño et al. 2015; Chen et al. 2015; Houel et al. 2015).

In breeding programs, markers linked to determined traits can be used to increase the chance of selecting those individual plants from the progeny of a cross that have the best-possible combination of desired properties. Molecular markers offer the possibility to select for a character at a stage long before it is expressed in the plant, since - for the analysis of molecular markers - usually only a small amount of DNA is needed. Another asset is the fact that markers tests can be applied at any time during the year and the plant development; also, they are not influenced by environmental noise and can be used even if the trait itself cannot be measured. In this work, linkage maps containing SSR and SNP markers were developed for a wine grape segregating F_1 progeny (Monastrell x Syrah), to perform quantitative analysis in combination with phenotypic data collected over different years.

2.2 Materials and Methods

2.2.1 Plant material

The segregating progeny of 229 plants resulting from hybridization between the wine grape cultivars Monastrell and Syrah was used, including both parents (see chapter 1).

2.2.2 Molecular marker analysis

Total DNA was isolated from 50 mg of young frozen leaves, using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) with extraction buffer supplemented with 1% polyvinylpyrrolidone to reduce polyphenols (Lodhi et al. 1995). The mapping population (229 F_1 individuals) and the parents were genotyped using SSR (Simple Sequence Repeat), SNPs (Single Nucleotide Polymorphism), and one CAPS (Cleaved Amplified Polymorphic Sequence).

Microsatellite primer sequences (SSR) were obtained from the UniSTS database of GeneBank (http://www.ncbi.nlm.nih.gov). The selection of

suitable markers was based on their presence over the 19 linkage groups in previous genetic linkage maps of *Vitis vinifera* (Adam-Blondon et al. 2004; Riaz et al. 2004; Doligez et al. 2006). The mapping population was genotyped for 104 SSR markers (Annex 1). The PCR amplifications were performed in 20-µl reactions containing 10-30 ng of template DNA, 0.2 µM of each primer (Applied Biosystems), 0.2 mM of each dNTP, 1x PCR buffer (Ecogen, Barcelona, Spain), 1.9 mM $MgCl_2$, and 0.25 units of *Taq* DNA polymerase (Ecogen, Barcelona, Spain). Amplification reactions were carried out in a 96-well block Thermal cycler (Eppendorf, Barcelona, Spain), using the following program: 5 min initial denaturation step at 94 ºC followed by 35 cycles (1 min denaturation at 94 ºC, 45 sec at the annealing temperature for the primer (Annex 1), and 1 min extension at 72 ºC), followed by 10 min final extension at 72 ºC. Primers failing to amplify were further tested using a touch-down PCR amplification program (Don et al. 1991), in which the initial annealing temperature (Ta) was reduced by 0.2 ºC per cycle for the following 14 cycles, followed by 20 cycles with an annealing temperature of Ta-3 ºC. All forward primers were labeled at their 5´-ends with fluorescent dyes (6-FAM, NED, VIC, or PET) and the PCR products were separated by capillary electrophoresis using the ABI Prism 3730 Genetic Analyzer sequencer (Applied Biosystems, Carlsbad, CA) in an external platform [Unidad de Genómica-Campus Moncloa del Parque Científico de Madrid (http://www.fpcm.es)]. Alleles were identified using GeneMapper software v3.7 (Applied Biosystems), and their sizes were determined using the internal size standard GS500LIZ (Applied Biosystems).

The mapping population was also genotyped for 238 SNP (Single Nucleotide Polymorphism) markers (Lijavetzky et al. 2007; Cabezas et al. 2011) using the Applied Biosystems SNPlexTM Genotyping System 48-plex

platform (De la Vega et al. 2005; Tobler et al. 2005) in the Centro Nacional de Genotipado (http://www.cegen.org).

In addition, new SNP-based markers were analyzed in the mapping progeny (Annex 3), after their identification and development by applying the candidate genes (CG) approach (Pflieger et al. 2001). These markers were identified and developed at the Centre for Research and Innovation, Fondazione Edmund Mach (FEM) (San Michele all'Adige, Italy) from the cultivars Monastrell, Syrah, and Pinot Noir, in collaboration with the research team of Dr. Stella Grando. These candidate genes were selected based on different QTL intervals found in common for some phenological and productivity traits located in two different progenies with Syrah as a common parent for both of them. The database of the FEM genome sequence (http://genomics.research.iasma.it/) was used to identify different CG. Thirty-five pairs of primers were designed using the NCBI tool (www.ncbi.nlm.nih.gov/tools/primer-blast/) and were tested on the parents. Three of them produced multiple bands and were discarded. The remainders were sequenced as described in Battilana et al. (2009) and sequences were analyzed with the software GAP 4 (www.gap-system.org), manually, to find informative SNPs in the three varieties under study. Then, the segregation was tested in a few hybrids to confirm an equilibrated segregation. For suitable polymorphisms new primers were developed and a mini-sequencing protocol was applied, employing the SNaPshot Multiplex Kit protocol reported at http://docs.appliedbiosystems.com/search.taf. Subsequently, they were scored by GeneMapper v3.7 (Applied Biosystems, Carlsbad, CA).

Moreover, the mapping population was also genotyped with the CAPS marker 20D18CB9, linked to berry color (Walker et al. 2007), as described in the first chapter of this manuscript.

2.2.3 Maps construction

Genetic maps were constructed using the JoinMap 3.0 software (Van Ooijen and Voorrips 2001), following the double pseudo-testcross strategy (Grattapaglia and Sederoff 1994) and applying the Kosambi mapping function (Kosambi 1944) to convert recombination rates into genetic distances. Both parental maps and a consensus map for the cross were constructed using double-haploid and cross-pollinated population types, respectively. Segregation patterns were assigned to each marker, following the JoinMap data entry notation: <abxaa>, <aaxab>, <abxab>, <abxac>, and <abxcd>. The segregation of each locus was tested for goodness-of-fit to the expected ratio using the chi-square test. Most markers showing distorted segregation were originally included in the map calculation unless they significantly affected the order of neighboring markers. For parental maps, markers of the <abxab> type were scored in the progeny as "ab" = missing data. Linkage groups (LGs) and marker order were determined using threshold values of 4.0 (2.0 for LGs 8 and 12 on female map) for LOD (logarithm of odds) and 0.4 for recombination rate. The marker order obtained was kept at around 2, but in some cases the marker order was fixed according to previously published maps and the position of the markers in the database of the grape genome sequences (www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/). The LGs were numbered from LG1 to LG19 according to the international agreement achieved within the IGGP (International Grape Genome Program; www.vitaceae.org). Female, male, and consensus genetic maps were aligned using MapChart v2.2 software (Voorrips 2002).

Framework maps (one map for each progenitor and one consensus map for the cross) were also developed using the most informative markers, with very reliable positions, evenly distributed along LGs and keeping an inter-marker distance of 5-20 cM when possible.

2.2.4 Genome length and map coverage

The observed genome length for each linkage map was calculated as the sum of all the linkage groups lengths (Gob). The estimated genome size (Ge) was determined according to Hulbert et al. (1988), with the modifications introduced by the method 3 of Chakravarti et al. (1991): Ge = N(N-1)X/K, where N is the total number of markers, X is the maximum observed distance between marker pairs above a threshold LOD, 4 in this study, and K is the number of loci pairs with LOD 4 or above. The confidence interval was calculated according to Gerber and Rodolphe (1994) with the formula: $I(Ge) = Ge(1 \pm 1.96K^{-1/2})^{-1}$, for α=5%. The expected genome map coverage was calculated by the formula of Lange and Boehnke (1982), adjusted for chromosomal ends: $Gcl = 1\text{-}e^{(-XN/1.25Ge)}$, where N is the number of markers and X is the maximum distance between two adjacent markers at a certain minimum LOD score (in this case, 4). The observed map coverage (Cob) was defined as the ratio between the observed genome and the estimated genome length (Gob/Ge).

2.3 Results

2.3.1 Molecular markers

One hundred and seventy-seven SSRs loci were initially analyzed in eight progeny individuals and both genitors. This allowed selection of 104 SSRs (59%) polymorphic for at least one parent (Annex 1; Table 2.1). Of the 238 SNPs previously identified by Lijavetzky et al. (2007) and studied in this work, 138 were polymorphic (58%) for at least one parent (Annex 2). In addition, in collaboration with the research team of Stella Grando (San Michele all'Adige, Italy), new SNP-based markers were analyzed in the mapping progeny, after their identification and development by applying the candidate genes (CG) approach (Pflieger et al. 2001). Of the 35 pairs of

primers designed and tested on the parental lines (see Material and Methods), eight new SNPs (representing six CG) were selected for the genotyping of the mapping progeny (Annex 2; Table 2.4).

Finally, the total number of molecular markers useful for linkage analysis in the MnxSy mapping progeny was 251 (104 SSRs, 146 SNPs, and 1 CAPS), of which 84% allowed discrimination between maternal and paternal inherited alleles (Table 2.1). One microsatellite marker (vmc5h11-200) showed a segregation pattern consistent with the presence of a null allele in 'Syrah' <aaxa0> and was re-coded as described by Doligez et al. (2002). A total of 166 markers (66%) were useful to generate the female map of Monastrell (segregation types abxaa, abxab, abxac, and abxcd) and a total of 196 markers (78%) were useful to generate the male map of Syrah (segregation types aaxab, abxab, aaxa0, abxac, and abxcd).

Table 2.1. Number and segregation type of the polymorphic markers genotyped in the progeny.

Marker type	Maternal 1:1 <abxaa>	Paternal 1:1 <aaxab>	1:2:1 <abxab>	1:3 <aaxa0>	1:1:1 <abxac>	1:1:1:1 <abxcd>	Total
SSRs	12	15	4	1*	49	23	104
SNPs	43	69	34	--	--	--	146
CAPS	--	--	1	--	--	--	1
Total	55	84	39	1	49	23	**251**

***** vmc5h11-200 for male parental line.

2.3.2 Genetic maps

The complete parental and consensus maps are shown in Figure 2.1. Of the 251 markers useful for the consensus map, ten loci (2 SSRs and 8 SNPs) could not be assigned to any linkage group (ungrouped) and three loci (2 SSRs and 1 SNP) could not be mapped (unpositioned), so they were discarded (Annex 1 and Annex 2). Finally, 238 markers (100 SSRs, 137 SNPs, and 1 CAPS) were assembled in the consensus map over the expected 19 LGs with

an average distance between loci of 5.23 cM (Figure 2.1; Table 2.2). The total number of positioned markers per LG was between 7 (LGs 10 and 16) and 18 (LGs 7 and 18). The average size of the linkage groups was 61.84 cM, ranging from 42.2 (LG 13) to 90.7 (LG 7) cM. The segregation of 52 loci (22%) was significantly distorted ($P<0.0001$) (Annex 1 and Annex 2).

This map had an observed size (Gob) of 1174.9 cM, which represents an observed coverage (Gob/Ge) of 76% (Table 2.3), and included only two intervals longer than 20 cM, the largest gap being 28.8 cM between VMC1E11 and VVMD5 in LG 16 (Figure 2.1). The consensus framework map (131 loci) covered 1190 cM, with three gaps > 30 cM and six gaps between 20 and 30 cM (data not shown).

Of the 166 markers useful for the female map (Table 2.1), one SNP could not be assigned to any LG (ungrouped) and five markers (4 SNPs and 1 SSR) were assigned to LGs but not mapped (unpositioned) (Annex 1 and Annex 2) because their inclusion in the linkage map led to inconsistencies in marker order. Accordingly, the Monastrell map was established on 160 markers (87 SSRs, 72 SNPs, 1 CAPS) which were positioned on 19 LGs with an average distance between loci of 7.02 cM (Figure 2.1; Table 2.2). The average LG size was 54.52 cM, ranging from 11.5 (LG 10) to 89 (LG 7) cM. The total number of positioned markers per LG was between three (LG 10) and 18 (LG 18). This map had an observed size (Gob) of 1035.40 cM, which represents an observed coverage (Gob/Ge) of 61% (Table 2.3), and included four intervals longer than 20 cM and two longer than 30 cM, the largest gap (37 cM) being between VMC1E11 and VVMD5 in LG 16 (Figure 2.1). The maternal framework map (101 loci) spanned a total of 1056 cM, with two gaps > 30 cM and eight gaps between 20 and 30 cM (data not shown).

Table 2.2. Summary of the information generated for the Monastrell x Syrah maps, by linkage group.

	Monastrell						Syrah						Consensus					
	nº of markers			Map length (cM)			nº of markers			Map length (cM)			nº of markers			Map length (cM)		
LG	Total	SSR	SNP	Total	average distance between adjacent markers	nº markers distorted	Total	SSR	SNP	Total	average distance between adjacent markers	nº markers distorted	Total	SSR	SNP	Total	average distance between adjacent markers	nº markers distorted
1	10	4	6	62.9	6.29	1	10	3	7	45.8	4.58	1	17	5	12	71.5	4.21	2
2	10*	5	4	52.9	5.29	0	11*	5	5	57	5.18	1	13*	6	6	62.3	4.79	4
3	5	4	1	44.9	8.98	1	9	5	4	41.3	4.59	0	10	5	5	51.7	5.17	1
4	11	5	6	56.6	5.14	2	13	5	8	57.7	4.44	0	17	5	12	58.5	3.44	4
5	11	5	6	63.7	5.79	1	11	5	6	43.5	3.95	1	15	5	10	60.8	4.05	4
6	9	5	4	52.5	5.83	0	12	5	7	64.1	5.34	3	16	6	10	68.1	4.26	2
7	11	6	5	89.4	8.12	5	16	6	10	88.5	5.53	3	18	6	12	90.7	5.04	5
8	4	3	1	58.4	14.6	1	17	5	12	60.5	3.56	0	17	5	12	60.5	3.56	1
9	8	5	3	75.4	9.42	3	5	3	2	37.5	7.5	0	10	5	5	74	7.4	2
10	3	2	1	11.5	3.83	3	7	4	3	51.1	7.3	5	7	4	3	51.3	7.33	5
11	7	5	2	53.7	7.67	3	8	4	4	44.7	5.59	2	9	6	3	59.6	6.62	6
12	7	4	3	62.5	8.93	2	8	4	4	45.3	5.66	2	11	5	6	54.5	4.95	2
13	8	4	4	21.3	2.66	1	4	3	1	48	12	2	9	4	5	42.2	4.68	2
14	9	5	4	68.1	7.57	3	11	5	6	66.7	6.06	0	14	5	9	73	5.21	5
15	10	4	6	43.4	4.34	0	9	4	5	56.8	6.31	0	11	4	7	57.9	5.26	5
16	5	4	1	59.7	11.94	0	5	4	1	51.7	10.34	0	7	5	2	61.2	8.74	0
17	8	4	4	44.3	5.54	0	5	4	1	46.1	9.22	0	9	4	5	46.3	5.14	0
18	18	8	10	69.2	3.84	2	16	8	8	76.6	4.78	1	18	8	10	78.8	4.38	8
19	6	5	1	45	7.5	1	9	6	3	56	6.22	8	10	7	3	52	5.2	7
Total	160	87	72	1035.4	7.02	29	186	88	97	1038.9	6.22	29	238	100	137	1174.9	5.23	65

*CAPS color; Segregation distortion $P<0.05$

Of the 196 markers useful for the male map (Table 2.1), five loci (2 SSR and 3 SNP) could not be assigned to any LG and five (1 SSR and 4 SNPs) could not be mapped (Annex 1 and Annex 2). Accordingly, the Syrah map was established on 186 markers (88 SSR, 97 SNP, and 1 CAPS) positioned on 19 LGs, with an average distance between loci of 6.22 cM (Figure 2.1; Table 2.2). The average LG size was 55 cM, ranging from 37.5 (LG 9) to 88.5 (LG 7) cM. The total number of positioned markers per LG was between 4 (LG 13) and 17 (LG 8). This map had an observed size (Gob) of 1038.9 cM, which represents an observed coverage (Gob/Ge) of 60% (Table 2.3), and included three intervals longer than 20 cM, the largest gap being 23.6 cM between VMC1E11 and VVMD5 in LG 16 (Figure 2.1). The paternal framework map (107 loci) spanned 1036 cM, with six gaps between 20 and 30 cM (data not shown).

Table 2.3. Main features of the parental and consensus genetic maps.

	Monastrell	Syrah	Consensus
Nº. Of mapped markers	160	186	238
Genome length (cM)			
Observed (Gob)	1035.4	1038.9	1174.9
Estimated (Ge)	1702.1	1741.6	1548.4
Confidence interval (95%) I(Ge)	1567.18 - 1862.54	1623.96 - 1877.81	1458.86 - 1649.63
Coverage (%)			
Expected (Gcl)	96.7	97.7	98.0
Observed (Gob/Ge)	61	60	76
Average map distance between loci (cM)	7.02	6.22	5.23
No. of gaps between 20 and 30 cM	4(LGs 3, 7, 8, 8)	3(LGs 13, 14, 16)	2 (LGs 14, 16)
No. of gaps >30 cM	2(LGs 14, 16)	0	0
LGs with distorted markers[a]	14	10, 19	1, 2, 4, 5, 7, 10, 11, 14, 15, 18, 19

Gob, sum of LG sizes; Ge, calculated by the method 3 of Chakravarti (Hulbert et al. 1988; Chakravarti et al. 1991); I (Ge), calculated by the method of Gerber and Rodolphe (1994); Gcl, calculated by the method of Lange and Boehnke (1982), adjusted for chromosomal ends.

[a] Linkage groups with two or more distorted markers at $P<0.0001$

Chi-square analysis revealed a distorted segregation ratio ($P<0.05$) for 18.1% of the polymorphic markers in Monastrell and 15.6% of the polymorphic markers in Syrah. Most of the distorted markers were randomly distributed

throughout the genome but some of them were located in clusters with a high distortion level (Figure 2.1). For the female map three clusters were located on LGs 7, 10, and 14; for the male map on LGs 10 and 19, and for the consensus map on LGs 7, 10, 14, 18 and 19. The marker order was generally consistent between parental and consensus homologs linkage groups, with local inversions of closely linked markers and proximal markers (Figure 2.1). A general consistency in marker order was found compared with other published maps, except for a few local inversions.

Figure 2.1. Parental and consensus complete linkage maps of the progeny Monastrell x Syrah.

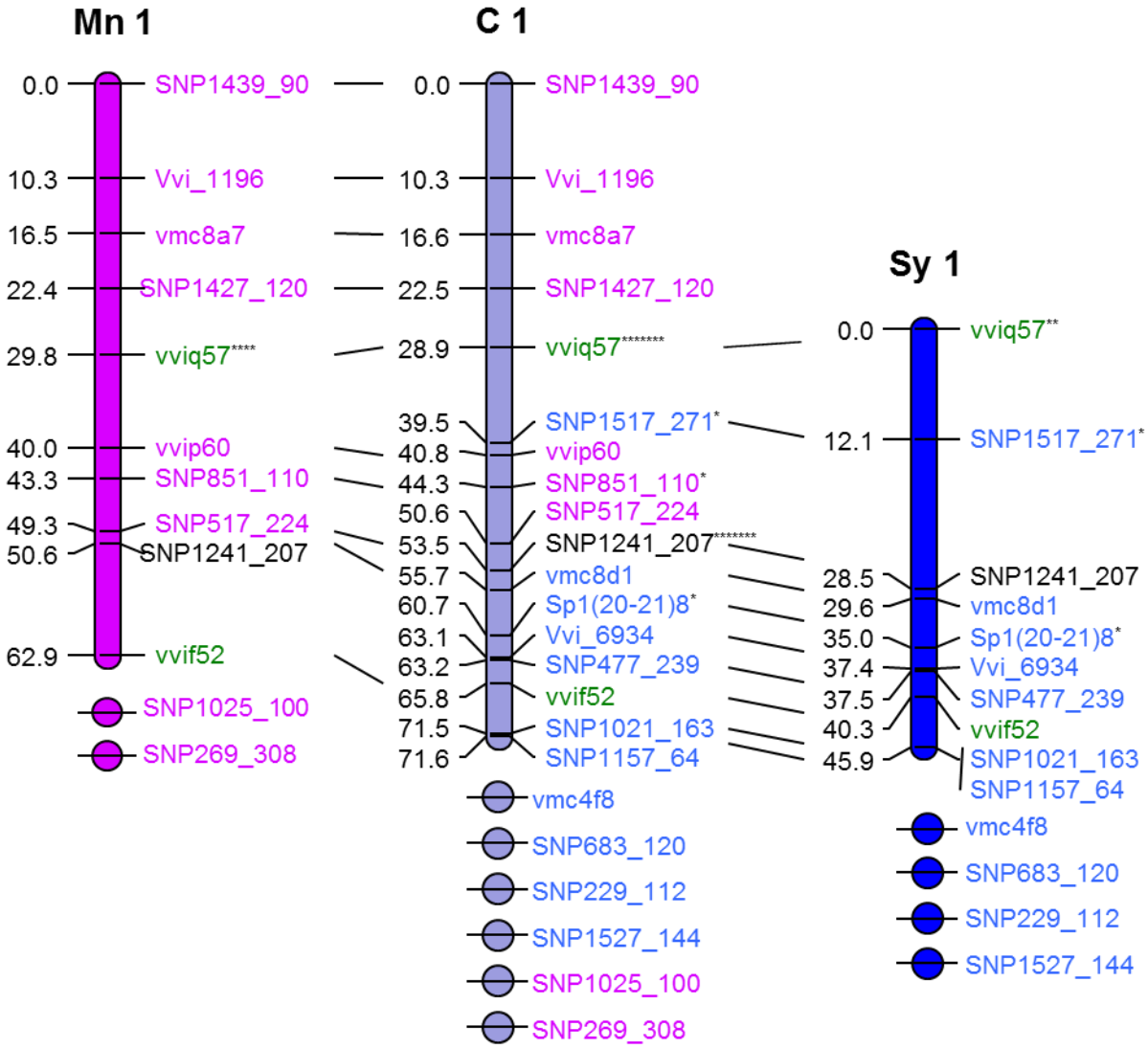


Figure 2.1. Continued

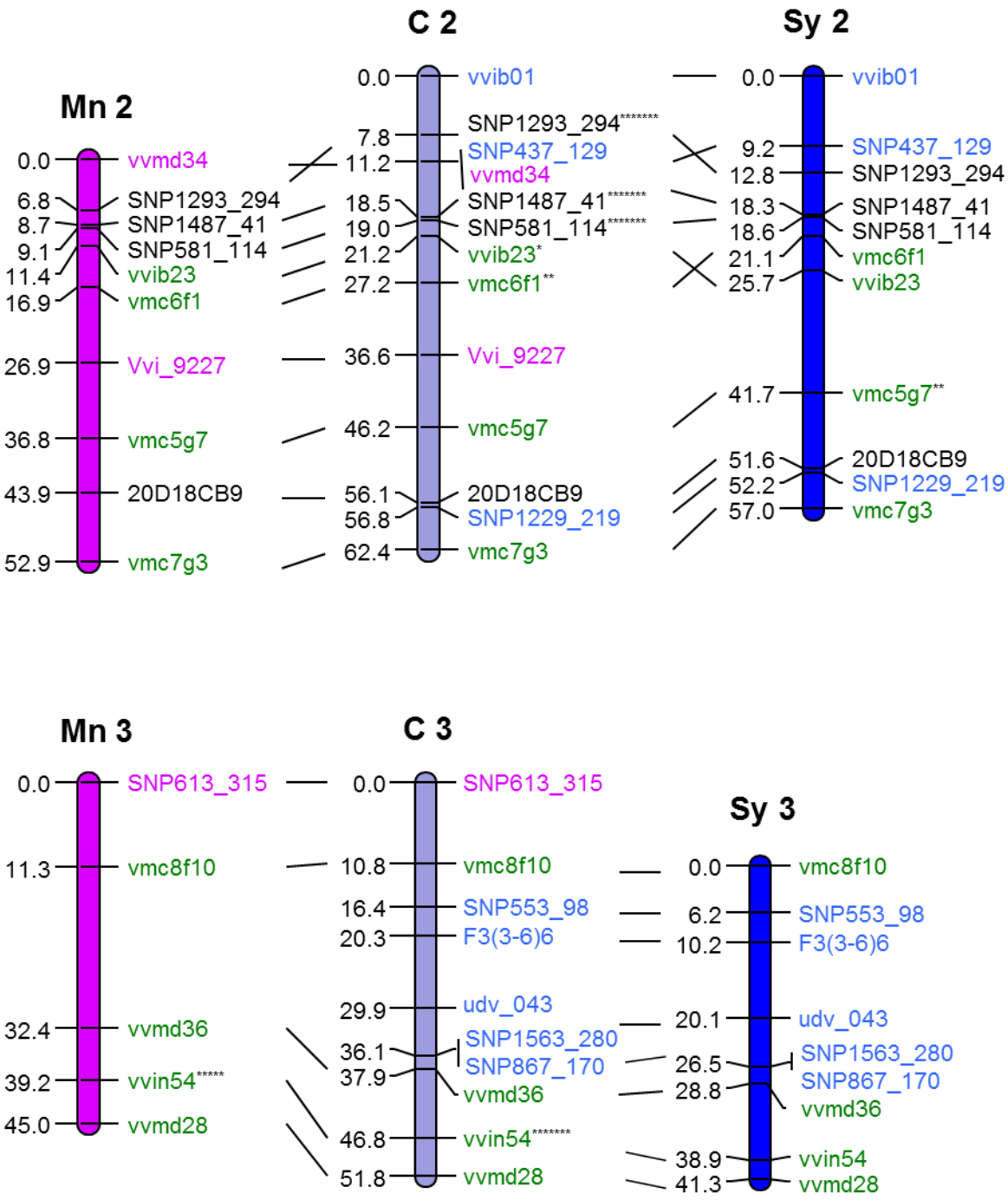


Figure 2.1. Continued

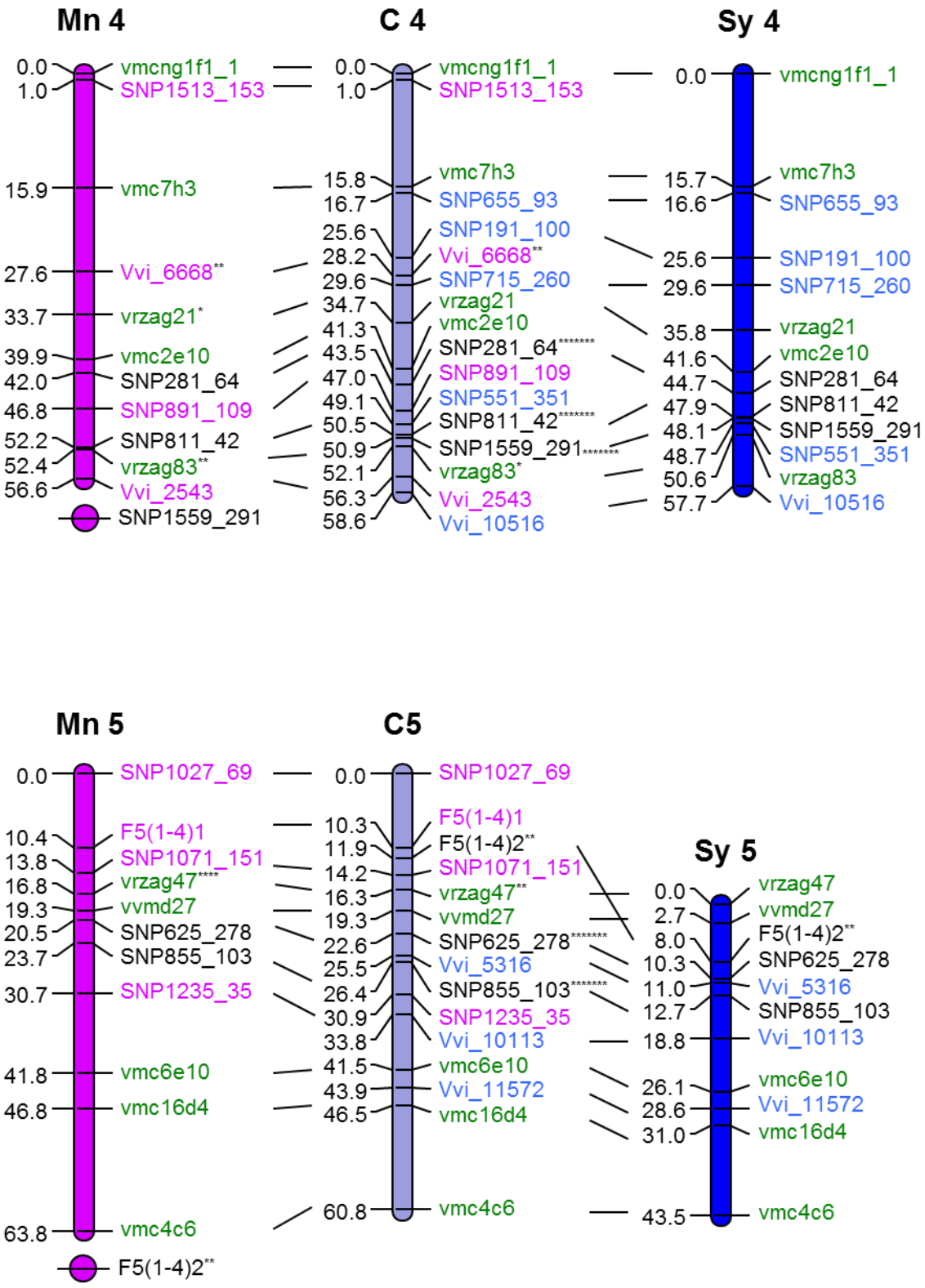


Figure 2.1. Continued

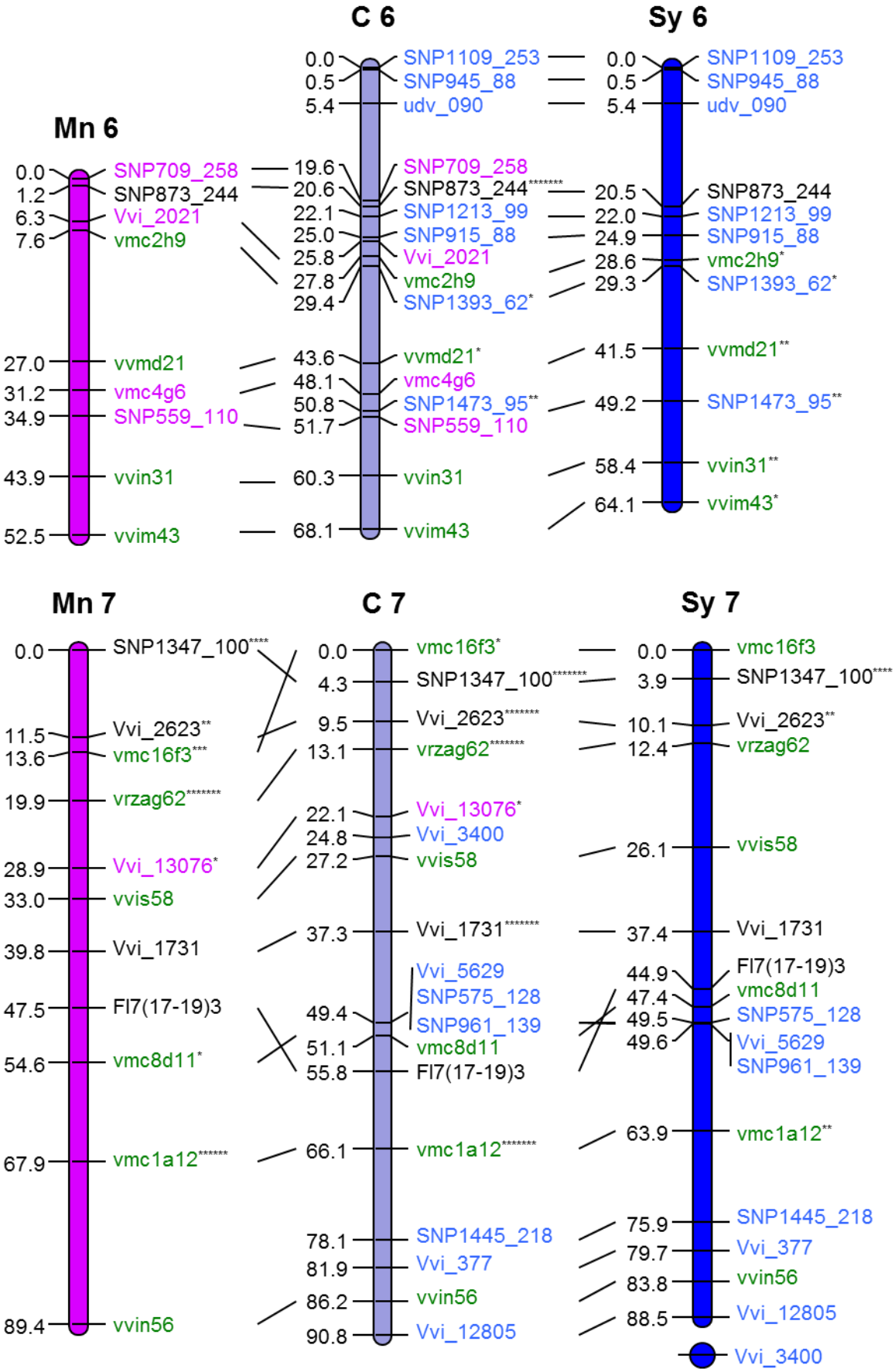


Figure 2.1. Continued

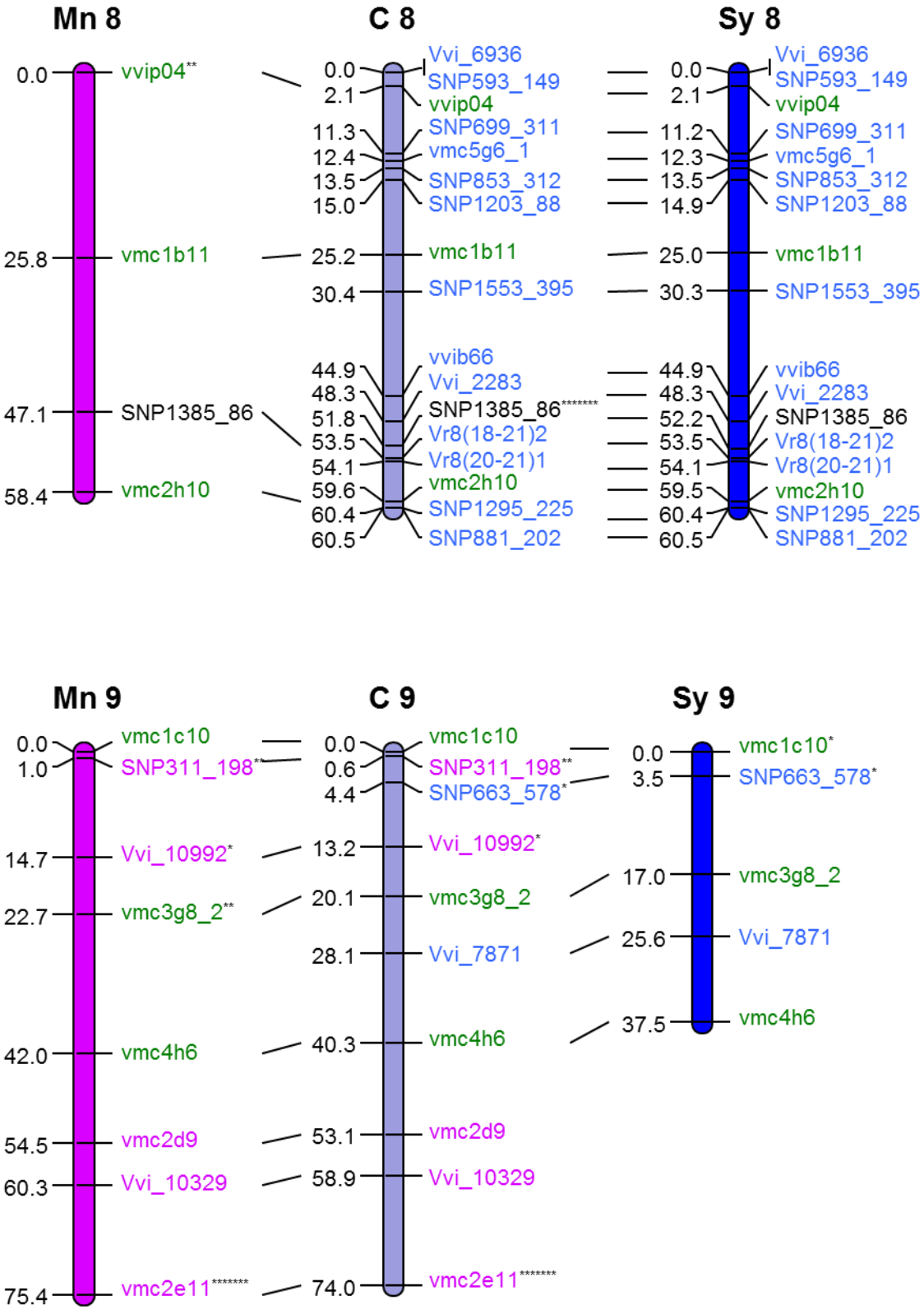


Figure 2.1. Continued

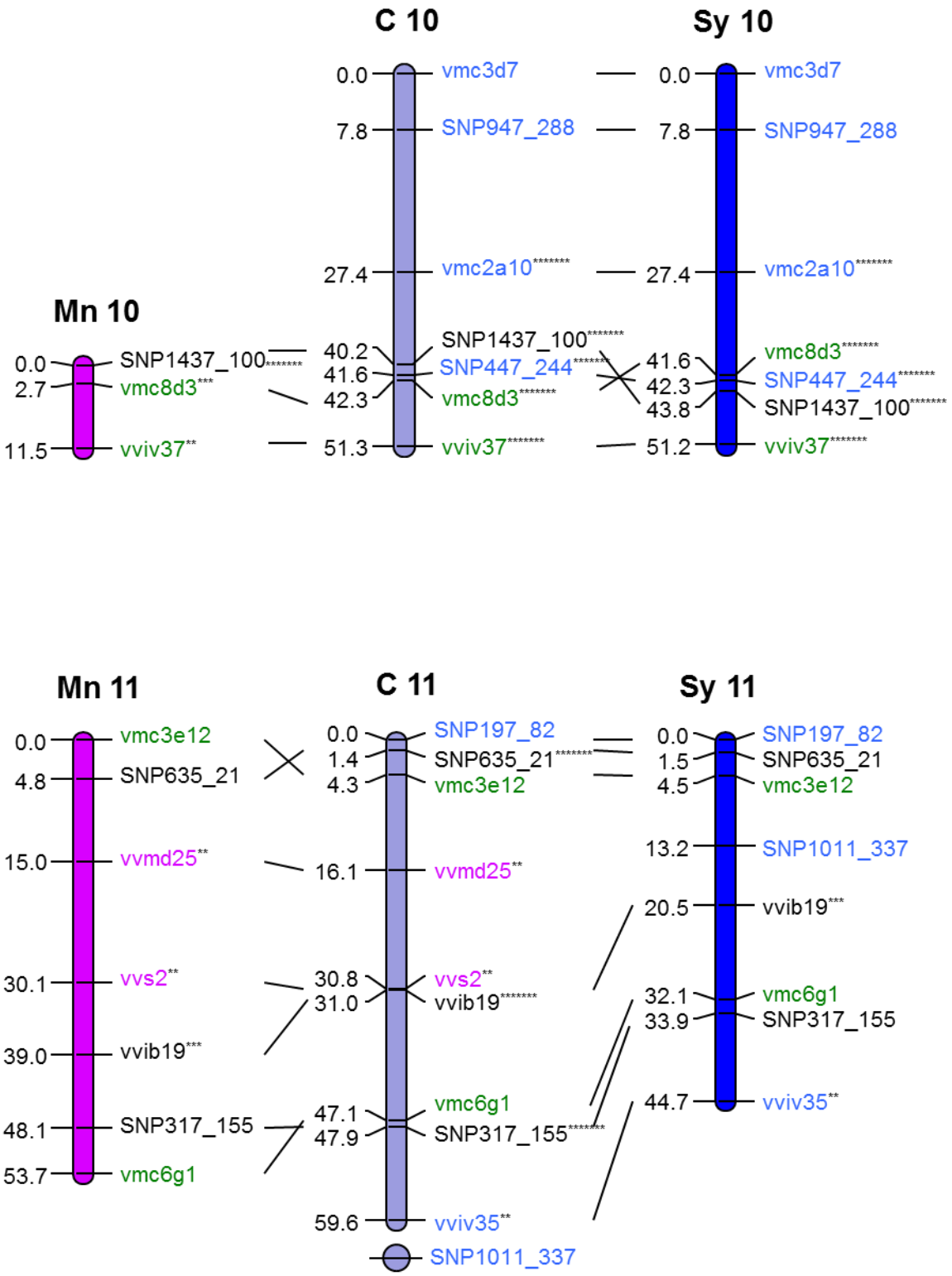
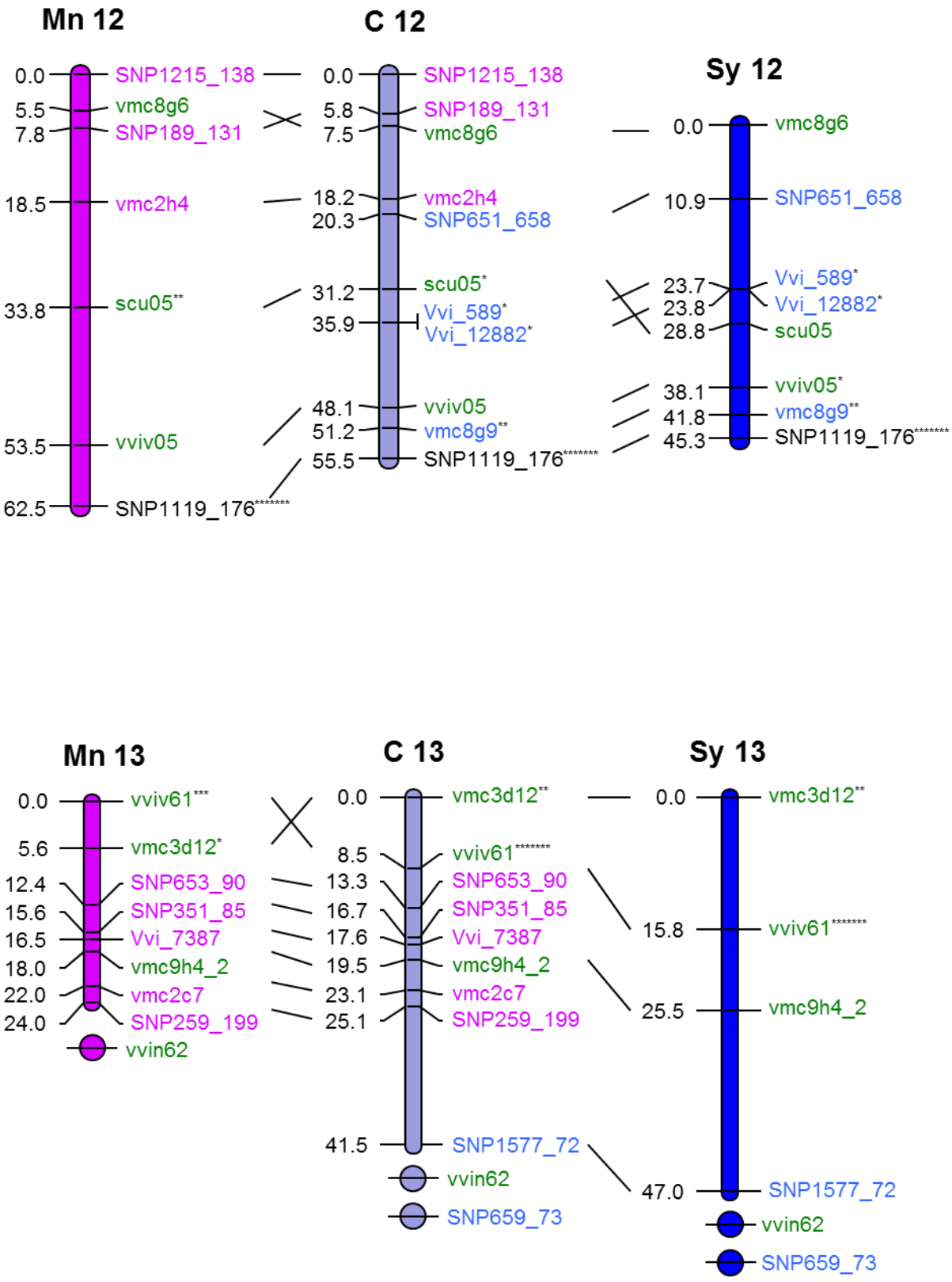


Figure 2.1. Continued



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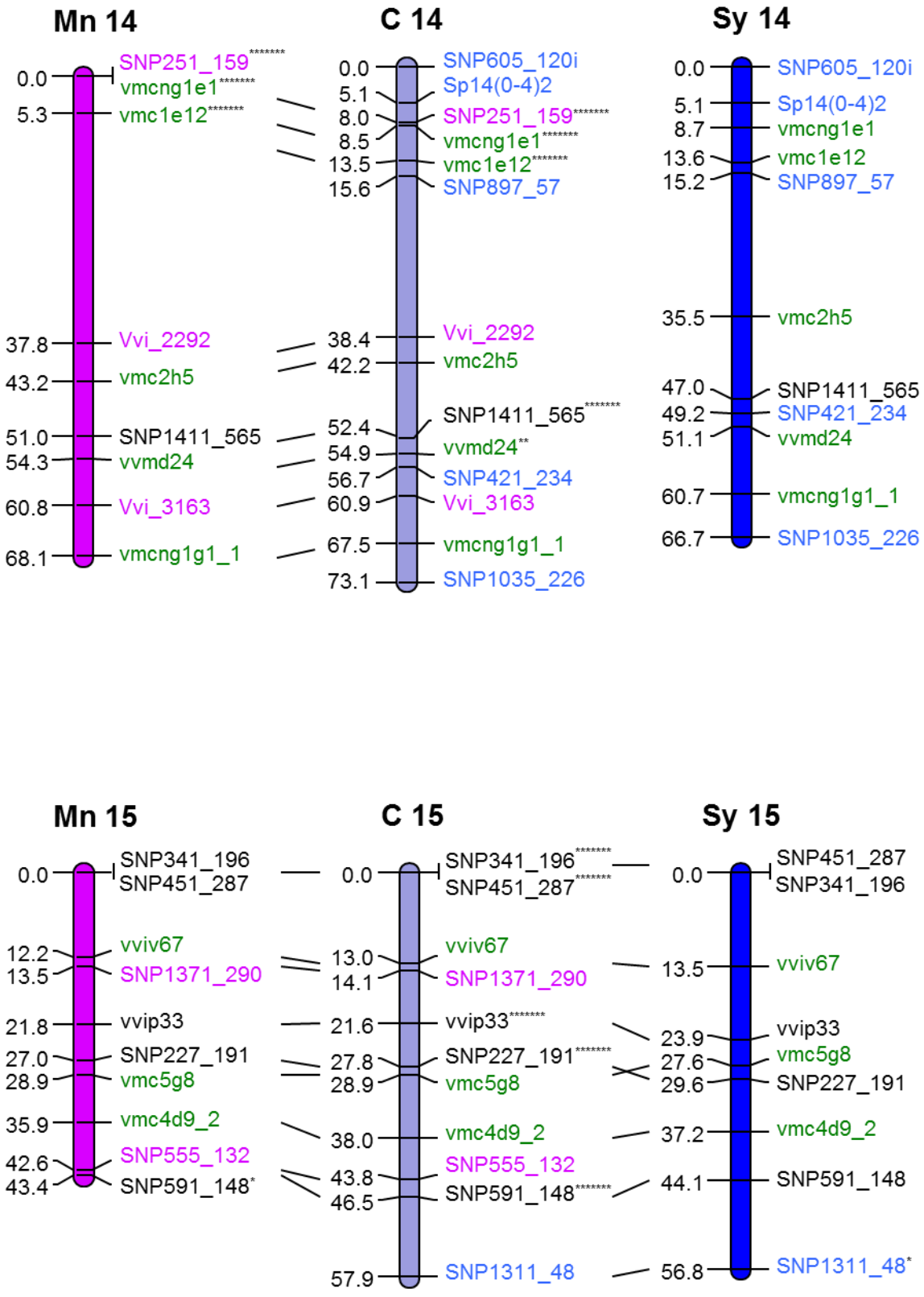
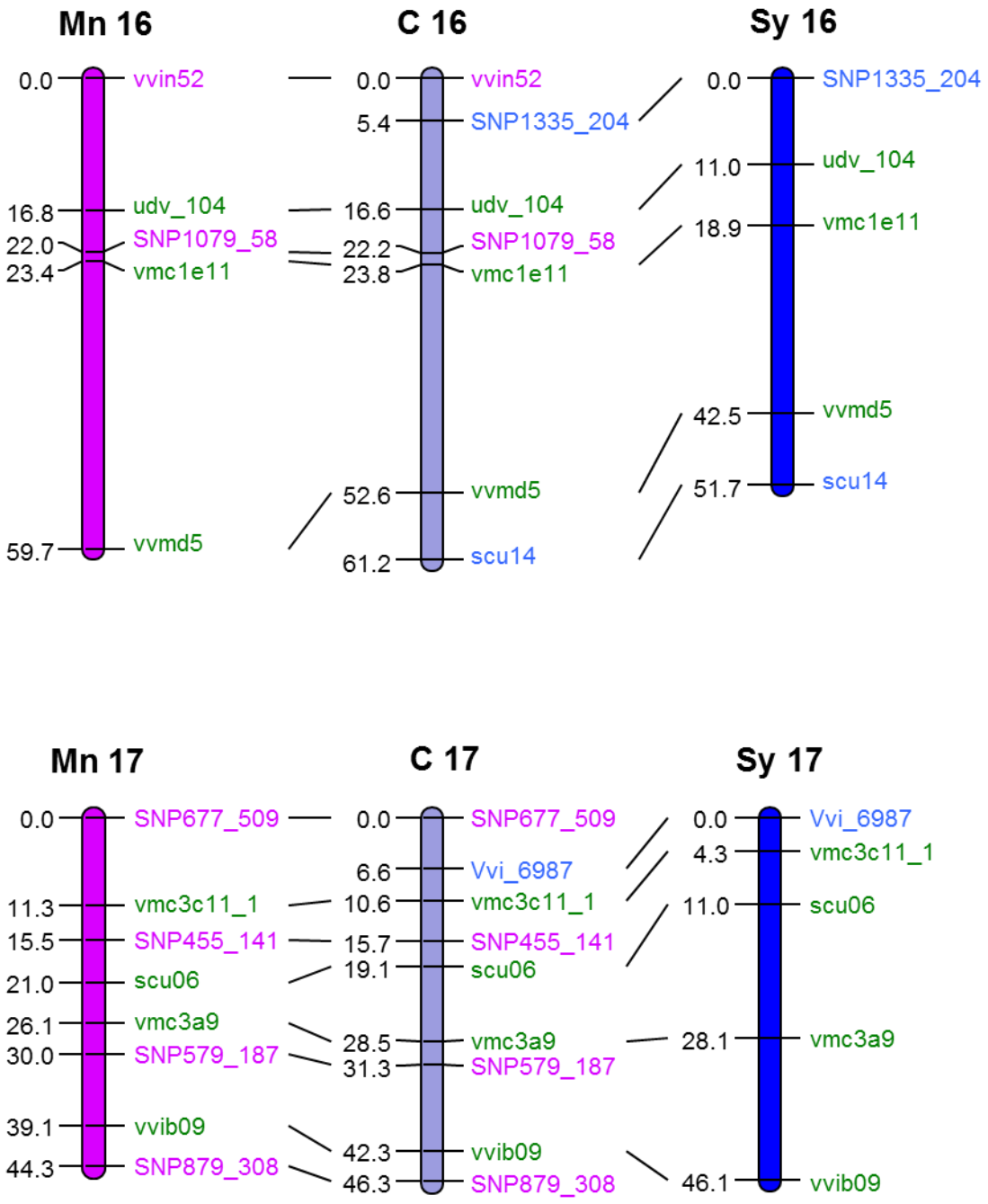


Figure 2.1. Continued



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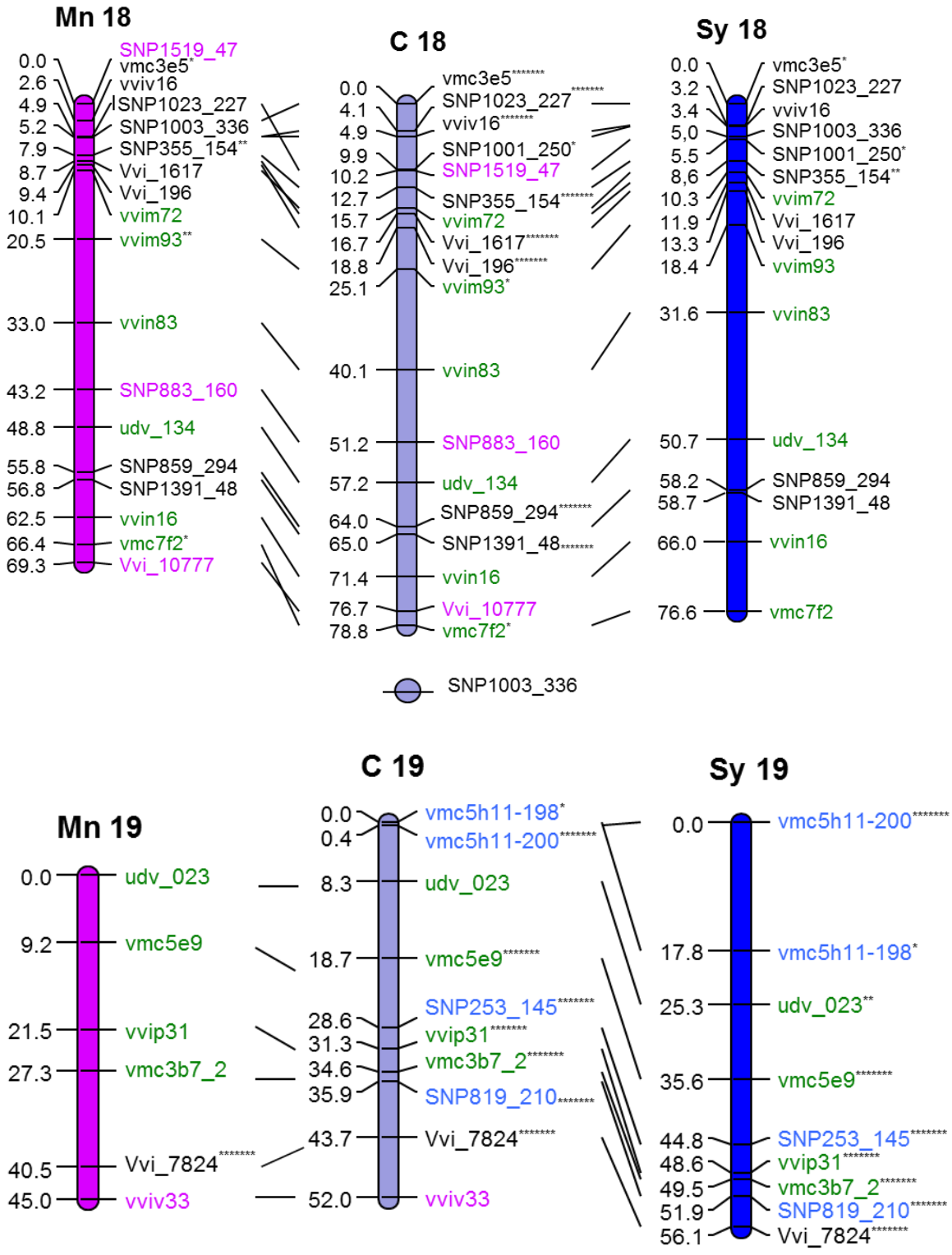

Mn, Monastrell; Sy, Syrah; C, consensus. Distances are in cM Kosambi. The asterisks indicate significantly distorted loci (*$P \leq 0.1$, **$P \leq 0.05$, ***$P \leq 0.01$, ****$P \leq 0.005$, *****$P \leq 0.001$, ******$P \leq 0.0005$, *******$P \leq 0.0001$). Numbering of the linkage groups was performed according to previous reference maps. Ø, unpositioned markers. Marker colors indicate the types of segregation: blue <aaxab>, pink <abxaa>, black <abxab>, and green <abxac>, <abxcd>.

The heterozygosity levels of the parents were calculated with the total number of markers analyzed in both progenitors (450: 177 SSRs + 273 SNPs), considering the total percentage of heterozygous (67%) versus homozygous (33%) markers. The level of heterozygosity detected was similar in both parents, irrespective of the markers used (45% in Monastrell and 52% in Syrah). If we compare the level of heterozygosity of the 177 SSRs versus the 273 SNPs for Monastrell and Syrah, a great difference between them is observed: 79-81% of the SSRs are heterozygous versus only 28-38% of the SNPs.

2.3.3 Linkage of candidate genes

We used the genomic sequence of Pinot Noir, available at the FEM (http://genomics.research.iasma.it/), to identify positional candidate genes in the proximity of the markers underlying the corresponding QTLs (Table 2.4). Gene prediction was based on *Vitis vinifera*, *Arabidopsis*, and other species. Six positional candidate genes co-located with the QTLs for sprouting, flowering, veraison, and fertility index in three years.

Table 2.4. Summary of eight new SNPs developed from six candidate genes.

Marker name	SNP	LG	Putative function
Sp1(20-21)8	tt x ct	LG 1	Transcriptional factor B3
F3(3-6)6	cc x cg	LG 3	Myb, DNA-binding
F5(1-4)1	ac x aa	LG 5	squamosa promoter-binding like protein
F5(1-4)2	at x at		regulation of transcription, DNA-dependent
Fl-7(17-19)3	ct x ct	LG 7	Homeobox domain, ZF-HD class
			DNA binding, regulation of transcription
Vr8(18-21)2	aa x at	LG 8	cell wall, xyloglucan:xyloglucosyl transferase activity
Vr8(20-21)1	cc x ct		
Sp14(0-4)2	tt x ct	LG 14	Myb, DNA-binding

Sp, sprouting; F, fertility index; Fl, flowering; Vr, veraison. The Monastrell x Syrah genotype is indicated (SNP), as well as the linkage group (LG) where the candidate genes were located.

Candidate genes were localized within the confidence interval of the QTLs for sprouting detected on LGs 1 and 14. The putative function of the gene located on LG1 is related to *APETALA2* and the transcriptional factor B3 in *Arabidopsis*. These two domains are present in the RAV family of genes (Related to ABI3/VP1), which are characterized by an N-Terminal DNA-binding AP2/EREBP domain and a C-terminal B3 domain. This family is involved in growth, development, and flowering time in *Arabidopsis* and other species (Romanel et al. 2009). Within the confidence interval of the QTL detected on LG 14, a *Myb*-like DNA binding domain was found (Table 2.4).

Two QTLs were detected for fertility index, on LG3 and LG5. Within the confidence interval of the QTL detected on LG3 was located a gene with a putative function related to a *Myb*-like DNA binding domain. These genes are key in several processes, such as: seed development, cell pigmentation, response to stresses, pathogen resistance, light-sensing responses, and sucrose related responses. These genes have been identified in several woody species (poplar, apple, and grape) and in grape are mainly linked with flavonoid synthesis. The R2R3 subfamily is the most abundant in plants and the C-terminal region interacts in the eukaryotic transcriptional machinery (Matus et al. 2008). The QTL detected on LG5 co-located with a *Squamosa* promoter binding (SBP) domain, a family of transcription factors with a MADS-box DNA binding domain that is involved in the identity of floral meristems and genetic control of flowering in *Antirrhinum majus* (Huijser et al. 1992). Moreover, recent studies showed that a microRNA156-Squamosa Promoter Binding Protein-Like3 (SPL3) regulates Flowering locus T (FT) expression in response to different temperatures in *Arabidopsis* (Kim et al. 2012).

A gene related to flowering time was co-located within the confidence interval of the QTL detected on LG7. This gene was a zinc finger-homeodomain (ZF-HD). The HD is a DNA-binding domain common in many transcription factors. There are many subfamilies with different

functions, such as: maintenance of meristematic cells, stem development, embryonic patterning, cell proliferation, or adaxial identity in leaves and embryos. The work by Tan and Irish (2006) showed that this subfamily (ZF) in *Arabidopsis* is expressed predominantly in floral tissue, playing a regulatory role in floral development.

Finally, a gene related to veraison, with two domains, co-located within the confidence interval of the QTL detected on LG8 (Table 2.4). One domain of this gene was related to a family of glycosyl hydrolases, and the other to a xyloglucan endo-transglycoxylase C-terminus. Xyloglucan is the major component of the primary cell wall and therefore is involved in its properties. The glycosyl hydrolases family is a wide family with a lot of biological functions in organisms, such as storage, structure, or signaling in plants (Davies and Henrissat 1995). Moreover, the hydrolysis of glycosidic bonds is crucial for energy supply in cell wall expansion and degradation. The two domains act together during seed germination, fruit ripening, and rapid wall expansion (Baumann et al. 2007).

The new SNPs developed were mapped and the QTLs were then re-analyzed. The primer sequence and the code of the Gene Ontology Consortium of the eight new SNPs developed are shown in Annex 3.

2.4 Discussion

The present work aims to contribute to our knowledge of the genetic determinants that control some of the most interesting characters related to the genetic improvement of wine grape. To address this goal, genetic maps covering most of the genome for a *Vitis vinifera* cross between two wine grape varieties, Monastrell and Syrah, were developed. The maps were constructed using molecular markers: SSR (Simple Sequence Repeat) and SNP (Single Nucleotide Polymorphism). First, the genetic maps ordered the molecular markers in linkage groups, based on their co-segregation in the mapping

population. Subsequently, the association of these markers with heritable characteristics will allow the prediction of the phenotype in the early stages of plant development and, therefore, the performance of a more efficient and faster markers-assisted selection of new varieties. Marker-assisted selection (MAS) may greatly increase the efficiency and effectiveness in plant breeding, compared to conventional breeding methods. The size of the population used to construct the linkage maps (n = 229) was suitable for a study of this type.

Complete parental and consensus genetic maps were developed using 238 informative molecular markers (100 SSRs, 137 SNPs, and 1 CAPS). The parental maps developed in this work reveal a similar length in both progenitors (1035.4 cM in Monastrell and 1038.9 cM in Syrah), with an observed coverage of 61% and 60%, respectively. The observed sizes (Gob) of the parental and consensus maps are within the range set by other published maps (Doligez et al. 2002; 2006; 2010; Grando et al. 2003; Riaz et al. 2004; 2006; Adam-Blondon et al. 2004; Doucleff et al. 2004; Cabezas et al. 2006; Lowe and Walker 2006; Di Gaspero et al. 2007; Welter et al. 2007; Costantini et al. 2008; Salmaso et al. 2008; Duchêne et al. 2009). Nevertheless, the comparison of maps developed by different research groups must be considered with due caution, since the fitting of the various parameters (LOD, REC, etc.), as well as the software used for their construction (JoinMap, Mapmaker, Cartographer, Carthagene, R/qtl, ...), may greatly influence the outcome of the map. Also, in the present work the position of some of the markers has been fixed based on the information available in the physical map of the grapevine. In general, a greater number of markers positioned usually produces a larger map. In the population of the present work, the observed size (Gob) of the saturated consensus map was greater than that corresponding to each parent. However, this is a trend that, as such, does not always hold true, as with the maps of Doligez et al. (2006b and 2010). This could be due to the presence of a greater number of distorted regions, which

would shorten the maps due to the variation that they cause in the recombination rates (Doligez et al. 2002; Costantini et al. 2008). The small number of intervals between markers greater than 25 cM indicates that the maps reported here are at a good level of saturation for the proposed objectives.

Based on markers common to other published maps, all the LGs were aligned to the 19 chromosomes of grape. Comparison of common markers among the three maps developed (Figure 2.1) showed a strong conservation of marker order with only minor changes detected. In some cases, this order was fixed following the physical database. The differences in the order of the markers may be caused by local variations in the frequency of recombination, by the segregation of specific markers in a single parent and by ruptures in the synteny of the parents (Salmaso et al. 2008). The changes of position in these maps are at sites of the genome in which they are more likely, such as the presence of markers with distorted segregation, or are partially informative in nature (abxab), as well as clusters of markers in a very narrow strip. A general consistency in marker order was also found, compared with other published maps.

Segregation distortion of markers has often been observed in fruit and forest species (Bradsaw and Settler 1994; Grattapaglia and Sederoff 1994; Lanaud et al. 1995; Viruel et al. 1995; Barrenche et al. 1998; Paglia and Morgante et al. 1998; Cervera et al. 2001; De la Rosa et al. 2003; Lambert et al. 2004). In this study, considering all the polymorphic markers, the distortion levels at $P<0.05$ found in the segregation of the alleles contributed by Monastrell was 18.1%, and 15.6% for Syrah. Of the useful markers for the construction of integrated map, 27.3% showed distortion in the segregation of the genotypes. These values are within the range presented by other genetic backgrounds of the genus *Vitis* that range from 3% to 20%. The ten LGs most susceptible to showing clusters of distorted markers are, in descending

order: 1, 17, 5, 14, 7, 18, 11, 4, 8, and 19 (Doligez et al. 2002; 2006; 2010; Grando et al. 2003; Riaz et al. 2004; 2006; Adam-Blondon et al. 2004; Doucleff et al. 2004; Cabezas et al. 2006; Lowe and Walker, 2006; Di Gaspero et al. 2007; Welter et al. 2007; Costantini et al. 2008; Salmaso et al. 2008; Duchêne et al. 2009).

In this work, regions containing at least two adjacent distorted markers were observed in the maps (LGs 1, 2, 4, 5, 7, 10, 11, 14, 15, 18 and 19). There are indications that the presence of these distorted regions may arise because they are located in genes that, under certain allelic combinations, cause a strong deleterious effect, or resistance to a pathogen or disease, so that only certain genotypes develop into adult plants. Duchêne et al. (2009) observed that all the LGs in which QTLs for terpenol content were detected, were affected by the distortion in the segregation of the markers. They argued that the negative effects of terpenes on seed germination and seedling growth could have amplified the inbreeding depression, as they used two populations obtained by backcrossing a parent. Welter et al. (2007) detected a QTL with a major effect for powdery mildew resistance in a region of LG 15 having a cluster of distorted markers. Finally, Cabezas et al. (2006) detected a QTL with a major effect on seedlessness in the lower part of LG18 in the map of Autumn Seedless, in which the markers involved exhibited distortion. This author discussed a possible link between the presence of the QTL and the distortion, suggesting that it could de due to the abortion of a high number of embryos of a seedless genotype. In this work, LG 18 in the consensus map and LG19 in the Syrah map were the LGs with the higher number of markers having distorted segregation (8 in each of them), in agreement with other authors (Cabezas et al. 2006; Troggio et al. 2007).

Grapevine is a species with a highly heterozygous genome, a characteristic which allows the construction of genetic maps using F_1 populations. The degree of heterozygosity of a genome, which can be

estimated by analysis of molecular markers, determines the efficiency of the construction of linkage maps. The level of heterozygosity of microsatellites (SSRs) analyzed in Monastrell (79%) and Syrah (81%) are within the range presented by other genetic backgrounds of the genus *Vitis* that range from 56% to 90% (Doligez et al. 2002; Riaz et al. 2004; 2006; Adam-Blondon et al. 2004; Cabezas 2004; Welter et al. 2007; Salmaso et al. 2008; Duchêne et al. 2009). The big difference of heterozygosity between the two types of markers used (79% and 81% for SSRs, 28% and 38% for SNPs) is probably due to the fact that, while SSRs are the types of sequence with the highest mutation rate (Moxon and Wills 1999; Oliveira et al. 2006), the SNPs were developed from ESTs (Lijavetzky et al. 2007), with a mutation rate far below that of the SSRs.

2.5 Conclusions

In this work, genetic maps were developed for a *Vitis vinifera* cross between two wine grape cultivars, Monastrell and Syrah, in order to identify the genetic determinants for given phenotypic traits. These maps will be used to carry out QTL detection for different traits of interest (see next chapter). Complete parental and consensus genetic maps were developed using 238 informative molecular markers (100 SSRs, 137 SNPs, and 1 CAPS). The observed sizes of the Monastrell, Syrah, and consensus maps were 1035.4 cM, 1038.9 cM, and 1174.9 cM, respectively, with observed coverages of 61%, 60%, and 76%, respectively. These results are in the range of other genetic maps developed for grapevine. Based on markers common to other published maps, we were able to align all the LGs to the 19 chromosomes of grapevine. A general consistency in marker order was found compared with other published maps. Furthermore, eight new SNPs, linked to six candidate genes that could be involved in the control of different traits of interest, were generated and mapped.

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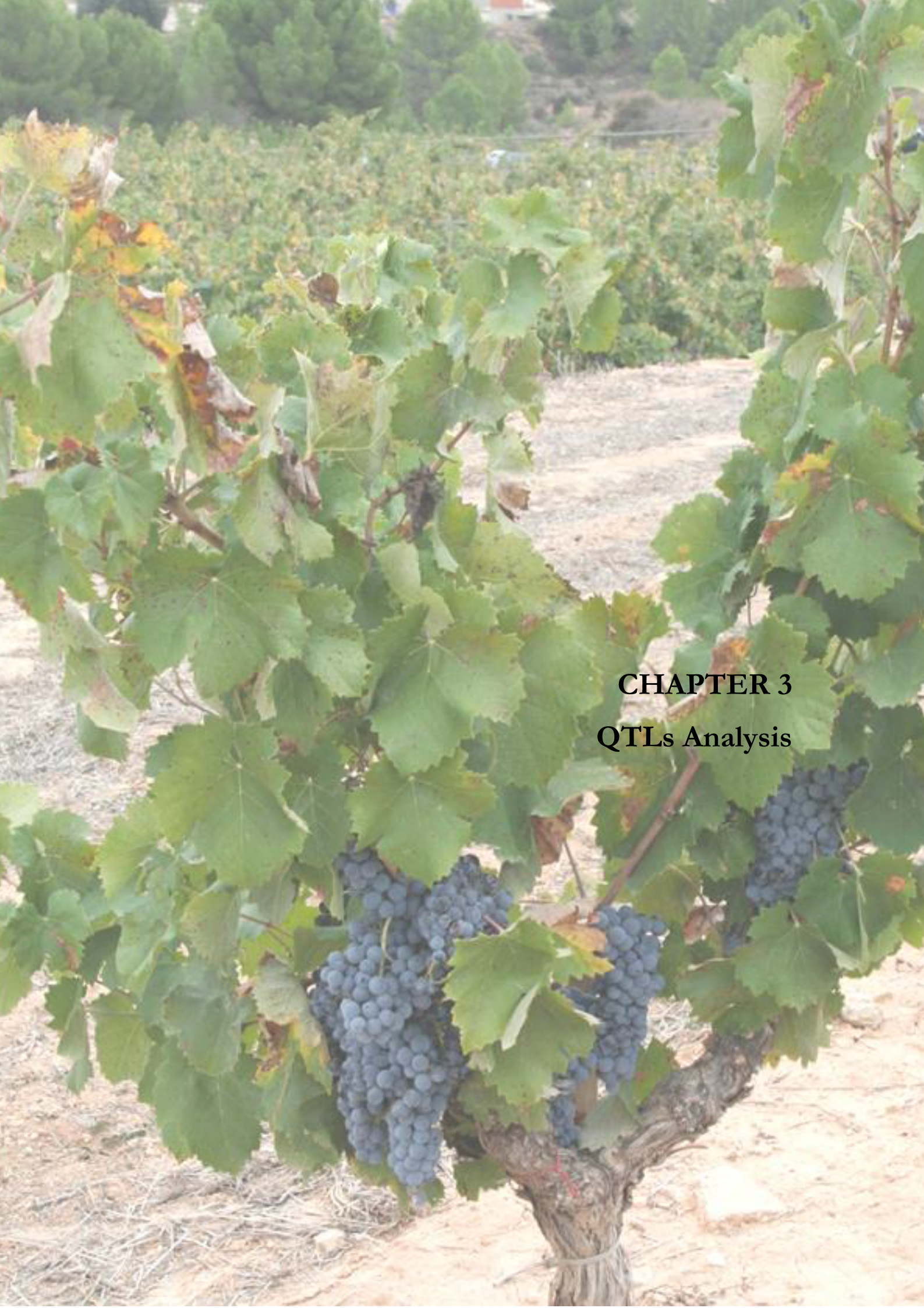


CHAPTER 3

QTLs Analysis

3.1 Introduction

Most of the characters of agricultural interest are known to be quantitative, polygenic, or complex traits. These traits are controlled by a large number of genes. The chromosomal regions involved in the genetic control of these traits are known as Quantitative Trait Loci (QTL). QTL mapping is the process used to identify the number and location of the genetic determinants responsible for the variation of the quantitative traits under study and their stability among different years (Collard et al. 2005). QTL analysis was initiated by the development of DNA or molecular markers in the 1980s and it is based on genetic maps and the phenotypic evaluation of a segregating progeny. The construction of linkage maps and QTL analysis take a considerable amount of time and effort. Nevertheless, the identification of markers linked to traits of interest allows improvement of the conventional breeding of grapevine, through more efficient marker-assisted selection (MAS).

QTL analysis is based on the principle of detecting an association between the phenotypic segregation and the genotypic polymorphisms of markers. When a marker is linked only loosely or is not linked to a QTL, there is independent segregation of the marker and QTL. Three methods used widely to detect QTLs are single-marker analysis, simple interval mapping, and composite interval mapping (Liu 1998; Tanksley 1993). Single-marker analysis is the simplest method for detecting QTLs associated with single markers. This method does not require a complete linkage map and can be performed with basic statistical software programs. The simple interval mapping (SIM) method makes use of linkage maps and analyzes intervals between adjacent pairs of linked markers along chromosomes simultaneously, instead of analyzing single markers (Lander and Botstein 1989). The use of linked markers for analysis compensates for recombination between the markers and the QTL, and is considered, statistically, more powerful than

single-point analysis. In general terms, an individual QTL may be described as major or minor, based on the proportion of the phenotypic variation explained by the QTL.

There are many factors that influence the detection of QTLs segregating in a population (Asíns 2002; Tanksley 1993). The main ones are the genetic properties of QTLs that control traits, environmental effects, population size, and experimental error. The genetic properties of QTLs that control traits include the magnitude of the effect of individual QTLs. Only QTLs with sufficiently large phenotypic effects will be detected; QTLs with small effects may fall below the significance threshold of detection. Another genetic property is the distance between linked QTLs: QTLs that are closely linked (≤ 20 cM) will usually be detected as a single QTL in typical population sizes (< 500 individuals) (Tanksley 1993). Environmental effects may have a profound influence on the expression of quantitative traits. Experiments replicated across sites and over time (for example, different seasons and years) may enable the determination of environmental influences on the QTLs affecting traits of interest. The most important experimental design factor is the size of the population used in the mapping study. The larger the population, the more accurate the mapping study and the more likely it is to allow detection of QTLs with smaller effects. An increase in population size provides gains in statistical power, estimates of gene effects, and confidence intervals of the locations of QTLs (Beavis 1998; Darvasi et al. 1993; Haley and Andersson 1997; Tanksley 1993).

The main sources of experimental error are mistakes in marker genotyping and errors in phenotypic evaluation. Genotyping errors and missing data can affect the order of the markers and the distance between markers within linkage maps (Hackett 2002). The accuracy of phenotypic evaluation is of the utmost importance for the accuracy of QTL mapping. A reliable QTL map can only be produced from reliable phenotypic data.

The selection of plants in a segregating progeny that contain the appropriate combination of genes is a critical component of plant breeding. Moreover, plant breeders typically work with hundreds or even thousands of populations. Marker-assisted selection may greatly increase the efficiency and effectiveness in plant breeding compared to conventional breeding methods. Once markers that are tightly linked to genes or QTLs of interest have been identified, prior to field evaluation of large numbers of plants, breeders may use specific DNA marker alleles as a diagnostic tool to identify plants carrying the genes or QTLs.

In this work, the progeny Monastrell x Syrah was used to map QTLs associated with the phenotypic variation in phenological, productive, morphological, and enological traits of interest in wine grape. Additionally, some putative candidate genes were also identified, based on the available molecular function annotation of grapevine and their colocation within the LOD-1 support intervals of the detected QTLs.

3.2 Materials and Methods

The phenotypic evaluation (phenological, productive, morphological, and enological traits) and linkage maps construction were described in the preceding chapters. Acidity and anthocyanin were measured three more years (2011-2013) with same protocols described before. The number of hybrids used was 76, 67 and 148, respectively.

3.2.1 QTL analysis

The QTL detection was carried out separately for the parental and consensus maps, using the software MapQTL v.4.0 (Van Ooijen et al. 2002) and the phenotypic data from each season. First, a non-parametric Kruskal-Wallis (KW) rank-sum test was applied to verify the global segregation of each locus and to detect putative QTLs (Lehmann 1975). The QTLs detected were

considered significant at $P > 0.0005$ and if they were identified in different maps and/or in different years. Second, a simple interval mapping (SIM, Lander and Botstein 1989) was performed to find regions with potential QTL effects; then, scored markers in these regions were used as co-factors (no more than five) in multiple QTL mapping (MQM, Jansen and Stam 1994). All the cofactors used were selected with the 'automatic cofactor selection' test implemented in MapQTL. Both linkage-group-wide (LGW) and genome-wide (GW) LOD thresholds corresponding to α=0.05 were used for both SIM and MQM detection of QTLs. The LOD thresholds were established through 1000 permutations of the phenotypic data. The QTLs detected with a LOD score higher than the GW threshold were considered as significant, while QTLs detected only with a LOD score higher than the LGW threshold were considered as putative QTLs. The QTLs detected for GW and LGW were analyzed separately. The percentage of variance explained for each QTL and for the combined effect of all QTLs detected in the same season, as well the QTL location, was estimated in the final MQM model. The QTL position was estimated from the location of the maximum LOD value and was indicated by the LOD-1 confidence interval and the cofactor. The LOD-1 support intervals were calculated using restricted MQM mapping. A QTL was considered stable when detected in at least two growing seasons.

3.2.2 Search for candidate genes

Candidate genes linked with the trait under study were looked for within each confidence interval of the QTLs detected in two or more years, based on the annotated molecular function available (NCBI, http://www.ncbi.nlm.nih.gov/projects/mapview/map_search.cgi?taxid=29760). The most proximal marker (SSR or SNP) was selected to delimit the confidence interval, and the position of these markers was identified in the

NCBI database. Only the most relevant sequences clearly linked with the trait were considered for the discussion of this work.

3.3 Results

3.3.1 Phenological QTLs

Significant associations between single marker genotypes and phenotypic data were found with the non-parametric KW test for sprouting on LGs 1, 8, 13, and 14, flowering on LGs 7 and 14, veraison on LGs 2, 8, and 11, ripening on LGs 2 and 17, and veraison-ripening period on LG2 (Annex 4). The QTLs detected at the GW for phenological data are shown in Table 3.1. One-LOD support confidence intervals of the main QTLs for phenological traits, detected with MQM, are represented in Figure 3.1. Five reliable QTLs for sprouting were detected, on LGs 1, 7, 8, 13, and 14 (Sp1, Sp7, Sp8, Sp13, Sp14) in the consensus and Mn maps. They individually explained between 6% and 12.6% of the total variance. The combined effect of all the QTLs detected in the same season explained up to 29.8% (in 2009) of the total variance in the consensus map. Only the Sp1 and Sp7 QTLs (in the Mn and consensus maps, respectively) were stable over years, explaining up to 11.3% of the total phenotypic variance. The Sp8 QTL was also detected only at the LGW level (putative QTL) in other seasons, in both the Mn and consensus maps, and the Sp13 region was detected in the consensus map (Annex 5). New unstable QTLs for sprouting were detected only at the LGW level (Sp2, Sp3, Sp11, Sp12, and Sp17) in the Sy and consensus maps (Annex 5).

For flowering time three QTLs were found, on LGs 5, 7, and 14 (Fw5, Fw7, and Fw14) in the Mn and consensus maps (Table 3.1), explaining individually up to 19% of the total phenotypic variance. The combined effect of all the QTLs detected in the same season explained up to 31% of the total

variance. Only the Fw7 QTL was consistent in the Mn map, explaining up to 17.4% of total phenotypic variance. Fw7 and Fw5 were also detected only at the LGW level in other seasons (Annex 5). New putative QTLs for flowering (Fw8 and Fw12) were detected in the Sy and consensus maps (Annex 5).

Table 3.1. QTLs identified for phenological traits in maps of the Monastrell x Syrah progeny.

Trait	Map	Year	LG	QTL	LOD max	cM	Confidence interval	Cofactor	GW LOD threshold	% variance QTL	% variance model
Sp	C	2008	14	Sp14	6.6	8.0	0-22	SNP251_159	4.1	12.6	12.6
		2009	1	Sp1	4.07	71.5	70-72	vvif52	4.0	7.8	29.8
			7	Sp7	4.88	37.3	30-45	Vvi_1731	4.0	8.4	
			8	Sp8	5.26	15.0	4-32	vmc1b11	4.0	11.3	
			13	Sp13	4.26	10.0	4-17	SNP653_90	4.0	6.9	
		2010	7	Sp7	4.94	37.3	31-42	Vvi_1731	4.2	11.3	11.3
	Mn	2008	1	Sp1	4.57	60.6	35-62	vvif52	2.7	8.9	17.8
			14	Sp14	5.90	5.0	0-24	vmc1e12	2.7	10.4	
		2009	1	Sp1	3.63	55.6	42-62	vvif52	2.7	6.4	27.2
			7	Sp7	4.23	39.8	28-54	Vvi_1731	2.7	7.2	
			8	Sp8	4.13	15.0	0-36	vmc1b11	2.7	8.8	
			13	Sp13	5.0	9.7	0-22	SNP653_90	2.7	8.0	
		2010	7	Sp7	3.99	39.8	35-52	Vvi_1731	2.7	8.7	8.7
Fw	C	2008	14	Fw14	5.02	13.5	9-18	vmc1e12	4.6	14.4	14.4
		2010	5	Fw5	4.85	14.2	0-15	SNP1071_151	4.0	9.5	31
			7	Fw7	8.86	22.1	7-49	Vvi_1731	4.0	19.3	
	Mn	2008	14	Fw14	4.52	5.0	0-21	vmc1e12	2.6	13.1	13.1
		2009	7	Fw7	3.1	39.8	35-52	Vvi_1731	2.1	10.1	10.1
		2010	5	Fw5	4.34	13.8	0-42	SNP1071_151	2.6	10.1	19.9
			7	Fw7	6.16	24.9	5-54	Vvi_13076	2.6	17.4	
Vr	C	2008	2	Vr2	5.08	21.2	14-25	vvib23	4.1	14.8	14.8
		2009	2	Vr2	7.19	19.0	0-33	SNP581_114	4.1	20.5	20.5
	Mn	2008	2	Vr2	5.04	11.4	0-48	vvib23	2.6	14.6	14.6
		2009	2	Vr2	7.74	9.1	0-26	SNP581_114	2.6	22.7	22.7
	Sy	2008	5	Vr5	2.9	43.5	38-43.5	vmc4c6	2.7	7.4	23.8
			8	Vr8	3.49	52.2	47-60	SNP1385_86	2.7	9.8	
			11	Vr11	3.09	20.5	19-30	vvib19	2.7	8.9	
Rp	C	2010	2	Rp2	6.86	51.2	38-60	20D18CB9	5.4	18.1	18.1
	Mn	2009	2	Rp2	2.91	31.9	24-48	vmc5g7	2.5	9.0	9.0
		2010	2	Rp2	5.5	31.9	18-50	vmc5g7	2.7	13.8	20.6
			17	Rp17	3.38	0.0	0-6	SNP677_509	2.7	7.7	
Vr_Rp	C	2010	2	Vr_Rp2	6.9	51.2	34-60	vmc5g7	5.4	18.5	18.5
	Mn	2008	2	Vr_Rp2	6.35	36.8	11-51	vmc5g7	2.6	16.7	27.5
			17	Vr_Rp17	4.01	0.0	0-8	SNP677_509	2.6	10.4	
		2009	2	Vr_Rp2	5.69	21.9	2-52	vmc5g7	2.5	17.2	17.2
		2010	2	Vr_Rp2	6.23	31.9	17-49	vmc5g7	2.7	15.5	22.4
			17	Vr_Rp17	3.25	0.0	0-5	SNP677_509	2.7	7.4	

The table shows the trait, the map, the year, and the linkage group (LG) in which the QTLs were identified. The QTLs are named using the LG number plus Sp, Fw, Vr, Rp, and Vr_Rp for sprouting, flowering, veraison, ripening, and veraison-ripening period, respectively. The QTL location is indicated by the position at which the highest LOD (LOD max) was detected (in cM), the LOD-1 confidence interval, and the cofactor. The QTLs considered are those with a maximum LOD value higher than that estimated for the genome-wide (GW) threshold, for a type I error rate of 5%. The percentage of the total variance explained by

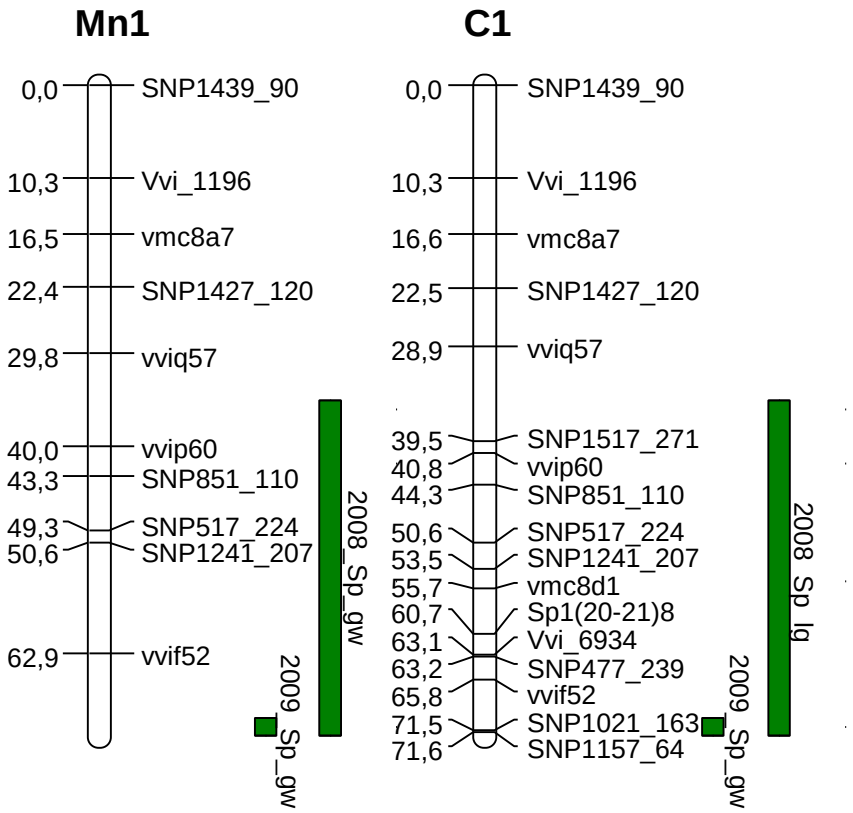
each QTL, and when considering the combined effect of all QTLs detected in a season (model), is indicated. C, consensus; Mn, Monastrell; Sy, Syrah.

Four significant QTLs were detected for veraison (Vr2, Vr5, Vr8, and Vr11). Only the Vr2 QTL was consistent in the Mn and consensus maps, explaining up to 22.7% of the total variance. The combined effect of the Vr5, Vr8, and Vr11 QTLs detected in the Sy map in the same season explained up to 23.8% of the total variance (Table 3.1). Nevertheless, they were also detected only at the LGW level in other seasons in the three maps, along with new putative QTLs (Vr11, Vr14, and Vr18) detected in the Mn and consensus maps (Annex 5).

Ripening date was under the control of two genomic regions (Rp2 and Rp17), which explained between 7% and 18% of total phenotypic variance. The combined effect of the Rp2 and Rp17 QTLs detected in the Mn map in the same season explained up to 20.6% of the total variance (Table 3.1). They were also detected only at the LGW level in other seasons, along with a new putative QTL (Rp18) detected in Sy only in one season (Annex 5).

For the veraison-ripening period two QTLs were found on LGs 2 and 17 (Vr_Rp2 and Vr_Rp17), collocating with Rp2 and Rp17, respectively (Table 3.1). The combined effect of both QTLs detected in the Mn map in the same season explained up to 27.5% of the total variance (Table 3.1). New putative QTLs (Vr_Rp5 and Vr_Rp19) were detected in the Sy map in one season (Annex 5).

Figure 3.1. One-LOD support confidence intervals of the main QTLs for phenological traits detected with MQM.



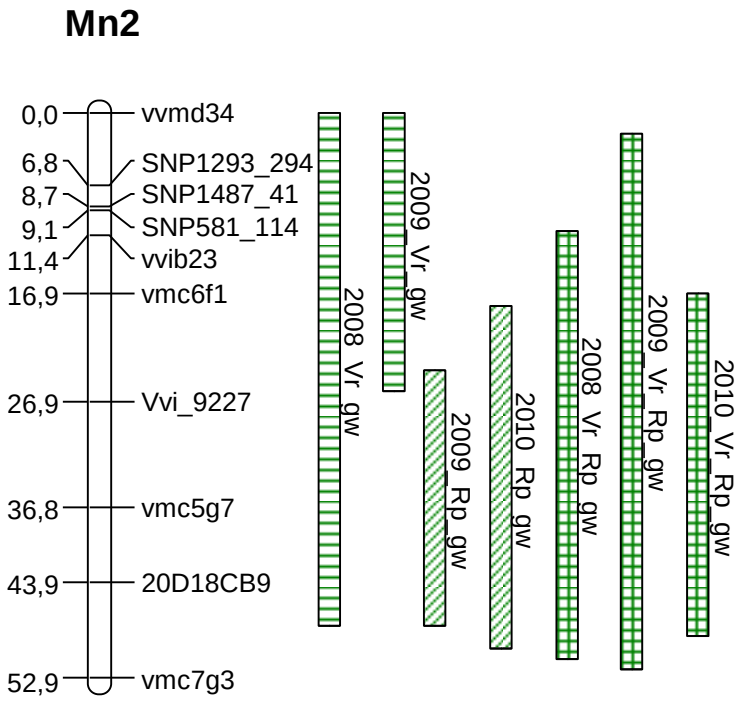


Figure 3.1. Continued.

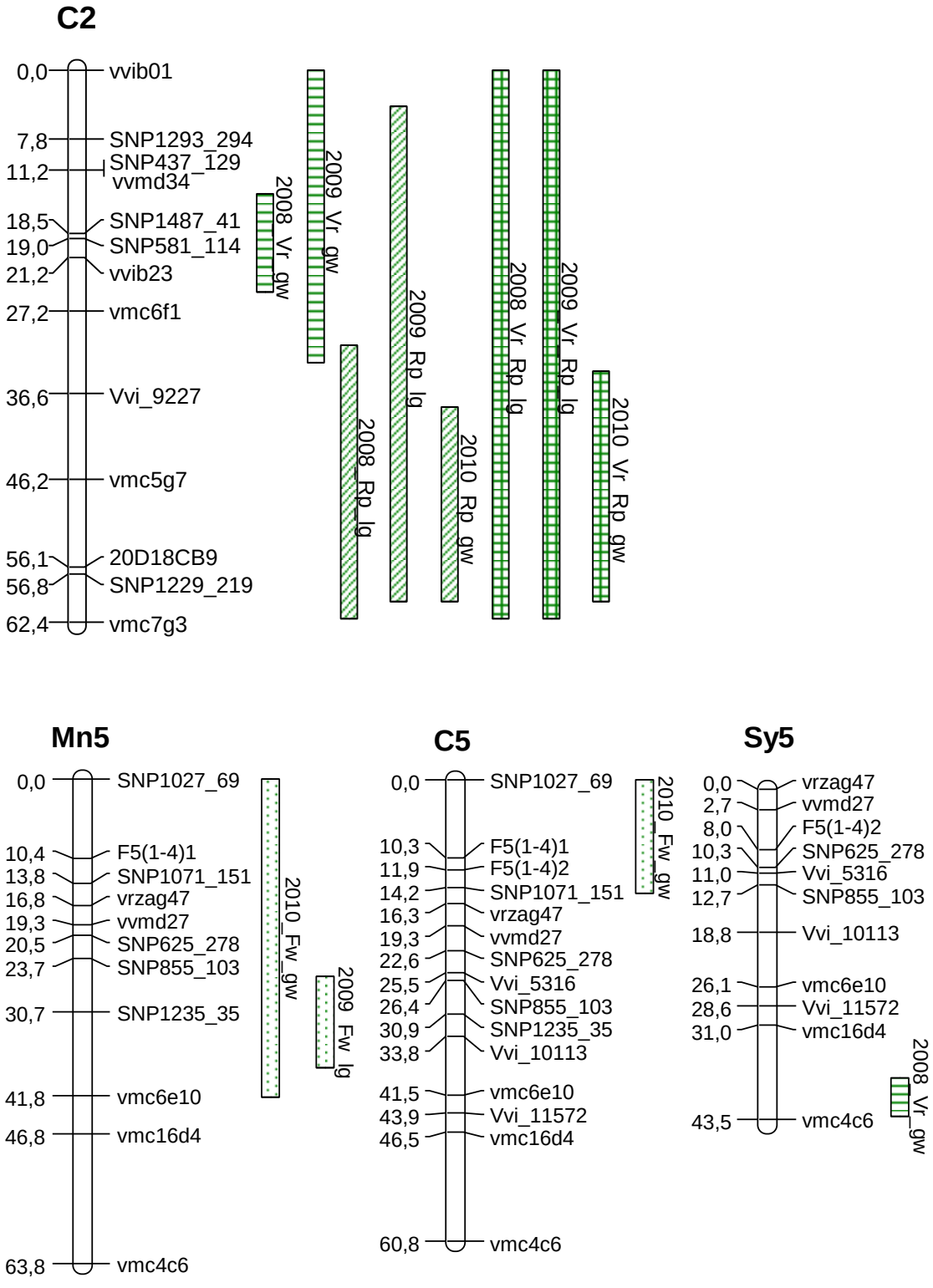


Figure 3.1. Continued.

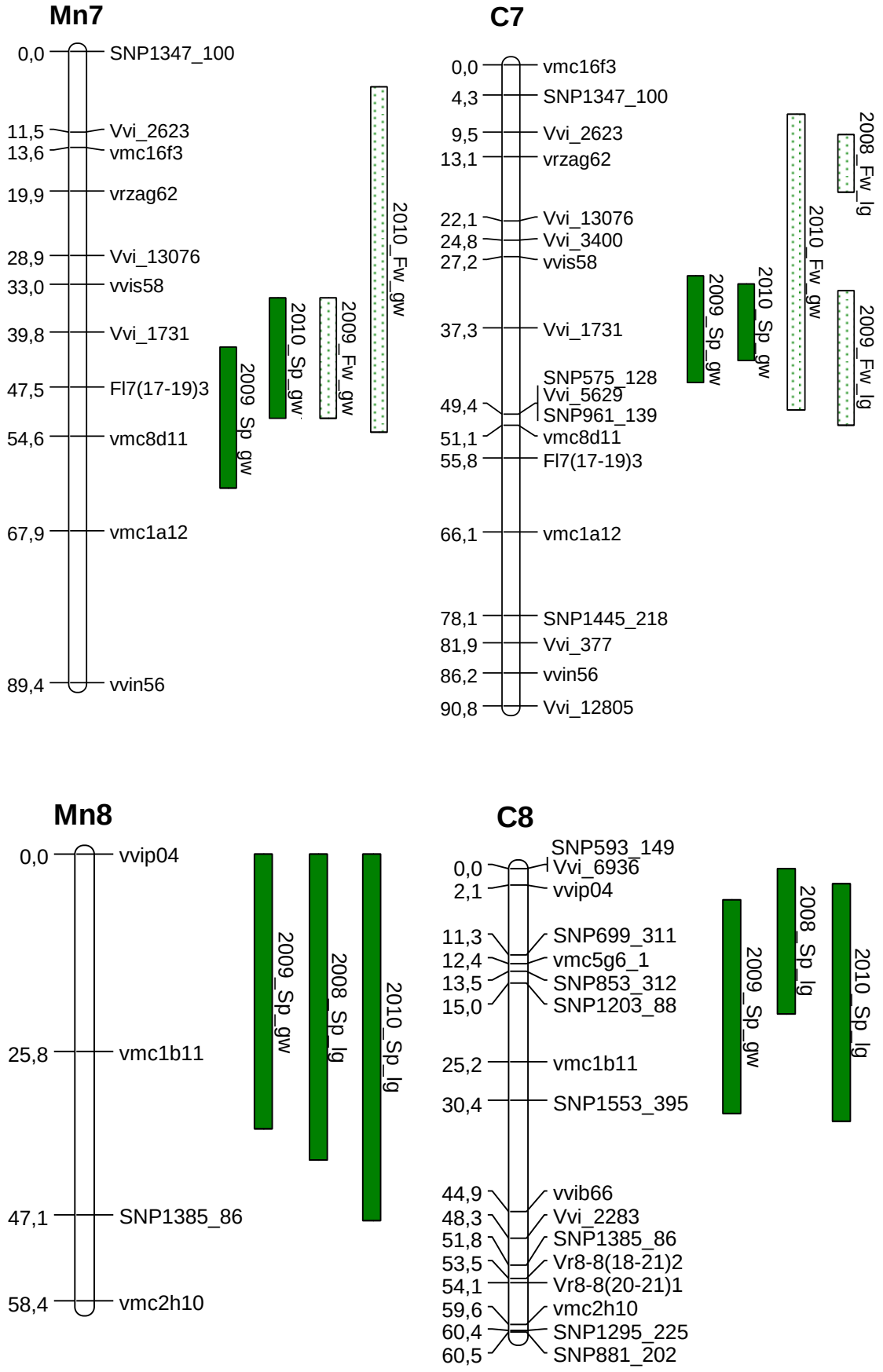


Figure 3.1. Continued.

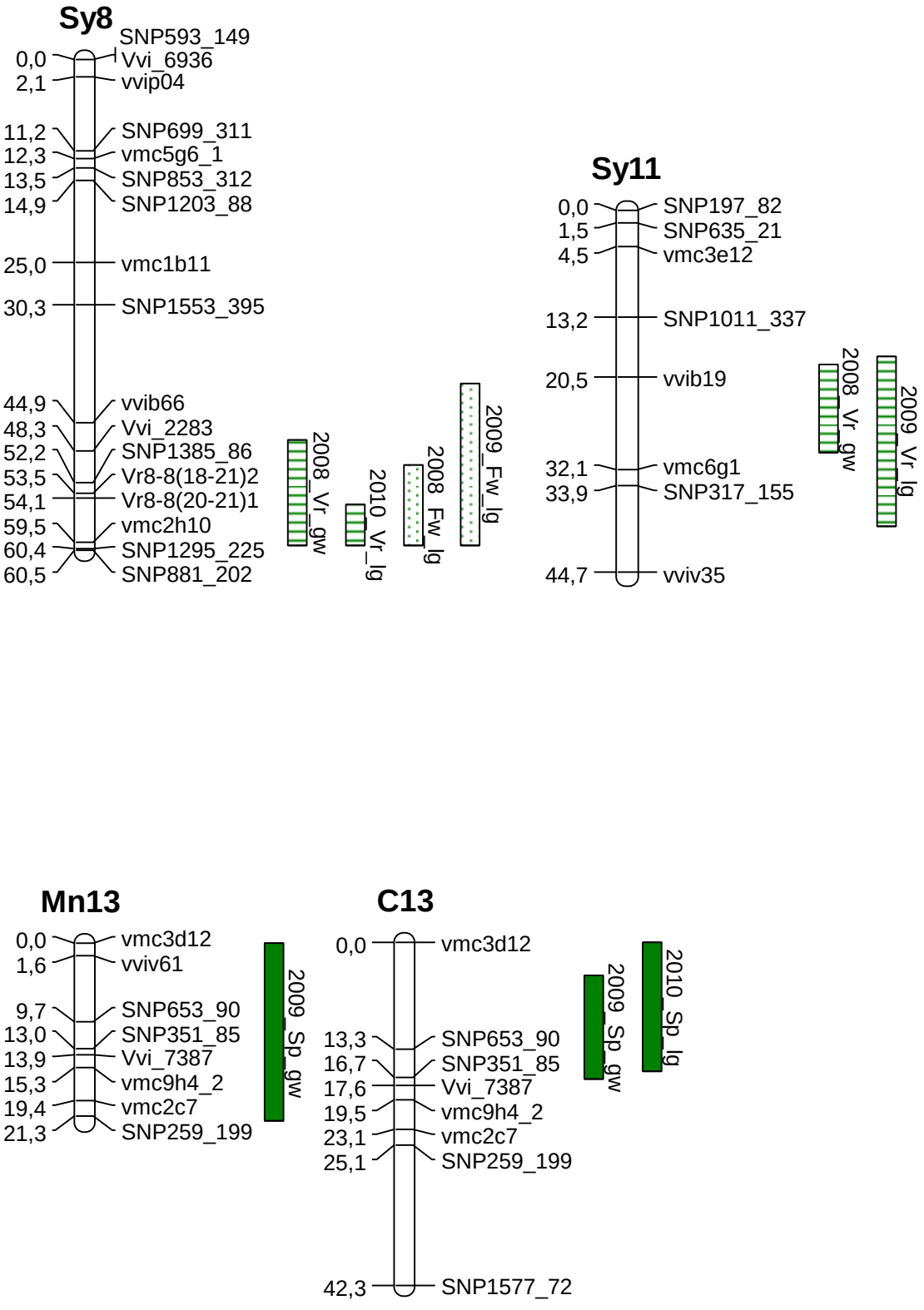


Figure 3.1. Continued.

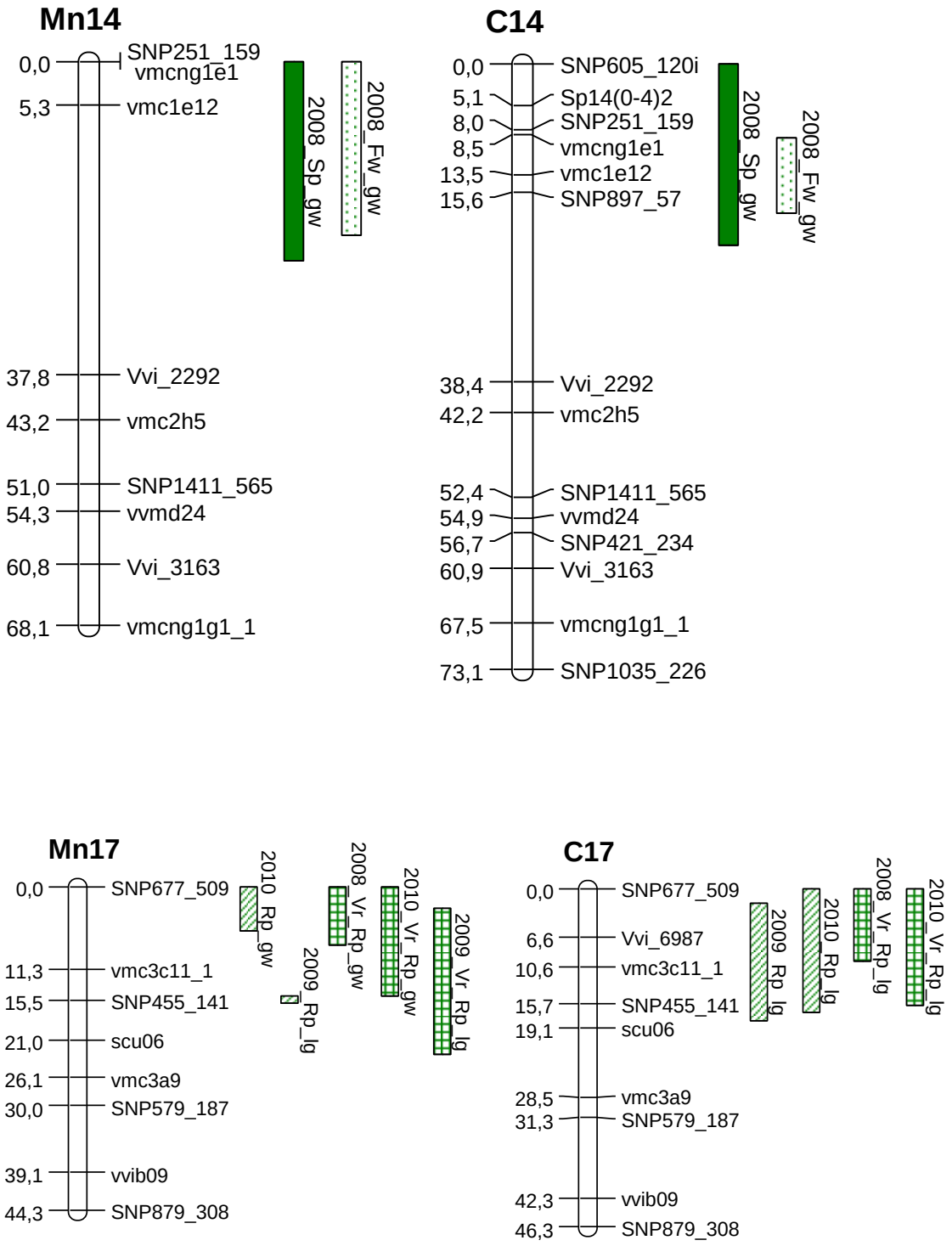
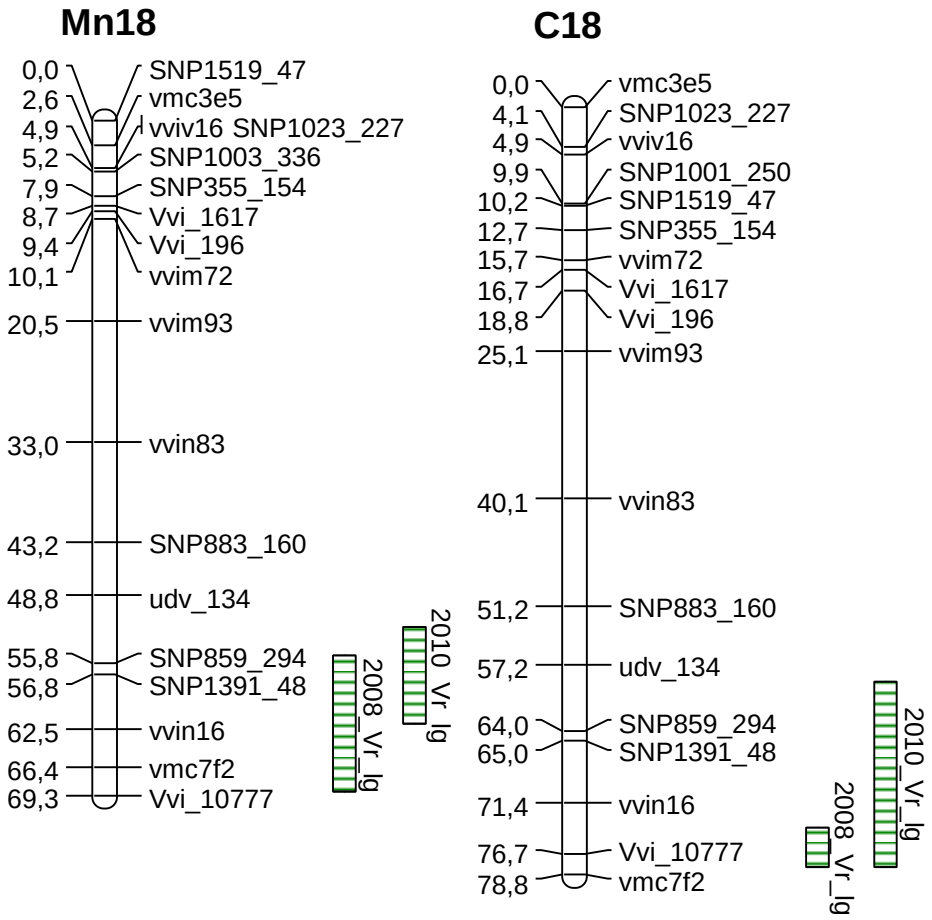


Figure 3.1. Continued.



The QTLs detected at the genome-wide level (gw) are represented, as are the QTLs detected only at the linkage-group-wide (lg) level but found in at least two years or in two maps. The putative QTLs detected only in one year but which co-located with the QTL interval found at the GW level are also represented. C, consensus; Mn, Monastrell; Sy, Syrah. Sp, sprouting; Fw, flowering; Vr, veraison; Rp ripening; Vr_Rp, veraison-ripening interval.

3.3.2 Productive and morphological QTLs

Significant associations between single marker genotypes and phenotypic data were found with the non-parametric KW test for fertility index on LGs 3 and 5, total production on LGs 5 and 8, cluster weight on LGs 2, 5, and 14, berry weight on LGs 5, 7, 14, and 17, and cluster compactness on LGs 2 and 5 (Annex 4). The QTLs detected at the GW level for productive and morphological data are shown in Table 3.2. The one-LOD support confidence intervals of the main QTLs for productive and morphological traits detected with MQM are represented in Figure 3.2.

Two significant QTLs were detected for fertility index on LGs 3 and 5 (Fi3 and Fi5). Only the Fi5 QTL was stable over years and was detected in the three maps, explaining up to 26.4% of the total phenotypic variance. The combined effect of the Fi3 and Fi5 QTLs detected in the same season (2008) explained 13.5% of the total variance in the Sy map. The Fi3 QTL was also detected only at the LGW level in other seasons, in both the Sy and consensus maps (Annex 6). New unstable QTLs for the fertility index were detected only at the LGW level (Fi8 and Fi11) in the consensus maps (Annex 6).

Total production was under the control of one genomic region (P8), which explained only 10% of the total phenotypic variance. This QTL was found only in one season in the Mn map at the GW level, but also was detected in other seasons at the LGW level in the Mn and consensus maps, explaining up to 13% of the total variance (Annex 5). Six new putative QTLs for total production (P1, P3, P5, P12, P14, and P17) were detected, and the combined effect of some of them in the same season explained up to 33.4% of the total variance (Annex 6). Similarly, one significant QTL was detected for cluster weight on LG19 (CW19), explaining only 9.5% of the total variance. This QTL was found in only one season in the Sy map (Table 3.2). Eight new putative QTLs for cluster weight (CW1, CW2, CW5, CW7, CW8, CW10, CW14, and CW17) were detected, and the combined effect of some of

them in the same season explained up to 41.9% of the total variance (Annex 6).

Table 3.2. QTLs identified for productive and morphological traits in maps of the Monastrell x Syrah progeny.

Trait	Map	Year	LG	QTL	LOD max	cM	Confidence interval	Cofactor	GW LOD threshold	% variance QTL	% variance model
Fi	C	2008	5	Fi5	9.08	0.0	0-16	SNP1027_69	4.8	21.6	21.6
		2013	5	Fi5	11.67	0.0	0-32	SNP1027_69	4.4	26.4	26.4
	Mn	2008	5	Fi5	4.31	0.0	0-7	SNP1027_69	2.7	8.4	8.4
		2009	5	Fi5	4.14	0.0	0-8	SNP1027_69	2.7	8.1	8.1
		2013	5	Fi5	7.22	0.0	0-17	SNP1027_69	2.7	14.3	14.3
	Sy	2008	3	Fi3	4.04	20.1	9-25	udv_043	2.8	7.4	13.5
			5	Fi5	3.79	12.7	0-21	Vvi_5316	2.8	7.2	
		2009	5	Fi5	3.22	8.0	5-16	Vvi_5316	2.6	6.6	6.6
		2010	5	Fi5	3.08	12.7	9-15	Vvi_5316	2.7	6.5	6.5
		2013	5	Fi5	3.38	0.0	0-12	vrzag47	2.7	6.9	6.9
P	Mn	2009	8	P8	2.64	40.8	36-49	SNP1385_86	2.5	10.1	10.1
CW	Sy	2008	19	CW19	2.96	1.9	50-56	SNP819_210	2.7	9.5	9.5
BW	C	2008	14	BW14	5.01	8.0	1-14	vmcng1e1	4.1	12.6	34.1
			17	BW17	4.56	10.6	9-13	vmc3c11_1	4.1	11.4	
			19	BW19	4.98	31.3	28-33	vvip31	4.1	12.5	
		2009	5	BW5	4.95	0.0	0-10	SNP1027_69	4.0	11.5	33.3
			7	BW7	5.28	27.2	22-48	vvis58	4.0	12.0	
			14	BW14	4.16	0.0	5-10	SNP251_159	4.0	9.4	
		2010	5	BW5	4.08	30.9	29-35	SNP1235_35	3.9	9.8	20.3
			17	BW17	4.05	10.6	9-16	vmc3c11_1	3.9	9.3	
	Mn	2008	5	BW5	3.94	19.3	0-26.5	vrzag47	2.8	11.6	20.5
			14	BW14	3.63	0.0	0-12	SNP251_159	2.8	10.7	
		2009	1	BW1	2.91	40.0	35-48	SNP851_110	2.7	6.2	34.0
			5	BW5	4.27	0.0	0-15	SNP1027_69	2.7	10.3	
			7	BW7	3.58	33.0	29.5-44	Vvi_1731	2.7	7.9	
			14	BW14	3.77	0.0	0-27	SNP251_159	2.7	7.9	
		2010	5	BW5	3.16	28.7	19-39	SNP855_103	2.6	8.7	8.7
	Sy	2009	7	BW7	2.63	37.4	36-38	Vvi_1731	2.6	9.1	9.1
		2010	17	BW17	3.57	4.3	0-8	vmc3c11_1	2.7	9.2	9.2
CN	Mn	2009	8	CN8	3.17	45.8	36-55	SNP1385_86	2.6	11.9	11.9
CC	C	2008	2	CC2	4.6	19.0	16-20	SNP581_114	4.5	16.9	16.9
		2010	5	CC5	7.5	5.0	0-8	SNP1027_69	6.9	28.3	28.3
	Mn	2010	5	CC5	2.95	5.0	0-8	SNP1027_69	2.6	9.7	9.7
	Sy	2010	5	CC5	3.25	0.0	0-5.5	vrzag47	2.8	8.9	8.9

The table shows the trait, the map, the year, and the linkage group (LG) in which the QTLs were identified. The QTLs are named using the LG number plus Fi, P, CW, BW, CN, and CC for fertility index, total production, cluster weight, berry weight, cluster number, and cluster compactness, respectively. The QTL location is indicated by the position at which the highest LOD (LOD max) was detected (in cM), the LOD-1 confidence interval, and the cofactor. The QTLs considered are those with a maximum LOD value higher than that estimated for the genome-wide (GW) threshold, for a type I error rate of 5%. The percentage of the total variance explained by each QTL, and when considering the combined effect of all QTLs detected in a season (model), is indicated. C, consensus; Mn, Monastrell; Sy, Syrah.

Berry weight was under the control of six genomic regions (LGs 1, 5, 7, 14, 17, and 19), which explained between 6% and 12% of the total variance (Table 3.2). Only three of them (BW5, BW14, and BW17) were stable over the years. The combined effect of some of them in the same season explained up to 34.1% of the total variance (Table 3.2). The BW1 and BW7 QTLs also were detected in other seasons at the LGW level in Mn and Sy maps, along with a new putative QTL (BW4) detected in the Mn map in one season (Annex 6).

For cluster number, one QTL was detected on LG8 (CN8), explaining 11.9% of the total variance. This QTL was found only in one season in the Mn map (Table 3.2) and in two seasons at the LGW level in the consensus map (Annex 6). Eight new putative QTLs for cluster number (CN1, CN3, CN5, CN6, CN11, CN12, CN14, and CN15) were detected, and the combined effect of some of them in the same season explained up to 21.2% of the total variance (Annex 6).

Two significant QTLs were detected for cluster compactness on LGs 2 and 5 (CC2 and CC5) only in one season, explaining 28.3% of the total variance (Table 3.2). Nevertheless, these QTLs were also detected only at the LGW level in other seasons, along with three new putative QTLs (CC16, CC17, and CC19) (Annex 6).

Figure 3.2. One-LOD support confidence intervals of the main QTLs for productive and morphological traits detected with MQM.

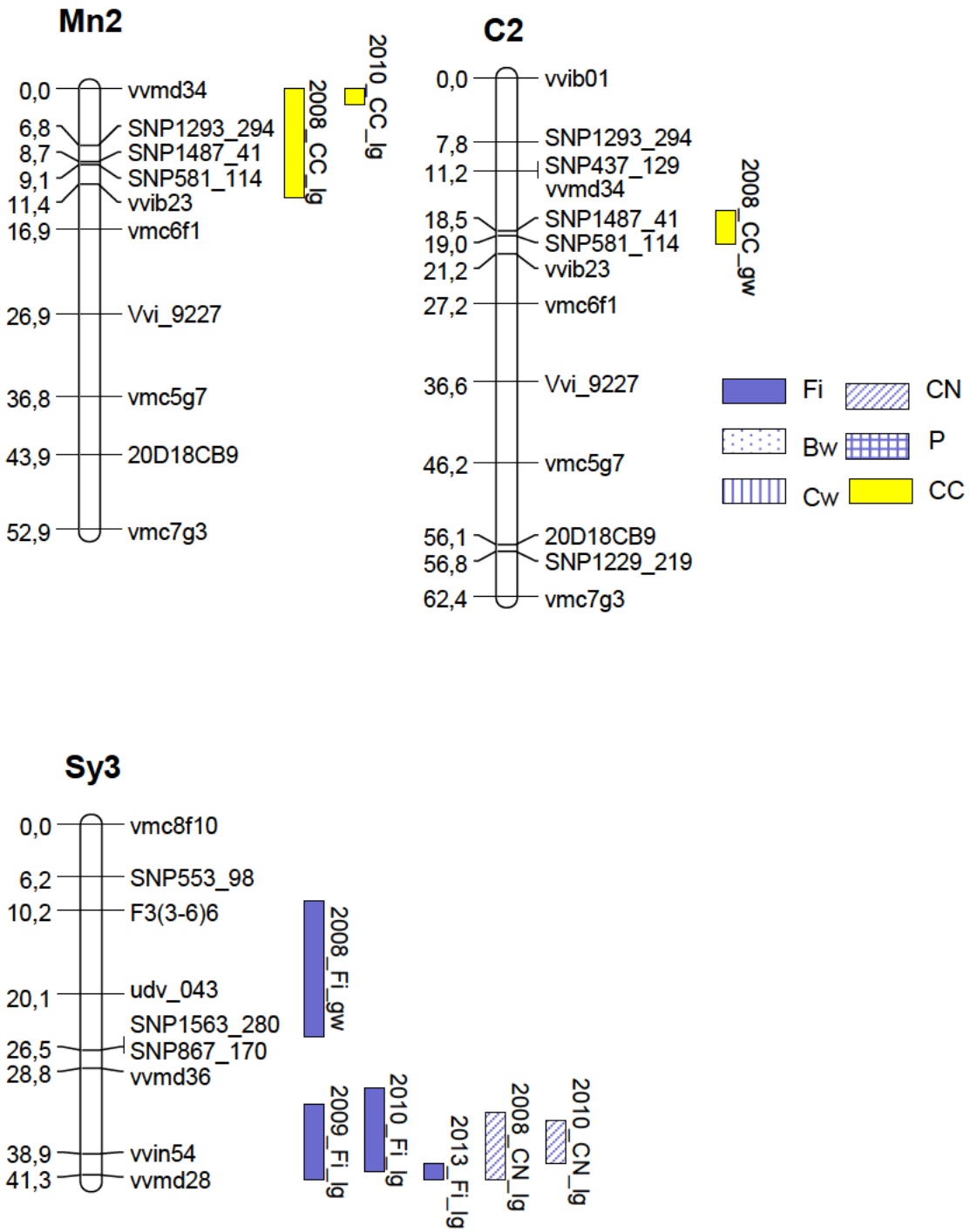


Figure 3.2. Continued.

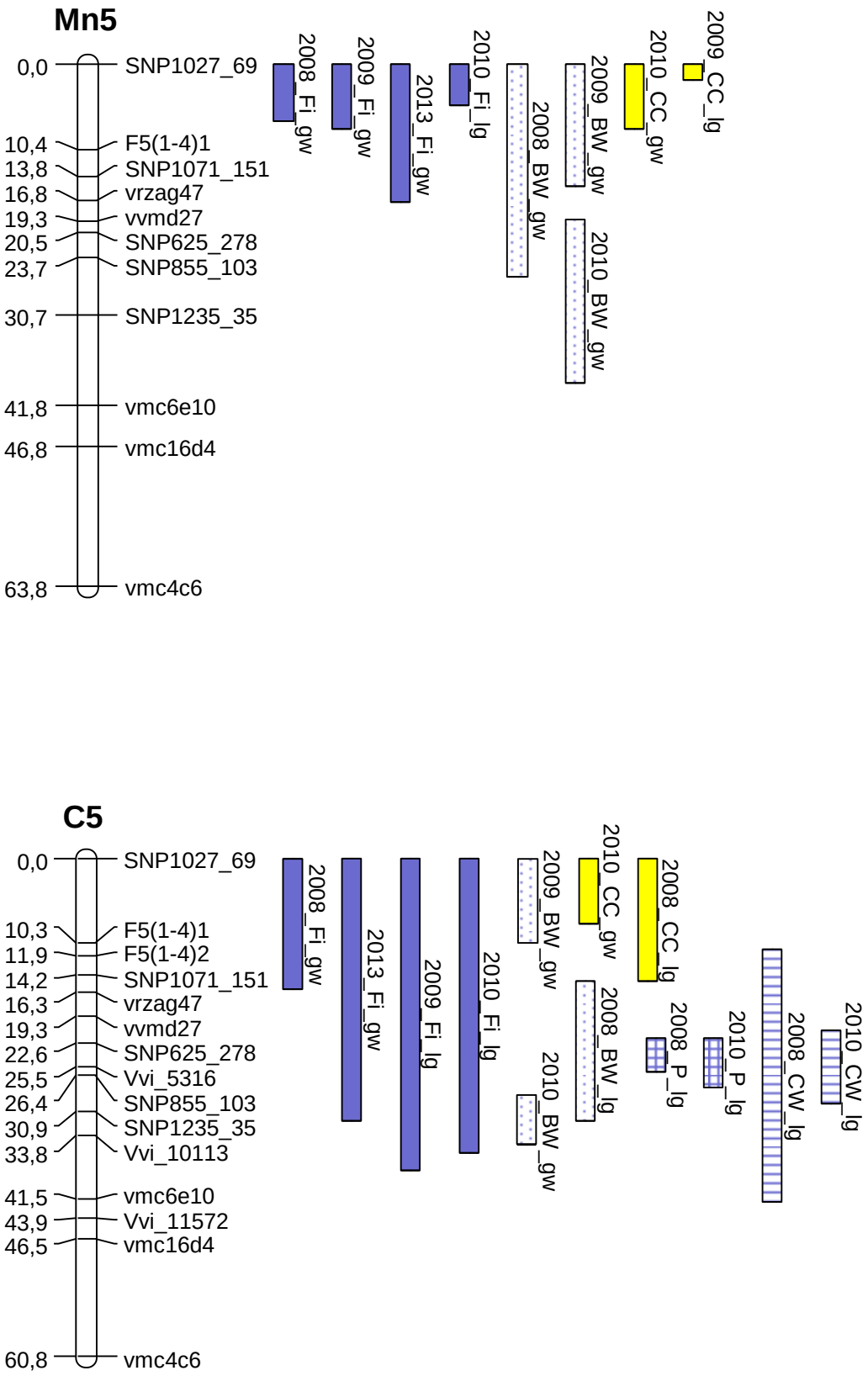


Figure 3.2. Continued.

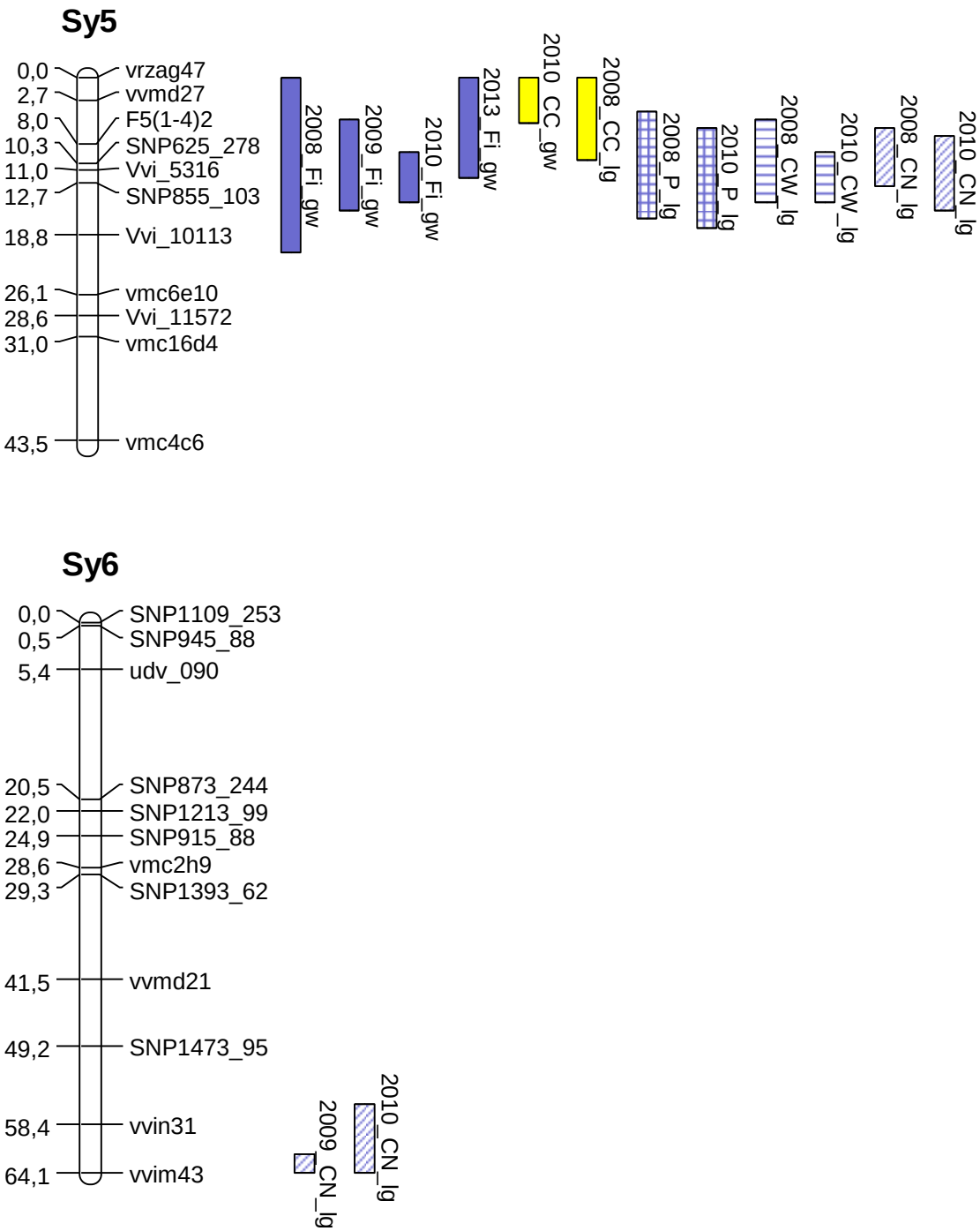


Figure 3.2. Continued

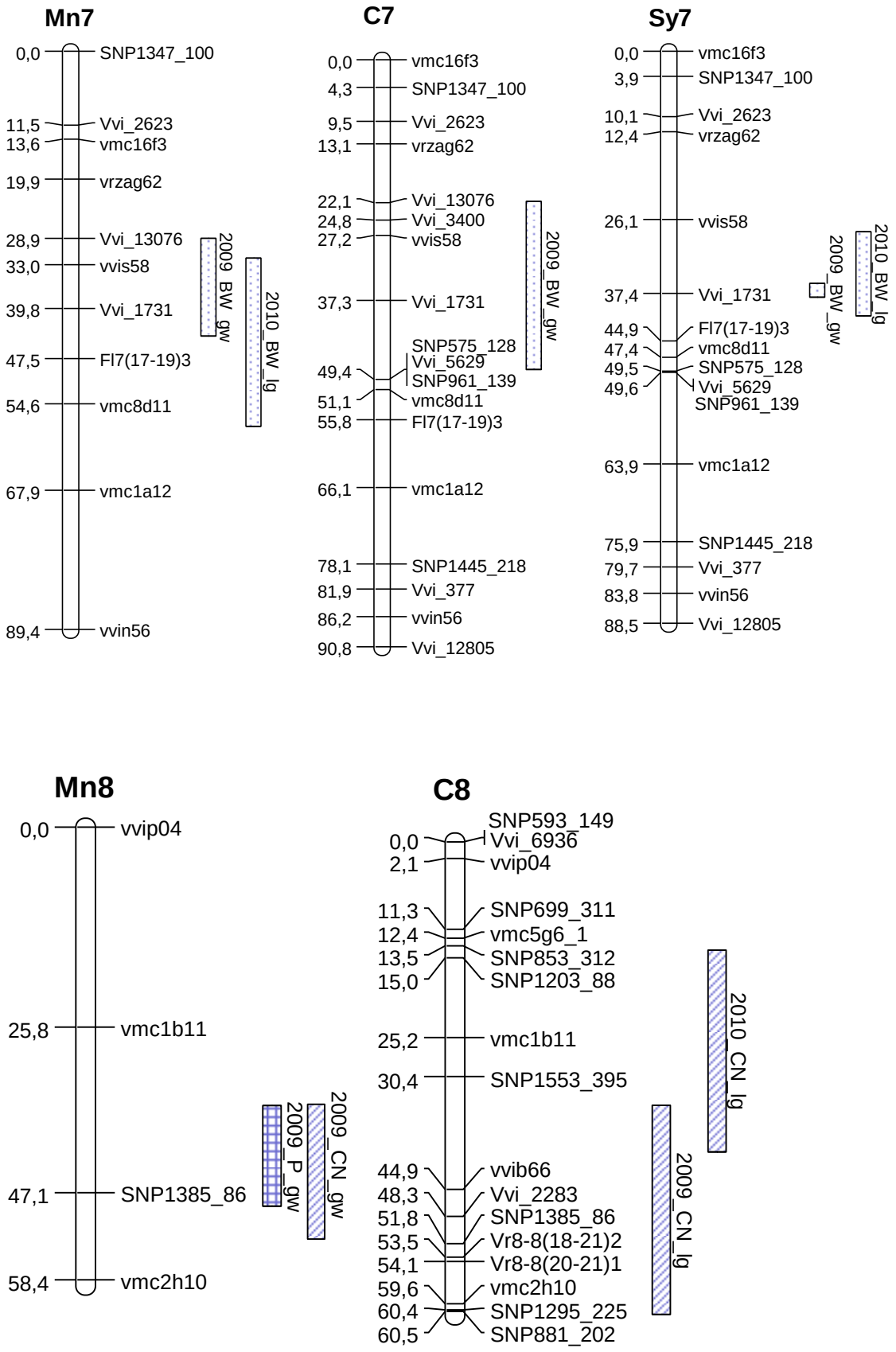


Figure 3.2. Continued

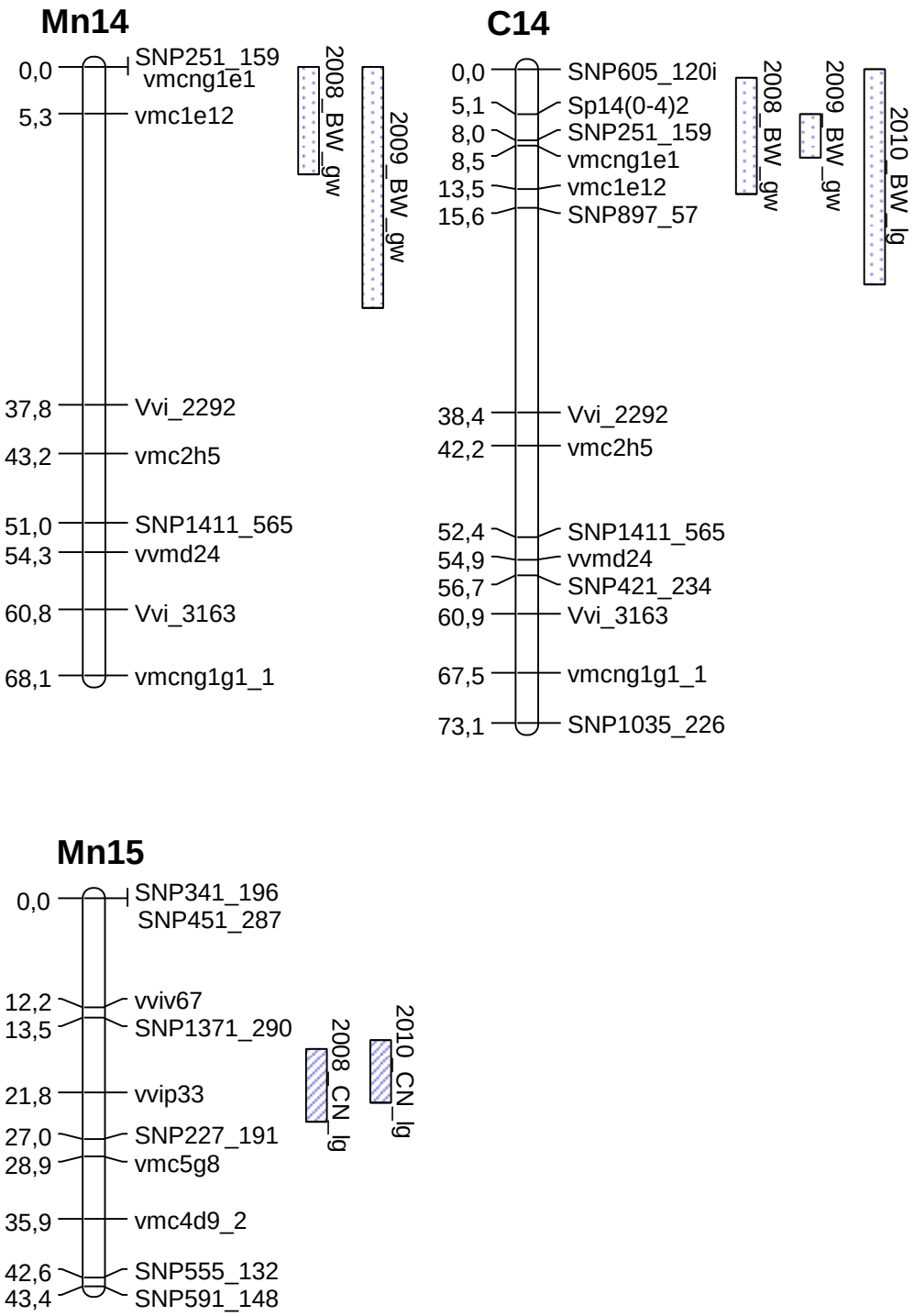
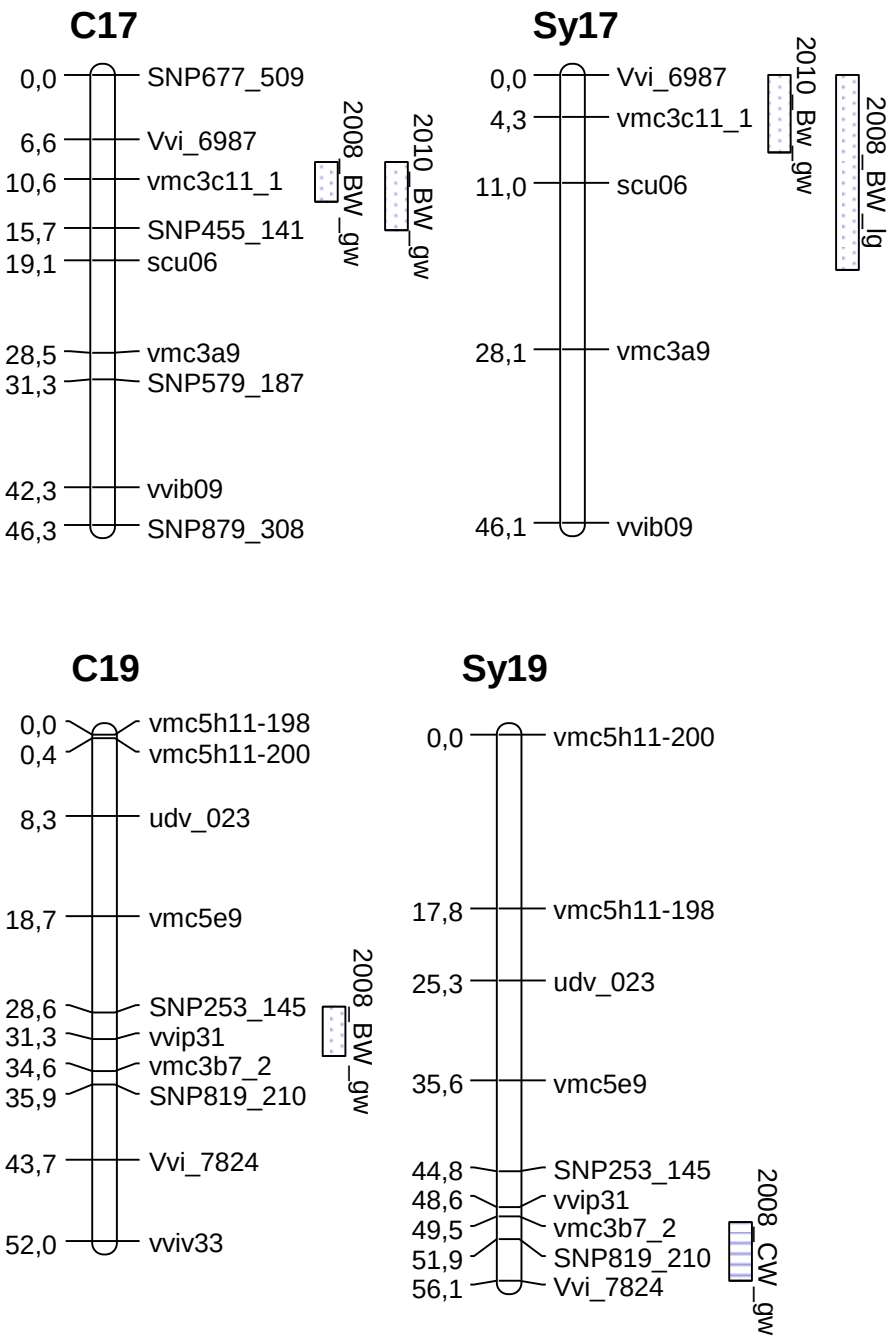


Figure 3.2. Continued



The QTLs detected at the genome-wide level (gw) are represented, as are the QTLs detected only at the linkage group-wide (lg) level in at least two years or in two maps. The putative QTLs detected only in one year were included only if they co-located with the QTL interval found at the GW level. C, consensus; Mn, Monastrell; Sy, Syrah. Fi, fertility index; BW, berry weight; CW, cluster weight; CN, cluster number; P, total production; CC, cluster compactness.

3.3.3 Enological QTLs

Significant associations between single marker genotypes and phenotypic data were found with the non-parametric KW test for total acidity on LGs 2 and 18, the ratio of total soluble solids to total acidity on LGs2 and 18, malic acid on LGs 2, 8, and 17, the ratio of tartaric to malic acid on LGs 1, 2, and 8, and total anthocyanins on LG 2 (Annex 4). The QTLs detected at the GW level for enological data are shown in Table 3.3. The one-LOD support confidence intervals of the main QTLs for the enological traits detected with MQM are represented in Figure 3.3.

For total acidity, two QTLs were detected on LGs 1 and 2 (Ac1 and Ac2), explaining between 7% and 18% of the total phenotypic variance. The Ac2 QTL was consistent only in the consensus map, but it was also located at the LGW level in other seasons in the Mn map and in one season in the Sy map (Annex 7). The Ac1 QTL was also detected at the LGW level in the Sy and consensus maps. New putative QTLs for total acidity were detected only at the LGW level (Ac4, Ac5, Ac6, Ac8, Ac9, Ac11, Ac12, Ac13, Ac16, Ac17, and Ac18). The combined effect of some of them in the same season explained up to 33.7% of the total variance (Annex 7). The Ac5 QTL was stable over the years in the Mn and Sy maps, and Ac18 QTL was stable in the Sy map.

Three significant QTLs were detected for the ratio of total soluble solids to total acidity on LGs 1, 2, and 4 (TSS/Ac1, TSS/Ac2, and TSS/Ac4), but only the QTL on LG2 was consistent, explaining up to 19% of the total phenotypic variance. The combined effect of the TSS/Ac2 and TSS/Ac4 QTLs detected in the same season explained up to 24% of the total variance in the Mn map (Table 3.3). The TSS/Ac4 QTL was also identified in other seasons in the Mn map at the LGW level (Annex 6). New putative QTLs for total acidity were detected only at the LGW level (TSS/Ac5, TSS/Ac8, TSS/Ac10, TSS/Ac11, TSS/Ac13, TSS/Ac14, TSS/Ac16, and TSS/Ac18).

Table 3.3. QTLs identified for enological traits in maps of the Monastrell x Syrah progeny.

Trait	Map	Year	LG	QTL	LOD max	cM	Confidence interval	Cofactor	GW LOD threshold	% variance QTL	% variance model
Ac	C	2009	2	Ac2	5.63	16.2	8-24	vvib23	4.0	18.5	18.5
		2010	2	Ac2	5.82	56.1	47-62	20D18CB9	4.1	15.7	15.7
	Mn	2009	2	Ac2	3.19	43.9	26-46	20D18CB9	2.7	11.0	11.0
	Sy	2010	1	Ac1	2.5	45.3	44-46	SNP1021_163	2.2	7.3	7.3
TSS/ Ac	C	2008	2	TSS/Ac2	4.45	56.8	54-58	20D18CB9	4.3	19.7	19.7
		2009	2	TSS/Ac2	4.71	51.2	48-57	20D18CB9	4.3	15.6	15.6
		2010	1	TSS/Ac1	5.37	71.6	67.5-71.6	SNP1157_64	4.0	16.0	16.0
	Mn	2008	2	TSS/Ac2	2.88	26.9	19-32	Vvi_9227	2.6	11.4	24.0
			4	TSS/Ac4	2.91	27.6	21-34	Vvi_6668	2.6	11.6	
		2009	2	TSS/Ac2	5.2	31.9	19-50	Vvi_9227	2.8	17.4	17.4
		2010	2	TSS/Ac2	3.73	43.9	26-51	20D18CB9	2.7	11.3	11.3
	Sy	2010	1	TSS/Ac1	3.81	45.3	36-46	SNP1157_64	2.7	11.0	11.0
Tar	Sy	2010	18	Tar18	2.79	18.3	16-20	vvim93	2.6	10.3	10.3
		2011	19	Tar19	2.93	49.5	49-51	vmc3b7_2	2.8	16.1	16.1
Ma	C	2010	5	Ma5	4.85	43.9	42-54	vmc16d4	4.3	15.5	25.6
			15	Ma15	4.56	56.5	48-58	SNP555_132	4.3	29.3	
		2013	8	Ma8	6.22	7.1	0-20	SNP699_311	4.2	19.6	19.6
	Mn	2011	4	Ma4	2.83	52.4	52-55	Vvi_2543	2.7	14.1	22.8
			9	Ma9	2.81	70.3	62-75	Vvi_10329	2.7	14.9	
		2012	17	Ma17	3.08	0.0	0-3.5	SNP677_509	2.7	17.3	32.3
			18	Ma18	2.92	2.6	2-4	vmc3e5	2.7	16.3	
	Sy	2010	5	Ma5	3.91	41.0	26-44	vmc4c6	2.7	13.0	24.0
			8	Ma8	3.52	13.5	6-18	SNP853_312	2.7	11.1	
		2013	8	Ma8	6.21	7.1	0-24	vvip04	2.6	19.5	19.5
Tar/ Ma	C	2013	8	Tar/Ma8	7.18	7.1	0-22	SNP699_311	4.1	19.5	31.1
			11	Tar/Ma11	4.7	9.3	5-18	SNP197_82	4.1	13.2	
	Sy	2010	5	Tar/Ma5	3.69	41.0	15-43.5	vmc4c6	2.6	12.2	23.6
			8	Tar/Ma8	3.52	7.1	0-22	vmc5g6_1	2.6	11.8	
		2013	8	Tar/Ma8	6.75	7.1	0-26	SNP699_311	2.7	21.1	21.1
Ant	C	2008	2	Ant2	15.95	56.1	20-62.4	20D18CB9	4.5	75.7	75.7
		2010	2	Ant2	36.15	51.2	12-62.4	SNP1229_219	4.8	79.4	79.4
		2011	2	Ant2	23.18	51.2	14-62.4	SNP1229_219	4.9	80.0	80.0
		2012	2	Ant2	11.43	62.4	36-62.4	vmc7g3	5.6	54.6	54.6
		2013	2	Ant2	43.04	56.8	12-62.4	SNP1229_219	4.7	77.5	77.5
	Mn	2009	2	Ant2	5.16	52.9	26-53	vmc7g3	2.9	21.8	21.8
		2010	2	Ant2	4.71	43.9	29-51	20D18CB9	2.7	17.6	17.6
		2013	2	Ant2	6.64	43.9	19-53	20D18CB9	2.8	21.5	21.5
	Sy	2008	2	Ant2	6.41	57.0	30-57	vmc7g3	2.9	53.1	53.1
		2009	2	Ant2	2.98	51.6	49-55	20D18CB9	2.7	13.3	13.3
		2010	2	Ant2	29.6	51.6	15-57	20D18CB9	2.8	69.8	69.8
		2011	2	Ant2	20.18	51.6	15-57	20D18CB9	2.9	73.0	73.0
		2012	2	Ant2	8.61	47.8	27-57	20D18CB9	2.6	47.8	47.8
		2013	2	Ant2	30.68	51.6	15-57	20D18CB9	2.9	67.2	67.2

The table shows the trait, the map, the year, and the linkage group (LG) in which the QTLs were identified. The QTLs are named using the LG number plus Ac, TSS/Ac, Tar, Ma, Tar/Ma, and Ant for total acidity, ratio of total soluble solids to total acidity, tartaric acid, malic acid, ratio of tartaric acid to malic acid, and total anthocyanins, respectively. The QTL location is indicated by the position at which the highest LOD (LOD max) was detected (in cM), the LOD-1 confidence interval, and the cofactor. The QTLs considered are those with a maximum LOD value higher than that estimated for the genome-wide (GW) threshold, for a type I error rate of 5%. The percentage of the total variance explained by each QTL, and when considering the combined effect of all QTLs detected in a season (model), is indicated. C, consensus; Mn, Monastrell; Sy, Syrah.

The combined effect of some of them in the same season explained up to 45.8% of the total variance (Annex 6). The TSS/Ac5 QTL was stable over the years in the three maps, the TSS/Ac11 QTL was stable in the Sy and consensus maps, and TSS/Ac14 was stable in the Mn map (Annex 6).

For tartaric acid two QTLs were detected on LGs 18 and 19 (Tar18 and Tar19) in the Sy map, explaining 10.3% and 16.1% of the total phenotypic variance, respectively (Table 3.3). New suggestive QTLs for tartaric acid were detected only at the LGW level (Tar2, Tar3, Tar4, Tar5, Tar7, Tar9, Tar15, and Tar16). The combined effect of some of them in the same season explained up to 15.2% of the total variance (Annex 7). The Tar16 QTL was stable over the years in the Sy map (Annex 7).

Malic acid was under the control of seven genomic regions (LGs 4, 5, 8, 9, 15, 17, and 18), which explained between 11% and 29% of the total phenotypic variance. The combined effect of some of them in the same season explained up to 32.3% of the total variance (Table 3.3). Only Ma8 in the Sy map was consistent at the GW level; it was also consistent in the three maps at the LGW level (Annex 7), and the Ma17 QTL was stable over the years at the LGW level in the Mn map. New suggestive QTLs for malic acid were detected only at the LGW level (Ma2, Ma7, Ma11, and Ma14). The combined effect of some of them in the same season explained up to 65.5% of the total variance (Annex 7). The Ma7 and Ma11 QTLs were stable over the years in the Sy and Mn maps, respectively (Annex 7).

For the ratio of tartaric acid to malic acid three QTLs were found, on LGs 5, 8, and 11, explaining between 11% and 21% of the variance. Only the Tar/Ma8 QTL was consistent in the Sy map (Table 3.3), explaining up to 21.1% of the total variance. New suggestive QTLs for the ratio between tartaric acid and malic acid were detected only at the LGW level (Tar/Ma2, Tar/Ma7, Tar/Ma9, Tar/Ma14, Tar/Ma15, Tar/Ma17, and Tar/Ma18). The combined effect of some of them in the same season explained up to 39% of

the total variance (Annex 7). The Tar/Ma17 QTL was stable over the years in the Mn map (Annex 7).

One significant and consistent QTL was detected for total anthocyanin, on LG2 (Ant2), explaining up to 80% of the total phenotypic variance. New unstable QTLs for total anthocyanin were found at the LGW level on LGs 3, 8, 10, 14, 16, 17, 18, and 19. The combined effect of some of them in the same season explained up to 51.6% of the total variance (Annex 7).

Figure 3.3. One-LOD support confidence intervals of the main QTLs for enological traits detected with MQM.

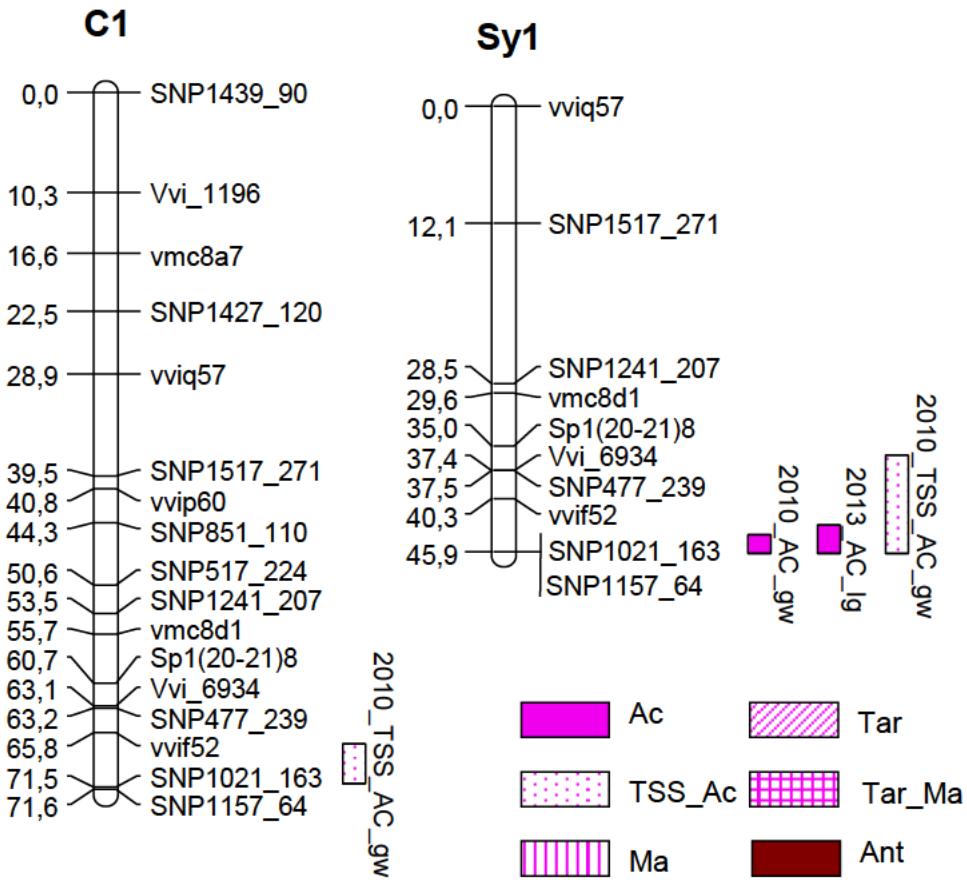


Figure 3.3. Continued.

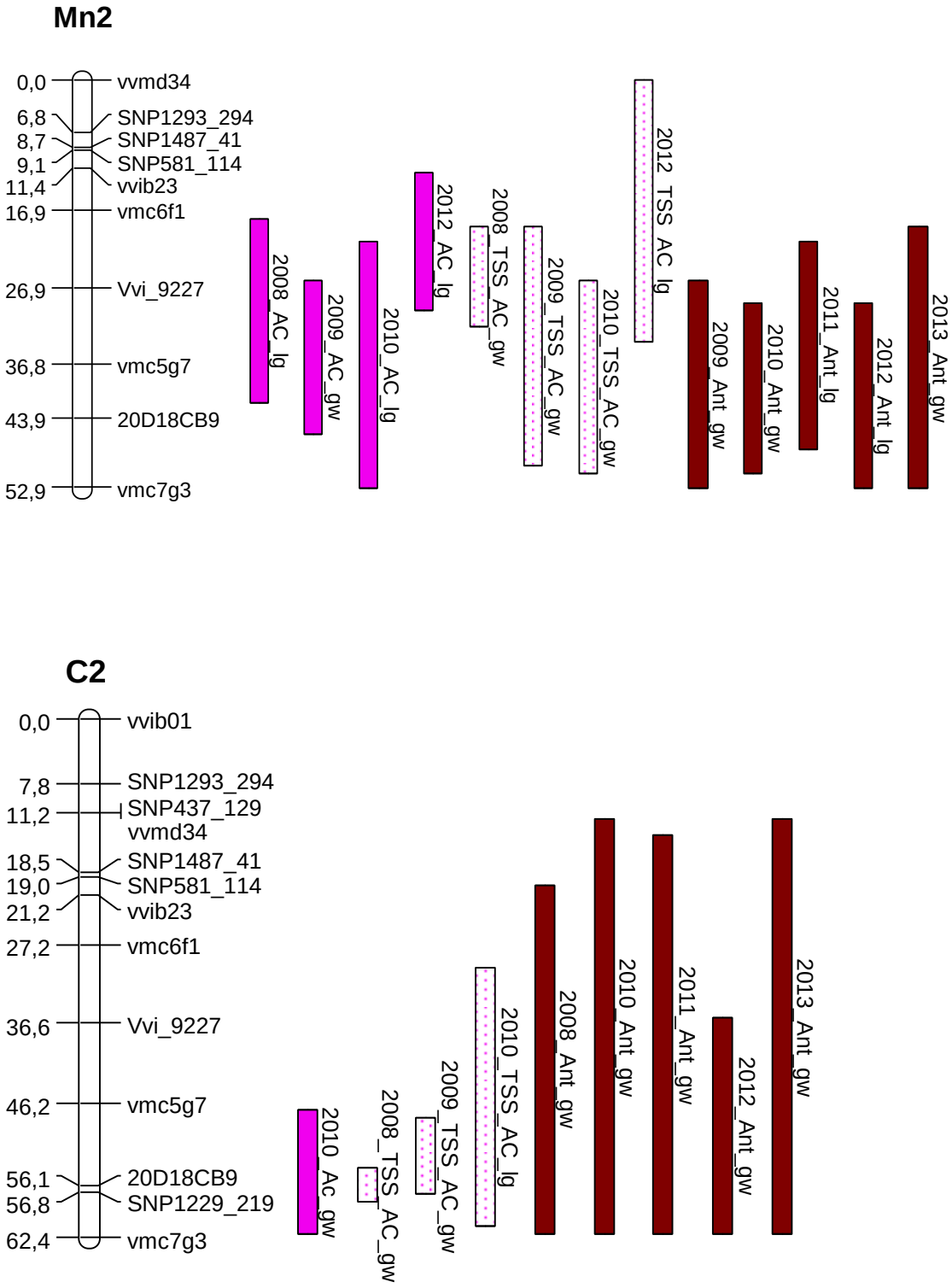
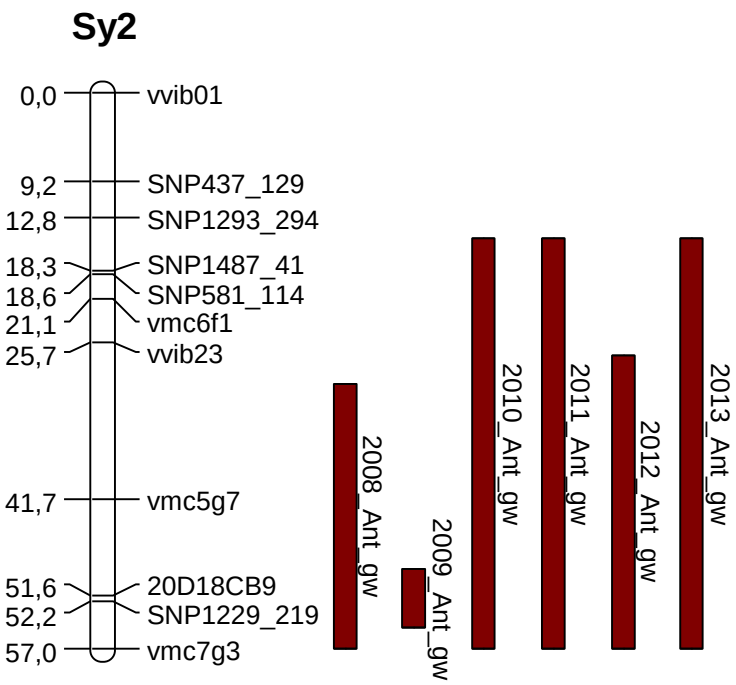


Figure 3.3. Continued.



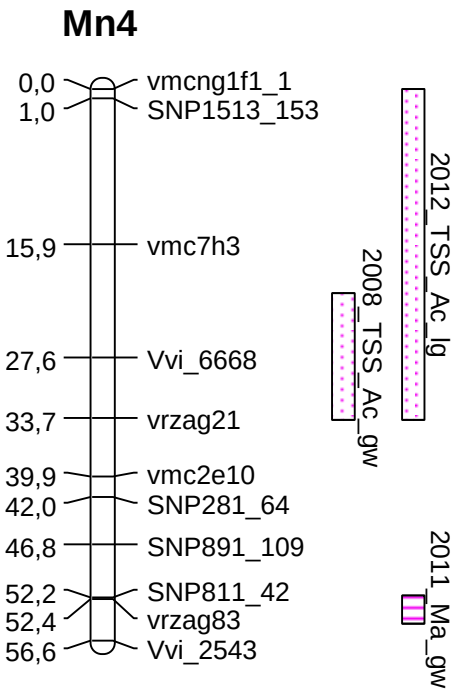


Figure 3.3. Continued.

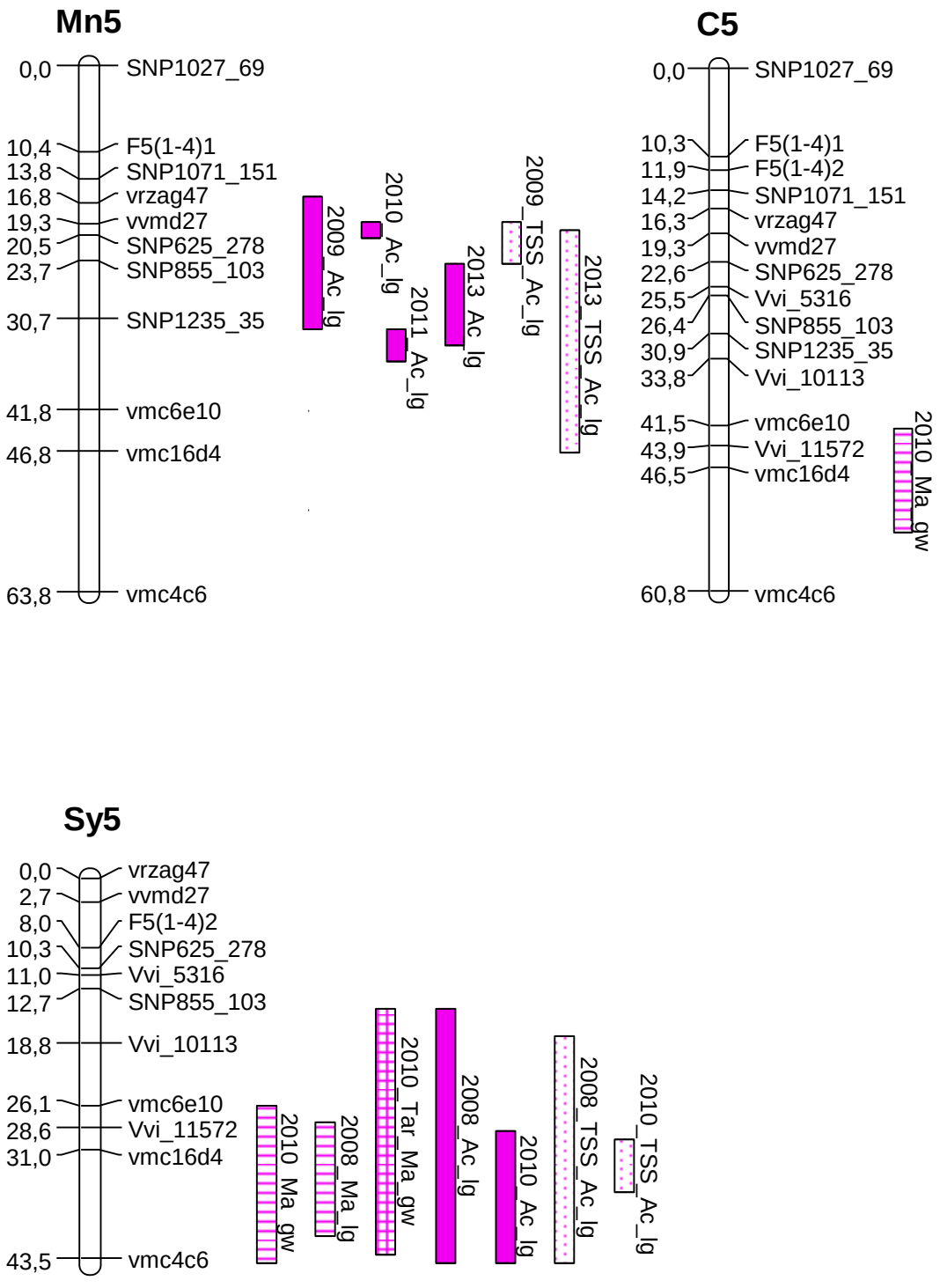


Figure 3.3. Continued.

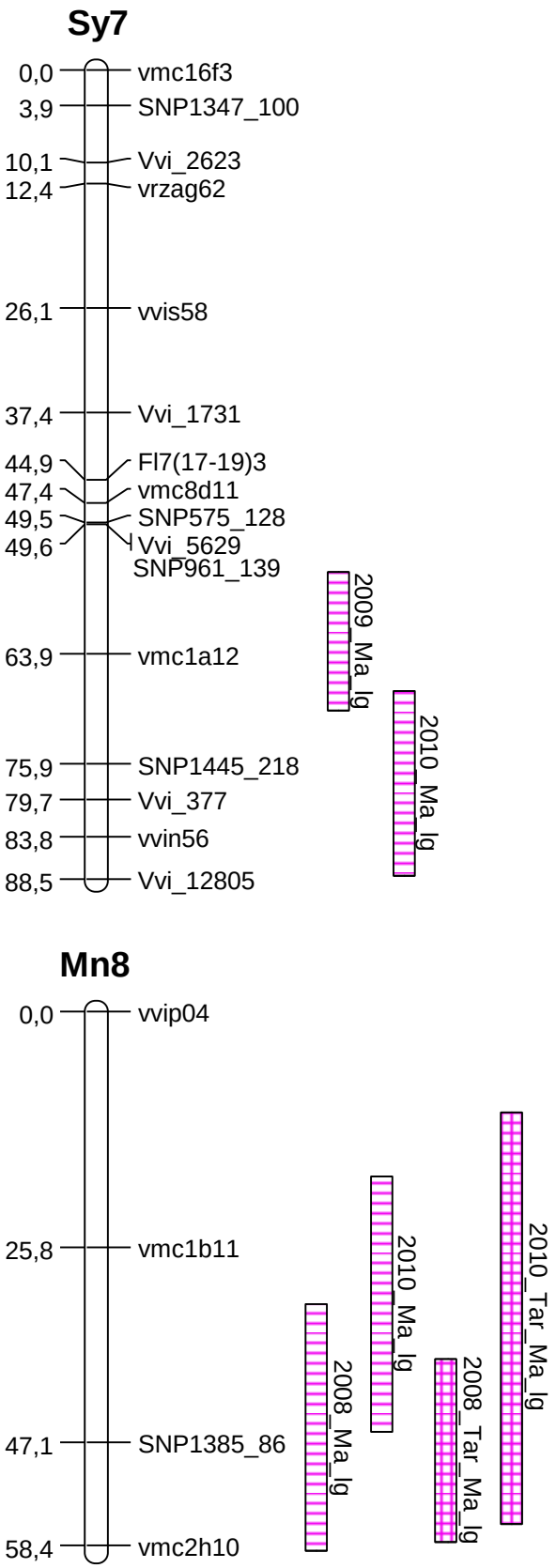
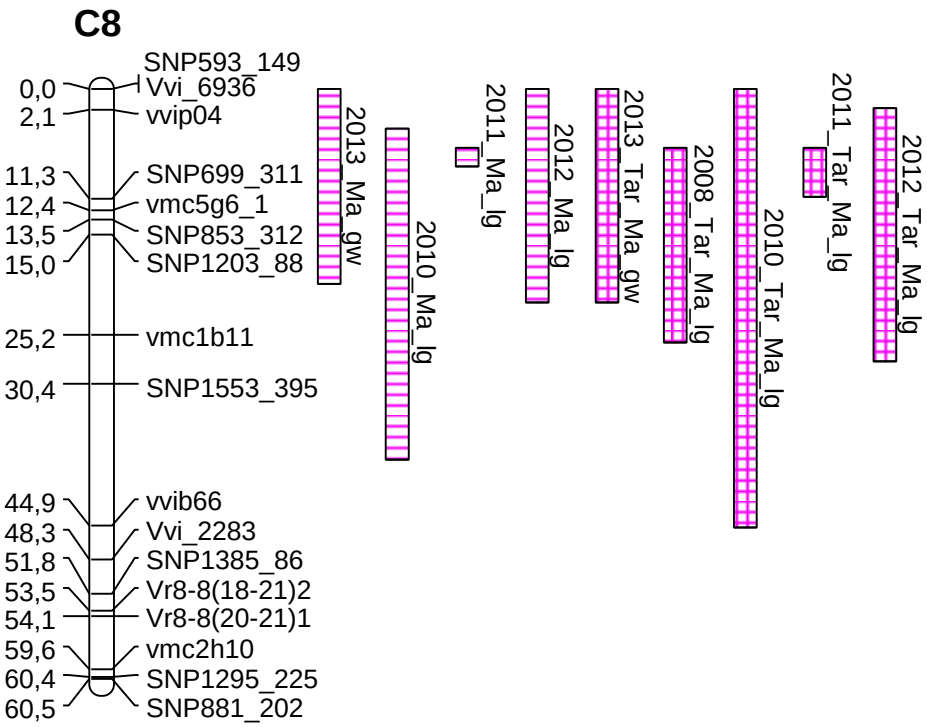


Figure 3.3. Continued.



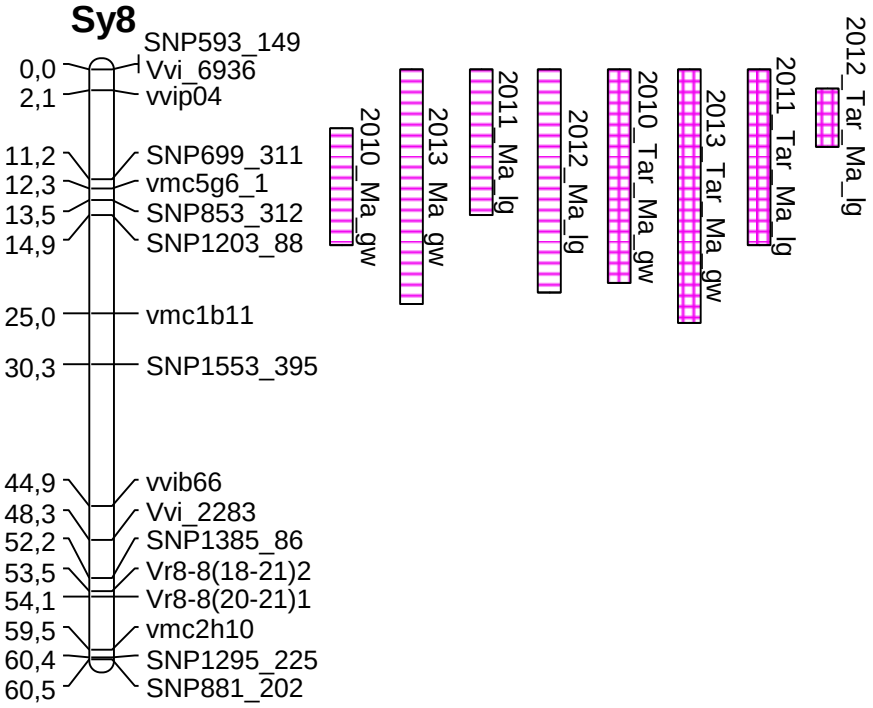


Figure 3.3. Continued.

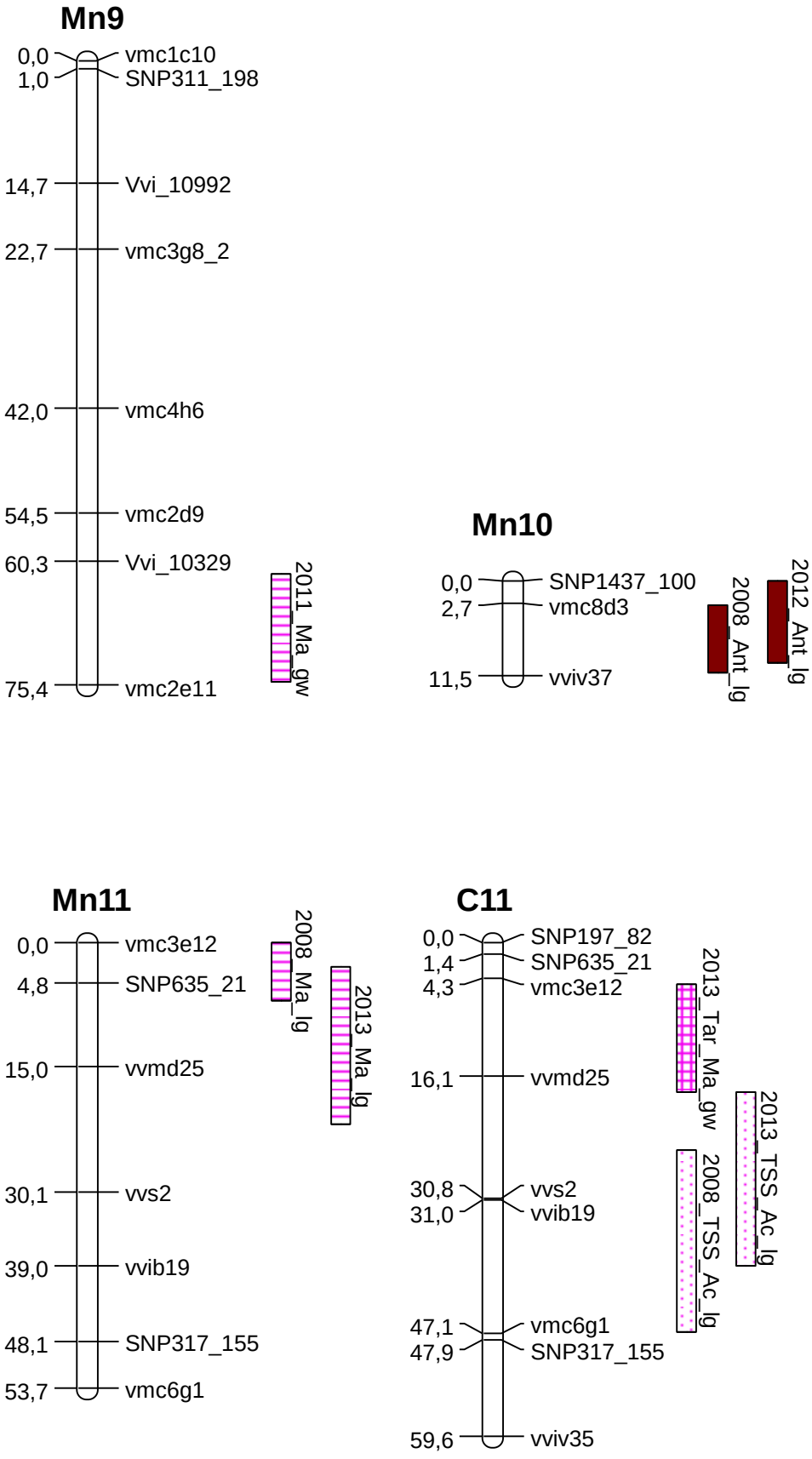


Figure 3.3. Continued.

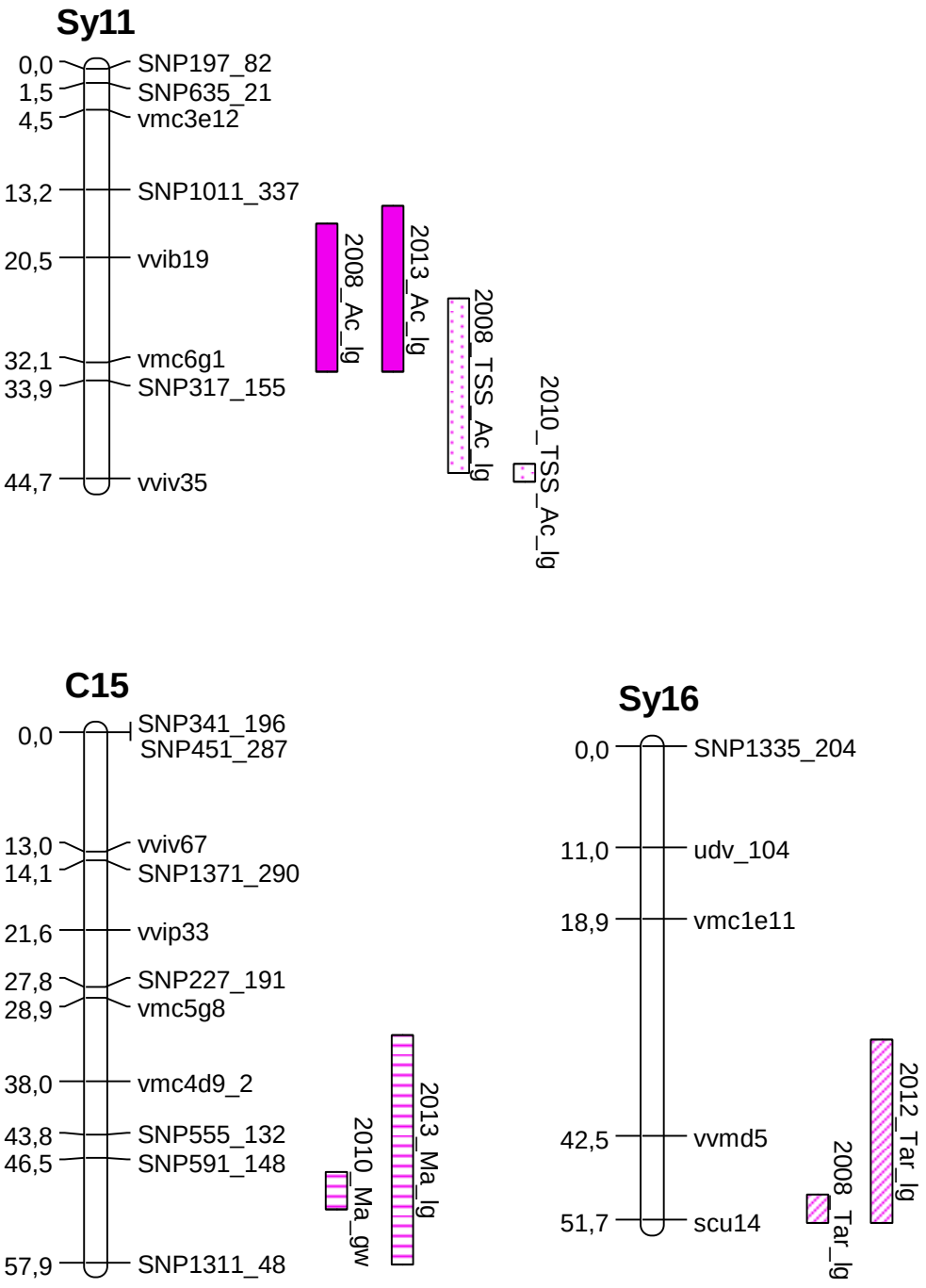


Figure 3.3. Continued.

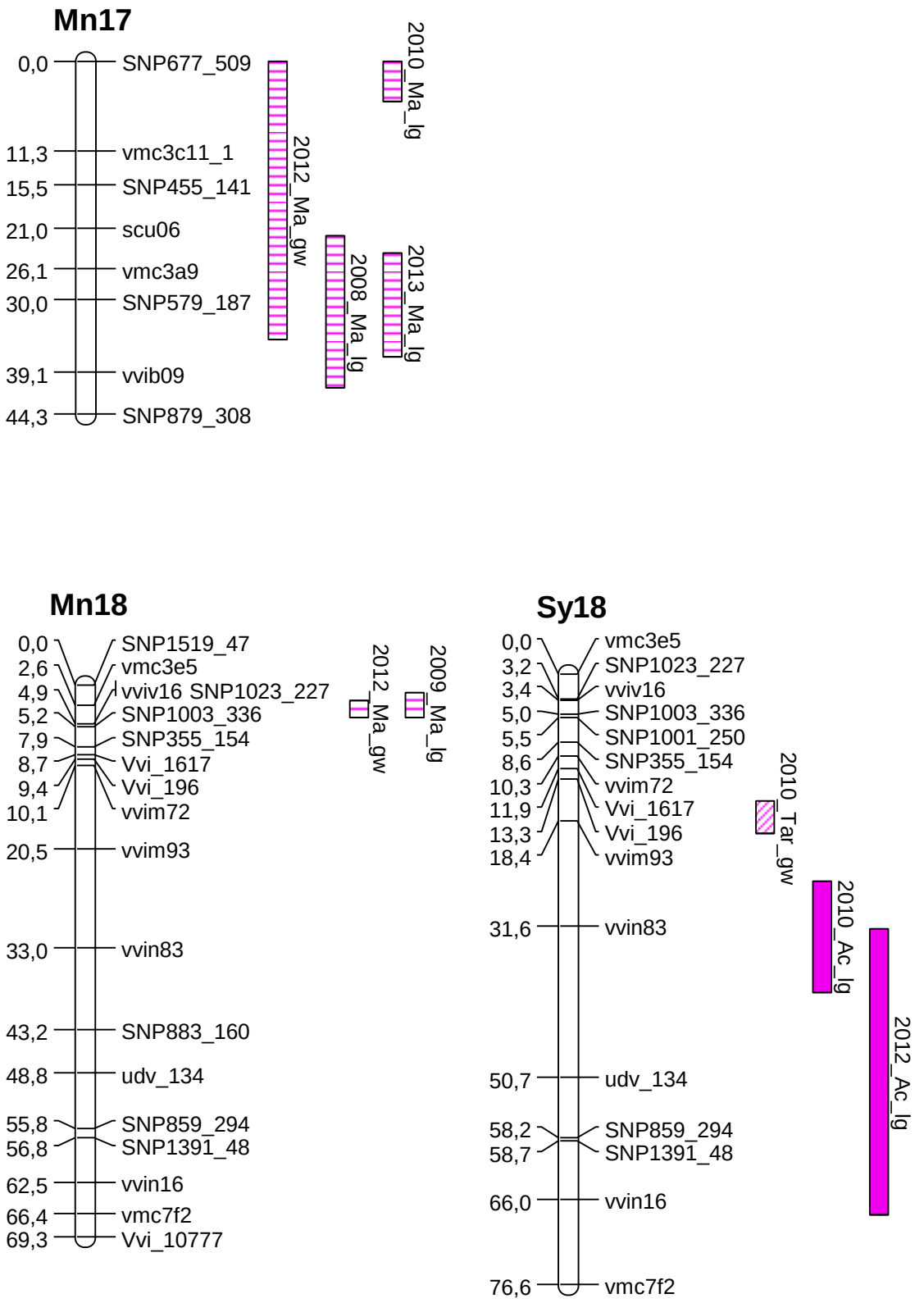
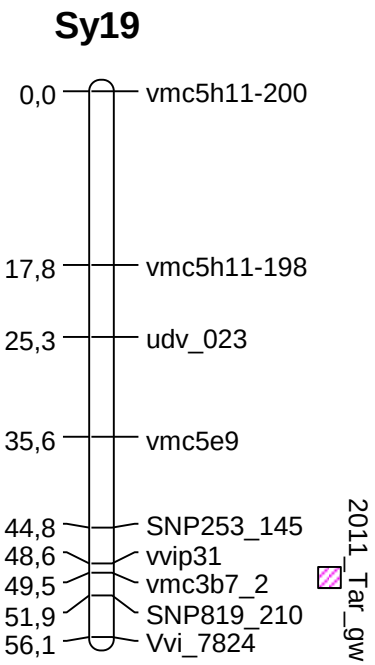


Figure 3.3. Continued.



The QTLs detected at the genome-wide level (gw) are represented, as are the QTLs detected only at the linkage group-wide (lg) level in at least two years or in two maps. The putative QTLs detected in only one year were included only if they collocated with the QTL interval found at the GW level. C, consensus; Mn, Monastrell; Sy, Syrah. Ac, Total acidity; TSS_Ac, Ratio of total soluble solids to total acidity; Ma, Malic acid; Tar, Tartaric acid; Tar_Ma, Ratio of tartaric acid to malic acid, and Ant, total anthocyanins.

3.4 Discussion

The QTL mapping methods rely on the assumption that the phenotype follows a normal distribution. In the present case, almost all the phenotype datasets displayed a non-normal distribution. Nevertheless, the SIM and MQM methods are quite robust against deviations from normality (van Ooijan 2009), therefore these methods were performed together with a maximum likelihood model and the permutation test based on the raw data. Moreover, recent work by Chen et al. (2015) produced similar QTL results with transformed and original data. On the other hand, most of the QTLs found by interval mapping were detected by KW tests, confirming that most

of the QTLs detected with interval mapping were not artefacts due to non-normal distribution of traits, large gaps, or segregation distortion.

The low number of genome-wide significant QTLs found could result from the limited size of the population analyzed in some seasons because of environmental conditions and disease incidence. In addition, the numbers of individuals analyzed for enological parameters were lower due to the minimal quantities of material necessary for these analyses.

In some cases significant QTLs (GW level) were detected only in a single year. This might be due to a limited power of detection because of a moderate population size, only one replicate vine, or alternatively to year effects and/or to genotype x year interactions. The environment affects the development of plants and modifies the harvest quality of the samples. All of these factors show the difficulties in QTL studies in fruit species. Nevertheless, some of the identified significant QTLs were also detected at the LGW level (putative QTLs) in other seasons and/or maps, providing additional evidence about their reliability. Also, the reliability of the results in some cases was supported by similar findings in other segregating populations, as discussed below.

Finally, the high number of QTLs detected for most of the traits analyzed shows the polygenic control of the respective traits. The percentage of total variance explained was low in most cases, indicating the existence of multiple genes involved in the control of the character. If a trait is composed of multiple QTLs the variance explained by each one is small. This complex genetic determinism, and the absence of major QTLs consistently explaining a large portion of the total phenotypic variance, makes it difficult to develop molecular markers for the genetic selection of the different traits studied. The markers significantly associated with the corresponding traits should be tested for their usefulness in marker-assisted selection.

In this work, some markers co-localized with QTLs and were significantly associated with the corresponding phenological, productive, morphological and enological traits in the KW analysis (Annex 4).

3.4.1 Phenological QTLs

Studies of phenology are important in the analysis of the behavior of a cultivar. For growers it is crucial to know these processes well enough to adequately perform cultural practices. There are four classical phenological stages (sprouting, flowering, veraison, and ripening). In grapevine the bud break is the first stage and starts when the required chilling hours are accumulated and the environmental conditions are favorable. To the best of our knowledge, there are only two reports of QTLs identified for sprouting in grapevine (Duchêne et al. 2012; Grzeskwiak et al. 2013), on LGs 4, 15, and 19. In Monastrell x Syrah progeny five new QTLs for bud break were found (Figure 3.1), explaining a low percentage of the phenotypic variance. However, only the Sp1 and Sp7 QTLs, found in the Mn and C maps, respectively, were stable over years.

Flowering is directly linked with the final production and involves complex processes. The initiation of the 'anlagen' starts in the year previous to the harvest and it is highly influenced by high temperatures and sun exposure. In the next season the final production depends on an effective fertilization, among other factors. In this work three significant QTLs for flowering time were found, but only Fw7 was consistent (Figure 3.1). This QTL has already been found in progenies from different crosses: Riesling x Gewurztraminer (Duchêne et al. 2012) and Syrah x Pinot Noir (Grzeskowiak et al. 2013). This locus contains several genes involved in the flowering process, such as *VvFT* (*Flowering Locus T*) and *VvSVP1* (*Short Vegetative Phase 1*): *VvFT* and *VvSVP1* cooperate to regulate flowering in *Arabidopsis* (Sreekantan and Thomas 2006; Carmona et al. 2007; Díaz-Riquelme et al. 2009). *VvSVP1* is expressed in

flowers and mediates the interaction with *FLC* (*Flowering Locus C*), according to the temperature. Moreover, a homolog of *VvSVP1* has been associated with a QTL of flowering in tomato (Jimenez-Gomez et al. 2007). The putative Fw8 QTL found on the Syrah map co-localized with the *VvAGL15.2* (*Agamous Like*) gene that is expressed in Arabidopsis flowers too. In the present work, the Fw14 QTL was found only in one year but in two maps (C and Mn), and has also been found in other progenies (Duchêne et al. 2012; Carreño 2012; Fechter et al. 2014) but in different positions. Other QTLs for this trait were found in other progenies on LGs 1, 2, 6, 15, and 18 (Costantini et al. 2008; Carreño 2012; Duchêne et al. 2012).

Veraison is composed of different processes such as softening and sugar and polyphenols accumulation. These results show only one stable QTL (Vr2), explaining up to 22% of the total phenotypic variance (Figure 3.1), in agreement with other authors (Costantini et al. 2008; Grzeskwiak et al. 2013). This QTL interval is close to the locus responsible for berry color, which includes the genes *VvMybA1*, *VvMybA2*, and *VvMybA3* involved in the regulation of anthocyanin biosynthesis (Kobayashi et al. 2004; Azuma et al. 2008; Fournier-Level et al. 2009). Significant, but not stable, Vr8 and Vr11 QTLs were confirmed by the KW test and have also been detected by Fischer et al. (2004). Moreover, recent work by Fechter et al. (2014) found the same QTL on LG11, in the same position. This work used a parent (Börner), derived by a cross between *V. riparia* and *V. cinerea*, with the objective of obtaining a pathogen-resistant rootstock. This result shows that the Vr11 QTL is present in different *Vitis* backgrounds. The putative Vr18 QTL, detected in two years in the present work, was found before by Duchêne et al. (2012). These authors found some genes in this interval related with sucrose (Gambetta et al. 2010), ABA, and a ripening-induced protein (Çakir et al. 2003). One marker associated with this QTL was VMC7F2; this is linked with the transcription factor *VvAGL11* (Mejía et al. 2011), related with seed

development. Other QTLs for this trait were found in different progenies on LGs 1, 3, 6, 15, 16, and 17 (Costantini et al. 2008; Carreño 2012; Duchêne et al. 2012; Grzeskowiak et al. 2013).

The results for the ripening date and veraison-ripening period were consistent in this study (LGs 2 and 17) (Figure 3.1). The transcription factors involved in anthocyanin synthesis, *VvMybA1*, *VvMybA2*, and *VvMybA3*, were located within the confidence interval of the Rp2 QTL. This QTL has also been detected by Costantini et al. (2008) and Grzeskowiak et al. (2013) in wine grape cultivars. The Rp17 QTL was also found by Mejía et al. (2007), and co-located with a QTL found for veraison by Carreño (2012), both in table grape progenies.

In this work, QTLs for sprouting and flowering co-localized on LG7 (in the Mn and consensus maps), and QTLs for flowering and veraison co-localized on LG8 (on the Sy map). These results suggest the implication of common genes in different developmental stages. In this sense, Sreekantan et al. (2010) proposed a group of candidate genes -regulated by the photoperiod- that could be involved in both dormancy and floral initiation.

3.4.2 Productive and morphological QTLs

The fertility index is an important parameter in grapevine, linked with the yield of a variety. In this work, only the significant Fi5 QTL was consistent, explaining up to 26.4% of the total phenotypic variance (Figure 3.2). This QTL has already been found in progenies from different crosses, by Doligez et al. (2010) and by Carreño (2012), suggesting that it interacts little with the genetic background and/or environment. The significant Fi3 QTL, detected in only one year on the Syrah map, has also been detected in the progeny Syrah x Pinot Noir (Grzeskowiak et al. 2013). These authors found that this locus contains several genes involved in cell wall biosynthesis, cell division, secondary metabolism, and a broad range of biochemical pathways.

Thus, these genes may be vital for normal plant growth and development. Other QTLs for this trait have been found in different progenies, on LGs 8, 9, 12, 14, and 18 (Fanizza et al. 2005; Doligez et al. 2010; Carreño 2012; Grzeskowiak et al. 2013).

The total production is one of the goals for growers; the number and weight of clusters, cluster compactness, and berry weight being associated with this parameter. For total production, one unstable QTL at the GW level was detected on LG8 (P8), collocating with CN8. Stable QTLs for total production were found only at the LGW level, on LGs 1, 5, and 8, and explained a low percentage of the phenotypic variance (Figure 3.2). Similarly, one significant QTL was detected for cluster weight, on LG19 (CW19) in the Sy map, but only in one year. Stable QTLs for this trait were found only at the LGW level, on LGs 1 and 5. Putative CW5 and CW17 QTLs, found only in one year in this work, have already been found by Fanizza et al. (2005).

Berry weight was under the control of six genomic regions, which explained up to 12% of the total variance. Three of these QTLs (BW5, BW14, and BW17) were stable over the years (Figure 3.2) and were confirmed by the KW test. The BW5 QTL showed different, but overlapping, confidence intervals through the years. It cannot be stated for sure whether there is one or more QTLs in this region or if it is the same QTL previously found by Fanizza et al. (2005), on LG5, for berry weight. The BW17 QTL has already been found by Doligez et al. (2013), and co-localized with three candidate genes: a cytochrome P450 78A-like protein linked with fruit weight in tomato, a WRKY transcription factor, and another transcription factor linked with the pre-veraison process. Although the BW7 QTL was detected only in one year, it has been reported also by Houel et al. (2015), who detected a major and stable QTL for berry weight on LG7, in both the green- and mature-grape phases and at different (controlled) temperatures.

Finally, two significant QTLs were detected for cluster compactness (CC2 and CC5) only in one season (Figure 3.2), and were confirmed by the KW test (Annex 4). These QTLs explained up to 28.3% of the total variance and were also detected in other seasons, but only at the LGW level. There are no previous publications concerning QTLs for this character.

It is worth highlighting the importance of LG5 in the control of all the production-related parameters studied in this work; QTLs for Fi and CC and for CW and BW were detected in similar QTL intervals on LG5 (Figure 3.2). The coincident interval between Fi and CC could be associated with the floral load - that affects both parameters. In this sense, Correa et al. (2014) studied the cluster architecture and found six QTLs for different parameters and ratios related to this trait in LG5. Also, some phenology and productivity QTLs co-localized, such as sprouting and berry weight on LG14 or ripening and berry weight on LG17.

3.4.3 Enological QTLs

The total acidity is the combination of both volatile (readily removed by steam distillation) and fixed (weakly volatile) acidity. The role of the acids in maintaining a low pH is crucial for the color stability of red wines. In this work, total acidity - expressed in tartaric acid equivalents - was under the control of two genomic regions (Ac1 and Ac2), which explained between 7% and 18% of the total phenotypic variance. Only the Ac2 QTL found in the consensus map was stable over years and was confirmed by the KW test. Although the Ac1 QTL was detected only in one year at the GW level, Viana et al. (2013) found a QTL for pH on LG1 in a table grape progeny. A consistent Ac18 QTL was found only at the LGW level and was confirmed by the KW test in this work. This result is in agreement with the results obtained by Chen et al. (2015) for total acidity.

Table 3.4. Genes located within the QTL intervals for total acidity.

Chromosome position	Gene Descriptor	Gene ID or Locus Tag	Position	Reference
Ch2:5062420..17197897	calcineurin B-like protein 01	GSVIVT00011978001	5592160..5598339	1
Ch5:17185187..24864931	2-isopropylmalate synthase 2, chloroplastic-like	100249820	20967342..21013384	2
	2,3-dimethylmalate lyase-like	GSVIVT00033586001	20385249..20410269	3
	probable UDP-sugar transporter protien SLC35A5-like	VIT_00013580001	21137773..21161143	
	fructokinase-2-like	GSVIVT00001970001	22616311..22620258	4
	3β-hydroxysteroid-dehydrogenase/decarboxylase isoform 3-like	VIT_00010844001	23306676..23315434	
	probable hydroxyacylglutathione hydrolase 2, chloroplast-like	GSVIVT00010300001	23317514..23328535	
Ch8:3320174..11073859	pyruvate decarboxylase 1	GSVIVT00001355001	8219848..8222883	5
	CBL-interacting protein kinase 07	VITISV_040419	10381868..10385364	6
Ch16:13708473..21845048	L-idonate dehydrogenase	GSVIVT00012394001	15651418..15653988	7
Ch17:0-9000000	malate synthase, glyoxysomal-like	100261216	1351757..1354948	8

The table shows the chromosome position of the QTL interval delimited by the closest markers, gene description, gene ID, gene position, and reference: (1) Cuéllar et al. 2013; (2) de Kraker et al. 2007; (3) Schnarrenberger and Martin 2002; (4) Pego and Smeekens 2000; (5) Or et al. 2000; (6) Weinl and Kudla 2009; (7) DeBolt et al. 2006, and (8) Schnarrenberger and Martin 2002.

In grapes, two dicarboxylic acids (tartaric and malic) often compose more than 90% of the fixed acidity (Jackson 2000; Conde et al. 2007). Malic acid may constitute about half the total acidity of grapes and wine. It is known that the principal synthesis of malic acid is linked with sugar metabolism (Sweetman et al. 2009). For that reason we looked for QTLs related to the total soluble solids/acidity ratio. A consistent QTL for the TSS/Ac ratio was found on LG2; it was confirmed by the KW test and co-located with Ac2 (Figure 3.3). In this region the *CBL01* (*calcineurin B-like protein 01*) gene was found (Table 3.4). This protein acts as a complex with a CBL-interacting

protein kinase (CIPK) in the activation of a K^+ channel of the Shaker family, *VvK1.2*. Cuéllar et al. (2013) found that the expression of the *VvK1.2* gene is induced in veraison and during ripening, and that the CBL/CIPK complex is involved in this activity. The CBL and CIPKs families develop several functions as stress responses: H^+ transport, K^+ homeostasis, or nitrate responses (Weinl and Kudla 2009). Two putative QTLs (Ac5 and TSS/Ac5) co-located on the Monastrell and Syrah maps and several genes were found within these QTLs intervals, related with the sugar and tricarboxylic acid (TCA) cycle. One gene encoded a probable UDP-sugar transporter (LOC100855157) and another a fructokinase-2-like activity (LOC100245852), both linked with sugar metabolism. Another candidate gene encoded a 2,3-dimethylmalate lyase-like activity (LOC100244697), involved in the TCA cycle and with citric acid. All of these could be associated with malic acid because it is a source of energy when sugar has been accumulated in the vacuoles (Table 3.4).

Two significant, but not stable, QTLs on LGs 18 and 19 were found for tartaric acid (Figure 3.3). Viana et al. (2013) also found a QTL for tartaric acid on LG19 of table grape. They used a progeny with *V. rupestris* and *V. arizonica* as grandparents. So, this QTL could have an ancestral origin in the genus *Vitis*. In this work, the QTL analysis showed the presence of ten QTLs at the LGW level, but only the putative Tar16 was stable on the Syrah map. In this QTL interval the gene encoding L-idonate dehydrogenase was found. This is the key enzyme in the conversion of L-idonate to 5-keto D-gluconic acid - that produces oxalic acid (DeBolt et al. 2006). It is highly expressed in the first phase of berry development, pre-veraison, when tartaric acid is accumulating. Because of the low degree of explanation of the phenotypic variance provided by this QTL, other pathways for tartaric acid production have to be presented in grapes. In this sense, Houel et al. (2015) found two

QTLs for tartaric acid, on LGs 4 and 7. In the current study, putative QTLs were also found on these LGs, but only for one year and in one parental map.

Consistent Ma8 and Tar/Ma8 QTLs were found (Figure 3.3) and were also confirmed by the KW test. Within these QTL intervals two candidate genes were found, for pyruvate decarboxylase 1 (*PDC1*) and a CBL-interacting protein kinase. *PDC1* was cloned in order to clarify the ethanol production in berries (Or et al. 2000). The increase in ethanol is linked with the pyruvate production derived from malic decarboxylation. *PDC* is the key enzyme in the fermentative metabolism and in the ethanol production. A malate synthase gene (LOC100261216) was located within the Ma17 and Tar/Ma17 QTLs intervals. This gene is up-regulated in the first phase of berry development, when malic acid is synthesized (Terrier et al. 2005). Finally, the putative Ma7 found in this work was also located by Houel et al. (2015) in another progeny.

Regarding the total anthocyanin content, one stable and significant QTL (Ant2) was found in the three maps (Figure 3.3); this was confirmed by the KW test. This QTL explained up to 80% of the total phenotypic variance. The transcription factors involved in anthocyanin synthesis, *VvMybA1*, *VvMybA2*, and *VvMybA3*, were located within this region. Recently, Costantini et al. (2015) have shown that almost all the LGs have a QTL linked with a specific anthocyanin. Other work detected QTLs related with anthocyanins in LGs 1, 8, and 14 (Azuma et al. 2015; Ban et al. 2014). In agreement with these results, in the present work one putative QTL, found on LG10 in two years on the Monastrell map (Figure 3.3), explained 12% of the phenotypic variance. Also, other putative QTLs were found on LGs 3, 8, 14, 16, 17, 18, and 19 (Annex 7), but only in one year.

3.5 Conclusions

Several QTLs have been identified for phenological, productive, morphological, and enological traits. The low number of genome-wide significant QTLs found could result from the limited size of the population analyzed in some seasons because of environmental conditions and disease incidence. Another reason could be the effect of the environment on the traits analyzed. The high number of QTLs detected for most of the traits analyzed shows the polygenic control of the respective traits. This complex genetic determinism, and the absence of major QTLs consistently explaining a large portion of the total phenotypic variance, makes it difficult to develop molecular markers for the genetic selection of the different traits studied.

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IV. GENERAL CONCLUSIONS

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In this work genetic maps were developed for a *Vitis vinifera* cross between two wine grape varieties, Monastrell and Syrah. These maps were used to carry out QTL detection for phenological stages and productive and fruit quality traits, contributing to our knowledge of the genetic determinants that control these traits of interest. The results allowed the following conclusions to be drawn:

(Chapter 1)

1. Crossing Monastrell and Syrah generated a large phenotypic variability that may be useful in the development of new cultivars with improved attributes.
2. Most of the phenotypic parameters evaluated showed transgressive and continuous variation within the progeny, suggesting a polygenic inheritance - in agreement with previous studies.
3. The year effect was significant in all the traits under study except for fertility index, visual color, cluster shape, total acidity, and total and extractable anthocyanins.
4. Weak or no correlation among traits indicated that traits such as fertility index, berry shape, cluster shape, cluster density, and tartaric acid may be genetically independent.
5. The genotypic evaluation of the color could be an important tool in the pre-selection of hybrids with a high content and extractability of anthocyanins.

(Chapter 2)

6. Parental and consensus genetic maps were developed using 251 informative markers (104 SSR, 146 SNP, and 1 CAPS). The observed sizes of the Monastrell, Syrah and consensus maps were 1035 cM, 1038 cM, and 1174 cM, respectively, with an observed coverage of 96%, 97%, and 98%. These results are within the range of other genetic

maps developed for grapevine.

7. Based on markers common to other published maps we were able to align all the LGs to the 19 chromosomes of grape. A general consistency in marker order was found compared with other published maps.
8. Eight new SNP markers, linked to six candidate genes that could be involved in the control of phenological stages and fertility index, were generated and mapped.

(Chapter 3)

9. Five QTLs were identified for sprouting, but only the QTLs on LG1 and LG7 were consistently significant at the genome-wide level, explaining up to 11.3% of the total phenotypic variance.
10. Three QTLs were located for flowering, but only the QTL on LG7 was consistent, explaining up to 19% of the total phenotypic variance. The *VvFT* gene was found within the confidence interval of this QTL.
11. The significant QTL detected on LG2 for veraison was consistent and explained up to 22.7% of the total variance.
12. Ripening time was under the control of two genomic regions on LGs 2 and 17, explaining up to 18% of the total variance. These QTLs co-located with the QTL detected for the veraison-ripening period.
13. Two significant QTLs were detected for fertility index on LGs 3 and 5. Only the QTL on LG5 was stable over years, explaining up to 26.4% of the total phenotypic variance.
14. For total production and number of clusters, one significant QTL was found on LG8, explaining up to 11.9% of the total variance. Similarly, one significant QTL was detected for cluster weight on LG19, explaining only 9.5% of the total variance.
15. Berry weight was under the control of six genomic regions, explaining up to 12% of total variance. Only the QTLs on LGs 5, 14, and 17 were

stable over the years.

16. Two significant QTLs were detected for cluster compactness on LGs 2 and 5, only in one season, explaining up to 28.3% of total variance. Nevertheless, these QTLs were also detected only at the LGW level in other seasons.
17. For total acidity two QTLs were detected on LGs 1 and 2, explaining up to 18% of total phenotypic variance. The QTL on LG2 co-located with the stable QTL detected for the ratio of total soluble solids to acidity, explaining up to 19% of total phenotypic variance. The *CBL01* gene was found within the confidence interval of this QTL.
18. Two QTLs were found for tartaric acid on LGs 18 and 19, explaining up to 16.1% of total phenotypic variance.
19. Malic acid was under the control of seven genomic regions, explaining up to 29% of total phenotypic variance. Only the QTL detected on LG8 was stable at the GW level, and co-located with the QTL detected for the ratio of tartaric acid to malic acid. The *PDC1* gene was found within the confidence interval of this QTL.
20. One significant and consistent QTL was detected for total anthocyanins, on LG2, explaining up to 80% of total variance. The transcription factors *VvmybA1*, *VvmybA2*, and *VvmybA3*, which control the berry color in grapevine, were located within this region.

ANNEXES

Annex 1. The 104 polymorphic SSRs and one CAPS used for the genotyping of the MnxSy progeny.

Locus	Segregation type	Mn genotype	Sy genotype	LG	Position	Distorsion	Tª (ºC)
vmc8a7	<abxaa>	156 158	158 158	1	16.554	-	61
vviq57	<abxac>	169 172	167 172	1	28.919	*******	54-51
vvip60	<abxaa>	315 319	315 315	1	40.836	-	55
vmc8d1	<aaxab>	196 196	200 210	1	55.699	-	63-60
vvif52	<abxcd>	259 268	257 279	1	65.824	-	61
vmc4f8	<aaxab>	115 115	116 121	1	unpositioned		56
vvib01	<aaxab>	292 292	292 296	2	0.000	-	56
vvmd34	<abxaa>	221 237	237 237	2	11.160	-	63-60
vvib23	<abxac>	281 283	283 289	2	21.176	*	57
vmc6f1	<abxac>	129 137	131 137	2	27.196	**	54-51
vmc5g7	<abxac>	197 209	197 215	2	46.226	-	54-51
20D18CB9[δ]	<abxab>	213 248	213 248	2	56.074	-	65
vmc7g3	<abxac>	115 117	115 131	2	62.369	-	58
vmc8f10	<abxac>	197 233	212 233	3	10.830	-	60
udv_043	<aaxab>	159 159	159 180	3	29.920	-	58
vvmd36	<abxcd>	260 266	250 289	3	37.862	-	63-60
vvin54	<abxac>	97 115	99 115	3	46.823	*******	54-51
vvmd28	<abxcd>	242 255	216 226	3	51.794	-	63-60
vmcng1f1_1	<abxac>	151 161	153 161	4	0.000	-	52
vmc7h3	<abxac>	133 136	135 162	4	15.812	-	54-51
vrzag21	<abxcd>	199 203	189 205	4	34.678	-	63-60
vmc2e10	<abxac>	56 58	69 58	4	41.267	-	56
vrzag83	<abxac>	191 201	195 201	4	52.058	*	65-62
vrzag47	<abxac>	155 165	165 167	5	16.323	**	61-58
vvmd27	<abxac>	179 188	188 190	5	19.326	-	62
vmc6e10	<abxcd>	90 115	104 107	5	41.502	-	58
vmc16d4	<abxcd>	154 170	168 205	5	46.464	-	55-52
vmc4c6	<abxcd>	165 168	159 177	5	60.824	-	50
udv_090	<aaxab>	145 145	143 171	6	5.437	-	57
vmc2h9	<abxcd>	114 120	116 155	6	27.819	-	56
vvmd21	<abxac>	240 246	245 263	6	43.593	*	61
vmc4g6	<abxaa>	119 123	132 132	6	48.082	-	55-52
vvin31	<abxac>	176 194	187 194	6	60.292	-	54-51
vvim43	<abxac>	81 99	81 97	6	68.067	-	54-51
vmc16f3	<abxcd>	177 184	180 188	7	0.000	*	55
vrzag62	<abxac>	189 204	189 194	7	13.131	*******	65-62
vvis58	<abxac>	303 305	303 292	7	27.162	-	56
vmc8d11	<abxac>	122 141	124 141	7	51.102	-	58
vmc1a12	<abxac>	118 137	137 150	7	66.109	*******	54
vvin56	<abxac>	161 172	169 171	7	86.236	-	57-54

Annex 1. Continued.

Locus	Segregation type	Mn genotype	Sy genotype	LG	Position	Distorsion	Tª (ºC)
vvip04	<abxcd>	85 126	96 103	8	2.059	-	53
vmc5g6_1	<aaxab>	138 138	114 148	8	12.373	-	55
vmc1b11	<abxac>	171 187	165 187	8	25.222	-	63-60
vvib66	<aaxab>	103 103	99 103	8	44.859	-	58-55
vmc2h10	<abxcd>	105 115	103 127	8	59.554	-	58
vmc1c10	<abxac>	168 171	159 171	9	0.000	-	63-60
vmc3g8_2	<abxac>	165 175	161 175	9	20.092	-	59-57
vmc4h6	<abxac>	157 161	161 181	9	40.347	-	59
vmc2d9	<abxaa>	79 89	89 89	9	53.094	-	54-51
vmc2e11	<abxaa>	102 106	106 106	9	74.049	*******	61
vmc3d7	<aaxab>	166 166	166 174	10	0.000	-	59
vmc2a10	<aaxab>	116 116	108 124	10	27.373	*******	60-57
vmc8d3	<abxac>	161 167	167 171	10	42.316	*******	56
vviv37	<abxac>	164 170	162 164	10	51.334	*******	52
vmc3e12	<abxac>	115 136	115 153	11	4.317	-	59
vvmd25	<abxaa>	239 261	239 239	11	16.062	**	63
vvs2	<abxaa>	130 150	130 130	11	30.799	**	58
vvib19	<abxab>	311 391	311 391	11	30.987	*******	54-51
vmc6g1	<abxac>	177 191	169 191	11	47.082	-	62-59
vviv35	<aaxab>	161 161	102 161	11	59.647	**	54
vmc8g6	<abxac>	136 170	166 170	12	5.825	-	55
vmc2h4	<abxaa>	215 227	215 215	12	18.259	-	55
scu05	<abxac>	129 178	129 168	12	30.247	*	63
vviv05	<abxac>	361 363	363 398	12	47.055	-	57
vmc8g9	<aaxab>	175 175	158 184	12	50.193	**	54
vmc4f3_1	<aaxab>	178 178	171 204	12	ungrouped		57
vmc3d12	<abxac>	200 205	200 216	13	0.000	**	57
vviv61	<abxac>	168 182	168 186	13	8.515	*******	56
vmc9h4_2	<abxac>	278 280	280 286	13	19.470	-	57
vmc2c7	<abxaa>	140 142	140 140	13	23.107	-	61-58
vvin62	<abxac>	356 360	354 360	13	unpositioned		55
vmcng1e1	<abxcd>	98 106	93 124	14	8.503	*******	63
vmc1e12	<abxac>	240 244	240 254	14	13.543	*******	63
vmc2h5	<abxac>	98 104	104 109	14	42.249	-	57
vvmd24	<abxac>	209 218	208 214	14	54.916	**	61-58
vmcng1g1_1	<abxcd>	176 180	218 223	14	67.546	-	54-51
vviv67	<abxcd>	355 362	359 379	15	12.955	-	55
vvip33	<abxab>	392 394	392 394	15	21.599	*******	56
vmc5g8	<abxcd>	301 317	309 315	15	28.910	-	56

Annex 1. Continued.

Locus	Segregation type	Mn genotype	Sy genotype	LG	Position	Distorsion	Tª (ºC)
vmc4d9_2	<abxcd>	226 234	228 238	15	37.993	-	62
vvin52	<abxaa>	84 100	84 84	16	0.000	-	56
udv_104	<abxcd>	186 216	155 221	16	16.646	-	56
vmc1e11	<abxcd>	187 193	195 205	16	23.781	-	60
vvmd5	<abxac>	224 238	224 230	16	52.566	-	54-51
scu14	<aaxab>	182 182	168 182	16	61.231	-	63
vmc3c11_1	<abxac>	110 115	106 110	17	10.566	-	58
scu06	<abxac>	174 178	172 174	17	19.057	-	58
vmc3a9	<abxac>	83 141	81 141	17	28.471	-	57
vvib09	<abxcd>	277 279	270 274	17	42.329	-	54-51
vvin73	<aaxab>	265 265	263 265	17	ungrouped		55-52
vmc3e5	<abxab>	108 110	108 110	18	0.000	*******	57
vviv16	<abxab>	102 104	102 104	18	4.931	*******	56
vvim72	<abxcd>	321 334	316 346	18	15.707	-	52-49
vvim93	<abxcd>	115 122	108 126	18	25.051	*	57-54
vvin83	<abxac>	235 237	233 235	18	40.131	-	56
udv_134	<abxac>	215 223	171 223	18	57.186	-	58
vvin16	<abxac>	152 158	150 152	18	71.361	-	52
vmc7f2	<abxac>	198 202	198 200	18	78.808	*	58
vmc5h11-198	<aaxab>	- -	-198	19	0.000	*	58
vmc5h11-200	<aaxa0>	200 -	200 -	19	0.377	*******	58
udv_023	<abxac>	180 200	196 200	19	8.301	-	58
vmc5e9	<abxcd>	211 225	215 219	19	18.730	*******	58
vvip31	<abxcd>	178 190	181 188	19	31.333	*******	58-55
vmc3b7_2	<abxac>	100 103	96 103	19	34.578	*******	58-55
vviv33	<abxaa>	339 353	343 343	19	51.994	-	56

δ CAPS marker.
For every locus the table shows the segregation type, the maternal (Mn, Monastrell) and paternal (Sy, Syrah) genotypes, the linkage group (LG), the position (cM) in the consensus map, the level of distortion (- none, *P<0.1, **P<0.05, *******P<0.0001), and the annealing temperature used for the amplification (Tª, ºC).
The ungrouped markers were assigned to a LG based on a previous reference map (Doligez et al. 2002).

Annex 2. The 146 polymorphic SNPs used for the genotyping of MnxSy progeny.

Locus	Segregation type	Mn genotype	Sy genotype	LG	Position (cM)	Distorsion
SNP1439_90	<abxaa>	AG	AA	1	0.000	-
Vvi_1196	<abxaa>	CT	TT	1	10.336	-
SNP1427_120	<abxaa>	AG	GG	1	22.490	-
SNP1517_271	<aaxab>	AA	AC	1	39.502	*
SNP851_110	<abxaa>	AG	AA	1	44.260	*
SNP517_224	<abxaa>	AG	AA	1	50.602	-
SNP1241_207	<abxab>	CT	CT	1	53.464	*******
Sp1(20-21)8$^{\delta}$	<aaxab>	TT	CT	1	60.668	*
Vvi_6934	<aaxab>	GG	CG	1	63.092	-
SNP477_239	<aaxab>	GG	CG	1	63.217	-
SNP1021_163	<aaxab>	GG	AG	1	71.542	-
SNP1157_64	<aaxab>	TT	AT	1	71.562	-
SNP1025_100	<abxaa>	CT	CC	1	unpositioned	
SNP1527_144	<aaxab>	TT	CT	1	unpositioned	
SNP229_112	<aaxab>	CC	AC	1	unpositioned	
SNP269_308	<abxaa>	AG	AA	1	unpositioned	
SNP683_120	<aaxab>	GG	AG	1	unpositioned	
SNP1293_294	<abxab>	AG	AG	2	7.830	*******
SNP437_129	<aaxab>	TT	CT	2	11.153	-
SNP1487_41	<abxab>	AG	AG	2	18.541	*******
SNP581_114	<abxab>	AG	AG	2	19.033	*******
Vvi_9227	<abxaa>	AT	TT	2	36.569	-
SNP1229_219	<aaxab>	CC	CG	2	56.758	-
SNP613_315	<abxaa>	CT	CC	3	0.000	-
SNP553_98	<aaxab>	AA	AG	3	16.405	-
F3(3-6)6$^{\delta}$	<aaxab>	CC	CG	3	20.283	-
SNP1563_280	<aaxab>	CC	CT	3	36.118	-
SNP867_170	<aaxab>	GG	CG	3	36.131	-
SNP1513_153	<abxaa>	CT	CC	4	1.004	-
SNP655_93	<aaxab>	CC	CT	4	16.680	-
SNP191_100	<aaxab>	CC	CT	4	25.597	-
Vvi_6668	<abxaa>	CG	CC	4	28.194	**
SNP715_260	<aaxab>	CC	CT	4	29.576	-
SNP281_64	<abxab>	AT	AT	4	43.490	*******
SNP891_109	<abxaa>	AG	GG	4	47.004	-
SNP551_351	<aaxab>	CC	CT	4	49.090	-
SNP811_42	<abxab>	AT	AT	4	50.503	*******

Annex 2. Continued.

Locus	Segregation type	Mn genotype	Sy genotype	LG	Position (cM)	Distorsion
SNP1559_291	<abxab>	CG	CG	4	50.927	*******
Vvi_2543	<abxaa>	AT	TT	4	56.256	-
Vvi_10516	<aaxab>	CC	AC	4	58.578	-
SNP1027_69	<abxaa>	CT	CC	5	0.000	-
F5(1-4)1$^{\delta}$	<abxaa>	AC	AA	5	10.327	-
F5(1-4)2$^{\delta}$	<abxab>	AT	AT	5	11.949	**
SNP1071_151	<abxaa>	CT	CC	5	14.164	-
SNP625_278	<abxab>	AC	AC	5	22.601	*******
Vvi_5316	<aaxab>	CC	AC	5	25.545	-
SNP855_103	<abxab>	CT	CT	5	26.400	*******
SNP1235_35	<abxaa>	CT	CC	5	30.888	-
Vvi_10113	<aaxab>	AA	AG	5	33.775	-
Vvi_11572	<aaxab>	CC	CT	5	43.939	-
SNP1109_253	<aaxab>	TT	CT	6	0.000	-
SNP945_88	<aaxab>	AA	AG	6	0.485	-
SNP709_258	<abxaa>	CT	CC	6	19.583	-
SNP873_244	<abxab>	CT	CT	6	20.590	*******
SNP1213_99	<aaxab>	TT	GT	6	22.067	-
SNP915_88	<aaxab>	AA	AC	6	24.978	-
Vvi_2021	<abxaa>	CT	CC	6	25.830	-
SNP1393_62	<aaxab>	GG	GT	6	29.402	*
SNP1473_95	<aaxab>	CC	CT	6	50.800	**
SNP559_110	<abxaa>	AG	GG	6	51.672	-
SNP1347_100	<abxab>	AG	AG	7	4.348	*******
Vvi_2623	<abxab>	GT	GT	7	9.462	*******
Vvi_13076	<abxaa>	AT	TT	7	22.071	*
Vvi_3400	<aaxab>	TT	GT	7	24.782	-
Vvi_1731	<abxab>	AC	AC	7	37.292	*******
Vvi_5629	<aaxab>	CC	CT	7	49.371	-
SNP575_128	<aaxab>	CC	CT	7	49.378	-
SNP961_139	<aaxab>	CC	CT	7	49.393	-
Fl7(17-19)3$^{\delta}$	<abxab>	CT	CT	7	55.838	-
SNP1445_218	<aaxab>	AA	AG	7	78.053	-
Vvi_377	<aaxab>	TT	GT	7	81.874	-
Vvi_12805	<aaxab>	CC	CT	7	90.771	-
SNP1011_337	<aaxab>	TT	CT	7	unpositioned	-
Vvi_6936	<aaxab>	TT	AT	8	0.000	-
SNP593_149	<aaxab>	TT	CT	8	0.001	-
SNP699_311	<aaxab>	TT	CT	8	11.262	-
SNP853_312	<aaxab>	AA	AT	8	13.534	-

Annex 2. Continued.

Locus	Segregation type	Mn genotype	Sy genotype	LG	Position (cM)	Distorsion
SNP1203_88	<aaxab>	TT	AT	8	14.969	-
SNP1553_395	<aaxab>	AA	AG	8	30.403	-
Vvi_2283	<aaxab>	GG	AG	8	48.262	-
SNP1385_86	<abxab>	GT	GT	8	51.832	*******
Vr8(18-21)2$^{\delta}$	<aaxab>	AA	AT	8	53.517	-
Vr8(20-21)1$^{\delta}$	<aaxab>	CC	CT	8	54.118	-
SNP1295_225	<aaxab>	CC	CT	8	60.407	-
SNP881_202	<aaxab>	AA	AG	8	60.522	-
SNP311_198	<abxaa>	AC	AA	9	0.600	**
SNP663_578	<aaxab>	GG	AG	9	4.433	*
Vvi_10992	<abxaa>	AT	AA	9	13.245	*
Vvi_7871	<aaxab>	CC	CT	9	28.111	-
Vvi_10329	<abxaa>	CT	TT	9	58.915	-
SNP947_288	<aaxab>	GG	AG	10	7.801	-
SNP1437_100	<abxab>	AG	AG	10	40.211	*******
SNP447_244	<aaxab>	TT	CT	10	41.590	*******
SNP197_82	<aaxab>	AA	AC	11	0.000	-
SNP635_21	<abxab>	AG	AG	11	1.446	*******
SNP317_155	<abxab>	GT	GT	11	47.855	*******
SNP1215_138	<abxaa>	CT	TT	12	0.000	-
SNP189_131	<abxaa>	CT	TT	12	7.653	-
SNP651_658	<aaxab>	CC	CT	12	19.039	-
Vvi_589	<aaxab>	CC	CT	12	34.779	*
Vvi_12882	<aaxab>	CC	CT	12	34.787	*
SNP1119_176	<abxab>	AC	AC	12	54.502	*******
SNP653_90	<abxaa>	CG	GG	13	13.286	-
SNP351_85	<abxaa>	CT	TT	13	16.688	-
Vvi_7387	<abxaa>	AG	AA	13	17.586	-
SNP259_199	<abxaa>	AT	TT	13	25.085	-
SNP1577_72	<aaxab>	CC	CT	13	42.261	-
SNP659_73	<aaxab>	CC	CT	13	unpositioned	
SNP605_120i	<aaxab>	--	-A	14	0.000	-
Sp14(0-4)2$^{\delta}$	<aaxab>	TT	CT	14	5.147	-
SNP251_159	<abxaa>	AG	GG	14	8.009	*******
SNP897_57	<aaxab>	TT	AT	14	15.558	-
Vvi_2292	<abxaa>	AG	AA	14	38.409	-
SNP1411_565	<abxab>	AT	AT	14	52.366	*******
SNP421_234	<aaxab>	AA	AG	14	56.743	-
Vvi_3163	<abxaa>	AG	AA	14	60.885	-
SNP1035_226	<aaxab>	TT	CT	14	73.083	-

Annex 2. Continued.

Locus	Segregation type	Mn genotype	Sy genotype	LG	Position (cM)	Distorsion
SNP341_196	<abxab>	CG	CG	15	0.000	*******
SNP451_287	<abxab>	CG	CG	15	0.018	*******
SNP1371_290	<abxaa>	AC	AA	15	14.130	-
SNP227_191	<abxab>	AC	AC	15	27.801	*******
SNP555_132	<abxaa>	AC	AA	15	43.756	-
SNP591_148	<abxab>	AT	AT	15	46.461	*******
SNP1311_48	<aaxab>	CC	CT	15	57.895	-
SNP1335_204	<aaxab>	CC	CT	16	5.391	-
SNP1079_58	<abxaa>	AG	GG	16	22.166	-
SNP677_509	<abxaa>	GT	GG	17	0.000	-
Vvi_6987	<aaxab>	GG	GT	17	6.626	-
SNP455_141	<abxaa>	CT	TT	17	15.657	-
SNP579_187	<abxaa>	CT	TT	17	31.254	-
SNP879_308	<abxaa>	AG	AA	17	46.250	-
SNP1023_227	<abxab>	CT	CT	18	4.061	*******
SNP1001_250	<aaxab>	GG	AG	18	9.941	*
SNP1519_47	<abxaa>	CT	CC	18	10.236	-
SNP355_154	<abxab>	CT	CT	18	12.714	*******
Vvi_1617	<abxab>	AC	AC	18	16.731	*******
Vvi_196	<abxab>	GT	GT	18	18.810	*******
SNP883_160	<abxaa>	CG	GG	18	51.249	-
SNP859_294	<abxab>	CT	CT	18	63.969	*******
SNP1391_48	<abxab>	AG	AG	18	64.999	*******
Vvi_10777	<abxaa>	CT	CC	18	76.702	-
SNP1003_336	<abxab>	AC	AC	18	unpositioned	-
SNP253_145	<aaxab>	CC	AC	19	28.640	*******
SNP819_210	<aaxab>	AA	AT	19	35.937	*******
Vvi_7824	<abxab>	AG	AG	19	43.684	*******
Vvi_2319	<abxab>	CT	CT	19	ungrouped	

For every SNP the table shows the segregation type, the maternal (Mn, Monastrell) and paternal (Sy, Syrah) genotypes, the linkage group (LG), the position (cM) in the consensus map, and the level of distortion (- none, *P<0.1, **P<0.05, *******P<0.0001). The ungrouped markers were assigned to a LG based on previous maps (Cabezas et al. 2011).
δ Locus selected by CG systems

Annex 3. Primer sequences of the eight new SNPs developed using six candidate genes.

Marker name	Primer sequence	GO_ID
Sp1(20-21)8	Fwd: GACAGTGCCGCCATTAAGCT	GO:0006355
	Rev: AAAATGGCTGCCCATCGTGA	
F3(3-6)6	Fwd: TCCCTCTTGCACTCATGCCTA	GO:0045449
	Rev: CTCGCCTTTGAGGAGTCACC	
F5(1-4)1	Fwd: CGGTGCAGGTGTTAATGTGAC	GO:0005634
	Rev: TGCCCATGAGGTGGGGTATT	
F5(1-4)2	Fwd: TCGGCTCTATGGGATTGGGG	
	Rev: GTCACATTAACACCTGCACCGA	
Fl-7(17-19)3	Fwd: GTGCAGCAGTTCTGTAGCGA	GO:0003677
	Rev: AAATCACAAGCACACGCACG	
Vr8(18-21)2	Fwd: GGCTTGACAGTACCTCAGGTCT	GO:0005618
	Rev: GCTTCCCGAATGTGGTTCCA	
Vr8(20-21)1	Fwd: GGCTTGACAGTACCTCAGGTCT	
	Rev: GCTTCCCGAATGTGGTTCCA	
Sp14(0-4)2	Fwd: GAGGGCTAGTGGAGAGGCTT	GO:0005634
	Rev: GGTCATGCCACACACCTTCA	

The marker name refers to each trait; Sp (Sprouting), F (Fertility index), Fl (Flowering), Vr (Veraison). Forward (Fwd) and reverse (Rev) sequences were used for the specific amplification. GO_ID, code of the Gene Ontology Consortium.

Annex 4. QTLs detected using non-parametric Kruskal-Wallis (KW) analysis.

Trait	LG	Map	Year	Marker	Kw
Sp	1	Mn	08, 09	vvif52	6, 7
		Mn/C	09	vvip60	6
	8	Mn/C	08/09	vmc1b11	7
	13	Mn/C	09	SNP653_90	7/6
	14	Mn/C	08	SNP251_159	7
				vmcng1e1	7
				vmc1e12	7
Fw	7	Mn/C	10	Vvi_13076	7
	14	Mn/C	08	SNP251_159	7
				vmcng1e1	7
				vmc1e12	7
Vr	2	Mn/C	08 ,09	vvmd34	7
				vvib23	7
			08, 09/09	vmc6f1	6, 7/6
			08	Vvi_9227	7
		Mn/Sy/C	09	SNP581_114	6
	8	Sy/C	08	SNP853_312	6
	11	Mn/Sy	08	vvib19	6
Rp	2	Mn/C	09, 10	vmc5g7	6, 7
			10	Vvi_9227	7
		Mn/Sy/C	09, 10	20D18CB9	6, 7/6, 7/7
	17	Mn/C	09	SNP455_141	6
Vr_Rp	2	Mn/C	08,09	vmc6f1	6, 7
			08, 09, 10	Vvi_9227	6, 7, 7
				vmc5g7	7
			09	vvib23	7
				vvmd34	7
		Mn/Sy/C	08, 09, 10	20D18CB9	7
		C	08, 10	vmc7g3	7

Annex 4. Continued.

Trait	LG	Map	Year	Marker	Kw
Fi	3	Sy/C	08	udv_043	6
	5	Mn/C	08, 09, 13	SNP1027_69	7
			13	F5(1-4)1	6
				SNP1071_151	7
		Sy/C	09, 13/08, 09, 13	vrzag47	6/7
			08,09/08	Vvi_5316	6
			08	Vvi_10113	6
		C	08, 09, 13	vvmd27	7
P	5	Mn/Sy/C	08	SNP625_278	6
		Sy/C	10	Vvi_5316	6
	8	Mn/Sy/C	09	SNP1385_86	6
CW	2	Mn/Sy	08	SNP1487_41	6
	5	Mn/Sy/C	08	SNP625_278	6
	14	Mn/C	09	SNP251_159	6
BW	5	Mn/C	09	SNP1027_69	6
			09, 10/09	SNP1235_35	6
		Mn/Sy/C	10	SNP855_103	6
	7	Mn/Sy/C	10	Vvi_1731	6
	14	Mn/C	09	SNP251_159	6
				vmcng1e1	7/6
	17	Sy/C	10	Vvi_6987	7
				vmc3c11_1	7/6
CC	2	Mn/Sy/C	08	SNP1487_41	6
				SNP581_114	7/7/6
	5	Mn/C	10	SNP1027_69	6
		Sy/C	10	vvmd27	6

Annex 4. Continued.

Trait	LG	Map	Year	Marker	Kw
Ac	2	Mn/C	09	Vvi_9227	6
		Mn/Sy/C	09/09/10	20D18CB9	6/7/7
	18	Mn/Sy/C	12	vmc3e5	6
TSS/Ac	2	Mn	09, 10	vmc5g7	7, 6
		Mn/Sy/C	09, 10/09, 10/08, 09	20D18CB9	7, 6/7/7
		Mn/C	09	Vvi_9227	7/6
	18	Mn/Sy/C	12	vmc3e5	6
Ma	2	Mn/C	10	Vvi_9227	6
	8	Sy/C	13	Vvi_6936	7
				SNP593_149	7
				vvip04	7
				SNP699_311	7
				SNP1203_88	7
			10, 13	vmc5g6_1	6, 7
				SNP853_312	6, 7
	17	Mn/C	12	SNP677_509	6
Tar/Ma	1	Mn/Sy/C	09/08/09	SNP1241_207	6/6/7
	2	Mn/C	10	Vvi_9227	6
	8	Sy/C	10, 13	SNP699_311	6, 7
				vmc5g6_1	6, 7
				SNP853_312	6, 7
			13	Vvi_6936	7
				SNP593_149	7
				vvip04	7
				SNP1203_88	7
Ant	2	Mn/Sy/C	13	SNP1487_41	6
			10/10-13/08,10-13	vmc6f1	6/7/7,7,7,6,7
			09-13/08,10-13/08-13	vmc5g7	7,7,6,6,7/6,7,7,7,7/7
			09-13/09-13/08-13	20D18CB9	7
			09,13/08-13/09-13	vmc7g3	7,6/7,6,7,7,7,7/7
		Mn/C	13	Vvi_9227	6
		Sy/C	10-13/08,10-13	vvib23	7
			08,10-13/08-13	SNP1229_219	7/7,6,7,7,7,7
		Sy	11, 13	SNP437_129	6
		C	11, 13	SNP1293_129	6

Markers statistically associated with traits are shown: Sp, sprouting; Fw, flowering; Vr, veraison; Rp, ripening; Vr_Rp, veraison ripening period; Fi, fertility index; P, total production; CW, cluster weight; BW, berry weight; CC, cluster compactness; Ac, total acidity; TSS/Ac, ratio of total soluble solids to total acidity; Ma, malic acid; Tar/Ma, ratio of tartaric acid to malic acid; Ant, total anthocyanins. The linkage group (LG), map (Mn, Monastrell; Sy, Syrah; C, Consensus), year (08, 2008; 09, 2009; 10, 2010; 11, 2011; 12, 2012, 13, 2013), and KW significance (6 = 0.0005; 7 = 0.0001) are shown.

Annex 5. Putative QTLs identified for phenological traits in maps of the Monastrell x Syrah progeny.

Trait	Map	Year	LG	QTL	LOD max	cM	Confidence interval	Cofactor	LGW LOD threshold	% variance QTL	% variance model
Sp	C	2008	1	Sp1	4.51	55.7	35-72	vvif52	2.6	6.6	13.4
			8	Sp8	3.71	7.1	0-19	vvip04	2.6	6.6	
		2009	3	Sp3	3.85	42.9	39-52	vvmd28	2.4	9.1	13.0
			17	Sp17	3.33	10.6	0-15	vmc3c11_1	2.5	6.1	
		2010	2	Sp2	3.29	21.2	11-24	SNP581_114	2.6	5.5	21.7
			8	Sp8	3.23	13.5	2-33	vmc1b11	2.7	7.2	
			11	Sp11	3.16	52.9	47-58	SNP317_155	2.5	6.5	
			13	Sp13	3.08	5.0	0-16	SNP653_90	2.3	5.8	
	Mn	2008	8	Sp8	2.98	10.0	0-40	vvip04	1.3	8.0	8.0
		2010	8	Sp8	2.0	10.0	0-48	vvip04	1.3	5.6	5.6
	Sy	2008	12	Sp12	2.45	45.3	33-45	vmc8g9	1.4	5.3	5.3
		2009	3	Sp3	2.7	41.3	21-41	vvmd28	1.4	5.3	5.3
		2010	2	Sp2	1.65	25.7	24-32	vvib23	1.4	3.1	7.4
			11	Sp11	1.84	38.9	33-44	SNP317_155	1.3	4.3	
Fw	C	2008	7	Fw7	3.61	13.1	10-18	vrzag62	2.8	9.4	19.2
			8	Fw8	3.51	54.1	49-60	Vr8(18-21)2	2.6	9.4	
		2009	7	Fw7	3.49	37.3	32-51	Vvi_1731	2.6	12.2	12.2
	Mn	2009	5	Fw5	1.41	30.7	26-38	SNP1235_35	1.2	5.2	5.2
	Sy	2008	8	Fw8	2.48	54.1	50-60	Vr8(18-21)2	1.6	7.4	7.4
		2009	8	Fw8	1.81	52.2	40-60	Vr8(20-21)1	1.2	5.5	5.5
		2010	8	Fw8	1.94	60.4	60-60.4	SNP1295_225	1.6	4.8	10.2
			12	Fw12	2.3	38.1	33-42	vviv05	1.4	5.7	
Vr	C	2008	5	Vr5	3.24	51.5	43-60	vmc4c6	2.6	9.4	25.1
			8	Vr8	2.93	7.1	0-20	SNP853_312	2.6	8.2	
			18	Vr18	2.75	76.4	74-78.8	Vvi_10777	2.7	7.3	
		2009	11	Vr11	3.82	31	28-47	vvib19	2.6	10.8	21.2
			14	Vr14	4.55	65.9	48-72	vmcng1g1_1	2.7	14.2	
		2010	18	Vr18	3.32	65	59-78	SNP1391_48	2.5	9.5	9.5
	Mn	2008	14	Vr14	2.2	5.3	2-16	vmc1e12	1.5	6.2	12.9
			18	Vr18	1.98	66.4	55-69	vmc7f2	1.5	5.6	
		2009	11	Vr11	2.67	39	34-44	vvib19	1.4	9.2	9.2
		2010	18	Vr18	3.11	56.8	52-62	Vvi_10777	1.7	9.1	9.1
	Sy	2009	5	Vr5	1.92	8.0	5-8	F5(1-4)2	1.5	5.6	11.6
			11	Vr11	1.93	25.5	18-39	vmc6g1	1.3	6.8	
		2010	8	Vr8	2.16	59.5	55-60	vmc2h10	1.5	5.6	5.6
Rp	C	2008	2	Rp2	3.80	46.2	31-62.4	vmc5g7	2.5	11.6	
		2009	2	Rp2	5.12	36.6	4-60	Vvi_9227	2.5	16.7	23.5
			17	Rp17	3.02	15.7	2-18	SNP455_141	2.5	7.8	
		2010	17	Rp17	4.49	0.0	0-17	SNP677_509	2.6	12.3	12.3
	Mn	2009	17	Rp17	1.83	15.5	15-16	SNP455_141	1.8	5.4	5.4
	Sy	2008	2	Rp2	1.68	46.7	32-57	20D18CB9	1.4	5.8	5.8
		2010	18	Rp18	1.78	36.6	22-46	vvin83	1.2	5.6	5.6
Vr_Rp	C	2008	2	Vr_Rp2	8.37	41.6	0-62	vmc5g7	2.6	24.3	33.5
			17	Vr_Rp17	4.7	0.0	0-10	SNP677_509	2.4	12.7	
		2009	2	Vr_Rp2	6.55	32.2	0-62	vmc6f1	2.7	20.5	20.5
		2010	17	Vr_Rp17	3.94	0.0	0-16	SNP677_509	2.3	10.9	10.9
	Mn	2009	17	Vr_Rp17	1.55	21.0	3-23	scu06	1.3	4.7	4.7
	Sy	2008	2	Vr_Rp2	2.44	21.1	0-57	vmc6f1	1.4	7.7	7.7
		2010	5	Vr_Rp5	1.91	17.7	9-25	Vvi_10113	1.3	5.2	9.2
			19	Vr_Rp19	1.63	0.0	0-2	vmc5h11-200	1.5	4.2	

The table shows the trait, the map, the year, and the linkage group (LG) in which the QTLs were identified. The QTLs are named using the LG number plus Sp, Fw, Vr, Rp, and Vr_Rp for sprouting, flowering, veraison, ripening, and veraison-ripening period, respectively. The QTL location is indicated by the position at which the highest LOD (LOD max) was detected (in cM), the LOD-1 confidence interval, and the cofactor. The QTLs considered are those with a maximum LOD value higher than that estimated for the linkage-group-wide (LGW) threshold, for a type I error rate of 5%. The percentage of the total variance explained by each QTL, and when considering the combined effect of all QTLs detected in a season (model), is indicated. C, consensus; Mn, Monastrell; Sy, Syrah.

Annex 6. Putative QTLs identified for productive and morphological traits in maps of the Monastrell x Syrah progeny.

Trait	Map	Year	LG	QTL	LOD max	cM	Confidence interval	Cofactor	LGW LOD threshold	% variance QTL	% variance model
Fi	C	2008	3	Fi3	4.24	29.9	18-52	udv_43	2.4	8.7	8.7
		2009	5	Fi5	7.84	0.0	0-38	SNP1027_69	2.6	17.7	17.7
		2010	5	Fi5	6.0	0.0	0-36	SNP1027_69	2.6	13.1	23.4
			8	Fi8	5.63	7.1	0-37	vmc1b11	2.4	11.8	
		2013	11	Fi11	3.6	16.1	10-24	vvmd25	2.5	9.1	9.1
	Mn	2010	5	Fi5	2.24	0.0	0-5	SNP1027_69	1.4	4.6	4.6
	Sy	2009	3	Fi3	2.05	38.9	33-42	vvin54	1.4	4.1	4.1
		2010	3	Fi3	2.57	38.9	31-41	vvin54	1.5	5.2	5.2
		2013	3	Fi3	2.1	41.3	40-42	vvmd28	1.3	4.3	4.3
P	C	2008	5	P5	2.86	22.6	22-26	SNP625_278	2.7	9.2	9.2
		2009	3	P3	2.67	42.9	42-47	vvin54	2.5	7.3	33.4
			8	P8	2.99	25.2	20-28	vmc1b11	2.4	6.3	
			14	P14	3.3	42.2	42-50	vmc2h5	2.9	7.3	
			17	P17	3.62	46.2	35-46.5	SNP879_308	2.4	8.8	
		2010	5	P5	3.4	25.5	22-28	SNP855_103	2.6	8.5	18.6
			8	P8	4.39	20.0	4-13	vmc1b11	2.5	13.0	
	Mn	2008	12	P12	1.88	33.8	29-49	scu05	1.3	6.0	6.0
		2009	1	P1	2.05	62.9	55-63	vvif52	1.4	5.5	15.4
			14	P14	1.74	0.0	0-7	SNP251_159	1.3	4.7	
			17	P17	1.87	44.3	40-45	SNP879_308	1.2	5.1	
		2010	1	P1	1.78	62.9	52-63	vvif52	1.4	4.5	9.8
			8	P8	1.87	10.0	0-52	SNP1385_86	1.1	6.7	
	Sy	2008	5	P5	2.37	10.3	4.5-17	SNP625_278	1.4	7.6	7.6
		2010	5	P5	2.02	12.7	6.5-18	SNP855_103	1.4	5.4	5.4
CW	C	2008	2	CW2	4.69	19.0	11-42	SNP581_114	2.6	10.3	41.9
			5	CW5	3.21	22.6	20-28	SNP625_278	2.5	1.9	
			10	CW10	3.83	0.0	0-3	vmc3d7	3.4	19.2	
		2009	1	CW1	3.34	40.8	36-42	vvip60	2.6	9.3	20.3
			14	CW14	3.56	42.2	35-42	vmc2h5	2.8	9.2	
		2010	1	CW1	3.51	63.2	55-71.5	SNP477_239	2.7	7.6	26.9
			5	CW5	3.48	25.5	21-30	SNP855_103	2.5	7.9	
			17	CW17	3.52	0.0	0-29	SNP677_509	2.4	8.9	
	Mn	2008	5	CW5	1.86	19.3	8-27	vvmd27	1.5	6.0	6.0
		2009	1	CW1	2.55	60.6	35-63.5	vvif52	1.5	7.6	14.3
			14	CW14	2.63	0.0	0-15	SNP251_159	1.4	7.3	
		2010	1	CW1	2.44	60.6	57-63.5	vvif52	1.4	5.9	21.3
			7	CW7	2.36	11.5	6-19	vmc16f3	1.5	6.1	
			8	CW8	1.69	47.1	35-58	SNP1385_86	1.3	4.8	
			17	CW17	2.26	26.1	20-29	vmc3a9	1.5	5.0	
	Sy	2008	5	CW5	2.05	10.3	5-15	SNP625_278	1.4	6.3	12.3
			10	CW10	1.57	0.0	0-4	vmc3d7	1.3	4.7	
		2010	5	CW5	1.79	12.7	9-15	SNP855_103	1.4	4.6	11.1
			17	CW17	2.35	0.0	0-12	Vvi_6987	1.2	6.0	
BW	C	2008	5	BW5	3.24	16.3	15-32.5	vrzag47	2.5	10.7	10.7
		2009	1	BW1	3.68	39.5	35-48	vvip60	2.7	11.7	11.7
		2010	14	BW14	5.0	5.1	2-9	Sp14(0-4)2	2.5	9.2	9.2
	Mn	2008	1	BW1	1.64	34.8	32-45	SNP851_110	1.5	7.0	7.0
		2010	4	BW4	2.31	56.6	49-56.6	Vvi_2543	1.5	5.8	12.9
			7	BW7	2.84	44.8	32-58	Fl7(17-19)3	1.6	8.5	
	Sy	2008	17	BW17	2.27	0.0	0-26	Vvi_6987	1.3	7.2	12.7
			19	BW19	1.59	44.8	44-48	SNP253_145	1.4	5.2	
		2010	7	BW7	1.74	37.4	28-41	Vvi_1731	1.5	5.1	5.1

Annex 6. Continued.

Trait	Map	Year	LG	QTL	LOD max	cM	Confidence interval	Cofactor	LGW LOD threshold	% variance QTL	% variance model
CN	C	2008	3	CN3	3.41	46.8	41-50	vvin54	2.5	10.8	10.8
		2009	3	CN3	3.26	29.9	18-34	udv_43	2.5	10.5	21.2
			8	CN8	3.86	44.9	34-61	SNP1385_86	2.7	15.1	
		2010	8	CN8	3.49	20.0	14-40	vmc1b11	2.5	13.0	13.0
	Mn	2008	15	CN15	1.96	21.8	17-25	vvip33	1.3	6.7	6.7
		2009	3	CN3	1.63	21.3	11-33	vmc8f10	1.3	6.0	9.8
			11	CN11	2.05	20.0	11-30	vvmd25	1.4	7.5	
		2010	14	CN14	1.46	54.3	53-55	vvmd24	1.4	3.5	9.5
			15	CN15	1.68	21.8	16-23	vvip33	1.4	4.7	
	Sy	2008	3	CN3	2.07	41.3	34-42	vvin54	1.3	6.2	11.6
			5	CN5	1.68	8.0	6-13	SNP625_278	1.4	5.2	
		2009	6	CN6	1.63	64.1	62-64	vvim43	1.4	4.6	9.7
			12	CN12	1.66	38.1	32-40	vviv05	1.3	4.7	
		2010	1	CN1	1.4	0.0	0-2	vviq57	1.3	3.2	17.2
			3	CN3	1.56	38.9	35-40	vvin54	1.3	3.6	
			5	CN5	1.91	11.0	7-16	Vvi_5316	1.4	4.4	
			6	CN6	2.05	63.4	56-64	vvim43	1.5	4.8	
CC	C	2008	5	CC5	3.84	0.0	0-15	F5(1-4)2	2.5	23.8	21.5
			16	CC16	2.6	52.6	48-54	vvmd5	2.5	8.6	
		2009	19	CC19	2.92	34.6	32-36	vmc3b7_2	2.5	9.7	9.7
	Mn	2008	2	CC2	2.01	0.0	0-13	vvmd34	1.5	6.7	15.0
			17	CC17	1.96	39.1	34-41	vvib09	1.4	6.5	
		2009	2	CC2	1.89	43.9	36-53	vmc7g3	1.4	6.6	10.9
			5	CC5	1.59	0.0	0-2	SNP1027_69	1.4	5.1	
		2010	2	CC2	1.62	0.0	0-2	vvmd34	1.4	4.6	4.6
	Sy	2008	5	CC5	1.99	7.7	0-10	vvmd27	1.3	8.1	8.1

The table shows the trait, the map, the year, and the linkage group (LG) in which the QTLs were identified. The QTLs are named using the LG number plus Fi, P, CW, BW, CN, and CC for fertility index, total production, cluster weight, berry weight, cluster number, and cluster compactness, respectively. The QTL location is indicated by the position at which the highest LOD (LOD max) was detected (in cM), the LOD-1 confidence interval, and the cofactor. The QTLs considered are those with a maximum LOD value higher than that estimated for the linkage-group-wide (LGW) threshold, for a type I error rate of 5%. The percentage of the total variance explained by each QTL, and when considering the combined effect of all QTLs detected in a season (model), is indicated. C, consensus; Mn, Monastrell; Sy, Syrah.

Annex 7. Putative QTLs identified for enological traits in maps of the Monastrell x Syrah progeny.

Trait	Map	Year	LG	QTL	LOD max	cM	Confidence interval	Cofactor	LGW LOD threshold	% variance QTL	% variance model
Ac	C	2008	4	Ac4	3 5	34 7	17-37 5	vrzag21	2 7	13 3	27 5
			5	Ac5	3 27	60 8	54-61	vmc4c6	2 6	12 3	
		2009	6	Ac6	3 92	25 0	13-35	SNP1213_99	2 7	11 1	20 9
			16	Ac16	4 05	61 2	32-61	vvmd5	2 4	16 0	
		2010	1	Ac1	2 97	55 7	48-58	vmc8d1	2 5	8 9	8 9
		2011	12	Ac12	3 32	24 0	11-30	SNP651_658	2 6	22 8	22 8
		2012	18	Ac18	3 33	0 0	0-2	vmc3e5	2 8	23 0	23 0
	Mn	2008	2	Ac2	2 13	26 9	18-42	Vvi_9227	1 4	7 1	33 7
			4	Ac4	3 18	11 0	0-56	Vvi_6668	1 5	12 7	
			9	Ac9	1 66	75 4	65-75	vmc2e11	1 5	5 4	
			17	Ac17	2 23	0 0	0-7	SNP677_509	1 3	7 5	
		2009	5	Ac5	2 44	23 7	16-32	SNP855_103	1 5	8 1	12 9
			13	Ac13	1 77	9 7	4-12 5	SNP653_90	1 3	5 3	
		2010	2	Ac2	2 12	41 8	21-53	20D18CB9	1 3	6 3	9 9
			5	Ac5	1 33	19 3	19-21	vvmd27	1 3	3 6	
		2011	5	Ac5	1 63	35 7	32-36	SNP1235_35	1 6	10 2	10 2
		2012	2	Ac2	2 32	21 9	12-30	Vvi_9227	1 5	13 7	29 1
			18	Ac2	2 88	2 6	0-7	vmc3e5	1 3	17 4	
		2013	5	Ac5	1 71	28 7	24-34	SNP1235_35	1 5	5 9	5 9
	Sy	2008	5	Ac5	2 71	36 0	15-44	vmc4c6	1 3	12 8	17 8
			11	Ac11	2 61	25 5	17-33	vmc6g1	1 4	7 7	
		2009	2	Ac2	1 95	47 0	44 5-57	vmc7g3	1 5	6 2	6 2
		2010	5	Ac5	1 5	36 0	29-44	vmc16d4	1 3	4 4	8 2
			18	Ac2	1 59	31 6	26-40	vvin83	1 4	4 3	
		2011	12	Ac12	2 33	28 8	25-40	scu05	1 3	11 9	21 2
			16	Ac16	2 26	0 0	0-10	SNP1335_204	1 2	11 6	
		2012	18	Ac18	2 0	58 7	32-68	vvin16	1 4	14 2	14 2
		2013	1	Ac1	1 64	45 9	43-46	SNP1021_163	1 5	4 5	15 0
			8	Ac8	1 78	7 1	1-14	SNP699_311	1 3	5 4	
			11	Ac11	1 68	32 1	25-33	vmc6g1	1 3	4 6	
TSS/Ac	C	2008	4	TSS/Ac4	3 61	34 6	17-40	vrzag21	2 6	9 7	39 3
			5	TSS/Ac5	3 94	60 8	52-61	vmc4c6	2 6	12 6	
			11	TSS/Ac11	2 97	36 0	25-47	vviv35	2 5	10 8	
		2009	5	TSS/Ac5	3 02	26 4	24-28	SNP855_103	2 8	11 1	11 1
		2010	2	TSS/Ac2	4 0	56 8	30-61	SNP1229_219	2 6	11 4	11 4
		2012	18	TSS/Ac18	3 47	0 0	0-2	vmc3e5	2 8	23 8	23 8
		2013	8	TSS/Ac8	3 09	25 2	16-28	vmc1b11	2 6	8 5	17 6
			11	TSS/Ac11	3 22	26 1	18-39	vvib19	2 5	11 8	
	Mn	2009	5	TSS/Ac5	1 67	19 3	19-24	vvmd27	1 6	4 7	15 9
			13	TSS/Ac13	1 7	9 7	4 5-13	SNP653_90	1 2	4 8	
			14	TSS/AC14	2 03	30 3	14-48	vmc2h5	1 4	7 9	
		2010	16	TSS/Ac16	1 59	10 0	0-18	udv_104	1 2	5 5	5 5
		2012	2	TSS/Ac2	3 64	21 9	0-34	Vvi_9227	1 4	16 9	45 8
			4	TSS/Ac4	1 72	0 0	0-1	vmcng1f1_1	1 5	6 7	
			14	TSS/AC14	2 07	68 1	63-68	vmcng1g1_1	1 4	8 3	
			18	TSS/Ac18	2 3	2 6	1-4	vmc3e5	1 5	10 0	
		2013	5	TSS/Ac5	1 86	35 7	20-47	SNP1235_35	1 4	6 2	6 2
	Sy	2008	5	TSS/Ac5	2 36	36 0	18-44	vmc4c6	1 4	10 9	19 8
			11	TSS/Ac11	2 41	43 9	15-44	vviv35	1 4	10 1	
		2009	2	TSS/Ac2	2 09	30 7	21-44	vvib23	1 5	8 1	8 1
		2010	5	TSS/Ac5	1 58	31 0	30-36	Vvi_11572	1 4	4 3	8 5
			11	TSS/Ac11	1 59	44 7	43-45	vviv35	1 4	4 4	
		2011	11	TSS/Ac11	2 11	0 0	0-17	SNP197_82	1 3	12 1	12 1
		2013	8	TSS/Ac8	1 96	14 9	2-26	SNP1203_88	1 5	5 7	10 6
			10	TSS/Ac10	2 25	48 8	21-51	vviv37	1 3	7 4	

Annex 7. Continued.

Trait	Map	Year	LG	QTL	LOD max	cM	Confidence interval	Cofactor	LGW LOD threshold	% variance QTL	% variance model
Tar	C	2008	7	Tar7	2 80	55 8	54-58	Fl7(17-19)3	2 7	12 9	12 9
		2009	15	Tar15	3 20	0 0	0-8	SNP341_196	2 5	18 7	18 7
		2010	18	Tar18	2 91	25 1	24-26	vvim93	2 8	10 6	10 6
		2011	19	Tar19	2 92	34 6	30-48	vmc3b7_2	2 5	16 2	16 2
		2012	9	Tar9	3 41	58 9	54-68	Vvi_10329	2 8	34 6	34 6
		2013	2	Tar2	3 35	21 2	18-37	vvib23	2 7	10 0	10 0
	Mn	2010	3	Tar3	1 42	0 0	0-1	SNP613_315	1 4	5 6	5 6
		2011	5	Tar5	1 86	30 7	26-36	SNP1235_35	1 5	11 4	11 4
		2013	2	Tar2	2 92	21 9	3-35	Vvi_9227	1 4	9 9	13 8
			4	Tar4	2 49	32 6	23-53	vrzag21	1 4	7 4	
	Sy	2008	2	Tar2	1 82	0 0	0-4	vvib01	1 5	7 7	15 2
			16	Tar16	1 54	51 7	49-52	scu14	1 4	6 5	
		2009	15	Tar15	2 61	0 0	0-33	SNP5451_287	1 4	14 2	14 2
		2010	7	Tar7	1 62	36 1	35-38	Vvi_1731	1 6	6 6	14 7
			16	Tar16	2 05	16 0	6-28	udv_104	1 3	7 6	
		2012	16	Tar16	1 54	47 5	32 5-52	vvmd5	1 3	10 8	10 8
Ma	C	2008	8	Ma8	3 75	59 1	30-60 5	vmc2h10	2 5	14 4	52 8
			9	Ma9	3 32	74 0	64-74	vmc2e11	2 6	23 9	
			14	Ma14	3 93	13 5	10-24	vmc1e12	2 6	14 7	
		2010	8	Ma8	4 07	15 4	4-38	SNP853_312	2 5	15 4	15 4
		2011	8	Ma8	2 85	7 1	6-8	SNP699_311	2 8	19 5	19 5
		2012	8	Ma8	4 02	12 4	0-22	SNP699_311	2 6	20 4	65 5
			17	Ma17	5 41	0 0	0-9	SNP677_509	2 7	27 2	
			18	Ma18	5 68	0 0	0-8	vmc3e5	2 6	29 2	
		2013	11	Ma11	4 31	14 3	0-25	vvmd25	2 7	14 4	24 2
			15	Ma15	3 94	38 0	33-58	vmc4d9_2	2 5	11 0	
	Mn	2008	8	Ma8	2 34	57 1	32-59	vmc2h10	1 3	9 6	23 9
			11	Ma11	1 68	0 0	0-7	vmc3e12	1 5	6 4	
			17	Ma17	2 08	26 1	22-41	vmc3a9	1 5	8 1	
		2009	18	Ma18	1 68	2 6	1-4	SNP1519_47	1 5	8 1	8 1
		2010	2	Ma2	2 77	31 9	17-51	vmc5g7	1 5	10 1	20 1
			8	Ma8	1 85	30 8	18-46	vmc1b11	1 3	7 2	
			17	Ma17	1 92	0 0	0-5	SNP677_509	1 3	6 3	
		2013	11	Ma11	2 58	15 0	3-22	SNP635_21	1 4	6 8	16 4
			15	Ma15	2 01	40 9	31-44	vmc4d9_2	1 4	5 8	
			17	Ma17	1 73	30 0	24-37	SNP579_187	1 3	4 7	
	Sy	2008	5	Ma5	2 12	41 0	28-41	vmc4c6	1 4	10 5	10 5
		2009	7	Ma7	1 82	63 9	55-70	vmc1a12	1 5	8 3	8 3
		2011	7	Ma7	2 35	88 5	68-88 5	SNP1445_218	1 5	11 9	21 1
			8	Ma8	2 08	7 1	0-15	SNP699_311	1 6	11 5	
		2012	8	Ma8	2 44	2 1	0-23	vvip04	1 5	15 4	15 4
		2013	11	Ma11	1 64	0 0	0-3 5	SNP197_82	1 3	5 1	5 1
Tar/ Ma	C	2008	8	Tar/Ma8	2 88	12 4	6-26	vmc5g6_1	2 5	15 8	15 8
		2009	7	Tar/Ma7	2 57	49 4	45-50	Vvi_5629	2 4	11 9	11 9
		2010	2	Tar/Ma2	3 6	5 0	0-60	vvib01	2 6	15 4	39 0
			5	Tar/Ma5	3 11	51 5	31-61	vmc16d4	2 6	8 4	
			8	Tar/Ma8	5 09	11 3	0-45	vmc5g6_1	2 5	15 2	
		2011	8	Tar/Ma8	2 59	11 3	6-11	SNP699_311	2 5	15 7	15 7
		2012	8	Tar/Ma8	3 39	20 0	2-28	SNP1203_88	2 6	25 3	25 3
		2013	15	Tar/Ma15	2 55	38 0	37-42	vmc4d9_2	2 4	7 7	7 7
	Mn	2008	8	Tar/Ma8	1 67	58 4	38-58	SNP1385_86	1 2	6 7	20 5
			14	Tar/Ma14	2 28	15 7	4-28	vmc1e12	1 3	22 1	
			17	Tar/Ma17	2 03	39 1	0-41	vvib09	1 3	8 6	
		2010	2	Tar/Ma2	2 75	31 9	0-49	vmc5g7	1 4	9 3	22 1
			8	Tar/Ma8	2 37	30 8	11-56	vmc1b11	1 3	8 9	
			17	Tar/Ma17	2 05	0 0	0-16	SNP677_509	1 2	6 7	
		2011	9	Tar/Ma9	1 81	11 0	1-25	Vvi_10992	1 5	11 0	11 0
		2012	17	Tar/Ma17	2 02	0 0	0-7	SNP677_509	1 4	12 0	24 8
			18	Tar/Ma18	2 30	2 6	0-8	vmc3e5	1 5	13 6	
	Sy	2011	8	Tar/Ma8	2 18	7 1	0-18	SNP699_311	1 5	12 9	12 9
		2012	8	Tar/Ma8	1 53	2 1	2-8	SNP1203_88	1 5	10 0	10 0
		2013	11	Tar/Ma11	1 48	1 5	0-3	SNP635_21	1 3	4 8	4 8

Annex 7. Continued.

Trait	Map	Year	LG	QTL	LOD max	cM	Confidence interval	Cofactor	LGW LOD threshold	% variance QTL	% variance model
Ant	C	2009	2	Ant2	10 53	56 8	52-62 4	SNP1229_219	2 6	33 2	51 4
			3	Ant3	3 18	20 3	16-26	F3(3-6)6	2 3	9 3	
		2013	16	Ant16	3 12	22 2	17-40	vmc1c11	2 4	10 4	10 4
	Mn	2008	8	Ant8	2 25	0 0	0-11	vvip04	1 3	16 6	
			10	Ant10	1 89	11 5	3-11 5	vviv37	0 9	11 5	51 6
			17	Ant17	2 53	44 3	40-44 5	SNP879_308	1 5	19 4	
		2010	14	Ant14	2 03	42 8	21-48	Vvi_2292	1 5	7 3	13 5
			19	Ant19	2 13	21 5	16-36	vvip31	1 4	7 4	
		2011	2	Ant2	2 43	41 8	21-48	20D18CB9	1 4	14 7	14 7
		2012	2	Ant2	2 0	36 8	29-53	vmc5g7	1 2	9 4	
			10	Ant10	2 56	2 7	0-10	vmc8d3	1 0	12 2	36 5
			18	Ant18	2 53	48 8	35-62	udv_134	1 4	12 1	
	Sy	2009	3	Ant3	2 63	10 2	0-36	F3(3-6)6	1 4	11 7	11 7
		2013	16	Ant16	1 99	23 9	16-39	vmc1e11	1 3	8 5	8 5

The table shows the trait, the map, the year, and the linkage group (LG) in which the QTLs were identified. The QTLs are named using the LG number plus Ac, TSS/Ac, Tar, Ma, Tar/Ma, and Ant for total acidity, ratio of total soluble solids to total acidity, tartaric acid, malic acid, ratio of tartaric acid to malic acid, and total anthocyanins, respectively. The QTL location is indicated by the position at which the highest LOD (LOD max) was detected (in cM), the LOD-1 confidence interval, and the cofactor. The QTLs considered are those with a maximum LOD value higher than that estimated for the linkage-group-wide (LGW) threshold, for a type I error rate of 5%. The percentage of the total variance explained by each QTL, and when considering the combined effect of all QTLs detected in a season (model), is indicated. C, consensus; Mn, Monastrell; Sy, Syrah.