# Development of a genetic linkage map for molecular breeding of chestnut

D. Torello-Marinoni[1,*], S. Nishio[2,*], E. Portis[1], N. Valentini[1], C. Sartor[1], F. Dini[1], P. Ruffa[1], S. Oglietti[1], G. Martino[1], A. Akkak[3] and R. Botta[1,a]

[1]DISAFA-Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università degli Studi Torino, Italy; [2]Pear and Chestnut Breeding Unit, Institute of Fruit Tree and Tea Science, NARO, Tsukuba, Japan; [3]Dipartimento di Scienze Agrarie, degli Alimenti e dell'Ambiente, Università degli Studi di Foggia, Italy.

***Abstract***

**In the last 50 years the nut production of *Castanea sativa* in Europe has strongly declined due to various factors: the aging of the population in the areas of growing, changes in the structure of society, and the spread of diseases and pests, including the chestnut gall wasp (*Dryocosmus kuriphilus,* Yasumatsu). Yet, recently, the interest for the planting of new and modern orchards has grown in many areas of Europe and there is an increasing demand for cultivars and rootstocks more tolerant to biotic factors and adapted to the changing climate. The spread of the gall wasp in Italy has promoted breeding strategies and studies on resistance to the insect. In this frame the *Castanea sativa* cultivar 'Madonna' and the Eurojapanese hybrid 'Bouche de Bétizac' (*C. sativa* × *C. crenata*), susceptible and resistant to gall wasp respectively, were used to create progenies for selecting new cultivars, investigating the mechanism of resistance to the cynipid and studying agronomical and technological traits. A progeny of 250 plants segregating for resistance to the pest and for phenological, morphological and nut traits were obtained. 148 plants of the progeny, currently 8 years old, were analysed at 132 SSR loci, and for agronomic traits such as time of budburst, tree habit, and resistance to *D. kuriphilus*. Data were statistically analysed to obtain two genetic linkage maps and identify putative QTL regions and markers associated to the resistance trait. This work represents an initial step in the identification of chromosomal regions carrying genes of interest, useful for breeding programs and to develop marker-assisted selection.**

**Keywords:** *Castanea*, SSR, MAS, QTL, *Dryocosmus kuriphilus*

## INTRODUCTION

Chestnut (*Castanea sativa* Mill.) is a species of major social, economic and environmental importance in Italy and Europe. For centuries, it has been closely associated with human activities and can be truly considered a multipurpose tree, providing food, fuel, tannin and wood for construction, as well as being a dominant component in the Mediterranean landscape and ecosystems.

Chestnut is valued for the health benefits of its fruits, since nuts have a high nutraceutical value, a characteristic that allows them to be inserted among the so-called 'functional food', product that can positively influence the organism, if consumed regularly.

The nut production of *C. sativa* in Europe has strongly declined due to various factors: changes in land use, abandonment of rural areas (mainly in the mountains), and spread of several pests and diseases, including ink disease (*Phytophthora* spp.), chestnut blight (*Cryphonectria parasitica* (Murr.) Barr), *Gnomoniopsis castaneae* G. Tamietti (Visentin et al., 2012) and gall wasp (*Dryocosmus kuriphilus* Yasumatsu). Nowadays, interest for the planting of modern orchards has grown and farmers are interested in planting local cultivars of *C. sativa*, but they need new rootstocks resistant to ink disease, adapted to dry and cold areas

[a]E-mail: roberto.botta@unito.it

*Both authors contributed equally to this work

and easy to be propagated. The spread of the gall wasp in Italy has promoted breeding strategies and studies on resistance to the insect. The construction of genetic linkage maps and the identification of molecular markers linked to traits of interest would greatly facilitate breeding programs and the development of marker-assisted selection (MAS). Many important agronomic and quality traits, such as time of bud burst, 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 frame the *C. sativa* cultivar 'Madonna' and the Eurojapanese hybrid 'Bouche de Bétizac' (*C. sativa* × *C. crenata*), susceptible and resistant to gall wasp respectively, were used to create progenies for selecting new cultivars, investigating the mechanism of resistance to the cynipid and studying agronomical and technological traits.

Objective of this paper was the construction of a linkage map, to study the genetic basis of the resistance trait and develop QTLs related to time of bud burst and plant growth habit.

## MATERIALS AND METHODS

The $F_1$ progeny (250 seedlings) was obtained by crossing the hybrid (*C. sativa* × *C. crenata*) cultivar 'Bouche de Bétizac' (female parent) with the *C. sativa* cultivar 'Madonna' (male parent). The seedlings were verified for either being resistant or susceptible, by visual assessment, after controlled infestation with *D. kuriphilus*.

The following phenotypic traits were evaluated: time of bud burst and growth habit. Time of bud burst was recorded, in 2013, 2014, 2016 and 2017, at the first leaf appearance out of the bud, when the veins of leaves are evident (''stage C3-D'', Fernández López et al., 2002). 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 (2015) guidelines. Tree growth habit was recorded in 2016 and 2017 in ordinal categorical scores (1= very upright, 2= upright, 3= semi-upright, 4= spreading, 5= very spreading), slightly modified by UPOV (2015).

DNA was extracted from young leaves of seedlings with CTAB method (Doyle and Doyle, 1987). Each individual was preliminarily checked to confirm the parentage by genotyping using ten SSR loci (Steinkellner et al., 1997; Buck et al., 2003; Marinoni et al., 2003). 148 $F_1$ individuals of the progeny were then genotyped with 132 microsatellite markers identified from *C. sativa*, *C. mollissima*, *C. crenata*, *Quercus rubra* and *Q. petraea* (Steinkellner et al., 1997; Kampfer et al., 1998; Aldrich et al., 2002; Buck et al., 2003; Marinoni et al., 2003; Nishio et al., 2011; Kubisiak et al., 2013; Akkak, pers. commun.). Amplification products were analysed on a 3130 Genetic Analyzer capillary sequencer (Applied Biosystems, USA). The internal GeneScan TM size standard 500 LIZ was included in each run. Allele sizes in the output were called using GeneMapper v4.0 software (Applied Biosystems).

JoinMap® 4 (Van Ooijen, 2006) software was used to construct the maps of 'Bouche de Bétizac' and 'Madonna' with the pseudo-testcross mapping strategy in the $BC_1$ mode (Grattapaglia and Sederoff, 1994), applying the Kosambi (1944) mapping function. For both maps, linkage groups (LGs) were established based on a threshold logarithm of odds (LOD) ratio of 3.0. The LG names were assigned according to the *C. mollissima* linkage maps (Kubisiak et al., 2013).

The two separate parental maps were used to assign putative QTL by performing the simple interval mapping procedure (Lander and Botstein, 1989), using the MapQTL® 6 software (Van Ooijen, 2009). Linkage maps and QTL positions were drawn using MapChart (Voorrips, 2002).

## RESULTS AND DISCUSSION

### Map construction

A set of 120 and 84 SSR markers was used for constructing the linkage maps of 'Bouche de Bétizac' and 'Madonna', respectively. A graphical representation of the genetic maps are shown in Figure 1. The linkage map for 'Bouche de Bétizac' consisted of 12 linkage

groups, for a total genetic length of about 650 cM, with a mean inter-marker distance of 5.4 cM; eight gaps >15 cM were present. The map was successfully aligned to the *C. mollissima* genetic map (Kubisiak et al., 2013). The linkage map of 'Madonna' consisted of 13 LG, and covered a total genetic length of 590.0 cM with a mean inter-marker distance of 6.8 cM; eight gaps >15 cM were present. The map was aligned with the map of 'Bouche de Bétizac', resulting that LG A of the map of 'Madonna' was divided into two groups (LG A1 and LG A2).

For the resistance, out of the 148 seedlings evaluated, 53% individuals were classified as resistant type, while 47% as susceptible type. The traits of resistance to *D. kuriphilus* were confirmed to follow a simple Mendelian segregation, with a segregation ratio of 1.06:0.94. The resistance to *Dryocosmus kuriphilus* (*Dk*) was mapped on LG K of 'Bouche de Bétizac'.

**Phenotypic evaluation and QTL detection**

Time of bud burst frequency, observed across four years, showed a roughly normal distribution, with year-to-year variations probably due to the environment influence. The tree growth habit frequency had as well a normal distribution: 42% of plants showed a semi erect habit, while 10 and 6% plants had very erect or very spreading habit, respectively (Figure 2).

Separate QTL analysis was performed in each year and for each parent ('Bouche de Bétizac' and 'Madonna'). The QTLs that explained over 10% of the phenotypic variance (PV) are hereafter referred to as 'major' QTL. Overall, 11 major QTL were identified for time of bud burst and 5 for plant growth habit.

For time of bud burst, we identified 6 major QTLs on the female map and 5 major QTLs on the male map. One major QTL for time of leaf budburst, stably expressed in all years (2013, 2014, 2016 and 2017) was responsible for 28-38% of PV and was identified on LG L of 'Bouche de Bétizac'. Other two major QTLs were found in only one season on LG B and LG G of 'Bouche de Bétizac' (10% of PV). On the male map of 'Madonna', one stable major QTL, responsible for 10-14% of PV, was detected on LG C in all years of observation; another major QTL was detected on LG I of 'Madonna' in one season.

Concerning tree growth habit, 3 major QTLs were detected on the female map and 2 major QTLs on the male map. In 'Bouche de Bétizac' map, one major QTL, expressed in all seasons, was detected on LG L (15-25% of PV), while in one season (2016) a major QTL (11% of PV) was detected on LG B. Finally, 2 major QTLs were identified on LG A of 'Madonna' (10% of PV).

**CONCLUSIONS**

This work presents two draft linkage maps for the Eurojapanese hybrid 'Bouche de Bétizac' (*C. sativa* × *C. crenata*), and the *C. sativa* cultivar 'Madonna'. The resistance to *Dryocosmus kuriphilus* (*Dk*) was mapped on the LG K of 'Bouche de Bétizac'. Sixteen significant QTLs were detected for time of bud burst and tree growth habit. This work represents an initial step in the identification of chromosomal regions carrying genes of interest. To get reliable QTL detection and to develop markers useful for breeding programs, these preliminary maps will be saturated using SNPs markers.

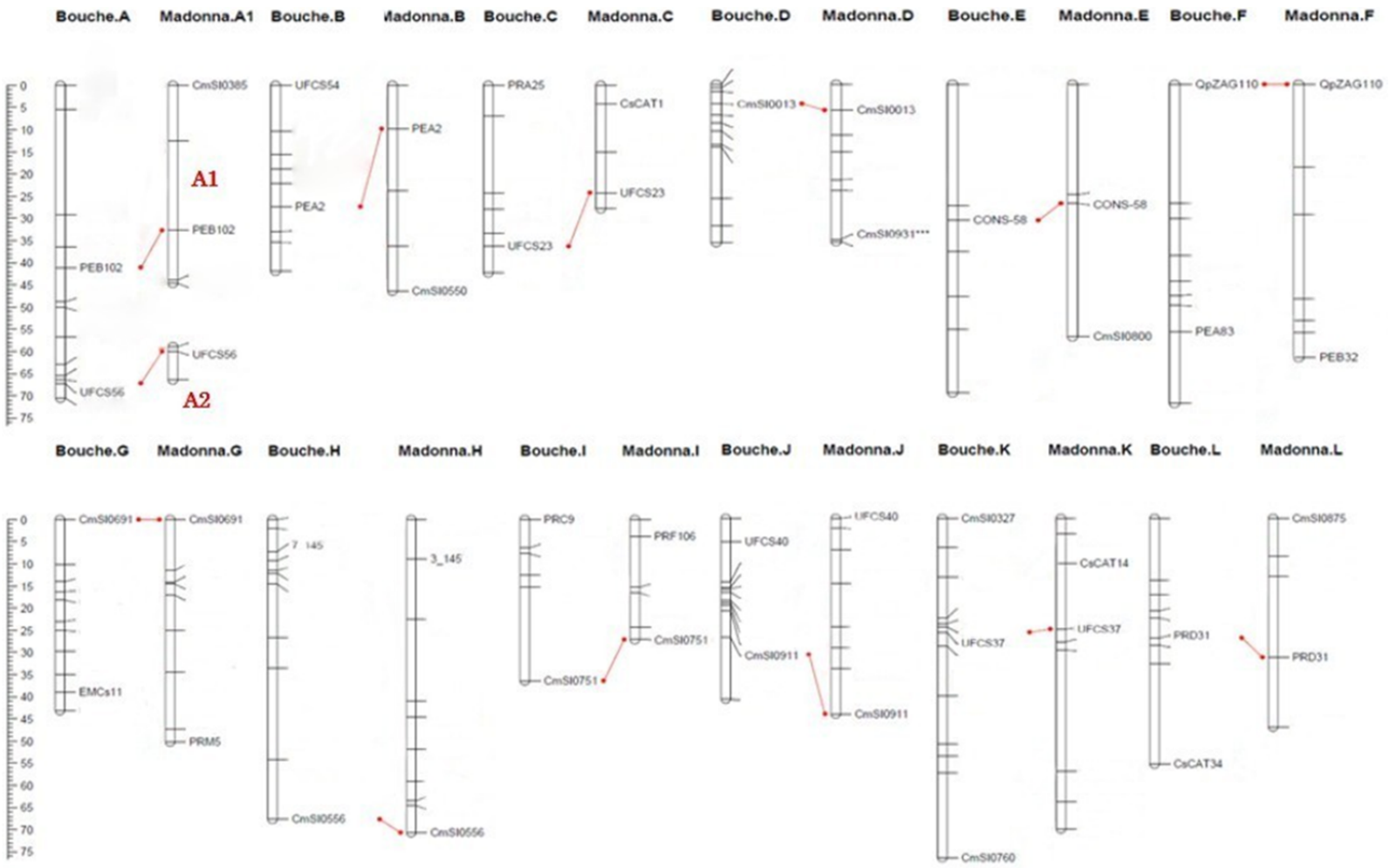


Figure 1. Genetic maps of the Eurojapanese hybrid 'Bouche de Bétizac' (*C. sativa* × *C. crenata)* and 'Madonna' (*C. sativa*), aligned on the basis of common markers.

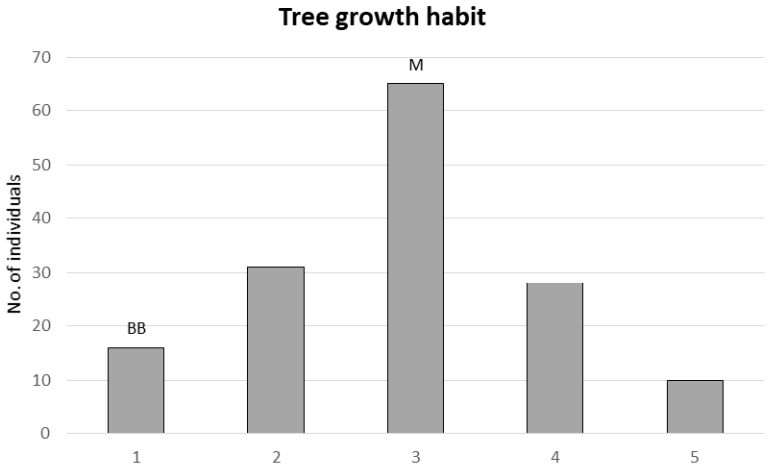


Figure 2. Distribution of tree growth habit in the progeny derived from the Eurojapanese hybrid 'Bouche de Bétizac' (*C. sativa* × *C. crenata*) and 'Madonna' (*C. sativa*) in 2016. Data are grouped in classes: very erect (1), erect (2), semi-erect (3), spreading (4), very spreading (5). BB and M indicate the parents 'Bouche de Bétizac' and 'Madonna', respectively.

**ACKNOWLEDGEMENTS**

The research was funded by the Cooperation program Italy-France Alcotra 2007-2013 project "Salvaguardia dell'ecosistema Castagno", by Regione Piemonte Administration and by the University of Torino – Local Research 2016.

## Literature cited

Aldrich, P.R., Michler, C.H., Sun, W., and Romero-Severson, J. (2002). Microsatellite markers for northern red oak (Fagaceae: *quercus rubra*). Mol. Ecol. Notes *2* (*4*), 472–474 https://doi.org/10.1046/j.1471-8286.2002.00282.x.

Buck, E.J., Hadonou, M., James, C.J., Blakesley, D., and Russell, K. (2003). Isolation and characterization of polymorphic microsatellites in European chestnut (*Castanea sativa* Mill.). Mol. Ecol. Notes *3* (*2*), 239–241 https://doi.org/10.1046/j.1471-8286.2003.00410.x.

Collard, B.C.Y., Jahufer, M.Z.Z., Brouwer, J.B., and Pang, E.C.K. (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: the basic concepts. Euphytica *142* (*1-2*), 169–196 https://doi.org/10.1007/s10681-005-1681-5.

Doyle, J.J., and Doyle, J.L. (1987). A rapid DNA isolation procedure from small quantities of fresh leaf tissue. Phytochem. Bull. *19*, 11–15.

Fernández López, J., Zás, R., and Díaz, R. (2002). Recording adaptive traits in chestnut field trials (Spain: CIFA Lourizán, Xunta de Galicia).

Grattapaglia, D., and Sederoff, R. (1994). Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: mapping strategy and RAPD markers. Genetics *137* (*4*), 1121–1137. PubMed

Kampfer, S., Lexer, C., Glössl, J., and Steinkellner, H. (1998). Characterization of $(GA)_n$ microsatellite loci from *Quercus robur.* Hereditas *129* (*2*), 183–186 https://doi.org/10.1111/j.1601-5223.1998.00183.x.

Kosambi, D.D. (1944). The estimation of map distances from recombination values. Ann. Hum. Genet. *12*, 172–175.

Kubisiak, T.L., Nelson, C.D., Staton, M.E., Zhebentyayeva, T., Smith, C., Olukolu, B.A., Fang, G.C., Hebard, F.V., Anagnostakis, S., Wheeler, N., et al. (2013). A transcriptome-based genetic map of Chinese chestnut (*Castanea mollissima*) and identification of regions of segmental homology with peach (*Prunus persica*). Tree Genet. Genomes *9* (*2*), 557–571 https://doi.org/10.1007/s11295-012-0579-3.

Lander, E.S., and Botstein, D. (1989). Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics *121* (*1*), 185–199. PubMed

Marinoni, D., Akkak, A., Bounous, G., Edwards, K.J., and Botta, R. (2003). Development and characterization of microsatellite markers in *Castanea sativa* (Mill.). Mol. Breed. *11* (*2*), 127–136 https://doi.org/10.1023/A:1022456013692.

Nishio, S., Yamamoto, T., Terakami, S., Sawamura, Y., Takada, N., Nishitani, C., and Saito, T. (2011). Novel genomic and EST-derived SSR markers in Japanese chestnuts. Sci. Hortic. (Amsterdam) *130* (*4*), 838–846 https://doi.org/10.1016/j.scienta.2011.09.012.

Steinkellner, H., Fluch, S., Turetschek, E., Lexer, C., Streiff, R., Kremer, A., Burg, K., and Glössl, J. (1997). Identification and characterization of (GA/CT)n-microsatellite loci from *Quercus petraea.* Plant Mol. Biol. *33* (*6*), 1093–1096 https://doi.org/10.1023/A:1005736722794. PubMed

Van Ooijen, J.W. (2006). JoinMap®4: Software for the Calculation of Genetic Linkage Maps in Experimental Populations (Wageningen: Kyazma B.V.).

Van Ooijen, J.W. (2009) MapQTL® 6, Software for the Mapping of Quantitative Trait Loci in Experimental Populations of Diploid Species (Wageningen: Kyazma B.V.).

UPOV. (2015). Chestnut: *Castanea crenata* Sieold & Zucc.; *Castanea mollissima* Blume; *Castanea sativa* Mill.: Guidelines for the Conduct of Tests for Distinctness, Uniformity and Stability. Doc. No. TG/124/4. (Geneva, Switzerland: UPOV - International Union for the Protection of New Varieties of Plants).

Visentin, I., Gentile, S., Valentino, D., Gonthier, P., Tamietti, G., and Cardinale, F. (2012). *Gnomoniopsis castanea* sp. nov. (Gnomoniaceae, Diaporthales) as the causal agent of nut rot in sweet chestnut. J. Plant Pathol. *94*, 411–419 https://doi.org/10.4454/JPP.FA.2012.045.

Voorrips, R.E. (2002). MapChart: software for the graphical presentation of linkage maps and QTLs. J. Hered. *93* (*1*), 77–78 https://doi.org/10.1093/jhered/93.1.77. PubMed