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Genetic mapping and QTL analysis in European hazelnut (*Corylus avellana* L.)

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Abstract

The European hazelnut (*Corylus avellana* L.) is the most economically important nut species in the Betulaceae family. Despite the need for new improved hazelnut cultivars, few breeding programs are carried out because of the large plant size, the long life cycle of the plant, and the expense and time required.

To date, there are no reports of maps with Quantitative Trait Loci (QTL) in hazelnut. Our objective in the present study was to identify QTL associated with vegetative traits to allow marker-assisted selection (MAS). A F_1 progeny (275 plants) of 'Tonda Gentile delle Langhe' X 'Merveille de Bollwiller' obtained in 2009 was used to develop a QTL linkage map for vigour, sucker habit and time of bud burst, after three years of observations. A set of 163 plants were analysed with 152 microsatellite markers. A map of 11 linkage groups was obtained, covering 663.1 cM and 15 QTLs were identified and mapped for the examined traits. Among them, 10 were 'major' QTL, including a stably expressed region on LG_02 for leaf budburst. At least one major QTL for each year underlays the variation in each trait and a clustering of QTL for *tc* and *s/tc* ratio with a high inter-trait correlations was observed on LG_05, suggesting a single pleiotropic locus.

This research represents an initial step for the future identification of chromosomal regions carrying genes of interest, important for breeding programs and MAS.

Key words

SSR – MAS – vegetative traits – leaf budburst – sucker habit - vigour

Introduction

The genus *Corylus* (Betulaceae) is spread throughout the temperate regions of the Northern Hemisphere, extending from Japan, Korea, China and the Russian Far East to the Caucasus, Turkey, Europe and North America (Kasapligil 1972). The European hazelnut (*Corylus avellana* L.) is the most economically important nut species in the Betulaceae family with a worldwide production of about 872,000 t of in-shell nuts and a cultivated area of approximately 604,000 ha (average 2008-2012, FAOstat, 2015), resulting the sixth most important commodity among the tree nuts behind cashew (*Anacardium occidentale* L.), walnut (*Juglans regia* L.), almond (*Prunus dulcis* (Miller) D.A. Webb), chestnut (*Castanea sativa* Miller), and pistachio (*Pistacia vera* L.). The major producers are Turkey (598,158 t) and Italy (104,577 t), followed by USA (32,399 t), Azerbaijan (30,035 t), Georgia (25,020 t), Iran (20,833 t), China (19,700 t) and Spain (16,239 t) (average 2008-2012, FAOstat 2015). It is estimated that over 90% of the hazelnut crop is destined to processing.

C. avellana is diploid ($2n = 2x = 22$), monoecious, wind-pollinated, dichogamous, and shows sporophytic incompatibility. Because of the large plant size, the long life cycle of the plant, and the expense and time required, very few breeding programs are currently being carried out in the world. The major one is underway at Oregon State University (OSU). On the other hand, there is a strong demand of plant material for new plantings in several countries and interest in new hazelnut cultivars with higher yield and tolerance/adaptation to particular pathogens or environmental conditions is very high.

The construction of genetic linkage maps and identification of molecular markers linked to trait of interest could allow marker-assisted selection (MAS), thereby enriching the population of seedlings planted in the field for those carrying desired traits. Many important agronomic and quality traits, such as time of bud burst, flowering time, yield and fruit quality are controlled by many genes: genomic regions containing these genes are known as Quantitative Trait Loci (QTL) (Collard et al. 2005). In this case, the MAS procedure involves the construction of a genetic map and identifies markers tightly linked to the major genes or QTL of agronomic interest (Francia et al. 2005). This would allow selection at the seedling stage. Application of MAS and QTL mapping in fruit and forestry tree breeding programs has made considerable progress during the last two decades (Rai and Shekhawat 2014). Molecular markers associated with traits of interest have

been identified for a wide variety of traits, including disease resistance genes in apples (Bus at al. 2010), grapevine (Riaz et al. 2011), apricot (Soriano et al. 2008), chestnut (Kubisiak et al. 2013), poplar (Jorge et al. 2005), Norway spruce (Lind et al. 2014), eucalyptus (Zarpelon et al. 2015) and abiotic stress tolerance in apple (Virlet et al. 2015), oak (Parelle et al. 2007), and poplar (Tschaplinski et al. 2006). Various QTL controlling morphological and qualitative fruit traits, maturity, flowering time, bud phenology, have also been identified in different species, including apples (Chagne et al. 2012, Sun et al. 2015, Virlet et al. 2015), apricot (Campoy et al. 2011), cranberry (Schlautman et al. 2015), grapevine (Chen et al. 2015), peach (Martínez-García et al. 2013, Bielenberg et al. 2015), pear (Zhang et al. 2013), pomegranate (Harel-Beja et al. 2015), sweet cherry (Castède et al. 2015), chestnut (Casasoli et al. 2004), oak (Scotti-Saintagne et al. 2004), pine (Yang et al. 2015), and poplar (Rohde et al. 2011, Du et al. 2015).

A high density linkage map of hazelnut was previously constructed using RAPD (Random Amplified Polymorphic DNA) and microsatellite (SSR - Simple Sequence Repeat) markers (Mehlenbacher et al. 2006) by crossing two selections developed at OSU, and was improved by the addition of 150 SSRs by Gürcan and Mehlenbacher (2010a) and Gürcan et al. (2010). The reference map was developed by the OSU hazelnut breeding program aimed to identify molecular markers to be used in MAS for selecting individuals bearing resistance to Eastern Filbert Blight (EFB), a disease caused by *Anisogramma anomala* (Peck) E. Müller (Mehlenbacher et al. 2004); a second objective of the work was to find markers linked to the alleles at the S-locus that controls incompatibility (Mehlenbacher et al. 2006), to aid the identification of S-alleles in cultivars and selections. More recent work carried out at OSU led to the identification of 9 RAPDs, 31 SSRs and 2 AFLPs (Amplified Fragment Length Polymorphisms) markers linked to EFB resistance loci, 4 RAPDs and 4 SSRs markers linked to the S locus, and 1 SSR marker linked to style colour (Chen et al. 2005; Sathuvalli et al. 2011; Sathuvalli and Mehlenbacher 2011; Sathuvalli et al. 2012; Sathuvalli and Mehlenbacher 2013; Ives et al. 2014; Colburn et al. 2015). To date, there are no reports of QTL analysis in hazelnut. Our objective in the present study was to identify QTL associated with three traits. In this paper, we present the results of three years of observations of young seedlings, a new linkage map, and the QTL analysis for vigour, sucker habit and time of bud burst.

Materials and Methods

Plant materials

A progeny of 275 F_1 individuals were obtained by crossing 'Tonda Gentile delle Langhe' (syn. 'Tonda Gentile Trilobata', female parent, hereafter TGdL) with 'Merveille de Bollwiller' (syn. 'Hall's Giant' male parent, MB). In December 2007, a TGdL plant was emasculated and then caged with a nonwoven tissue to avoid uncontrolled pollination. At the same time, pollen was collected from a MB plant and stored at -20°C until TGdL female flowering time. The controlled

pollination was manually made using a paintbrush in February 2008. The cage was removed at the end of female flowering when the stigmas were dry and dark brown.

Nuts were collected from the plant in early August 2008 when the shells were at least halfway brown but before they fell to the ground. They were then stored in a mesh bag at 4°C for about three months. In early November, nuts were removed from storage and soaked for two days in a bucket filled with water, changing the water after 24 h. After the soaking, nuts were stratified using an equal volume of moistened vermiculite in a plastic box. The box was covered with a polyethylene bag not hermetically closed, and stored in a cooler at 4°C for 150 days.

At the end of March 2009, the plastic box was removed from the cooler and left at room temperature (about 20°C) for a week to promote germination. Only seeds that showed root tips were transferred to pots (5cm x 5cm x 25cm) filled with a substrate composed by peat and perlite (3:1 ratio). The pots were kept under a glass greenhouse until plants reached a height of about 20 cm. In summer 2009, the plants were kept in a tunnel, covered with a shading net and with drip irrigation. Nutrients were provided using irrigation lines.

The 275 seedlings and three individuals obtained from rooted suckers of each of the two parent cultivars were planted in November 2010 and evaluated over the years 2011, 2012, 2013 and 2014. The field is located at the campus of University of Torino, Department of Agricultural, Forest and Food Sciences (45°07'N; 7°58'E; 293 m a.s.l.). The plants were trained in an open vase system with a spacing of 4 x 4 m. From 2010, water was supplied using an integral PC drip line (UniRam^{TM} 20010 AS, Netafim) from mid-June to mid-September. Fertilization was supplied by foliar sprays using Hascon 12 (Green, Italia) or through the drip lines every two weeks from mid-June to late July. Soil was maintained covered with grass that was mowed and chopped during the growing season; before harvest, the grass was killed in the area below the tree canopy using broadleaf active contact herbicide (SPOTLIGHT PLUS®, carfentrazone-ethyl 60 g/L EO, Belchim Crop Protection Italia). A copper-based bactericide was applied in autumn, while thiophanate-methyl fungicide was applied at the beginning of leaf drop. No insecticide treatments were applied during the trial.

During the trials, weather data (temperature, relative humidity, rainfall) were recorded using an automatic weather station located near the field (data collected by Regione Piemonte - Rete Agrometeorologica Regionale). The sensors of the weather stations were installed 2 m above the ground, according to World Meteorological Organization (WMO) guidelines.

SSR analyses

DNA was extracted from young leaves collected in the spring following Doyle and Doyle (1987). Each individual was preliminarily checked to confirm the parentage by genotyping with 7 SSR loci selected from Boccacci et al. (2005) and

Bassil et al. (2005a). A set of 163 individuals, planted in the core of the field, was then used for SSR analysis at this first stage of the study.

The 163 F_1 individuals of the progeny were genotyped with 152 microsatellite markers (including 15 EST-SSRs) identified by Bassil et al. (2005a,b), Boccacci et al. (2005), Gürcan et al. (2010), Gürcan and Mehlenbacher (2010a,b) and Boccacci et al. (2015) (Online Resource 1).

PCR reactions were performed in a volume of 15 µl containing 1X PCR buffer, 2.25 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM of reverse primer and 5'-labelled M13 tail, 0.1 µM of forward primer (with complementary M13 tail sequence added to 5'-end), 0.1 U of Taq polymerase (Bioline, USA) and 40 ng of template DNA. The thermal cycler program had two stages: after an initial 3 min at 94°C, the first stage comprised 30 cycles of 30 sec at 94°C, 45 sec at 56/58/60°C, 45 sec at 72°C; the second stage consisted of 10 cycles of 30 sec at 94°C, 45 sec at 54°C, 45 sec at 72°C and a final elongation step of 10 min at 72°C (Schuelke 2000). Amplification products were analysed on a 3130 Genetic Analyzer capillary sequencer (Applied Biosystems, USA). The internal GeneScan™ size standard 500 LIZ® was included in each run. Allele sizes in the output were called using GeneMapper v4.0 software (Applied Biosystems).

Linkage analysis and consensus map construction

All SSR loci were used for the construction of a consensus linkage map with JoinMap v4.0 (Van Ooijen 2006), applying the Kosambi (1944) mapping function: in particular loci belonging to the segregation classes 1:2:1 (the same pair of alleles segregating in each parent) and 1:1:1:1 (different alleles segregating in each parent) were used as 'bridge markers'. Differences between observed and expected segregation ratios were assessed using a χ^2 test. Markers associated with a χ^2 value $\leq \chi^2_{\alpha=0.1}$ or with only a minor deviation ($\chi^2_{\alpha=0.1} < \chi^2 \leq \chi^2_{\alpha=0.01}$) were used for map construction and for estimation of genetic distance, if their presence did not alter the local marker order in the linkage group (LG). LGs were established based on an initial threshold logarithm of odds (LOD) of 6.0, with the following parameters set to determine locus order and distances between loci: Rec=0.40, LOD=1.0, Jump=5.

Markers with highly significant deviation from Mendelian expectation ($\chi^2 > \chi^2_{\alpha=0.01}$) were subsequently added with a LOD threshold of 4.0: they were checked one by one and placed in their most likely position within LGs without forcing, in order to avoid artefacts.

Markers deviating in their segregation only marginally from the expected Mendelian ratio were identified with one ($\chi^2_{\alpha=0.1} < \chi^2 \leq \chi^2_{\alpha=0.05}$), two ($\chi^2_{\alpha=0.05} < \chi^2 \leq \chi^2_{\alpha=0.01}$), three ($\chi^2_{\alpha=0.01} < \chi^2 \leq \chi^2_{\alpha=0.005}$), four ($\chi^2_{\alpha=0.005} < \chi^2 \leq \chi^2_{\alpha=0.001}$) and five ($\chi^2 > \chi^2_{\alpha=0.001}$) asterisks. LGs were numbered according to Gürcan et al. (2010) map order.

Evaluation of phenotypic traits

The progeny segregates for several traits, including vigour, number of suckers, time of leaf bud burst.

In years 2011 to 2014 trunk circumference, suckering index and time of bud burst were recorded. Trunk circumference (*tc*) was measured in mm at about 20 cm from the ground to evaluate vigour. Suckers were removed by manual pruning and counted in June of each year; suckering index was calculated as the number of suckers/trunk circumference (*s/tc*). Time of leaf bud burst (*lb*) was recorded at the first leaf appearance out of the bud ("stage C1", Germain and Sarraquigne 2004). Time of bud burst was expressed using the classification in levels of expression of the trait from very early (1) to very late (9), by UPOV (1979) guidelines.

Trait evaluation and QTL detection

Population means, standard deviations, ranges, distribution histograms and trait correlations were calculated using R software (R Development Core Team 2006). For analyses of variance, each season was treated as an independent replicate. Broad sense heritability (H^2_{BS}) was estimated with the formula $H^2 = \sigma^2_g/(\sigma^2_g + \sigma^2_e/y)$, where σ^2_g is the genetic variance, σ^2_e is the error variance, and y is the number of years. The difference in traits between two parents was compared using a *t* test ($P<0.05$). Correlations between traits were calculated using the Spearman coefficient, while normality, Kurtosis and Skewness measures were assessed with the Shapiro-Wilks test ($\alpha=0.05$). Segregation was considered as transgressive where at least one F_1 individual recorded a trait value higher or lower than the parental value by at least two standard deviations.

The consensus map was used at first to assign putative QTL locations by performing a Kruskal-Wallis (KW) non-parametric test in conjunction with the simple interval mapping procedure (SIM) (Lander and Botstein 1989), based on the CP (cross-pollination) algorithm implemented within MapQTL v4.0 software (Van Ooijen et al. 2002). Next, one linked marker per putative QTL was identified using interval mapping, after which it was treated as a co-factor in the approximate multiple QTL model (MQM) (Jansen and Stam 1994). For the MQM, a backward elimination procedure was applied to select the appropriate co-factors (e.g. significantly associated with each trait at $P<0.02$). The LOD thresholds for QTL significance were confirmed using a permutation test comprising 1,000 replications, which implies a genome-wide significance level of 0.05 (Churchill and Doerge 1994). Only those QTL associated with a LOD greater than either the genome-wide threshold or the threshold for that linkage group were considered, and 1-LOD support intervals were determined for each LOD peak (Van Ooijen 1992). The proportion of the overall phenotypic variance (PV) associated with each QTL was estimated from the MQM model. Linkage maps and QTL positions were drawn using MapChart (Voorrips 2002). Each QTL was designated by an abbreviated version of the trait name as a prefix, followed by the relevant linkage group and the suffix indicating the year of its expression. So for example "*s/tc*_01_14" indicates

the QTL underlying the ratio suckers/trunk circumference, mapping to the linkage group 01 by analysing data of the year 2014.

Results

Genotyping and linkage analysis

Of the 152 microsatellite loci, 101 segregated in the F_1 population for both parents: 89 in a 1:1:1:1 ratio, and 12 in a 1:2:1 ratio. The other 51 loci segregated 1:1, 28 from TGdL and 23 from MB (Online Resource 1).

The analysis of genotype frequencies showed that about 7% of the loci produced distorted segregation ratios. Four loci (B788, CaC-A014a, CaC-B108, KG800) showed the presence of null alleles: these loci were not discarded but analysed twice with JoinMap 4, considering them both segregating for one parent (1:1 ratio), and segregating for both parents with a dominant genotype (1:2:1 ratio).

Three loci (B719, Cac-B101 and CaT-B503) showed χ^2 values slightly deviating from expectation ($\chi^2_{\alpha=0.1} < \chi^2 \leq \chi^2_{\alpha=0.05}$), while three others (KG815, A614 and KG801) showed significant segregation distortion ($\chi^2_{\alpha=0.01} < \chi^2 \leq \chi^2_{\alpha=0.005}$, $\chi^2_{\alpha=0.005} < \chi^2 \leq \chi^2_{\alpha=0.001}$, $\chi^2 > \chi^2_{\alpha=0.001}$, respectively), and were initially set aside. The subsequent addition of these six loci did not alter the initial marker order; moreover, they showed linkage only to a single LG and with a LOD score ≥ 4 (Online Resource 2): for these reasons they were included in the final map construction.

The 152 SSRs segregating in the F_1 population were used to generate a map of 11 LGs (each with four or more loci), for a total genetic length of 663.1 cM, with a mean inter-marker distance (discounting completely co-segregating ones) of 4.45 cM (Fig. 1 and Table 1). LG length varied from 33.8 cM (LG_06) to 98.9 cM (LG_01). The number of markers per chromosome was the highest in LG_09 (25) and the lowest in LG_06 (4) (Table 1). The majority (85%) of map intervals were less than 10 cM, but some large gaps remained. In particular 8 gaps >15cM were present (2 in LG_01, 1 in LG_02, 1 in LG_03, 1 in LG_05, 2 in LG_07, 1 in LG_11). For each of the marker pairs involved, the specific LOD in support of the two-point placement was checked, to confirm the robustness of the LGs (Online Resource 3). Only B720 was ungrouped: the strongest linkage for it was with CaC-A014b on LG_04 with a LOD value of 2.8. Due to this low LOD value, it was not considered for the map construction, nevertheless Gürcan et al. (2010) placed B720 on LG_05.

Genic and EST-SSRs were distributed in six LGs (Fig. 1). In particular, both LG_01 and LG_09 had 4 EST-SSRs (Corav1232, BPT6452, Corav2208, AG4314 and AG4765, AG3754, Corav2564, Corav1859, respectively). The loci created noticeable clustering only on LG_11 (CD278264 and Corav2560 clustered with 1.4 cM distance). The other EST-SSRs were on LG_02 (AJ417975b and BP0585), LG_03 (AG4395), LG_10 (Corav2241 and Corav6822) and LG_11 (Corav1576).

The six loci that showed segregation distortion mapped to LGs 05, 06 and 09 (Fig. 1): in particular, B719 and CaC-B101 (α= 0.1) clustered in a central position on LG_09 with a distance < 4 cM, while A614 and KG801 (α= 0.001 and α= 0.005 respectively) grouped at 5.6 cM on LG_06. We also note that KG815 (α= 0.01) was placed at the end of LG_09.

LG numbers were those assigned by Gürcan et al. (2010), based on a cross between genotypes from a maternal parent (OSU 252.146) susceptible and a male parent (OSU 414.062) resistant to eastern filbert blight. The two maps have 111 SSR loci in common, but 5 loci were assigned to a different LG compared to the map by Gürcan et al. (2010): B640 (LG_04 instead of LG_08), CaT-B504 (LG_02 instead of LG_07), CaC-B005 (LG_02 instead of LG_07), B660 (LG_02 instead of LG_07), CaC-B101 (LG_09 instead of LG_07). The others 106 SSR loci were distributed across all of the 11 LGs (3 to 15 present on each LG) allowing to identify each LG (Table 1). Marker order and genetic separation are comparable between the two maps, except for LG_09 and LG_10. Initially by JoinMap analysis, a single linkage group was found for markers belonging to LG_09 and LG_10; in particular, a set of 25 markers was grouped together by the first two rounds of attempts for comparing the goodness-of-fit of the calculated map for each tested position, while another 14 markers were added to the LG only after the third round without the constraints of maximum allowed reduction in goodness-of-fit and no negative distances. It was noticed that the first two rounds grouped together all the markers belonging to the Gürcan et al. (2010) LG_09, while during the third one, all LG_10 markers were added. For this reason, we chose to maintain the two groups separated, creating a linkage map of 11 LGs, corresponding to the haploid chromosome number for hazelnut ($n = x = 11$) (Fig. 1).

Of the 152 SSRs used for mapping, 40 were here placed for the first time on a hazelnut genetic map.

Phenotypic variation and inter-trait correlations

A summary of the phenotypic and statistical values are listed in Table 2. There were significant ($P < 0.1$) phenotypic differences between TGdL and MB for each trait, except for the suckering index (*s/tc*) in 2012 and 2013 (Table 2). TGdL was less vigorous (*tc*) and had a lower suckering index and earlier leaf bud burst (*lb*) than MB, which confers the higher value for each trait. The F_1 progeny phenotype distribution was intermediate between the two parents for *lb* (Fig. 2c) and *s/tc* (considering for *s/tc* the year 2014, when parents showed significant differences; Fig. 2b) while for *tc* the mean of the population was below the mid-parent value in 2013 and 2014 (Fig. 2a). Significant inter-trait correlations ($P < 0.05$) were detected both within and between years (Online Resource 4): in particular *tc* and *s/tc* showed positive correlations between them in 2012 (0.346) and 2013 (0.175). The correlation of a trait between years were significant and ranged from 0.540 to 0.962. Transgressive segregations (Fig. 2) were observed for *s/tc* (positive transgression for two plants) and for *tc* (positive transgression for seven plant) in 2012; for *lb* (negative transgression for one plant and positive transgression for four plants) and *tc* (negative transgression for 85 plants and positive transgression for six plants) in 2013; for *lb* (positive

transgression for one plant), *s/tc* (negative transgression for five plants and positive transgression for six plants) and *tc* (negative transgression for 106 plants and positive transgression for one plant) in 2014. H^2_{BS} was relatively high ranging from 0.60 (*tc*) to 0.85 (*s/tc*) (Table 2).

QTL identification

A separate QTL analysis was performed in each year. Overall, 15 QTL were identified and mapped onto seven genomic regions dispersed on six of the 11 LGs: 1, 2, 5, 8, 10 and 11. Ten of these QTL explained more than 10% of the phenotypic variance (PV) and are hereafter referred to as "major" QTL. Table 3 documents the properties of each of the QTL: maximum LOD value, location on the genetic map and proportion of phenotypic variance explained. In year 2012, five QTL (3 major) were identified; in 2013 four QTL (3 major) were found, while in 2014 six were detected (4 major). Among the QTL detected, three were expressed in all years, one in both 2013 and 2014, while four were only detected in one year. The genomic locations of these QTL are shown in Fig. 1. At least one major QTL for each year underlays the variation in each trait. Clustering of QTL associated with different traits was observed on LG_05, harbouring QTL for *tc* and *s/tc* ratio. The high inter-trait correlations between the traits controlled by this cluster (Online Resource 4) suggests either a set of closely linked loci or, more likely, a single pleiotropic locus.

Trunk circumference. Two regions harboured *tc* loci (Table 3), one of which was expressed in all three years and the other was detected only in 2012. The former was located on LG_05 (linked closely to three SSR loci) and over the three years explained 11.4 to 13.6% of the PV. The latter was a minor one, located on LG_08 and accounted for 8.5% of the PV in 2012 only.

Suckers/trunk circumference. QTL associated with *s/tc* were detected in five genomic regions, of which one was detected in all three years, one in two years, and three were detected in only a single year.

The SSR locus A602 mapped on LG_10 and was stably associated with *s/tc* trait across the three years and explained from 8.7% to 14.9% of the PV. The QTL mapped on LG_01 were expressed in both 2013 and 2014 explained from 8.1 to 10.9% of the PV. The other QTL were expressed in one season only, the most interesting of them was a major one, accounting for 17.1% of the PV, that mapped to the LG_05 region where *tc* QTL were also located, with overlapping LOD confidence intervals.

Leaf bud burst. One major *lb* QTL was detected in all three years. It explained 45-53% of the PV and mapped in the vicinity of the SSR locus AJ417975b on LG_02.

Discussion

Genetic map construction

A genetic map was constructed on a F_1 progeny using a two-way pseudotestcross approach. The parent cultivars showed several phenotypic differences. Previous studies showed a very high level of genetic variation and heterozygosity in hazelnut, attributable in part to the sporophytic incompatibility that enforces cross pollination (Mehlenbacher 1991; Mehlenbacher et al. 2006), and the clonal propagation of superior genotypes.

SSR markers were analysed as they are ideal for linkage mapping for their robustness, polymorphism, co-dominance and conservation (Wu et al. 2004), even if they are based on relatively low-throughput technologies, which tend to limit marker density across the genome (Bowers et al. 2012). The development of new high-throughput sequencing technologies enable to achieve high-density linkage maps by direct analyses of sequence variations, including single nucleotide polymorphisms (SNPs) (Huang et al. 2014). Markers such SNPs are more abundant and genome wide distributed and can be analysed multiplexing hundreds of markers (Salazar et al. 2015). Nevertheless, SSRs are found to be more polymorphic and are considered as the best marker system for construction of framework linkage map (Jones et al. 2007).

The map consisted of 151 markers distributed across 11 LG (Fig. 1); length and average density (663.1 and 4.45 cM respectively) were lower than those found by Gürcan et al. (2010). The presence of an unlinked marker and 8 gaps >15 cM (Online Resource 3) suggested that there are parts of the genome not covered yet, remaining under-represented (Sargent et al. 2004; Portis et al. 2009).

Only 7% of the markers showed segregation distortion (Online Resource 2), consistent with observations (Lorieux et al. 1995a; Lorieux et al. 1995b; Portis et al. 2012) that distortion at co-dominant marker loci such as SSRs occurs at a lower frequency than at dominant marker loci. Segregation distortion has been reviewed by Lyttle (1991) to indicate the excessive transmission of one allele at a heterozygous locus to the progeny without following Mendelian proportion. Segregation distortion has been associated with statistical bias or errors in genotyping, but also with biological factors (such as chromosome/locus loss, zygotic survival, self-incompatibility alleles) that can affect meiosis, fertilization and embryogenesis (Bradshaw et al. 1994; Jenczewski et al. 1997) or with the presence of null alleles (Pekkinen et al. 2005). Markers were included even when their segregation distortion was significant, as their presence did not alter marker order. Cervera et al. (2001) and Doucleff et al. (2004) reported that it was appropriate to include markers with <1% distortion, as their exclusion can lead to a failure of analysis in significant parts of a linkage group. Moreover, some mapping studies

demonstrated a positive effect of inclusion of highly distorted markers in linkage analyses (Kuang et al. 1999; Fishman et al. 2001).

Four markers showed null alleles and were analysed as dominant markers. Null alleles at SSR loci are not uncommon, in hazelnut (Gürcan et al. 2010). They are probably due to amplification failure for one allele in heterozygotes, resulting in a loss of data and a higher apparent number of homozygotes (Pekkinen et al. 2005, Gürcan et al. 2010). Apart from biological factors such as inbreeding, reasons for the occurrence of null alleles can be sequence mismatch or deletion that affected primer annealing, the competition which often favours amplification of the smaller allele, or poor quality/quantity of the DNA template (Dakin and Avise 2004). In this situation possible solutions are to disregard the affected loci, to score segregation in the same way as for a dominant marker (Rodzen and May, 2002), to redesign primers (Shaw et al. 1999; Van Oosterhout et al. 2004), or to adjust allele frequencies relating to a global estimate of the frequency of null alleles (Portis et al. 2012).

A total of sixteen genic/EST-SSR markers were mapped on six linkage groups. Boccacci et al. (2015) developed fourteen of them from sequences of alder, birch and hazelnut. BLASTx analyses and functional annotation by Blast2Go programme suggested their involvement in biological processes associated with stress response, signal transduction and processes regulation. AJ417975b and CD278264, derived from *Corylus* and *Betula* expressed sequences, respectively, were developed by Gürcan and Mehlenbacher (2010b) but were not previously mapped. In particular, AJ417975b from the Lox gene, coding for a lipoxygenase (LOXs) and was mapped on the LG_02, the same as AJ417975c, a marker designed from the same sequence and mapped by Gürcan and Mehlenbacher (2010a). CD278264 was a sequence from a root tissue cDNA library (Johansson et al. 2004).

The use of 111 markers in common with the map developed at OSU (Mehlenbacher et al. 2006; Gürcan et al. 2010; Gürcan and Mehlenbacher 2010a) allowed the comparison between corresponding linkage groups and the assignment of the same LG numbers (Fig. 1). Marker order was similar, with some exceptions. These discrepancies could reflect genetic differences between the pairs of mapping parents and/or be statistical artefacts resulting from the use of different mapping parameters (Barchi et al. 2012). In fact, re-ordering of closely linked markers is relatively commonplace (Cervera et al. 2001; Jeuken et al. 2001; Lespinasse et al. 2000; Sebastian et al. 2000). For this reason, variation in stringency (LOD threshold), marker choice, genotyping errors, excess of missing values and the mapping of distorted markers can cause mapping inconsistency (Portis et al. 2009; Hackett and Broadfoot 2003).

The choice of these markers was done to enable the future merging of the two maps, developed with the same mapping strategy. Integration of mapping data from several crosses on a single integrated map would be useful to determine the relative positions of transferable markers, independent of the heterozygous stated in either parents of the cross (Doligez

et al. 2006). In this way, it would be possible to integrate 40 SSRs not mapped by Gürcan et al. (2010) as well as the genes/QTL responsible for the phenotypic variation in the different progenies (Aranzana et al. 2003; Doligez et al. 2006). Preliminary results in linkage map construction showed only 10 LGs rather than the expected 11 (the haploid chromosome number for hazelnut), for merging of markers located on LG_09 and LG_10 at the third round of JoinMap analyses. It is possible to speculate about the presence of a reciprocal translocation event during meiosis: in fact using standard linkage analysis, translocations usually lead to "pseudo-linkage" with the creation of a single linkage group showing a mix of markers from the chromosomes involved in the phenomenon (Livingstone et al. 2000; Farré et al. 2011). Recombination is severely suppressed near the translocation breakpoints, so reordering markers in those regions is not feasible (Farré et al. 2011). This type of event has been described in different hazelnut cultivars, including TGdL, by Salesses (1973) and Salesses and Bonnet (1988).

Phenotyping, QTL mapping and clustering of agronomic traits

The QTL approach allows analysis of the genetic basis of variation in quantitative traits and identification of genomic regions on a linkage map (Paterson et al. 1988), not yet been published for hazelnut. Agronomic traits are often quantitative in nature and are under polygenic control. This type of study has not been published yet for hazelnut, so this is the first report identifying QTL for agronomic traits in the species.

Concerning the parental characteristics for the examined traits, according to the literature, they differ for trunk circumference and leaf bud burst (IPGRI, FAO and CIHEAM 2008), while the suckering habit (and suckers/trunk circumference ratio, consequently) was more evident in MB than TGdL, although no significant differences were highlighted during the first two years of study (Fig. 2 and Table 2). It is noteworthy that the presence of suckers is undoubtedly a genetic character linked to the cultivar. Nevertheless their amount is also influenced by climatic and soil factors, and by propagation and cultural systems. For instance sucker production increases for plants growing in sandy soil (Germaine and Serraquigne 2004) or those originating as rooted suckers (as the parents in our study) (Radicati et al. 1994).

Phenotypic trait distributions were not normal and changed over the years, especially between 2012 and 2013 (Fig. 2). This could be due to the development of plants in the progeny during growth: in fact characters such suckering habit become stable only after some years after plantation (Germain and Sarraquigne 2004). Distribution of traits in 2013 and 2014 were more similar, suggesting a stabilization of the characters. Leaf bud burst data was similar over the three years (Fig. 2c). Only in 2014 the distribution was less uniform than the previous years. A temperature decrease recorded after stage 3 suggested a possible effect on leaf bud burst. Indeed, temperature influence on timing of bud burst is evident in forest trees and fruit trees (Howe et al. 2003; Cooke et al. 2012).

The H^2_{BS} was 0.74 for *lb* and 0.85 for *s/tc*, while it was 0.60 for *tc* (Table 2). High heritability estimates have been reported for phenological traits in many other crops, even though the populations growing conditions and measurement methods are different (Dicenta et al. 1993; Tancred et al. 1995). Heritability studies of phenological traits such as leaf bud burst and vigour have been reported for several forest trees, including poplar (Bradshaw and Stettler 1995; Frewen at al. 2000, Rohde et al. 2011), chestnut (Casasoli et al. 2004; Casasoli et al 2006), oak (Scotti-Saintagne et al. 2004), Douglas-fir (Jermstad et al. 2001; Jermstad et al. 2003), and eucalyptus (Bundock et al. 2008). Only a preliminary work on QTL analyses was published for *Betula platyphylla* Suk, with the identification of two QTL for stem circumference (Zhang et al. 2012).

Bradshaw and Stettler (1995) reported H^2_{BS} estimates of 0.67 for vigour and 0.98 for spring bud flush. Several studies in forest trees have shown that bud phenology is highly heritable, with values ranging from 0.50 to 0.80, (Bradshaw and Stettler 1995; Jermstad et al. 2001; Scotti-Saintagne et al. 2004; Billington and Pelham 1991). Heritability estimates for growth range from low to high (0.20 to 0.80). (Bradshaw and Stettler 1995; Byrne et al. 1997, Scotti-Saintagne et al. 2004). These values indicate strong genetic control of bud phenology (Li and Adams 1993; Bradshaw and Stettler 1995). For *s/tc* it is not possible to compare H^2_{BS} of hazelnut with other species since suckering is not a commonly studied trait. Work on tobacco (*Nicotiana tabacum* L., Julio et al. 2006) and sugarcane (*Saccarum officinarum* L., Jordan et al. 2004) attempted to identify QTL for suckering. In both cases, high sucker production was negatively correlated with yield. The estimate in tobacco. (H^2_{BS} =0.69) was high, as in our work.

Transgression, both positive and negative, was present for all the three traits studied during the three years (Fig. 2). Transgressive genotypes derived from the combination of alleles from both parents with effect on the same direction (De Vicente and Tanksley 1993). Transgression was evident for the examined traits also in other studies (Bradshaw and Stettler 1995; Julio et al. 2006). The effect of these allele combinations will be investigated, keeping in mind that traits such as sucker habit and tree vigour in hazelnut can be considerably influenced by environment (Valentini et al. 2004). These considerations will be accounted also for the suggesting semi-dominance for poor vigour, highlighted by the mean value of *tc* in the progeny minor of the mid-parent value in 2013 and 2014. In fact, it is well accepted that traits such as growth can be considered as developmental traits, and show a temporal shift for what concern their action (Kremer 1992). This work identified 15 QTL, of which 10 are major and stably expressed over years (Collard et al. 2005; Li et al. 2001; Pilet-Nayel et al. 2002). At least one major QTL was identified for each of the three traits studied in hazelnut: *tc*_05, *s/tc*_10 and *lb*_02 which were detected in all three years with LOD values of 3.3 and 5.3. Particularly striking was *lb*_02 which explained around 50% of the PV with a LOD score > 20. This QTL is associated to the SSR locus AJ417975b, an EST-SSR derived from a *Betula* sequence of the Lox gene: it coded for lipoxygenases (LOXs), a class of widespread dioxygenases that catalyse the addition of oxygen to polyunsaturated fatty acids containing a cis,cis-1,4-pentadiene

structure. This reaction produces hydroperoxides that are the starting point for a series of other enzymatic reactions for the synthesis of a group of biologically active compounds named oxylipins, playing diverse functions in several physiological processes such as growth, development, and response to biotic and abiotic stresses (Santino et al. 2003).

The other QTL were identified for *tc* and *s/tc*, of which four were year-specific. The presence of year-specific QTL for *tc* and *s/tc* agrees with observation on other forest trees. The higher proportion of year-specific QTL for growth, compared to phenology, was explained by Wu et al. (2002) who considered growth as a trait determined by loci expressed at a specific stage of development. Previous work detected QTL for growth varying from one developmental stage to another (Plomion et al. 1996; Emebiri et al. 1998; Tsarouhas et al. 2002).

The number of QTL identified in other forest trees varied among the different species studied. In poplar Bradshaw and Stettler (1995) identified 2 QTL for growth and 5 QTL for leaf bud burst; in chestnut Casasoli et al. (2004) found 11 QTL for bud phenology and 4 for growth; 32 and 33 QTL were identified for leaf bud burst in oak and Douglas-fir, respectively (Scotti-Saintagne et al. 2004; Jermstad et al. 2001), and 3 QTL were found for growth in eucalyptus (Bundock et al. 2008). For sucker production, the number of QTL varied in two species: 3 QTL were found in tobacco and 14 in sugarcane (Julio et al. 2006; Jordan et al. 2004). The power to detect the QTL controlling a trait and to accurately estimate the magnitude of their effects is strongly influenced by the size of the mapping populations, the marker information and the accuracy of trait measures (Jermstad et al. 2001). In particular, simulation studies have shown that with a decrease in sample size there is a reduction in QTL detection power and an overestimation of their magnitude (Beavis 1995).

The clustering of QTL associated with different traits was observed on LG_05, which harboured QTL for *tc* and *s/tc* ratio (Fig. 1 and Table 3). Co-localization of QTL is very important for practical applications in breeding programs (Portis et al. 2012). If the traits are highly correlated, plant breeders can select for both traits with the same markers. This leads to maximum genetic gain for both traits in segregating populations (Carter et al. 2011). Our data showed a positive correlation between *tc* and *s/tc* in 2012 and 2013, while leaf bud burst showed no correlation with other traits (Online Resource 4). Valentini et al. (2004) studied progenies obtained from the same female parent used in this work, but from different male parents. In their work, early time of bud burst showed a positive correlation with tree vigour, and a negative correlation with the amount of suckers; in addition, the amount of suckers showed negative correlation with tree vigour.

In our research, correlation data (Online Resource 4) showed that vigour and *s/tc* ratio had a positive correlation only during the first two years, and values decreased over time. In particular, *s/tc* in 2014 showed a negative correlation with vigour in 2012 and 2014, although these values were not significant. It could be speculated that *tc* and *s/tc* traits require time for stabilization of the character and thus further observations of these traits during the next years will show if this trend is confirmed.

Conclusion

This work presents the first QTL analyses for hazelnut. Fifteen QTLs were identified, including at least one major QTL for each of the three traits. A major QTL on LG_02 for time of leaf bud burst explained about 50% of the PV. This is an initial step in the identification of chromosomal regions carrying genes of interest, which will be important for breeding programs and allow marker-assisted selection.

Conflict of interest

The authors declare that they have no conflict of interest.

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List of Tables

Table 1 Characteristics and alignment of the TGdL x MB consensus linkage map

Linkage group name	Size (cM)	Total markers	Marker density (cM/No. markers)	Gaps (> 15 cM)	Alignment with OSU 252.146 x OSU 414.062 map [1]	
					Aligned LGs	Shared markers
LG_01	98.9	14	7.06	2	LG1S + LG1R	8
LG_02	58.4	17	3.44	1	LG2S + LG2R	8
LG_03	48.8	11	4.88	1	LG3S + LG3R	7
LG_04	71.7	15	4.78	0	LG4S + LG4R	13
LG_05	52.3	14	3.74	1	LG5S + LG5R	9
LG_06	33.8	4	8.45	0	LG6S + LG6R	3
LG_07	51.8	11	4.71	2	LG7S + LG7R	10
LG_08	47.0	15	3.36	0	LG8S + LG8R	15
LG_09	74.8	25	3.25	0	LG9S + LG9R	14
LG_10	66.6	15	4.76	0	LG10S + LG10R	12
LG_11	59.0	14	4.54	1	LG11S + LG11R	7
Total	**663.1**	**155**		**8**		**106**
Average	**60.28**	**13.55**	**4.45**	**0.73**		**9.64**

[1] Alignment with the maps published by Gürcan et al. 2010 and Gürcan and Mehlenbacher 2010a and based on a cross between genotypes from a maternal parent (OSU 252.146) susceptible and a male parent (OSU 414.062) resistant to eastern filbert blight

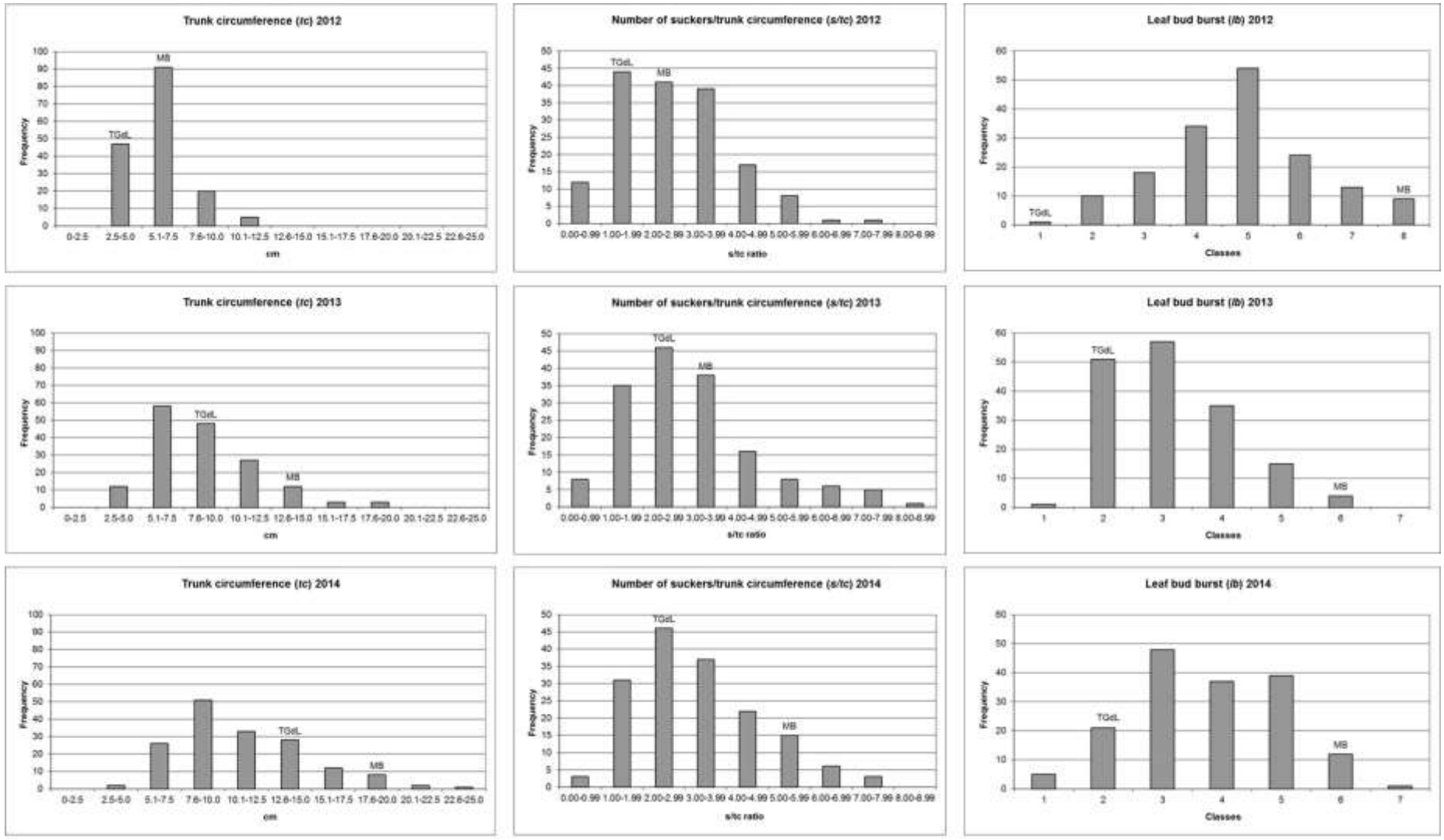
Table 2 Parent means (±SD) and population mean (±SD) and range for the traits. Skewness and Kurtosis (±SE) and values of broad sense heritability for the traits are also listed

Trait	Trait code	Year	Parent means ± SD		Significant mean difference among parental values (wilcoxon test)	F_1 population mean ± SD	Range	SE	Skewness	SE	Kurtosis	SE	Heritability H^2_{BS}
			TGdL	MB									
Trunk circumference (cm)	*tc*	2012	4.55 ± 0.7	7.07 ± 1.1	Yes: p<0.1	5.90 ± 1.57	3.14 – 10.99	0.12	0.85	0.19	0.89	0.38	0.60
		2013	9.50 ± 0.7	13.25 ± 1.0	Yes: p<0.05	8.84 ± 2.99	4.00 – 18.00	0.23	0.92	0.19	0.53	0.38	
		2014	13.25 ± 0.4	18.50 ± 2.8	Yes: p<0.1	11.25 ± 3.79	4.50 – 25.00	0.30	0.83	0.19	0.54	0.38	
No. of suckers/ trunk circumference (No./cm)	*s/tc*	2012	1.67 ± 1.8	2.79 ± 1.5	ns	2.71 ± 1.30	0.00 – 7.35	0.10	0.56	0.19	0.16	0.38	0.85
		2013	2.79 ± 2.8	3.77 ± 2.8	ns	3.03 ± 1.62	0.00 – 8.67	0.13	0.96	0.19	0.98	0.38	
		2014	2.20 ± 0.5	5.28 ± 0.4	Yes: p<0.05	3.26 ± 1.47	0.25 – 7.37	0.15	0.62	0.19	-0.09	0.38	
Leaf bud burst (rating: 1 to 9)	*lb*	2012	1.0 ± 0.0	8.0 ± 0.0	Yes: p<0.01	4.83 ± 1.50	1.0 – 8.0	0.12	0.08	0.19	-0.13	0.38	0.74
		2013	2.0 ± 0.0	5.5 ± 0.7	Yes: p<0.01	3.15 ± 1.60	1.0 – 6.0	0.08	0.64	0.19	-0.18	0.38	
		2014	1.5 ± 0.7	6.0 ± 0.0	Yes: p<0.01	3.76 ± 1.28	1.0 – 7.0	0.10	0.01	0.19	-0.61	0.38	

Table 3 QTL detected in the mapping population for trunk circumference (*tc*), number of suckers/trunk circumference ratio (*s/tc*) and leaf bud burst (*lb*). Each QTL name is formed by the abbreviated form of the trait followed by the relevant LG. The table indicates genome-wide LOD thresholds (GW) as determined by a permutation test at $P \leq 0.05$, the closest linked markers (Locus) and their map position in cM, the estimated LODs at the QTL peak (LOD), and the proportions (%) of the total phenotypic variance (PV) explained

			2012					2013					2014				
Trait code	LG	QTL	GW	Position	Locus	LOD	PV	GW	Position	Locus	LOD	PV	GW	Position	Locus	LOD	PV
tc	05	tc_05	3.1	16.4	B625	4.0	11.4	3.3	25.2	KG800b	3.8	12.7	3.2	26.3	KG800a	3.3	13.6
	08	tc_08		26.9	B773	3.1	8.5		-	-	-	-		-	-	-	-
s/tc	01	s/tc_01	3.6	-	-	-	-	3.3	77.8	KG857	3.4	8.1	3.5	77.8	KG857	4.5	10.9
	05	s/tc_05		-	-	-	-		-	-	-	-		17.9	CaT-B503	6.5	17.1
	08	s/tc_08		-	-	-	-		-	-	-	-		0.0	B726	4.0	9.8
	10	s/tc_10		11.4	A602	5.3	14.9		11.4	A602	3.5	10.1		11.4	A602	3.5	8.7
	11	s/tc_11		59.0	B652	3.8	9.3		-	-	-	-		-	-	-	-
lb	02	lb_02	4.2	12.1	AJ417975b	21.4	45.4	4.8	12.1	AJ417975b	26.0	52.0	4.7	12.1	AJ417975b	26.9	53.2

Fig. 2 Frequency distributions of trunk circumference (a), number of suckers/trunk circumference ratio (b), leaf budburst (c) for the progeny derived from TGdL x MB in 2012, 2013 and 2014. Data are grouped in classes. Means for the parents TGdL and MB are shown for each histogram



a b c

Fig. 1 Consensus genetic linkage map of TGdL x MB progeny and locations of QTLs for trunk circumference (*tc*), number of suckers/trunk circumference ratio (*s/tc*) and leaf budburst (*lb*) in 2012, 2013 and 2014 using the multiple QTL mapping method. Marker names are shown to the right of each LG, with map distance (in cM) to the left. Markers showing significant levels of segregation distortion are indicated by asterisks (0.1>*P>0.05; 0.05>**P>0.01; 0.01>***P>0.005; 0.005>****P>0.001; *****P>0.001). Black bars represent QTLs: each is indicated by trait code_LG number_year. 1-LOD support intervals of each QTL are marked by thin bars