Title

Fine mapping of a QTL region controlling the variations of flowering date in Sweet cherry *(Prunus avium* L.*)*

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Presentation of the INRA Bordeaux structure

The National Institute of Agronomic Research (INRA) is the first agronomic research institute in Europe, the second largest in agricultural science. It is involved in the production and dissemination of scientific knowledge. Always with the same dynamics of research to fit the interest of our current societies, INRA is made up of 17 centers throughout France and has its headquarters in Paris. Its creation took place after the Second World War with the objective of achieving food self-sufficiency. The institute still retains research dynamics on agriculture, but has expanded its field of action taking into account the environmental, ecological, plant biology and physiology.

The internship will be performed at the INRA Nouvelle-Aquitaine Bordeaux center led by Mr. Hubert de Rochambeau. This center is one of the five INRA's centers created in 1946. It includes different research sites (Villenave d'Ornon, Cestas-Pierroton, Saint Pée sur Nivelle, Bordeaux, Talence, Limoges) with several research units such as Biodiversity, Genes and Communities (BIOGECO), Fruit Biology and Pathology (BFP), Behavioural Ecology and Biology (ECOBIOP), Ecophysiology and Functional Genomics of Grapevine (EGFV), Animal Molecular Genetics (GMA), Soil Plant Atmosphere Interactions (ISPA), Mycology and Food Safety (MYCSA), Nutrition, Metabolism Aquaculture (NuMéA), Integrated Nutrition and Neurobiology (Nutri Neuro), Vineyard Health & Agroecology (SAVE), Unit of Research and Service in Plant an Environment Analysis (USRAVE) Mycoplasma and Chlamydia Infections in Humans (IHMC), Institute of Mechanics and Engineering (I2M) and Enology. This center also includes five experimental units in particular the Domaine des Jarres in Toulenne (Gironde) and the Domaine de la Tour de Rance in Bourran (Lot-et-Garonne) devoted to Fruit tree experiments. These two domains hold several collections of fruit species (peach, plum, cherry and walnut) and participate in the breeding of new varieties of sweet cherry.

More specifically, my internship will be performed in the team 'Adaptation of sweet cherry to Climate Change' (A3C) under the direction of Dr. Elisabeth Dirlewanger, attached to the research unit BFP led by Mr. Thierry Candresse. This team works mainly on the phenology and the fruit quality, two traits of the sweet cherry affected by global warming, but also leads a sweet cherry breeding program and conducts Distinctness, Uniformity and Stability expertise activities for the International Union for the Protection of New Varieties of Plants (UPOV).

My internship is titled "Fine mapping of a QTL region controlling the variation of flowering date in sweet cherry (*Prunus avium* L.)", started on January 8 and ended on June 29-2018.

Abbreviations

cM	CentiMorgan
CI	Confidence Interval
CP	Cross-pollinated
χ^2	chi-square
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization of the United Nations
GBS	Genotyping By Sequencing
KASP	Kompetitive allele specific PCR
LG	Linkage group
LOD	Logarithm of the Odds
MAB	Molecular Assisted Breeding
Map	Mapping
Max	Maximum
Min	Minimum
NGS	Next generation sequencing
PEV	PhenotypicVariation Explained
QTL	Quantitative Trait Loci
RosCOS	Rosaceae Conserved Orthologous Set
RxG	‘Regina’ x ‘Garnet’
RNAseq	RNA sequencing
RAPD	Random amplified polymorphic DNA
SSR	Single sequence repeat
SNP	Single nucleotide polymorphism

Summary

In the context of climate change, there are many questions relating to the adaptation of fruit species. In temperate zones, an increase in temperatures in autumn or winter leads to a slowdown in the dormancy process, causing phenological disorders and a decrease in production. Similarly, an increase in spring temperatures results in a significant advance in flowering and budburst dates leading to increased risk of frost damage. The phenology of sweet cherry tree (*Prunus avium* L.*)*, as for other perennial fruit species, depends directly on climatic conditions with an important effect of the temperatures. Therefore, there is a risk of a direct impact of climate change on productivity and on fruit quality. In the case of sweet cherry, chilling requirements for flowering are very important for almost all the varieties currently cultivated. Taking into account that climate change could endanger the production of cherries, and considering the time required for the releasing a new variety in this species (around 25 years), it is essential to integrate the criteria linked to the adaptation to climate change in the improvement programs that will help the choice of plants better adapted to future environmental conditions.

A segregation population from a cross between 'Regina', a late flowering date variety, and 'Garnet', an intermediate flowering date variety, was studied for many years for the flowering date and others traits such as the chilling and heat requirements. The regions of the genome (Quantitative trait Loci, QTL) involved in the control of the flowering date have been investigated and a QTL with high effect, explaining up to 36% of the variation observed for this trait, has been identified on the linkage group 4 (LG4) with a confidence interval of less than 1 cM (Castède *et al.*, 2014). This search of QTL was done using a linkage map with 700 markers (Castède *et al.*, 2014; Castède *et al.*, 2015). The objective of this work is to do a fine mapping of the QTL region (LG4) using a segregation progeny of 1526 individuals issued from the same cross. New QTL detection was performed using the flowering date evaluation of this year. New SNP markers issue from different strategies were identified in the QTL region and transformed into kompetitive allele specific PCR (KASP) markers to genotype all the progeny. Recombinant individuals were identified using these markers. Moreover, results indicated that theses KASPs can be used for sweet cherry Molecular Assisted Breeding (MAB).

Key works: climate change, flowering date, QTL analyzes, SNPs, *Prunus avium*

Introduction

All cherry species belong to the *Rosaceae* family and *Spiraeoideae* subfamily (Potter *et al.*, 2007), they are part of the Amygdaleae tribe and are *Prunus* species, including other stone fruits: almond (*Prunus amygdalus* Batsch), apricot (*Prunus armeniaca* L.), and peach/nectarine (*Prunus persica* (L.) Batsch), and plum (*Prunus domestica* L. and *Prunus salicina* Lindl.). Cherries are further placed within two subgenera (*Cerasus* Pers. and *Padus* (Moench) Koehne) (Rehder, 1947). The *Cerasus* Pers. subgenus and *Cerasus* Koehne section contain the diploid sweet cherry (2n = 2x = 16, *Prunus avium* L.) and the tetraploid sour cherry (2n = 4x = 32, *Prunus cerasus* L.) and ground cherry (*Prunus fruticosa* Pall.). These species and their interspecific hybrids (such as *Prunus x gondouinii*) constitute the Eucerasus section of the *Cerasus* subgenus, based on morphological criteria (Krussmann, 1978; Rehder, 1947). This classification has been confirmed by chloroplastic DNA variation analysis (Badenes and Parfitt, 1995) and microsatellite analysis (Tavaud *et al.,* 2004). *P. avium* includes sweet cherry trees, cultivated for human consumption and wild cherry trees used for their wood, also called mazzards (Webster, 1996). Sweet cherry has a small genome, 338 Mb, according to Arumuganathan and Earle in (1991) with flow cytometry. More recently, it was determined by k-mer analysis from the 'Satonishiki' cherry genome to 353 Mb (Shirassawa *et al.*, 2017).

The origin of *Prunus avium* is located in an area between the Black Sea and the Caspian Sea (De Candolle, 1883; Hedrick, 1915) and has been later spread by birds until the extremity of its present area of distribution across Europe, well before its domestication (Webster, 1996). Sweet cherry was probably domesticated in its own area of origin but several domestication events from different wild populations may have occurred (Tavaud, 2002). Several archeological findings based on pits discovered in Europe from the Neolithic Period to Bronze Age indicate that sweet cherry was consumed by early inhabitants of Europe 4,000 to 5,000 BC (Webster, 1996; Faust and Surányi, 1997). Theophrastus, the "father of botany" in 300 BC referred for the first time to cherry cultivation (Brown *et al.,* 1996) reflecting that its cultivation may be very old. Therefore, natural and human selection occurring during centuries, lead to numerous cherry landraces in Europe.

Sweet cherry has been cultivated for more than 2000 years and cherry breeding started around the early 1800s (Iezzoni *et al.*, 1990; Dirlewanger *et al.*, 2007). Genetic improvement of sweet cherry is relatively recent compared with other fruit species such as peach or apple and numerous breeding programs were initiated after the 1950s. Cherry breeding is currently carried out in almost every sweet cherry-producing country, through public and private programs, and sweet cherry cultivars are continuously being released (recently reviewed in Quero-García *et al.*, 2017a). More than 230 varieties

were released between 1991 and 2004, 116 bred in Europe, 71 in North America and 33 in Asia (Sansavini and Lugli, 2008). During the last 20 years, most of the new varieties were originated from USA breeding programs, 88 new varieties being registered during this period.

Sweet cherry pollination is mostly made by the European honeybee (*Apis mellifera* L.) which is the most common pollinator used in cherry orchards (Free, 1993). As most sweet cherry cultivars are self-incompatible (Crane and Lawrence, 1929; Crane and Brown, 1937), the entomophily pollination is fundamental for the sweet cherry production. Given that self-incompatibility is genetically determined by a single multi allelic locus called S locus. This genetic factor that determines pollen tube growth self-incompatibility operating in *Prunus spp*. is named S-RNase-based gametophytic self-incompatibility (Tao and Iezzoni, 2010).

Traditionally, breeding objectives on sweet cherry have been: large, attractive and good- flavored fruits; short juvenile phase; large and constant yields; reduced susceptibility to fruit cracking; self-compatibility; improved resistance or tolerance to diseases, especially bacterial canker induced by *Pseudomonas* mors pv. *prunorum* and *P. syringae*; Climate adaptation (low chilling requirements) required for the growing conditions. In order to break bud dormancy, sweet cherries need to meet minimum chilling requirements in the autumn and winter (Orden and Nybom, 1968). Consequently, varieties with low chilling requirements are needed to face the new climatic condition.

Sweet cherry is one of the most popular temperate fruit crops despite of its relatively high price. The fruits are attractive in appearance, because of their bright, shiny, skin color, and their subtle flavor and sweetness are appreciated by most consumers. The world production according to the FAO is the 2.2 M tones and it is increasing (FAOSTAT: http://www.fao.org/faostat).

However, sweet cherry production is challenging as it is highly dependent on climatic conditions. Several stages of growth are subjected to strict temperature control, including dormancy, flowering and fruit development (Allona *et al.,* 2008). Climatic adaptation has become one of the major breeding objectives for many fruit crops. Indeed, one of the main environmental problems of this century is the climate change (Campoy *et al.,* 2011). An increase of 0.3 to 0.7°C in temperature is predicted for the 21st century with specific variations at each region of the globe (Kirtman *et al.,* 2013). This increase may generate risk in the fruit tree production in various temperate and subtropical regions throughout the world due to the rapid climatic change and the reduction of chilling accumulation already observed in numerous temperate zones (Luedeling *et al.,* 2011).

Cultivars with low chilling requirements, well adapted to warm autumn and winter with high heat requirements to avoid early flowering and frost damage, are desired. Taking into account the fact that, as for all long- lived perennials, cherry orchards are installed for long periods, up to 20 years, the choice of new cultivars that will be planted is crucial. Genetic and genomic tools can help mitigate challenges by improving efficiency and reducing the time necessary to obtain new cultivars. In breeding programs, Molecular Assisted Breeding (MAB) approaches based on genetic maps have the potential to save time and resources needed to select new sweet cherry cultivars. Knowledge of linkage relationships between marker loci and important agronomical traits will facilitate and shorten the selection of promising individuals. Consequently, marker-assisted selection would be especially beneficial for sweet cherry selection (Iezzoni *et al.,* 2017; Quero-Garcia *et al.,* 2007b). Genetic linkage maps were initially constructed using random amplified polymorphic DNA (RAPD) markers (Stoclinger *et al*., 1996) and isoenzymes (Boskovic and Tobutt, 1998). Later, maps were constructed mainly using single sequence repeat markers (SSRs) (Dirlewanger *et al*., 2004; Clarke *et al*., 2009). Recently, single nucleotide polymorphisms (SNPs) have been used for sweet cherry linkage maps construction (Cabrera *et al*., 2012; Klagges *et al*., 2013; Guajardo *et al.,* 2015; Wang *et al*., 2015). Cabrera *et al.* (2012) used 81 SNPs derived from the Rosaceae Conserved Orthologous Set (RosCOS) for mapping in four sweet cherry F_1 populations. This was the first example of high-throughput SNP genotyping assay in sweet cherry. Later, Klagges *et al.* (2013) published a high density linkage maps using the RosBREED cherry 6K SNP array v1.0 (Peace *et al.,* 2012). This array contains 5,696 SNPs obtained from re-sequencing of 16 sweet and eight sour cherry accessions, with physical positions of each SNP based on the peach reference genome (Peach v1.0) as reference (Verde *et al*., 2013). These maps are useful for genetic analysis of relevant traits in sweet cherry breeding by quantitative trait loci (QTL) analysis (Rosyara *et al*., 2013; Castède *et al*., 2014; Campoy *et al.,* 2015). QTLs detection for traits of interest can be further used for MAB (Peace, 2017) or to identify candidate genes controlling these traits (De Franceschi *et al*., 2013; Castède *et al.,* 2015).

In sweet cherry, a progeny issued from a cross between 'Regina', a late flowering variety and 'Garnet', an intermediate flowering variety, were genotyped using single nucleotide polymorphism (SNP) markers from the RosBREED cherry 6K SNP array v1 (Peace *et al*., 2012). Then, a QTL with a major effect for flowering date and chilling requirement was found on the 'Regina' LG4 (Castède *et al.,* 2014). This QTL, explaining 36% of the variation observed for this trait, was highly stable between the years of evaluation, and with a narrow confidence interval (less than 1cM) (Castède *et al.,* 2015). A

second RosBREED cherry SNP array of 15K SNPs has recently been made available and is composed of the 6K SNPs included in the previous array plus 9K additional SNPs.

Recently, the 'Regina' genome was sequenced (Dirlewanger *et al.,* 2018). This variety was chosen because an unigen (https://lipm-browsers.toulouse.inra.fr/plants/P.avium) and RNAseq data are already available from this cultivar, and moreover, it is one of the parents of several mapping F_1 families. Additionally, a new genetic map was constructed using Genotyping By Sequencing (GBS) markers. For this analyses, DNA fragments, digested with the restricted enzyme *ApeKI,* were aligned to the 'Regina' genome sequence. SNPs were labeled according to the scaffold in the 'Regina' sequence. This new map indicates that the LG4 is covered by 7 scaffolds. The longest scaffold, scaffold 21, covers 16 370 K bases including the region of the QTL for flowering date detected by Castède *et al.* (2015).

Following the work described by Castède *et al.* (2014, 2015), based on the analysis of 117 individuals we analyzed more individuals (1526) of the same progeny. The objective is to obtain a fine map of the QTL on LG4 in order to reduce the confidence interval of this QTL and further, to identify a smaller number of candidate genes controlling the trait. To reach this objective, the strategy indicated in figure 1 was followed.

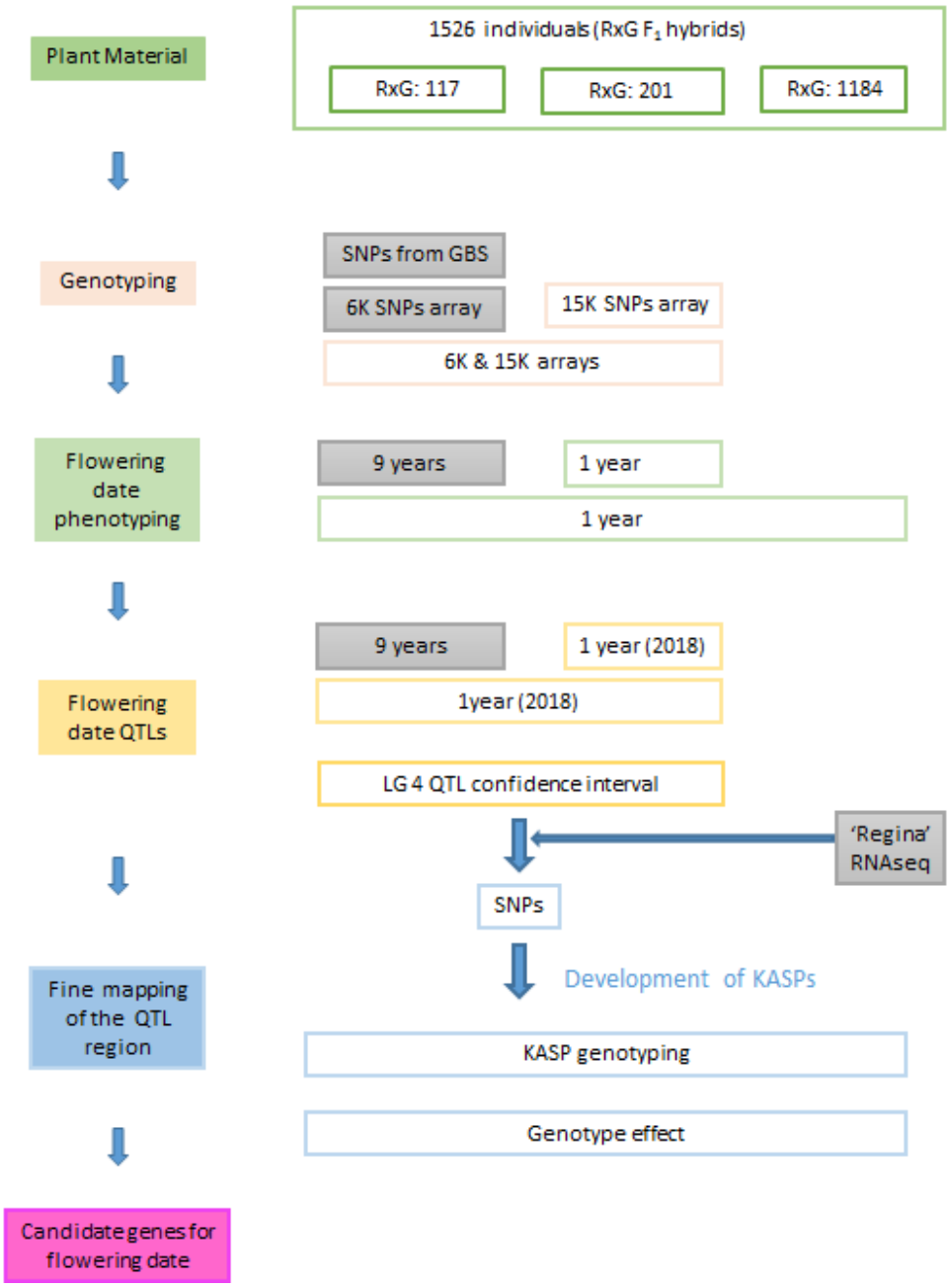

Figure 1: Strategy for the fine mapping of the QTL on LG4 controlling the flowering date.

Within a progeny of approximately 1526 F_1 hybrids from a cross between 'Regina' and 'Garnet' (RxG), three subsets of hybrids were analyzed, including 117, 201, and 1184 individuals. The two first set were genotyped with the 6K and 15K SNP arrays, respectively. The first set was also analyzed with SNPs derived from GBS. Phenotyping of flowering date were done for one or 9 years according to the set of individuals. QTL analysis were made on the RxG subset of 117 individuals (9 years of phenotyping data and analysis with 6K SNP array already available). New detections were made: with the subset of 201 (measurements of this year and mapping from the 15K SNP array), and using the two sub-populations. Fine mapping was done using new SNPs selected from de GBS analyses, the 15K SNP maps and the 'Regina' RNAseq already available. The selected SNPs were used to develop KASP markers (Kompetitive alleles specific PCR) that were used to genotype the whole progeny. Candidate genes for flowering date will be then performed on the QTL region.

Material and Methods

Plant Material

For this study, we analyzed an intraspecific sweet cherry F_1 family of 1 526 individuals, derived from a controlled cross- pollination of heterozygous parents 'Regina' and 'Garnet' called 'RxG' (Figure 1). 'Regina' is a German cultivar, with a late flowering date, while 'Garnet' is an American cultivar, with an intermediate flowering date. The self-incompatibility alleles of 'Regina' are S_1S_3 and those of 'Garnet' are S_1S_4.

This family was cultivated in the fruit tree Experimental Unit of the "Institut National de la Recherche Agronomique" (INRA) – Nouvelle Aquitaine-Bordeaux research center in Toulenne, near to Bordeaux in France (Figure 2). Three different types of trials exist: a first sub-set of this family, composed by 117 individuals, was created in 2000 and 2001 and planted in 2002 and 2003 on the full sibs' own roots. In 2010, these individuals were grafted on two clonal copies on rootstock MaxMa14 and one year later, they were planted in a different plot in a randomized complete block (RCB) design. Also in 2010, a new cross between cultivars 'Regina' and 'Garnet' allowed the enlargement of the initial family, and in 2012, 1409 new full-sibs were planted on their own roots, on the same plot than the grafted individuals. More precisely, two reciprocal crosses were made by using potted trees pollinated by bumble bees in confined tunnels, and 883 full-sibs come from the cross 'Regina' × 'Garnet' and 526 from the cross 'Garnet' × 'Regina'. Within the original family, 117 hybrids were previously analyzed with a 6K SNP array (Castède *et al*., 2014) and among these, 117 were genotyped by Genotyping By Sequencing (GBS) (Dirlewanger *et al*., 2018, Annex 1). A second set of 201 RxG hybrids from the second cross were genotyped during this study using the 15K Illumina Infinium® SNP array. The set of 117 RxG hybrids was phenotyped during 9 years for flowering date and QTLs analyses were already available. Among the 9 years of evaluation, 5 years were described by Castède *et al*. (2014). The 117 hybrids grafted on two copies and the 1409 hybrids recently planted on their own roots were phenotyped for bloom date for the first time in 2018.

Figure 2. The F_1 progeny located on Toulenne, France.

Genotyping and construction of new genetic linkage maps with RosBREED cherry 15K Illumina Infinium® SNP array

In the first step of this study we analyzed 201individuals of the RxG progeny. SNP genotypes were determined using Genome Studio® Genotyping Module (version 2.0) as described in Peace et al., (2012). Initial clustering was done using Genetrain2, a GenomeStudio® build-in clustering algorithm (Illumina, Inc., 2010 a,b). Following the clustering by Gentrain2, all SNPs were visually examined for appropriateness of clustering, cluster separation, number of clusters and presence of genotyping errors.

Construction of new genetic linkage maps

The genetic linkage maps were constructed using the cross-pollinated (CP) population option proposed in JoinMap® 5 (Van Ooijen, 2012). Heterozygous markers in only one of the parents were scored either <lmxll> or <nnxnp> and heterozygous markers in both parents were scored as <hkxhk>. Marker segregation distortion was determined by calculating chi-square (χ^2) using JoinMap®. Maternal and paternal maps construction was performed using the regression mapping algorithm. Markers were grouped using a minimum independence LOD score of 10 and LGs were established at a LOD score of 8.0 to 10 and maximum recombination frequency of 0.40. Map distance was estimated using Kosambi's mapping function. Maps were also constructed based on the analyses of the 318 hybrids using common SNP markers from the 6K and 15K SNP arrays. The graphical presentation of linkage maps was performed using MapChart software version 2.2 (Voorrips, 2002).

Detection of QTLs for the flowering date

QTL analysis were performed using MultiQTL V2.6 software (Haifa, Israel, 2005; http://www.multiQTL.com). According to the principle of multiple interval mapping, the QTL with the greatest effect is used as a cofactor to control the genetic background and then a search for additional QTL is carried out in a different position. This procedure was repeated until no other QTL was found. The proportion of the phenotypic variation explained by the QTL (EV) was calculated as EV= ¼ (d2/α ph2), where d2 is the estimated substitution effect of the QTL (d= X(A) – X(B), A and B are the two homozygote genotypes at the marker loci) and α ph is the phenotypic variance of the trait. Maps constructed with the SNPs from the GBS analyses or from the SNP arrays (6K or 15K) were used for the QTL detection.

Search of SNP markers in the region of the QTL (LG4)

Based on the smallest confidence interval of the QTL detected in the previous step in this study and in previous analysis (Castède *et al*., 2014) several strategies were followed to identify new SNP markers in this region:

1. Identification of SNP markers on the sweet cherry physical map using the RosBREED SNP arrays.
2. Identification of SNP markers from de GBS analyses.
3. Identification of new SNP markers based on the RNAseq data of 'Regina'. The RNAseq sequences were mapped on the 'Regina' scaffold 21 using TOPHAT (http://ccb.jhu.edu/software/tophat/index.shtml). SNPs were visualized with IGV® software (http://software.broadinstitute.org/software/igv/). Detection of heterozygous SNP markers in 'Regina' were searched within the QTL interval (Figure 3). SNPs with a depth of coverage higher than 30 were selected.

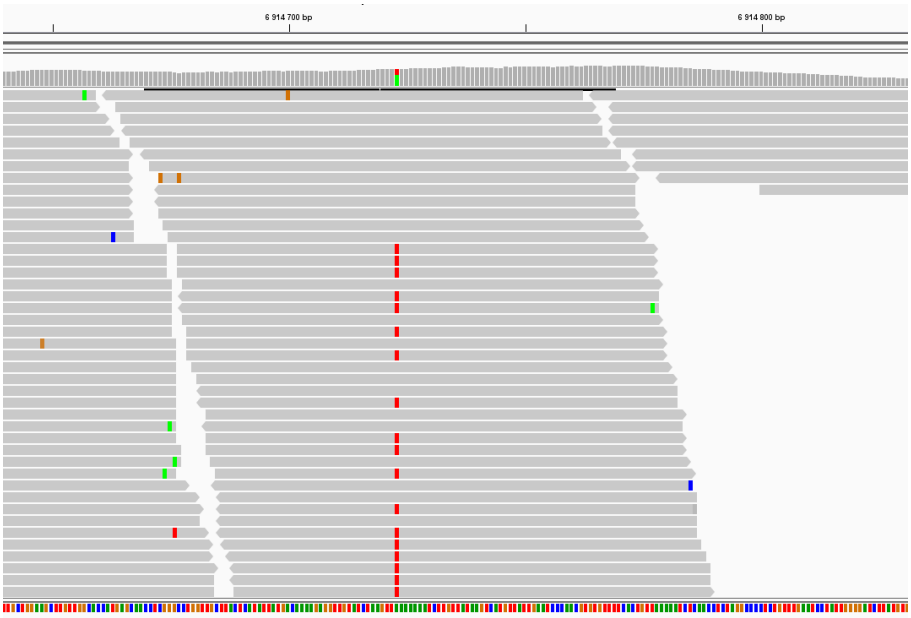

Figure 3. Visual SNPs detection using the IGV software.

Development of Kompetitive Allele Specific PCR (KASP) markers within the QTL confidence interval

KASP is a fluorescence assay that enables accurate bi-allelic discrimination of known SNPs (https://www.lgcgroup.com). This low cost technology offers high-throughput genotyping, suitable to screen thousands of breeding germplasm individuals for several SNPs linked to a trait.

SNPs spread in the confidence interval were selected to be transformed into KASP markers for genotyping the complete progeny (1526 individuals). Primers design was done using the BathPrimer3 v1 software (https://probes.pw.usda.gov/batchprimer3). Three primers (two forwards which are specific of each allele and one revers), were designed for each KASP marker. In total, primers were designed for 26 KASP markers chosen well distributed on the QTL region. Preliminary tests were performed on a subset of 30 individuals and 7 KASPs were selected to genotype the whole population for their highly easy readable profile.

Search of recombinant individuals

KASP markers located in the major QTL on LG4, were used to genotype all the 1526 'Regina' x 'Garnet' hybrids. The normality of the distribution of the flowering date within each set of individuals (homozygotes/heterozygotes) was tested with Schapiro test. To test if markers were linked to the flowering date, a Student test was used to compare the two mean values of flowering date of homozygous/ heterozygous individuals for each marker.

Results

Genetic linkage maps using the RosBREED cherry 15K SNP array

Using the RosBREED cherry 15K SNP array, a total of 13 563 SNPs were identified and were genotyped on the 201 RxG hybrids. From these SNPs, 2 631 were polymorphic (80.5% were monomorphic). Among them, 2 204 SNPs were used to construct the linkage maps (Table 1).

Table 1: summary of result from the RosBREED cherry 15K SNP array

	RosBREED cherry 15K SNP array			
		'Regina'	'Garnet'	'Regina' and 'Garnet'
Total SNPs	13 563			
Monomorphic SNPs (%)/	10 926 (80.5)	11 743	12 078	10 926
Polymorphic SNPs (%)	2.631 (19.40)	1 588	1 393	706
Used for mapping	2 204	841	657	

In a first step, 8 linkage groups (LG) were obtained as expected for *Prunus* (n=8), except for the 'Garnet' map for which LG6 was divided into two sub-groups. We tried to reduce the LOD threshold to obtain a single LG. However, by reducing the LOD, the markers from LG6 and LG8 were then included in the same LG. Consequently, trying to make a single LG6, markers located only on this group were

selected to construct the map of this LG6. Then, eight LGs were finally obtained. LGs included 140 to 221 SNPs for the 'Regina' map and 51 to 352 for the 'Garnet' map. The 'Regina' map had 1 401 SNPs covering 556.2 cM with an average distance between markers of 0.40 cM. For 'Garnet', 1 215 SNPs were mapped covering 650.0 cM with an average distance between markers of 0.52 cM (Table 2).

Markers on the bottom of LG6 were highly distorted in the 'Garnet' map. This was expected as the S self-incompatibility locus is located at this place. The self-incompatibility alleles of 'Regina' are S_1S_3 and those of 'Garnet' are S_1S_4, then all individuals of the progeny will be S_1S_4 or S_3S_4, only issued from the S_4 pollen of the male parent 'Garnet', which may explain this segregation distortion.

Maps constructed based on the analyses of the 316 hybrids using common SNP markers from the 6K and 15K arrays, allowed us to separate markers that were located at the same position, i.e.: two markers on position 20.9 cM on the 15K array were found on position 20.2 and 20.5 cM on the 6K + 15K maps. These markers are of interest because they are in the confidence interval of the QTL (Figure 4).

Table 2. Genetic map constructed from the RosBREED cherry 15K SNP array genotyping of 203 RxG genotypes. Number of SNP markers, genetic length, and average distance between markers, per linkage group (LG).

'Regina'					**'Garnet'**				
LG	Number of SNPs	Length (cM)	Average distance (cM)	Max gap (cM)	LG	Number of SNPs	Length (cM)	Average distance (cM)	Max gap (cM)
LG1	221	122.9	0.60	16.5	LG1	352	148.4	0.40	4.5
LG2	162	66.0	0.40	4.7	LG2	123	82.8	0.70	10.6
LG3	149	60.6	0.40	5.0	LG3	128	85.4	0.60	19.8
LG4	174	56.5	0.30	3.9	LG4	135	66.3	0.50	16.4
LG5	170	51.7	0.30	3.1	LG5	79	55.5	0.70	16.1
LG6	201	63.6	0.30	4.1	LG6	212	63.9	0.30	14.2
LG7	140	61.6	0.51	4.8	LG7	51	62.6	1.20	8.9
LG8	184	73.3	0.40	7.5	LG8	134	85.1	0.60	10.9
Max/group	221	122.9	0.60	18.0	Max/group	352	148.4	0.70	30.1
Min/group	149	51.7	0.30	3.1	Min/group	51	16.1	0.10	2.1
Total	**1,401**	**556.2**	**0.40**		**Total**	**1,214**	**650.0**	**0.52**	

The distribution of the SNPs in the maps constructed with the RosBREED cherry 15K SNP array along the chromosomes is represented in Fig. 5. These genetic maps will allow QTLs detection with high accuracy and the high number of SNPs available will be very helpful for using on fine mapping

approaches with the aiming to identify candidate genes and for the development of new molecular markers useable in MAS.

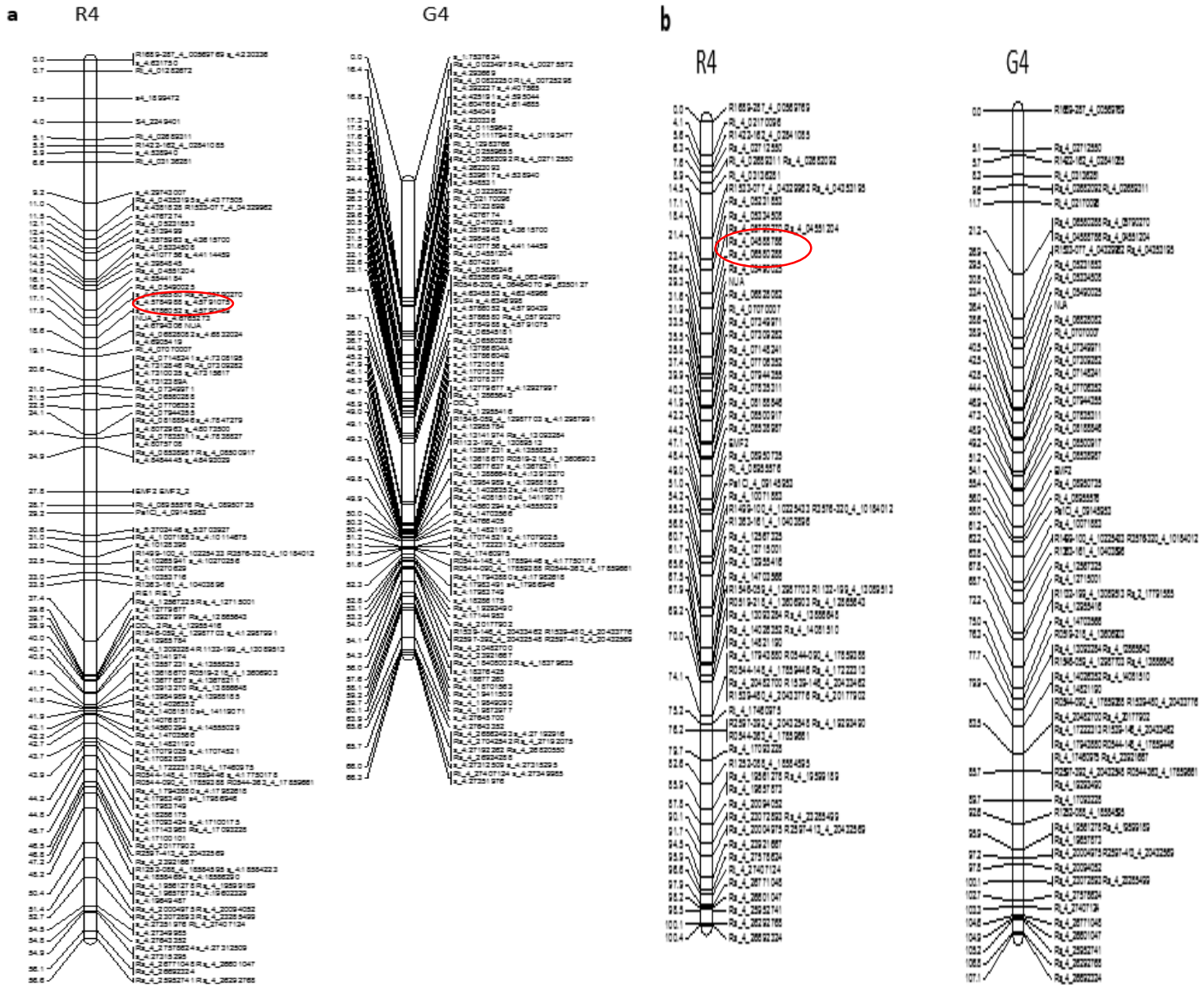

Figure 4: LG4 of 'Regina' (R4) and 'Garnet' (G4). a. Linkage maps issued from the 15K SNPs array on 201 hybrids. b. Linkage maps based on the analyses of the 316 hybrids using SNPs common markers from the 6K and 15K arrays. Markers within the confidence interval of the QTL LG4 in the same position using 201 individuals were separated using 316 individuals (red circle).

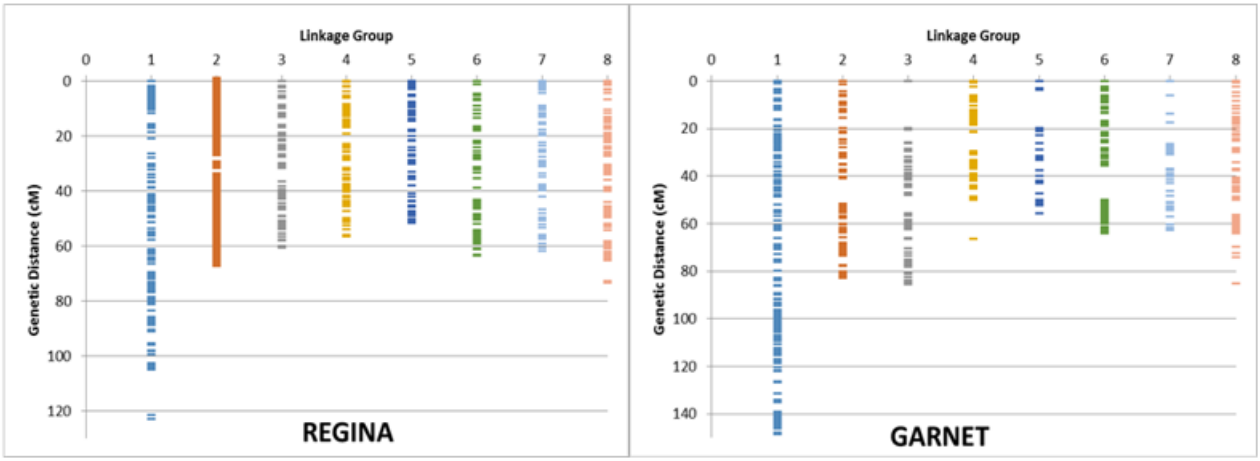

Figure 5. The distribution of the SNPs in the maps constructed with the RosBREED cherry 15K SNP array.

Analyses of QTLs for the flowering date

Based on the 9 years of flowering evaluation since 2008 on the RxG 117 hybrids on their own roots, QTLs were detected in nearly all the LGs of 'Regina' and 'Garnet', the one with higher effect located on the LG4 of 'Regina' with an EV of 34.9 % and a very small confidence interval (Table 3).

Base on the flowering date evaluated in 2018, on the RxG 117 hybrids grafted, three major QTLs in LG2, LG4 and LG7 of 'Regina' were detected. LG4 was the most significant with an EV of 31.5%. Using the 201 hybrids on their own roots, one major QTL on the LG4 of 'Regina' was detected with an EV of 6.7 %. Then, using the 318 hybrids (RxG 117 + RxG 201), two major QTLs were detected on LG4 and LG6 of 'Regina', with a large confidence interval (35,8 cM) and an effect of 11 % (LG4) and 4.3 % (LG6). Not significant QTL was detected in 'Garnet' with the evaluation done in 2018 (Table 3).

Table 3. QTL on LG4 from 117 (8 years and one years), 201 and 316 hybrids.

		LOD	L (cM)	CI (95%)	EV %.
9 years	117 RxG own roots	136,3	20,05	< 0.5 cM	34,9
2018	117 RxG grafted	13,62	23,92	9,6 cM	31,5
	201 RxG own roots	2,99	28,01	59,6 cM	6,7
	318 RxG own roots	7.727	32.63	35,8 cM	11

LOD: Lod Score, L: length from the top of the linkage group, CI: confidence Interval, EV %: percentage of explained variation

Several confidence intervals were defined, each one is delimited by two markers. The markers names in the 6k RosBREED SNP array correspond to the physical map positions in the peach genome sequence V1 while the names of the GBS markers correspond to their physical map of the sweet cherry

'Regina' genome sequence. (Table 4). The smallest confidence interval of the QTL (LG4) for flowering date was obtained with the 9 years of evaluation of the 117 hybrids on their own roots.

Table 4. Detail of the Markers which are the border of the QTLs detected on 117 and 316 hybrids.

QTL (LG4) detection	C.I (cM)	Markers/Origin	Marker	Physic Interval. Peach V1.	Physic Interval. Scaffold 21 (sweet cherry)
9 years/ 117 hybrids	< 0.5	6K RosBREED SNP array	Rs_4_07309282	7309282	7101147
			Rs_4_07944355	7944355	6211705
1-years/318 hybrids	35.8	15K RosBREED SNP array.	Rs_4_07148241	7148241	7273811
		6K RosBREED SNP array,	Rs_4_27578624	27578624	Outside

SNPs identified in the confidence interval of the QTL

As the smallest confidence interval of the QTL (LG4) for flowering date was obtained with the 9- years of evaluation of the 117 hybrids on their own roots, the SNPs were searched in the region of the peach physical region LG4-7309282-794435, corresponding to the Scaffold 21: 6211705-7101147. Twenty-six SNP markers were selected from the three approaches (Annex 2):

1. 4 SNPs were detected from the RosBREED SNP array
2. 7 SNPs were identified from the GBS analyses
3. More than one hundred SNPs were identified with the RNAseq analyses of 'Regina', only 15 were chosen to the next step.

KASP markers

From this pool of SNPs, KASP markers were developed (Table 5). The three primers per KASP marker are indicated in the Annex 3. They were tested on 23 individuals and 10 were selected to genotype all progeny (1526 RxG hybrids). Two groups corresponding to heterozygous and homozygous individuals were detected, corresponding to early or late flowering individuals (Figure 6).

Table 5. SNPs used to develop KASP markers.

SNPs	Origin SNP	Position on scaffold_21	Genotype 'Regina'	Genotype 'Garnet'	KASP name
Super-Scaffold_21_6184315	GBS	6184315	C/T		
RosBREED_snp_sweet_4_07944355	SNP 6K	6211705	T/C	T/T	KASP 1
Super-Scaffold_21_6248027	GBS	6248027	A/G		
Super-Scaffold_21_6280991	GBS	6280991	A/G	A/A	KASP 2
Super-Scaffold_21_6419113	RNA_seq	6419113	C/T		
Super-Scaffold_21_6435196	RNA_seq	6435196	A/G	G/G	KASP 3
Super-Scaffold_21_6480810	RNA_seq	6480810	A/C		
Super-Scaffold_21_6590224	GBS	6590224	A/G		
Super-Scaffold_21_6617951	RNA_seq	6617951	T/C		
Super-Scaffold_21_6643700	GBS	6643700	A/G	A/A	KASP 4
Super-Scaffold_21_6708069	RNA_seq	6708069	A/G		
Super-Scaffold_21_6914574	RNA_seq	6914574	A/T	A/A	KASP 5
Super-Scaffold_21_6943204	RNA_seq	6943204	A/G		
Super-Scaffold_21_6948399	RNA_seq	6948399	C/T		
RosBREED_snp_sweet_4_07349971	SNP 6K	7053286	T/C		
Super-Scaffold_21_7058361	RNA_seq	7058361	A/C		
Super-Scaffold_21_7078306	GBS	7078306	T/A		
Super-Scaffold_21_7078450	RNA_seq	7078450	A/G		
scaffold_4:7312389	SNP15K	7098164	A/G		
Super-Scaffold_21_7098254	RNA_seq	7098254	A/C		
Super-Scaffold_21_7099952	GBS	7099952	G/A	A/A	KASP 6
Super-Scaffold_21_7099961	RNA_seq	7099961	T/C		
RosBREED_snp_sweet_4_07309282	SNP 6K	7101147	T/C		
Super-Scaffold_21_7101797	RNA_seq	7101797	C/T	T/T	KASP 7
Super-Scaffold_21_7192553	RNA_seq	7192553	G/C		
Super-Scaffold_21_7260307	RNA_seq	7260307	A/G		

SNPs used to genotype the 1 526 hybrids.

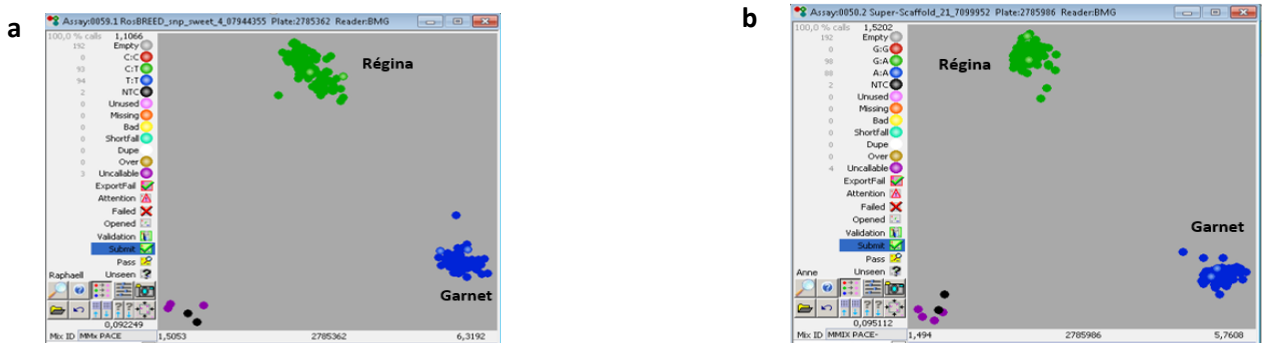

Figure 6: KASP marker profiles a: KASP 6, b: KASP 1. Green and blue corresponding to heterozygous (such as 'Regina') and homozygous (such as 'Garnet') individuals, respectively.

KASP genotyping

To genotype the progeny of 1526 'Regina' x 'Garnet', 10 KASP markers well distributed within the QTL (LG4) region, were selected. At the moment, the results of 7 KASPs are available, information of the remaining three additional will be only available at the end of June (Annex 4).

Based on the genotyping with the 7 KASPs, fifteen genotypes (G) were identified (Table 7). For all KASP markers, 'Garnet' was homozygote (named A), while 'Regina' was heterozygous (named B).

Among the 1385 individuals from which genotypes were identified, 1338 (96%) individuals have parental genotypes 639 were homozygous and 699 were heterozygous for all markers. In total, 47 recombinant individuals were detected. Recombinant events were detected between all markers except between the KASP 6 and 7, these two markers separated by the lowest distance (1.8Kb). Only one recombination was identified between the KASP 1 and 2 (G11), separated by 69Kb. Genotypes with single recombination were observed for 41 individuals:

- 1 individual (G9) with a recombination between KASP 2 and 3 (separated by 154 Kb),
- 20 individuals (G7 and G8) with a recombination between KASP 3 and 4 (separated by 208 Kb),
- 17 individuals (G5 and G6) with a recombination between KASP 4 and 5 (separated by 270 Kb),
- 3 individuals (G3 and G4) with a recombination between KASP 5 and 6 (separated by 185Kb).

Double recombinant events were observed for 3 individuals corresponding to G10, G11 and G15 genotypes (Table 6). Four recombinant events were observed for 2 individuals with G12 and G13 genotypes. For the individual with the G14 genotype, at least 3 recombinations occurred. The mean values for the flowering date for the G5 to G8 genotypes (37 recombinants), suggest that the region involved in the flowering date is may be delimited by the KASP 1 and KASP 3 marker. However, the mean values for the flowering date for the G3, G4 and G9 (4 recombinants), suggest that the region involved is delimited by the KASP 6 and covers the opposite side. Consequently, according to the phenotype values obtained this year, it is not possible to define a reliable region by using the recombinants data.

Table 6. Genotypic Determination of the 1526 progeny (RxG hybrids) by each KASP markers.

Source	KASP1 6K	KASP2 GBS	KASP3 RNAseq	KASP4 GBS	KASP5 RNAseq	KASP6 GBS	KASP7 RNAseq		Mean date flo.
Position in scaffold 21	6211705	6280991	6435196	6643700	6914574	7099952	7101797	n° ind	
G1 (Garnet)	A	A	A	A	A	A	A	639	97,43
G2 (Regina)	B	B	B	B	B	B	B	699	99,65
G3	A	A	A	A	A	B	B	2	99,00
G4	B	B	B	B	B	A	A	1	96,00
G5	A	A	A	A	B	B	B	10	97,70
G6	B	B	B	B	A	A	A	7	99,00
G7	A	A	A	B	B	B	B	8	97,75
G8	B	B	B	A	A	A	A	12	99,82
G9	A	A	B	B	B	B	B	1	99,00
G10	B	B	B	B	A	B	B	1	96,00
G11	B	A	B	B	B	B	B	1	103,00
G12	B	B	A	B	A	B	B	1	99,00
G13	B	-	A	B	A	B	B	1	94,00
G14	-	-	A	B	A	B	B	1	89,00
G15	B	-	B	A	B	B	B	1	94,00

KASP markers which their respectively source and position on the 'Regina' genomic sequence. Fifteen genotypes (G) were determined on 1526 hybrids of RxG. A: homozygous genotypes. B: heterozygous genotypes.

The recombination events shown in Table 6, associated with the phenotypic value of each genotype group, do not allow reducing the confidence interval of the QTL region. Consequently, the candidate genes list identified by Castède et al. (2014) is still valuable and none could be eliminated according to their physical position.

However, by comparing the mean value of the homozygous /heterozygous individuals for each marker, significant differences were observed for all markers: homozygous having earlier flowering date than heterozygous individuals. Two days of difference were detected between means, indicating the potential of prediction of the markers (Table 7). Genotypes effects for each marker are shown in figure 7. These results indicate that these seven markers could be used in MAS.

Table 7. Comparison of the means of flowering date of homozygous (A) and heterozygous (B) individuals for each marker

		KASP1		KASP2		KASP3		KASP4		KASP5		KASP6		KASP7	
		mean	Ind.	mean	Ind.	mean	Ind.	mean	Ind.	mean	Ind.	mean	Ind.	mean	Ind.
	A	97,44	654	97,45	667	97,42	662	97,47	658	97,46	657	97,48	662	97,47	661
	B	99,61	721	99,64	719	99,63	723	99,59	718	99,59	717	99,56	725	99,55	723
Numb of ind.			1375		1386		1385		1376		1374		1387		1384
Days		2.17		2.18		2.21		2.12		2.13		2.08		2.08	

KASP markers and they respectively mean by genotype. A: homozygous. B: heterozygous. Ind: number of individual by mean. Days: significate different between mean (P<0.05)

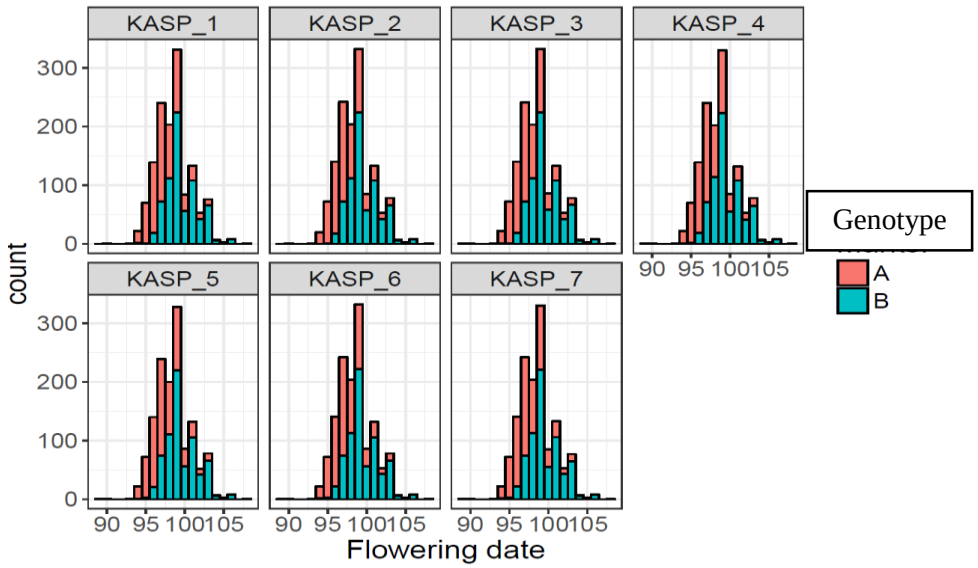

Figure 7. Genotype effect for each marker. A: homozygous. B: heterozygous. Axis x: classes by flowering date in Julien days. y: number of individuals.

Discussion

Considering the climate change and the reduction of chilling accumulation observed, concern about climate adaptation in temperate fruit trees has arisen. It is essential for plant cultivars to be well adapted to warmer winter, spring frosts and summer heat waves. For that reason, it is necessary to have a better understanding of the genetic control of flowering and of its environmental regulation. This will allow the evaluation of climate change impacts and the anticipation of future needs for production.

In this study we analyzed a QTL region on LG4 which was reported by Castède *et al*. (2014 and 2015) to be involved in the genetic control of flowering date and other related traits such as chilling and heat requirements. The presence of this QTL was detected in several years on 'Regina' in the RxG progeny. In the frame of the RosBREED project a new chip of 15K SNPs was developed and can be used to construct genetic linkage maps.

Construction of new genetic linkage maps

The RxG 201 hybrids were genotyped using the 15K RosBREED SNP array. Among a total of 13 563 SNPs, 10 926 were monomorphic and 2 631 were polymorphic in the progeny. These results are similar to those obtained using the 6K SNPs array analysis. Among the 5 696 SNPs, of the 6K SNPs array, a total of 1825 were polymorphic where the majority (63 %) were monomorphic (Castède *et al.*, 2014).

Parental maps of 'Regina' and 'Garnet' were constructed. The total coverage of the two parental maps were similar to those previously published for cherry trees (Kaggles *et al*., 2013; Castède *et al*., 2014). Markers with distortion segregation were located on the bottom of LG6 of 'Garnet'. This is in agreement with the results previously detailed by Castède *et al.,* (2014), who mentioned that these distortions could be associated with the presence of the gametophytic self-incompatibility *S* locus, located at the bottom of LG6 (Dirlewanger *et al.,* 2004; Tao and Iezzoni. 2010).

In addition, the fact that markers known to be on LG6 and LG8 on 'Garnet' were grouped in the same LG, may resulted of a translocation between these two groups previously reported by Jauregui *et al*. (2001) and Dirlewanger *et al*. (2004).

SNP array technology can be compared with GBS analysis. Using GBS analysis, 8 642 SNPs were identified on RxG 117 hybrids where 49.6% SNPs were monomorphic. The 'Regina' map includes 2 130 SNPs covering 714.6 cM with an average distance between markers of 0.34 cM. The 'Garnet' map includes 1 826 SNPs covering 768.1 cM whit an average distance between markers of 0.42 cM. (Dirlewanger et al., 2018). Using the 15K RosBREED SNP array, 80.5 % of the 13 563 SNPs were

monomorphic in the RxG progeny (details Table 2). The detection of high number of potential SNPs polymorphs could be more effective using a GBS approach. However, it needs bioinformatics analyses which are time consuming and require specific knowledge, making it not entirely possible for all the breeding programs. The SNP array technology is easier to analyze and not require specific knowledge.

SNPs selected to construct the Cherry SNP chip were polymorphic on a set of 16 sweet cherry cultivars and 8 sour cherries (Peace *et al*., 2012). Consequently, many SNPs moght not be polymorphic in other cultivars. Similar results were reported with the Peach IPSC 9K Infinium II array (Verde *et al*., 2013) were many SNPs were monomorphic and not usable for linkage map construction (Eduardo *et al*., 2013; Sánchez *et al.*, 2014). Another possible limit of these SNP chips is that included SNPs might not be true SNPs. Indeed, the depth of resequencing of the initial set of 24 cultivars was not too high, which increases the risk of false SNP detection. Hence, Campoy *et al*. (2016) studied a large sample of 150 different genotypes (both old landraces and modern cultivars) and found a relatively high number of monomorphic SNPs.

Analyses of QTLs for the flowering date

The smallest confidence interval of the QTL (LG4) for flowering date was obtained with the 9 years of evaluation of the 117 hybrids on their own roots. This confidence interval (less than 0.5 cM) corresponds to the one that was determined by Castède *et al.*, (2014). Using the two sub-populations of hybrids (RxG 117 + RxG 201) and phenotypic data from only this year, it was determined an interval of confidence with more amplitude (35,8 cM). Hence, the region of QTL LG4 was not reduced.

Several aspects can be considered to explain the differences between the confidence intervals found while working with different types of full-sibs subsets. First, the climatic conditions were particularly unusual in 2018: after a relatively mild winter (in particular during January) the bloom period was particularly fresh and rainy. More specifically, a high proportion of abnormal flowers and necrotic flowering buds (which never went through bud burst and hence to flowering) was observed on the 1 409 R×G and G×R individuals planted on their own roots. Although these phenotypic observations were also made on the grafted parents ‘Regina’ and ‘Garnet’, they may have been less frequent within the 117 grafted individuals. On the other hand, the 1 409 full-sibs may still be in a phase of transition between juvenility and adult life. Differences in the QTL detection for flowering date have already been observed in different *Prunus* species when working with juvenile versus adult trees (Dirlewanger et al., 2012). Taking this into account, it appears as highly necessary to have phenotypic data of several years. Finally, the fact of mixing the data from the 117 grafted individuals and the 203 individuals planted on their own

roots did not allow increasing the power of QTL detection as could have been expected. Indeed, one of the critical factors for QTL detection studies is the number of full-sibs studied. Indeed, it is known that flowering, but also maturity dates differ between grafted cherries and cherries planted on their own roots. The best strategy to dramatically increase QTL detection power would be to genotype the 1409 individuals with a set of molecular markers covering LG4 and to conduct new QTL analyses on this large subset.

SNPs identified in the confidence interval of the QTL and development of KASP markers

Many markers were found in the QTL region using these three strategies. However, more than one hundred SNPs were detected with the RNAseq approach. This strategy could be a good sources of SNPs all over the genome.

Twenty-six SNPs were successfully transformed to KASP markers, seven of them were tested by now on the RxG 1 500 progeny. High quality genotypes were obtained for all the KASPs, indicating that they could be used for sweet cherry MAB. All these 7 KASPs, covering an interval of 0.8 Mb, have similar effect: heterozygous genotypes flowering 2 days later than the homozygous. This difference is not so important based on the phenotypic evaluation of this year but we cannot rule out the possibility that this difference may be higher for year.

As the confidence interval of the QTL LG4 was not reduced, none of the previous selected candidate genes (Castède *et al.*, 2015) were eliminated. Once the confidence interval be reduced, i*n silico* candidate gene analysis will be performed again. The search could be easily done in the GDR database (https://www.rosaceae.org/), using the *Prunus avium* v1.0 genome.

Conclusion and perspective

Using all the genomic tools now available on sweet cherry, it is possible to develop and use markers for breeding programs. The strategy followed in this study can be used for other traits such as ‘large fruits’ and ‘firm fruits’. This is possible as soon as QTL covering a small region is available. The critical factor will be the quality of the trait evaluation, with a higher reliability when many years of observation are available. In this study we developed 7 KASP markers that can be useful to select genitors with early or late flowering date. In order to validate them, as it was done for the KASPs associated to fruit weight and firmness Dirlewanger, et al., (2018), these 7 KASPs will be tested on the whole cherry

germplasm collection and on other families issued from other parents. To continue the development of new KASPs, the strategy of using the ‘Regina’ RNAseq sequences mapped on the ‘Regina’ scaffolds is an infinity source of potential SNPs for different traits.

To search candidate genes involved in the control flowering date, in the present study, we followed a positional approach. Another complementary approach would be to look for genes differentially expressed between the two parents. This is in progress in the team using RNAseq data from flowering buds at different stages of development from the two parents. Genes differentially expressed between the two parents and located in the confidence interval will be good candidates. Another alternative strategy to identify and validate candidate genes would be to use the cherry core collection that is already phenotyped for several years for the flowering date. This collection was genotyped with the 6K SNPs but not significant association were detected for the moment. Additional markers may be need to find significant associations.

By using all these strategies, we can expect that new varieties, highly adapted to future climate and with good fruit quality will be selected in the future to meet producers and consumers demand.

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Annexes

Annex 1

Dirlewanger E, Le Dantec L, Fouché M, Zaracho Echagüe N, Campoy JA, Barreneche T, Wenden B, Flutre T, Iezzoni A, Quero-García J (2018) New sweet cherry genomic tools and their use in marker-assisted breeding. XX. International Horticultural Congress, August 12-16 2018 Istanbul, Turkey

New sweet cherry genomic tools and their use in marker-assisted breeding

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Abstract

Thanks to the new sequencing technologies, a vast amount of genomic data such as genome sequences, high density genetic maps and transcriptomic data, have become available for genetic studies in sweet cherries. Using all these resources, it is possible to detect QTLs covering a very small chromosomic region and to find molecular markers tightly linked to traits of interest. Moreover, based on fine mapping and RNASeq analyses, candidate genes can be easily identified with a higher accuracy. Hence, marker-assisted breeding (MAB) has now become a reality for this species. Given that sweet cherry has a long period of juvenility and that large areas are needed to evaluate thousands of new hybrids, MAB will allow breeders to increase the efficiency of their programs and plant only those hybrids with favorable allelic combinations for the most critical agronomic traits. The main goal of our team is to understand sweet cherry adaptive responses to climate change in order to create sweet cherry cultivars well adapted to the global warming, with a good yield and good fruit quality. We focus on complex traits such as chilling and heat requirements for flowering as well as fruit weight, firmness and additional fruit quality traits in order to meet farmer's needs. In this study, we will present the new 'Regina' genome sequence using a combination of sequencing strategies (PacBio RSII sequencing and BioNano optical mapping). The efficiency of the two genotyping technologies, 15K SNP arrays and Genotyping By Sequencing (GBS) will be compared for the construction of high density linkage maps. Moreover, new prospects offered by genomic selection approaches, aiming at selecting hybrids for difficult and expensive traits to phenotype, will be presented. These genomics tools will considerably decrease the cost and the duration of our sweet cherry breeding program.

Keywords:

Prunus avium, genomic, genetic, QTL, marker assisted breeding, adaptation to climate change

INTRODUCTION

Sweet cherry (*Prunus avium* L.) is an economically important fruit crop, highly appreciated in many countries around the world in temperate, Mediterranean and even subtropical regions. According to the Food and Agriculture Organization (FAO), the world annual sweet cherry production is about 2.2 million t, showing a slightly increasing tendency. The majority of cultivated cherry trees belongs to the diploid (2n=2x=16) sweet cherry and the segmental allotetraploid (2n=4x=32) sour cherry (*P. cerasus* L.) species. The genome size of sweet cherry was estimated at 338 Mb with flow cytometry (Arumuganathan and Earle, 1991), and, more recently, at 353 Mb estimated by k-mer analysis from the 'Satonishiki' cherry genome (Shirassawa et al., 2017). Numerous linkage maps have been constructed in sweet cherry using different types of markers, SSRs (Dirlewanger et al., 2004), SNPs (Cabrera et al., 2012; Klagges & Campoy et al.,2013; Guajardo et al., 2015), or Specific-Locus amplified Fragment (SLAF) (Wang et al., 2015). These maps are useful for genetic analysis of relevant traits in sweet cheery breeding by QTL analysis (Rosayara et al., 2013; Castède et al., 2014; Campoy et al., 2015). QTL for traits of interest can be further used for Molecular Assisted Breeding (MAB) (Peace 2017) or to identify candidate genes controlling these traits (De Franceschi et al., 2013; Castède et al., 2015). Breeding of perennial trees such as sweet cherry is a long and expensive process. Indeed, due to a long juvenile phase, it can take more than 20 years to create a new cultivar. Traditionally, the main sweet cherry breeding objectives have been to select for large and firm fruits with good-flavoured, abundant and consistent yields, reduced susceptibility to fruit cracking, self-compatibility and improved resistance or tolerance to diseases such as bacterial canker induced by *Pseudomonas mors* pv. *prunorum* and *P. syringae* (Proebsting et al., 1987; Kappel et al., 1996; Iezzoni, 2008; Kappel et al., 2012; Quero-Garcia et al., 2017a).

Due to rapid climate change and the reduction of chilling accumulation already observed in numerous temperate zones (Luedeling et al., 2011) concern about climatic adaptation has arisen (Campoy et al., 2011). Cultivars with low chilling requirements, well adapted to warm autumn and winter and with high heat requirements to avoid early flowering and frost damage, are desired. As for all long-lived perennials, cherry orchards are installed for long periods, up to 20 years; hence, the choice of new cultivars that will be planted is crucial. To increase the efficiency of breeding programmes and to reduce the cost of the tree evaluation on orchard, MAB approaches can be developed for all these important traits. This will allow meeting farmer's needs by creating new sweet cherry cultivars well adapted to the global warming, with good yield and fruit quality.

Here, we will give an over view of new genomic data, such as the new genome sequence from the 'Regina' cultivar, new high density SNP genetic maps and the development of Kompetitive Allele Specific PCR (KASP). Moreover, preliminary results on genomic prediction approaches used to select hybrids for highly complex traits, both in terms of genetic determinism and in terms of complexity of phenotyping, will be presented.

MATERIALS AND METHODS

Whole genome sweet cherry sequence

We have sequenced and assembled the genome of the 'Regina' sweet cherry cultivar, which is a late blooming cultivar. This cultivar was chosen for several reasons: a unigen (https://lipm-browsers.toulouse.inra.fr/plants/P.avium) and RNAseq data are already available from this cultivar, and additionally, it is one of the parents of several mapping F_1 families.

In order to optimize the sweet cherry genome assembly, DNA sequencing was performed with PacBio RSII to obtain long reads and an optical map was constructed with BioNano. The PacBio sequencing was done by the GeT-PlaGe INRA platform (http://get.genotoul.fr/) and the optical map was performed by INRA CNRGV (http://cnrgv.toulouse.inra.fr/).

For the PacBio RSII sequencing, we used 2g of young leaves and DNA was extracted according to the protocol developed by Mayjonade et al. (2016). For the BioNano optical map, cell nuclei were embedded in plug of agarose gel 1%, then nuclei membrane was digested using proteinase K. DNA plugs were migrated in pulsed field gel electrophoresis to eliminate degraded DNA. Purified high molecular weight DNA was recovered by digestion of the agarose plugs using agarose. Obtained DNA fragments ranged from 150 kb to more than one megabase. The Genomics Irys Technology of BioNano was used to analyze DNA labelled by incorporation of fluorescent nucleotides through nicking by the enzyme *Bss*S1.

PacBio long reads were de novo assembled using FALCON assembler and phased using FALCON UNZIP. BioNano optical whole genome maps were used for further scaffolding. Completeness of the assembly was assessed with sets of Benchmarking Universal Single-Copy Orthologues (BUSCO).

SNPs development using GBS analyses

115 hybrid progeny from the 'Regina' x 'Garnet' cross (RxG), and previously analysed with an Illumina 6K SNP chip (Castède et al., 2014) were genotyped by Genotyping By Sequencing (GBS). The aim was to generate a higher number of markers to test the feasibility of genomic selection approaches by working with this biparental population. DNAs were digested with the restricted enzyme *Ape*KI which recognizes a degenerate five base-pair sequence (GCWGC). This enzyme with partial sensitivity to DNA methylation promotes the exclusion of repetitive regions. GBS libraries were constructed following the protocol described for maize (Elshire et al., 2011). The DNA fragments were sequenced with the Illumina HiSeq3000. The reads were aligned to the sweet cherry 'Regina' genome. SNPs were extracted using a pipeline including several steps: quality control with FastQC, demultiplexing, trimming with CutAdapt, alignment with BWA, SNP calling and filtering with GATK. SNPs were labeled according to the scaffold in the 'Regina' sequence. Genotype imputation was performed with FImpute (Sargolzaei et al., 2014).

Genotyping with RosBREED cherry 15K Illumina Infinium® SNP array

A new cross between cultivars 'Regina' and 'Garnet' was conducted in 2010 in order to enlarge the initial population for QTL fine mapping purposes (Quero-Garcia et al., 2017b). Out of the 1500 new hybrids created, 203 were genotyped with SNP markers from a recently designed new cherry Illumina 15K SNP array. This array contains the 6K SNPs already present in the one used to build the first map of RxG, all the mapped GBS-derived SNPs from Guajardo et al. (2015), and polymorphic SNPs from the INRA ESTs (Vanderzande S., personal communication). Genotypes were scored with the Genotyping Module of the GenomeStudio Data Analysis software (Illumina, Inc., San Diego, CA, USA) using the default parameters.

The genetic map was constructed with the SNP markers using the software JoinMap 5 with the Regression mapping and Kosambi's distance calculation settings.

Development of Kompetitive Allele Specific PCR (KASP) markers linked to agronomical traits

QTLs for fruit weight and firmness had been previously detected on linkage groups 2 and 5 of 'Regina', by working with 'Regina' x 'Lapins' (RxL) and RxG crosses, and by using MultiQTL V2.6 software (Rosyara et al., 2013; Campoy et al., 2015). SNP markers located in the QTL regions controlling these traits were identified and used to design allele specific primers for the KASP PCR amplification. The primer design was done with BatchPrimer3 v1.0 and the end point genotyping was done with the KASP V4 2x Mastermix from LGC. To validate the trait predictiveness of these KASP markers, a genotyping assay was performed on a subset of the two F_1 families RxG and RxL (32 individuals of each family) and on a subset of 31 cultivars from the INRA sweet cherry collection which had already been genotyped with the 6K SNP array (Campoy et al., 2016) and which present contrasted phenotypes for the fruit weight or the fruit firmness.

First development for genomic prediction

Genomic prediction is being assessed on the RxG family (116 genotypes) genotyped by sequencing (GBS). Flowering date was chosen as the trait to predict. In a first stage, raw phenotypic data collected over several years were combined to obtain BLUPs of genotypic values via the *lme4* R package (Bates et al., 2015).

In a second stage, an infinitesimal model with additive and dominance SNP effects was used to predict the genotypic BLUPs from the SNP data via the *rgs3* R package (Flutre, 2018) which wraps the *GS3* software (Legarra et al., 2014).

RESULTS AND DISCUSSION

The sweet cherry genome sequence

Based on the protocol developed by Mayjonade et al. (2016), 40 µg of very pure DNA with high molecular weight were obtained (50 kb) from 'Regina'. Three libraries were produced with 40 kb mean size fragments. 30 single molecule real time sequencing (SMRT) cells were sequenced with the PacBio RSSII. A total of 2.5 million reads (27.9 billion bases) with a N50 of 17.5kb were obtained, corresponding to 82.5X genome coverage. PacBio long reads were *de novo* assembled (contig N50=1.23 Mb) and phased using FALCON Unzip (Table 1). Following the PacBio assembly, BioNano optical whole genome mapping was used for further scaffolding. Using *BssS*1 as the nicking enzyme, 433 contigs with a N50 of 1.7 Mb were obtained (Table 2). Finally, hybrid assembly between PacBio contigs and BioNano map produced a genome of 279 Mb (83% of the estimated genome size) with high contiguity (scaffold N50 = 5.96 Mb). BUSCO analysis of this assembly revealed a good completeness with 90.9% of complete orthologues (95.9% if the hybrid assembly was complemented with unscaffolded PacBio contigs and haplotigs). Some comparisons of this assembly with the published assembly of *Prunus avium* 'Satonishiki' (Shiraswa et al., 2017) are given in Table 3.

Table 1. 'Regina' PacBio assembly using Falcon assembler and phased with Falcon Unzip

	Primary contigs	Haplotigs
Number of contigs	1,078	1,289
N50 (Mb)	1.23	0.35
L50	68	150
Max Size (Mb)	10.5	1.83
Total Size (Mb)	323	177
% genome coverage (338 Mb)	95%	52%

Table 2. Number and length of contigs obtained with the BioNano Assembly

Number of contigs	433
Median length (Mb)	0.809
N50 (Mb)	1.7
Max Size (Mb)	9.9
Total Size (Mb)	535
% genome coverage (338 Mb)	158

Table 3. Comparison of the two sweet cherry genomes 'Regina'and 'Satonishiki' sequences

Cultivar	'Regina'	'Satonishiki' (Shirasawa et al, 2017)
Technology	PacBio RSII + BioNano	Illumina HiSeq
Number of scaffolds	92	10,148
Size of scaffolds	279 Mb (hybrid scaffold)	272 Mb
Scaffold N50	5.96 Mb	0.21 Mb
Longest scaffold	16.3 Mb	1.46 Mb
GC (%)	38.2	37.7

High density genetic maps from GBS data and 15K SNP RosBREED array

The GBS analysis lead to the identification of 8,642 SNPs that were genotyped on 115 RxG hybrids while 13,563 SNPs were identified with the 15K SNPs array that were genotyped on 203 RxG different hybrids (Table 4). From these SNPs, 49.6 % and 80.5 % were monomorphic among those obtained by the GBS and 15K array, respectively (Table 4). From the 4,358 polymorphic SNPs obtained by GBS analyses, 3,956 SNPs with expected Mendelian segregation and with less than 20% of missing data were used for mapping. Using the 15K SNP RosBREED array, 2,631 polymorphic SNPs were obtained and from these markers, 2,204 were used to construct the linkage maps (Table 4).

Table 4. Total number of SNPs and numbers of monomorphic, polymorphic and usable markers for mapping obtained by GBS analyses and by the 15K SNP RosBREED array

	GBS	**15K SNP RosBREED array**
Total SNPs	8,642	13,563
Monomorphic SNPs (%)	4,284 (49.6)	10,926 (80.5)
Polymorphic SNPs (%)	4,358 (50.4)	2,631 (19.40)
Usable for mapping	3,956	2,204

Among SNPs produced by GBS, 1,351 and 1,317 were heterozygous in 'Regina' and 'Garnet', respectively, and 1,690 SNPs were heterozygous in both cultivars. Using these markers, parental linkage maps of 'Regina' and 'Garnet' were constructed. The eight expected LGs contained between 205 to 326 SNPs for the 'Regina' map and between 100 to 320 SNPs for the 'Garnet' map. The 'Regina' map had 2,130 SNPs covering 714.6 cM, with an average distance between markers of 0.34 cM. For 'Garnet', 1826 SNPs were mapped covering 768.1 cM with an average distance between markers of 0.42 cM (Table 5).

With the SNPs from the 15K SNP RosBREED array, eight LGs were obtained for 'Regina' and 'Garnet' maps (Table 5). LGs included 149 to 221 SNPs for the 'Regina' map and 51 to 352 SNPs for the 'Garnet' map. The 'Regina' map had 1,401 SNPs covering 556.2 cM, with an average distance between markers of 0.40 cM. For 'Garnet', 1,214 SNPs were mapped covering 650 cM with an average distance between markers of 0.52 cM (Table 5). As the S-alleles for 'Regina' are S_1S_3 and those for 'Garnet' are S_1S_4, gametes from 'Garnet' can only carry the S_4 allele and hence, markers on the bottom of LG6 were highly distorted.

The distribution of the SNPs in the maps constructed with the GBS and the 15K RosBREED array technologies along the chromosomes is represented in Fig. 1. These genetic maps will allow detecting QTLs with high accuracy and the high number of SNPs available will be very helpful for fine mapping approaches aiming at identifying candidate genes and at developing new molecular markers useable in MAB.

Table 5. Number of SNP markers, genetic length, average distance between markers, per linkage group (LG) in 'Regina' and 'Garnet' maps constructed from the GBS or 15 K array genotyping

'Regina'					'Garnet'"				
LG	Number of SNPs	Length (cM)	Average distance (cM)	Max gap (cM)	LG	Number of SNPs	Length (cM)	Average distance (cM)	Max gap (cM)
Genetic map constructed from the **GBS** genotyping of 115 RxG genotypes									
LG1	322	112.0	0.35	6.3	LG1	320	152.9	0.48	10.7
LG2	276	99.4	0.36	3.8	LG2	264	109.1	0.41	5.1
LG3	253	82.0	0.32	2.6	LG3	275	100.2	0.36	4.1
LG4	326	79.3	0.24	4.9	LG4	293	74.9	0.25	3.6
LG5	205	83.1	0.40	3.5	LG5	100	73.7	0.73	14.2
LG6	244	93.4	0.38	6.3	LG6	244	85.4	0.35	6.0
LG7	225	78.2	0.34	6.1	LG7	150	92.5	0.61	14.9
LG8	279	87.3	0.31	6.3	LG8	180	79.4	0.44	2.8
Max/group	326	112.0	0.40	6.3	Max/group	320	152.9	0.73	14.9
Min/group	205	78.2	0.24	2.6	Min/group	100	73.7	0.25	2.8
Total	**2,130**	**714.6**	**0.34**		**Total**	**1,826**	**768.1**	**0.42**	
Genetic map constructed from the **15K SNPs** array genotyping of 203 RxG genotypes									
LG1	221	122.9	0.60	16.5	LG1	352	148.4	0.40	4.5
LG2	162	66.0	0.40	4.7	LG2	123	82.8	0.70	10.6
LG3	149	60.6	0.40	5.0	LG3	128	85.4	0.60	19.8
LG4	174	56.5	0.30	3.9	LG4	135	66.3	0.50	16.4
LG5	170	51.7	0.30	3.1	LG5	79	55.5	0.70	16.1
LG6	201	63.6	0.30	4.1	LG6	212	63.9	0.30	14.2
LG7	140	61.6	0.51	4.8	LG7	51	62.6	1.20	8.9
LG8	184	73.3	0.40	7.5	LG8	134	85.1	0.60	10.9
Max/group	221	122.9	0.60	18.0	Max/group	352	148.4	0.70	30.1
Min/group	149	51.7	0.30	3.1	Min/group	51	16.1	0.10	2.1
Total	**1,401**	**556.2**	**0.40**		**Total**	**1,214**	**650.0**	**0.52**	

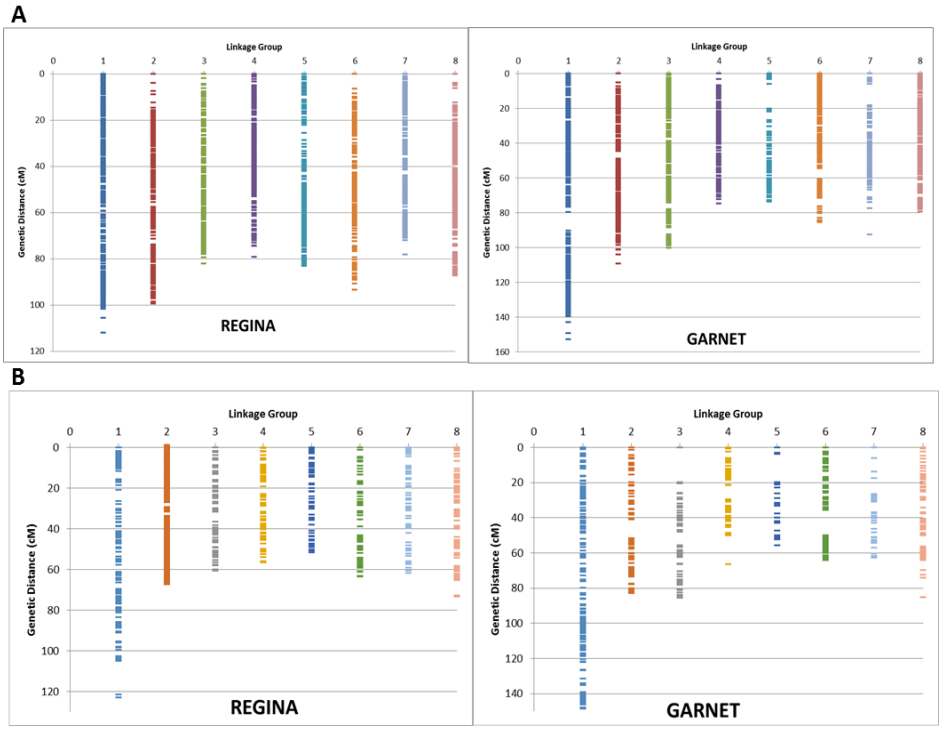

Figure 1. Genetic linkage maps of 'Regina' and 'Garnet' built with GBS genotyping (A) and 15 K SNPs array (B).

KASP markers for genotyping fruit quality

KASP markers have been developed for two traits so far: fruit weight and fruit firmness. The KASP maker developed for the QTL on linkage group 2 for fruit weight was able to discriminate three genotypic groups that were associated with fruit weight: individuals homozygous for "large fruit allele", individuals homozygous for "small fruit allele" and a third group with heterozygous genotypes. This discrimination was effective for individuals from the RxG and RxL families and for the individuals of the core collection (Fig. 2). The KASP marker developed for fruit firmness QTL on linkage group 5 could distinguish firm or soft fruit offspring in the segregating families but not among accessions of the core collection.

This preliminary analysis indicates that KASP markers could be used for sweet cherry MAB to develop cultivars with large and firm fruit. KASP markers linked to other traits should be soon available. KASP assays offer high-throughput genotyping at low cost that is suited to screen thousands of breeding germplasm individuals for just the few SNPs required to reveal trait locus genotypes with quick turnaround times. These KASP markers could also be used in fine mapping strategies for others traits.

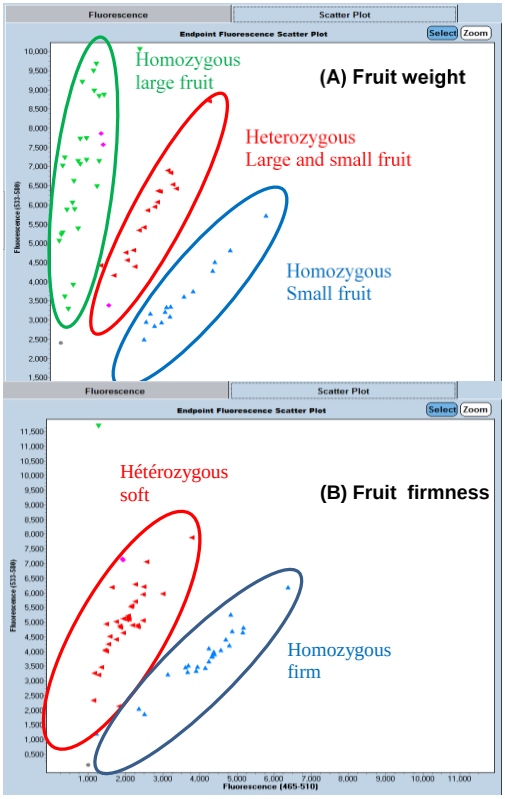

Figure 2. Scatterplots of fluorescence indexes of FAM and HEX fluorophores (A) KASP marker on LG2 for the fruit weight (B) KASP marker on LG5 for the fruit firmness.

Genomic prediction

Prediction accuracy for flowering date was assessed by 50-replicate 5-fold cross-validation, with, as a result, a mean Pearson correlation of 0.78 (std. dev. = 0,06). This result is promising given the relatively small size of the

RxG population studied, although it was not too surprising given the high heritability of the trait considered (Castède et al., 2014). Additional analyses are on-going for other important agronomic traits such as maturity date, fruit weight and fruit firmness. In the future, this methodology will be tested on several larger families involving more than two cultivars, in order to test its applicability in INRA sweet cherry breeding program. Several practical but challenging issues remain to be solved: to determine the minimum number of markers required; to choose the cheapest and most reliable genotyping platform and to establish analytical pipelines usable in routine by breeders.

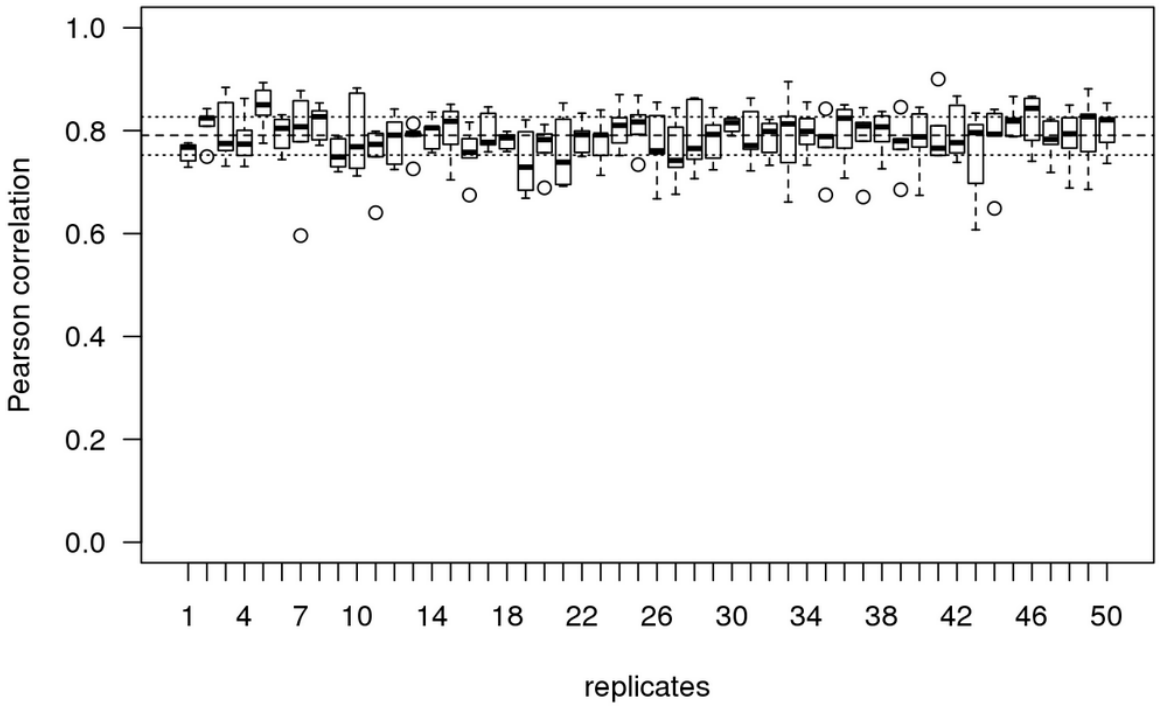

Figure 3. Prediction accuracy for the flowering date

CONCLUSIONS

All these new tools and genomic data recently available for sweet cherry will accelerate genetic analysis and breeding programs in this species but also in other fruit trees. Two high quality genome sequences and several high density linkage maps are now available. As QTLs for many traits, with a very small confidence interval, are already identified, low cost and high throughput markers can be developed within genomic regions for traits of interest, offering new perspectives for breeding programs. Additionally, with the genomic prediction methodology, breeding for traits difficult to phenotype or with polygenic determinism, becomes possible. In new breeding programs, a small number of hybrids can be selected for simple and complex traits at very early stages, from very large number of hybrids, highly increasing the efficiency of the selection.

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Annex 2: List of SNP markers used to develop KASP markers

SNPs	Orig in SNP	Position on scaffold_21	Genotype 'Regina'	Genotype 'Garnet'	Sequence
Super-Scaffold_21_6194315	GBS	6194315	C/T		TCTCCTTGTCATACACTTTCTTTGATACCAGAGTTTGCGTTGCCACTAGGCGTYGGAGGTCTGTTTAGGAAATCTAGTGGTGGGAAAAAGAGAAGCTGCAACACCATATTCT
RosBREED_snp_swee t_4_07944555	SNP 6K	6211705	T/C	T/T	TGGGGAAACCATAAGCACCAGTGCACAATATTGGGGCTGATTTAAAGCCATGCATACACTTTGCAAACCAATCACACTCCCATCTGATCCTTTCACTGACAACTCAGGAGG
Super-Scaffold_21_6248027	GBS	6248027	A/G		AACATGAAAAAGGACATGGAGTTGTTATAAACTGCCTTGGTTTCATTTGATTGATGTTTTTGARTTGATTATTCAGACATATATGTTTAGTACAAACTGCCTTTGATTTTA
Super-Scaffold_21_6280991	GBS	6280991	A/G	A/A	CGTCGGAGCGAGGCGGCAGCAAATCGCCTCAAGGCATCGTTTTGTTTCRCGACTTTGCACCTTTAGCACAGGGGAAGGCGTGAAGAGGCGTTTCAAGGCGCAGCGAGGTGGGGC
Super-Scaffold_21_6419113	RNA_seq	6419113	C/T		AGAAGGCTTTGCCAAAGGCATGAAATACCTGTGATACCCAACCACTGTAACAGATCTTCTCACCAGCYYTTTCTGTTACCATCTTAGCAAAAACCTTGCACAAATTTTCAGCTTAA
Super-Scaffold_21_6435196	RNA_seq	6435196	A/G	G/G	AAACAAAGACACCCTCTCAAAACACCAGTTGTAAATCTCTTATAAATCTGTGACAGATTCAGGCRGGGGCAAATCAGAGAACTTCTCCTGTGCTAAATCAAAAGCAGCAA
Super-Scaffold_21_6480810	RNA_seq	6480810	A/C	C/C	AGGGAGGGCTTATGTTGTTGTTAAATGGAGTCCTGGACACCCTTTCTGGTAGACTAATGGAAAGTTGTAGGTGAGGCCGCTATGGCTTGCGGGTTGGTTGAGGTCT
Super-Scaffold_21_6590224	GBS	6590224	A/G	A/A	ACCATATGATCAGCTGGTAAGCATATGGTAAGTACATTAGACCCATAGCCTCTTGCAGCCAGTAGTGGTCCTCRTCATATTTCATCATCTGTAAATTTAAAACGATGTACATGA
Super-Scaffold_21_6617951	RNA_seq	6617951	T/C		TCTCTTCATGTCCCAAGTGTATTATTGTGATTTCTTCGCAGACCCGGTCCTTGGTATGCTCTAATYCTTTTTTTGATGTTCCCCAGAGGCACCCTAAGATATTTCAGTGCCAATCCGT
Super-Scaffold_21_6643700	GBS	6643700	A/G	A/A	CCAAACACCAGAGTTTGCGTATGAAAATTGGACACCCAATTGAGGATTGATGATAATAAATTTGGTATTTAAAAATTGACAAATAACCATATAAATATATTGTATGG
Super-Scaffold_21_6708069	RNA_seq	6708069	A/G		ACTGAAGAGGAAAGCCAATTGGTGCTGTAGATAAAAAGCATAGTTAGRCTGACAAAGGCTGAAAGGAGGCTAAGAAAGTAAGAAAGAGGCTAAGAAACAAG
Super-Scaffold_21_6914574	RNA_seq	6914574	A/T	A/A	ATTTAAACACTTTACGAAGAAGTGTCGATGGGTGTGTCGGACTCGGCTCCCCATTCWGTCCAAGATCCATCATAGACTGCTACTTCAGGCTTACCGAGTCGATGAAGACCCT
Super-Scaffold_21_6943204	RNA_seq	6943204	A/G		CACTTGCAGATGTTTATGTGAATGATGCTTTTGGTATCTGCTCATAGGGCTCATGCATCAACTGARGGAGTGGCAAAGTTCTTAAGCCTTCCGTTGCTGGATTCCTTATGCAG
Super-Scaffold_21_6948399	RNA_seq	6948399	C/T		GAATGATCCTGAGCATGCAAAGAAGCTTGCCTCCTTAGCAGATCTTTATGTCAATGATGCATTTGGTACTGCCCATAGAGCTCACGCCTCAACTGAGGGGGTCACAAAATT
RosBREED_snp_swee t_4_07349971	SNP 6K	7053286	T/C		CATAACATATGCCGAAGTAAAGGAAACCCATTGAAATTGGAGGGGGAAAAGTTATATTGATCTTATAGCTTACATCCTTCTTTTTGGTTGCTGCATGAGCGAAAATAGAAG
Super-Scaffold_21_7058361	RNA_seq	7058361	A/C		CCAACTCAGTCTTAAACGGTTCCAACTCAGTTCTCACCTTCTTTAATCCGCCCTCAACTCGCCMCACATACTCACCCACCTTCACATTAGCATCCTTCAAATCAAACCCAG
Super-Scaffold_21_7078306	GBS	7078306	T/A		AAATTTTCCTATTTATTAAATATTTTAAATTTTGGCGCTAACTATGGAGCAGCCAGAGGTGTCGTTTCTGCCACACCCTAWGTTAAAGGTTAAAACTTTCGTCCCAA
Super-Scaffold_21_7078450	RNA_seq	7078450	A/G		GTGGATTTTTCGATTTCGGTCTCAGTGCTGGAGGGGTCCGGGGTAGGAGAAGGGTTAGGGTYAGGGGTTTGGGAGGGTTTTGGGTCTTGGGTCGTGAATGGTTTGAAGAGGGA
scaffold_4:7312389	SNP15K	7098164	A/G		AGTGAATTTGAAAACATACAGATAAAATATATCAAAAAGCTTAGTGTACTTTCATGATTGTCTAGTATTRCTATGATGATCTGAGCGCATGAAACAAAGCGAAAGCGTAATA
Super-Scaffold_21_7098254	RNA_seq	7098254	A/C		GTGCAAGATGGACAGGGGGAACACTTGCTACCGTAAGTACATGGTTGATGTTCTCTTMGGCATGTCTTGCTTCGAAAAGATAG
Super-Scaffold_21_7099932	GBS	7099932	G/A	A/A	TGAATGTCTTCCGCAAAATTTAGGCTGAGAATCCACATGGACCATTGAAACTCCAGCTGCTATTAATGGTGTGAGATGATAAGATCTACTRTCTCCTTCCACATCATTTCCAA
Super-Scaffold_21_7099961	RNA_seq	7099961	T/C		TTGACCAGGATCTTGGTTTTCCGCCTGTGATTGGAAATGATGTGGAAAGGAGAYAGTAGATCTTATCATCTCACACCATTAATAGCAGCTGGAGTTTCA
RosBREED_snp_swee t_4_07309282	SNP 6K	7101147	T/C		CTTCTTCAACAGGAAGATGGGTCATCAAATTACCATGATACAGCTGTAAAGGGACAGAGTGCTCTTTCCCCGAGGGGTAAAGTTTTTCAGGATCACTCCTCTTGCTGCAGTGAA
Super-Scaffold_21_7101797	RNA_seq	7101797	C/T	T/T	ATTAAATATCAGCTTCCTGGAGAGGATGTTGATGGCTTAGTTTCTGTATCTTGGCAGGAGGAATTTGCAGAATATGATGGAAGAATGGAAGACTAGAAGATAAAGAAGG
Super-Scaffold_21_7192553	RNA_seq	7192553	G/C		ATAGATAGAAATTTTATCAACGAAATTCAAGTATCATCACTCACCTGAAGCACAAACTGGCAAAGGTTCATCACATATGTCGCASAAACAGATCTTCCGGATGGCAAATT
Super-Scaffold_21_7260307	RNA_seq	7260307	A/G		GTTCCTGATATGAGTCTGCTTCTGGTGTTACTTATTTCAGGTATACGGCCTGTGTTCAAGTAATRCATGTTTAAGTGATAGCTAGCTCTGTAGGGGGTGACATTTTGCCACATC

Annex 3: Primers designed for the 26 KASPs

SNPs			Primers	
Super-Scaffold_21_6184315	1 FORWARD	T	GAAGGTGACCAAGTTCATGCTTGCGTTGCCACTAGGCGTT	FAM
	2 FORWARD	C	GAAGGTCGGAGTCAACGGATTTGCGTTGCCACTAGGCGTC	VIC
	REVERSE		TGCAGCTTCTCTTTTCCCACCACTA	
Super-Scaffold_21_6248027	1 FORWARD	G	GAAGGTGACCAAGTTCATGCTTTGGTTTCATTTGATTGATGTTTTTGAG	FAM
	2 FORWARD	A	GAAGGTCGGAGTCAACGGATTTTGGTTTCATTTGATTGATGTTTTTGAA	VIC
	REVERSE		CGGGTTTTAAAATCAAAGGCAGTTTG	
Super-Scaffold_21_6280991	1 FORWARD	G	GAAGGTGACCAAGTTCATGCTCCTCAAGGCATCGTTTGTTTCG	FAM
	2 FORWARD	A	GAAGGTCGGAGTCAACGGATTCCTCAAGGCATCGTTTGTTTCA	VIC
	REVERSE		TTCCCCGTGCTAAACGTGCAAGT	
Super-Scaffold_21_6419113	1 FORWARD	T	GAAGGTGACCAAGTTCATGCTCCACTGTACAGATCTTCTCACCAGCT	FAM
	2 FORWARD	C	GAAGGTCGGAGTCAACGGATTCACTGTACAGATCTTCTCACCAGCC	VIC
	REVERSE		GCTGAAATTTGTGCAAGGTTTTTGC	
Super-Scaffold_21_6435196	1 FORWARD	G	GAAGGTGACCAAGTTCATGCTCTCTTATAATCTGTGACAGATTCAGGCG	FAM
	2 FORWARD	A	GAAGGTCGGAGTCAACGGATTTCTCTTATAATCTGTGACAGATTCAGGCA	VIC
	REVERSE		GCACAGGAGAAGTTCTCTGATTTGC	
Super-Scaffold_21_6480810	1 FORWARD	A	GAAGGTGACCAAGTTCATGCTCGCTTTCTTGGTAGACTAATCGGAAA	FAM
	2 FORWARD	C	GAAGGTCGGAGTCAACGGATTCGCTTTCTTGGTAGACTAATCGGAAC	VIC
	REVERSE		AAATCCGATAGCGGCCTCACCTAC	
Super-Scaffold_21_6590224	1 FORWARD	G	GAAGGTGACCAAGTTCATGCTTGCAGCCAGTAGTCGTCCTCG	FAM
	2 FORWARD	A	GAAGGTCGGAGTCAACGGATTTTGCAGCCAGTAGTCGTCCTCA	VIC
	REVERSE		CATGTACATCGTTTAATTTACAGATGATGA	
Super-Scaffold_21_6617951	1 FORWARD	T	GAAGGTGACCAAGTTCATGCTCGCGTCCTTGGTATGCTCTAATT	FAM
	2 FORWARD	C	GAAGGTCGGAGTCAACGGATTCGCGTCCTTGGTATGCTCTAATC	VIC
	REVERSE		TATCTTAGGGTGCCTCTGGGGAACA	
Super-Scaffold_21_6643700	1 FORWARD	G	GAAGGTGACCAAGTTCATGCTTTGAGGATTGATGATAATAAATTTGGTATTATG	FAM
	2 FORWARD	A	GAAGGTCGGAGTCAACGGATTTTGAGGATTGATGATAATAAATTTGGTATTATA	VIC
	REVERSE		GCCATACAATATATTTATATGGTTATTTG	
Super-Scaffold_21_6708069	1 FORWARD	G	GAAGGTGACCAAGTTCATGCTCATTGGTGCTGTAGATAAAAGCATAGTTAGG	FAM
	2 FORWARD	A	GAAGGTCGGAGTCAACGGATTAAGTGGTGCTGCAGATAAAAGCATAGTTAGA	VIC
	REVERSE		TCTTAAGCTTTGCCCTCCTTTCAGC	
Super-Scaffold_21_6914574	1 FORWARD	A	GAAGGTGACCAAGTTCATGCTGACTGCGCTCCCCATTCA	FAM
	2 FORWARD	T	GAAGGTCGGAGTCAACGGATTGGACTGCGCTCCCCATTCT	VIC
	REVERSE		CGACTCGGTAAGCCTGAAGTAGCAG	
Super-Scaffold_21_6943204	1 FORWARD	G	GAAGGTGACCAAGTTCATGCTCATAGGGCTCATGCATCAACTGAG	FAM
	2 FORWARD	A	GAAGGTCGGAGTCAACGGATTCATAGGGCTCATGCATCAACTGAA	VIC
	REVERSE		ATCCAGCAACGGAAGGCTTTAAGAA	
Super-Scaffold_21_6948399	1 FORWARD	T	GAAGGTGACCAAGTTCATGCTTTAGCAGATCTTTATGTCAATGATGCATTT	FAM
	2 FORWARD	C	GAAGGTCGGAGTCAACGGATTAGCAGATCTTTATGTCAATGATGCATTC	VIC
	REVERSE		AGGCGTGAGCTCTATGGGCAGTAC	

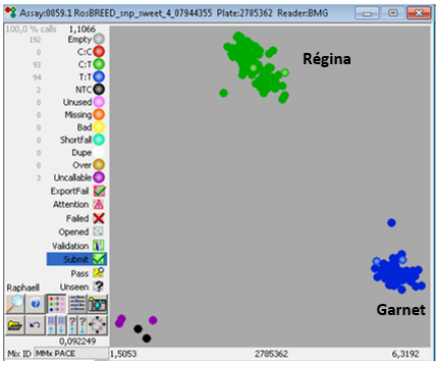
Annex 3 (followed)

SNPs			Primers	
RosBREED_snp_sweet_4_0734	1 FORWARD	T	GAAGGTGACCAAGTTCATGCTTTATATTGATCTTATAGCTTACATCCTTCTTTTT	FAM
	2 FORWARD	C	GAAGGTCGGAGTCAACGGATTATTGATCTTATAGCTTACATCCTTCTTTTC	VIC
	REVERSE		CTTCTATTTTCGTCATGCACCAACC	
Super-Scaffold_21_7058361	1 FORWARD	A	GAAGGTGACCAAGTTCATGCTCGCCCTCAACTCGCCA	FAM
	2 FORWARD	C	GAAGGTCGGAGTCAACGGATTCGCCCTCAACTCGCCC	VIC
	REVERSE		GGATGCTAATGTGAAGGTGGGTGAG	
Super-Scaffold_21_7078306	1 FORWARD	A	GAAGGTGACCAAGTTCATGCTTGTCGTTTCTGCCACACCCTAA	FAM
	2 FORWARD	T	GAAGGTCGGAGTCAACGGATTGTGTCGTTTCTGCCACACCCTAT	VIC
	REVERSE		CGCTTGGGACGAAAGTTTTAACCTT	
Super-Scaffold_21_7078450	1 FORWARD	T	GAAGGTGACCAAGTTCATGCTGGCGTAGGAGAAGGGTTAGGGTT	FAM
	2 FORWARD	C	GAAGGTCGGAGTCAACGGATTGGCGTAGGAGAAGGGTTAGGGTC	VIC
	REVERSE		CATTCAGACCCAAGACCCCAAAAC	
scaffold_4:7312389	1 FORWARD	G	GAAGGTGACCAAGTTCATGCTAAGCTTAGTGTACTTTCATGATTGTCTAGTATTG	FAM
	2 FORWARD	A	GAAGGTCGGAGTCAACGGATTAAGCTTAGTGTACTTTCATGATTGTCTAGTATTA	VIC
	REVERSE		TTGTTTCATGCGCTCAGATCATCAT	
Super-Scaffold_21_7098254	1 FORWARD	A	GAAGGTGACCAAGTTCATGCTAACTGAGTACATGGTTGATGGTTCTCTTA	FAM
	2 FORWARD	C	GAAGGTCGGAGTCAACGGATTAACTGAGTACATGGTTGATGGTTCTCTTC	VIC
	REVERSE		CTATCTTTTCGAAGCAAGACATGC	
Super-Scaffold_21_7099952	1 FORWARD	G	GAAGGTGACCAAGTTCATGCTTGCTATTAATGGTGTGAGATGATAAGATCTACTG	FAM
	2 FORWARD	A	GAAGGTCGGAGTCAACGGATTTGCTATTAATGGTGTGAGATGATAAGATCTACTA	VIC
	REVERSE		TGTGATTGGAAATGATGTGGAAGGA	
Super-Scaffold_21_7099961	1 FORWARD	T	GAAGGTGACCAAGTTCATGCTTGATTGGAAATGATGTGGAAGGAGAT	FAM
	2 FORWARD	C	GAAGGTCGGAGTCAACGGATTGATTGGAAATGATGTGGAAGGAGAC	VIC
	REVERSE		CCAGCTGCTATTAATGGTGTGAGATGA	
Super-Scaffold_21_7101797	1 FORWARD	T	GAAGGTGACCAAGTTCATGCTAGTTTCTGTATCTTGCGACGAGGATT	FAM
	2 FORWARD	C	GAAGGTCGGAGTCAACGGATTAGTTTCTGTATCTTGCGACGAGGATC	VIC
	REVERSE		TTCCATTCTTCCATCATATTCTGCA	
Super-Scaffold_21_7192553	1 FORWARD	T	GAAGGTGACCAAGTTCATGCTTGCCATCCCGAAGATCTTGTTTT	FAM
	2 FORWARD	C	GAAGGTCGGAGTCAACGGATTGCCATCCGGAAGATCTTGTTTC	VIC
	REVERSE		GCACAAACTGCAAAGGTTCATCACA	
Super-Scaffold_21_7260307	1 FORWARD	G	GAAGGTGACCAAGTTCATGCTTCAGGTATACGCCTGTGTTCAAGTAATG	FAM
	2 FORWARD	A	GAAGGTCGGAGTCAACGGATTTTTCAGGTATACGCCTGTGTTCAAGTAATA	VIC
	REVERSE		GCAAAATGTCACCCCCTACAGAGCT	

Sequence	fluorescence
GAAGGTGACCAAGTTCATGC	FAM
GAAGGTCGGAGTCAACGGAT	VIC

Annex 4: KASP marker profiles

RosBREED_07944355



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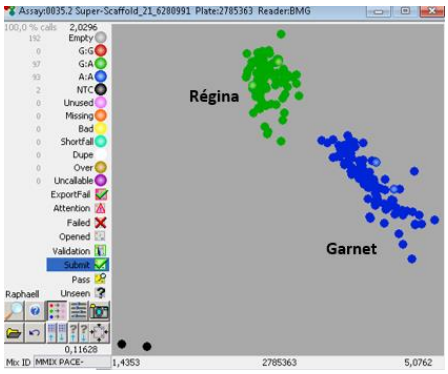

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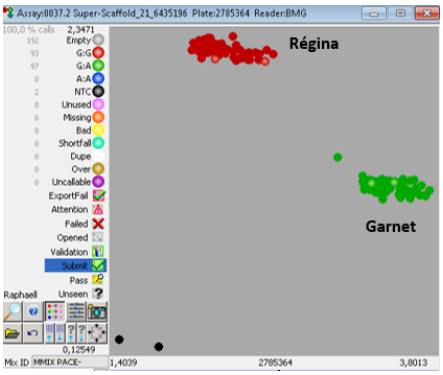
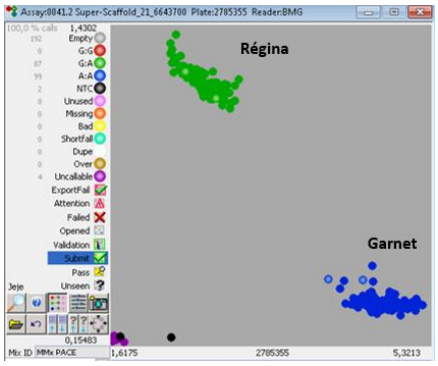
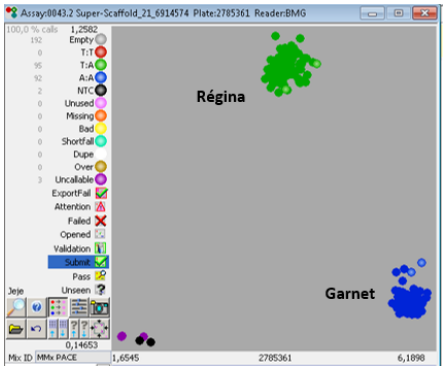


Super-Scaffold_21_6643700



Super-Scaffold_21_6914574



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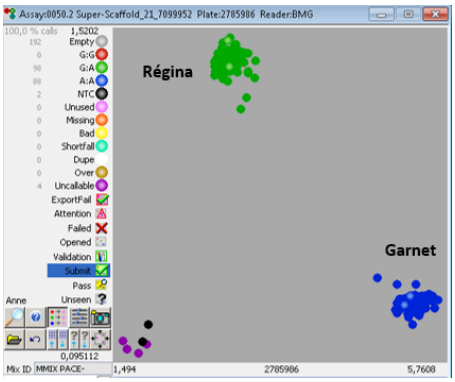
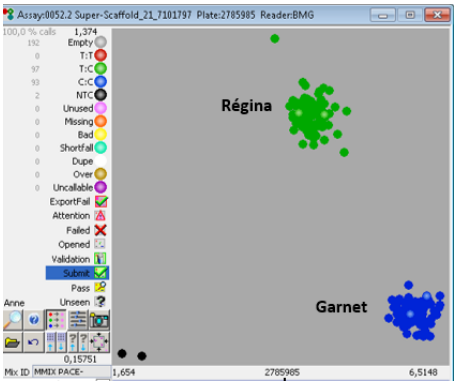


Super-Scaffold_21_7101797



Fine mapping of a QTL region controlling the flowering date in sweet cherry (*Prunus avium* L.)

In the context of climate change, there are many questions relating to the adaptation of fruit species. The phenology of sweet cherry tree, as for other perennial fruit species, depends directly on climatic conditions with an important effect of the temperatures. Therefore, there is a risk of a direct impact of climate change on productivity and on fruit quality. Taking into account that climate change could endanger the production of cherries, and considering the time required for the releasing of a new cultivar in this species, it is essential to integrate the criteria linked to the adaptation to climate change in the breeding programs that will help the choice of plants better adapted to future environmental conditions. In this work, a segregation population from a cross between 'Regina', a late flowering date variety, and 'Garnet', an intermediate flowering date variety, was studied. This population has been studied for many years for the flowering date and others traits such as chilling and heat requirements. The regions of the genome (Quantitative trait Loci, QTL) involved in the control of the flowering date have been investigated and a QTL on linkage group 4 (LG4) with high effect, explaining up to 36% of the variation observed for this trait, has been identified with a confidence interval of less than 1 cM (Castède *et al.*, 2014). The objective of this work is to do a fine mapping of the QTL region (LG4) using a segregation progeny of 1526 individuals issued from the same cross. New QTL detection was performed using the flowering date evaluation of this year. New SNP markers issue from different strategies were identified in the QTL region and transformed into kompetitive allele specific PCR (KASP) markers to genotype all the progeny. Recombinant individuals were identified using these markers. Moreover, results indicated that theses KASPs can be used for sweet cherry MAB.

Key works: climate change, flowering date, QTL analyzes, SNPs, *Prunus avium*

Cartographie fine d'une région QTL contrôlant la date de floraison chez la cerise douce (*Prunus avium* L.)

Dans le contexte du changement climatique, il existe de nombreuses questions relatives à l'adaptation des espèces fruitières. La phénologie du cerisier, comme pour les autres espèces de fruits vivaces, dépend directement des conditions climatiques avec un effet important des températures. Par conséquent, il existe un risque d'impact direct du changement climatique sur la productivité et la qualité des fruits. Tenant compte du fait que le changement climatique pourrait mettre en péril la production de cerises et compte tenu du temps nécessaire à la libération d'une nouvelle variété, il est essentiel d'intégrer les critères liés à l'adaptation au changement climatique dans les programmes d'amélioration choix de plantes mieux adaptées aux conditions environnementales futures. Dans ce travail, une descendance de ségrégation provenant d'un croisement entre 'Regina', une variété à date de floraison tardive, et 'Garnet', une variété à date de floraison intermédiaire, a été étudiée. Cette population a été étudiée pendant de nombreuses années pour la date de floraison et d'autres traits tels. Les régions du génome (QTL) impliquées dans le contrôle de la date de floraison ont été étudiées et un QTL sur le groupe de liaison 4 (LG4) avec un effet plus important, expliquant 36% de la variation observée pour ce caractère, a été identifiés avec un intervalle de confiance inférieur à 1 cM (Castède *et al*., 2014). L'objectif de ce travail est de faire une cartographie fine de la région QTL (LG4) en utilisant une de ségrégation de 1526 individus issus du même croisement. Une nouvelle détection de QTL a été réalisée en utilisant l'évaluation de la date de floraison de cette année. De nouveaux marqueurs SNP provenant de différentes stratégies ont été identifiés dans la région QTL et transformés en Kompetitive allele specific PCR (KASP) marqueurs pour génotyper toute la descendance. Les individus recombinants ont été identifiés en utilisant ces marqueurs. De plus, les résultats indiquent que ces KASP peuvent être utilisés pour le MAB de cerise douce.

Mots clés: changement climatique, floraison date, QTL analyse, SNP, *Prunus avium*