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A genetic linkage map of European chestnut (*Castanea sativa* Mill.) based on RAPD, ISSR and isozyme markers

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Abstract A genetic linkage map of European chestnut (*Castanea sativa* Mill.) based on RAPD, ISSR and isozyme markers was constructed using the two-way pseudo-testcross strategy. A total of 96 individuals from a F_1 full-sib family was genotyped with 381 molecular markers (311 RAPDs, 65 ISSRs, 5 isozymes). Markers in testcross configuration, segregating 1:1, were used to establish two separate maternal and paternal maps including 187 and 148 markers, respectively. The markers identified 12 linkage groups based on the haploid number of chestnut. The female and male framework maps reached a total length of 720 and 721 cM (Kosambi), respectively, representing a 76% and 68% coverage of the overall genome. A total of 46 markers, found in intercross configuration, segregating 3:1 and 1:2:1, were used to identify homologous linkage groups between parental maps; out of 12 linkage groups 11 could be joined. RAPD and ISSR markers showed a good and comparable reliability, allowing for the first time the establishment of a saturated linkage map for European chestnut. These maps will be a starting point for studies on the structure, evolution and function of the chestnut genome. Identification of QTLs for adaptive traits in chestnut will be the primary target.

Keywords *Castanea sativa* · Genetic map · RAPD · ISSR · Isozymes

Introduction

Among the 13 species belonging to the genus *Castanea*, sweet chestnut (*Castanea sativa* Mill.) is considered to be the only species native to Europe. Currently it is widespread throughout Europe and South-west Asia and is one of the multipurpose tree species of upmost economic importance for the Mediterranean region. Based on palynological data (Huntley and Birks 1983) chestnut was present during the Tertiary but disappeared from western Europe during Pleistocene glaciations, surviving mainly in South-west Asia. In the last 2,000 years, beginning during the Roman period, it has undergone a new rapid expansion towards Europe (Zohary and Hopf 1988). Its evolution appears, therefore, to be a complex mixture of long-range gene flow, natural and artificial selection and local effects of isolation by distance. This fact implies that the genetic diversity of the European chestnut varies consistently both in geographic distribution and level according to historical events, environmental variations and management practices.

The genetic variability in European chestnut has been assessed both in natural populations and in cultivated varieties (Fineschi et al. 1994; Villani et al. 1994, 1999; Pereira-Lorenzo and Fernandez-Lopez 1995). The general picture which can be drawn is that cultivation has caused a consistent decrease in the amount of genetic variation but there are still regions, acting as important natural sources of genetic diversity, which can be of great relevance for the study and the conservation of chestnut biodiversity and adaptive potential. Among the regions studied, Turkey represents the area with the highest genetic diversity. A particular high degree of genetic differentiation, close to interspecific level, was observed between two groups of populations: one, designated Hopa, from north-eastern Anatolia and the other designated Bursa, from western Anatolia. The overall genetic differentiation pattern observed in the Turkish populations was congruent with data referring to some morphometric and physiological traits (Villani et al. 1992). Some important adaptive traits as drought tolerance and phenology (i.e. bud burst) were further investigated (Lauteri et al. 1997).

The peculiar genetic and adaptive variation observed in the Turkish *Castanea sativa* populations makes them suitable genetic material on which to perform a genetic dis-

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section of loci underlying relevant quantitative traits by means of quantitative trait locus (QTL) analysis. A prerequisite of such a study is the availability of a genetic linkage map for this species. Therefore, the main objectives of the investigation reported here were: (1) to construct a genetic linkage map based on a two-way pseudo-testcross strategy and polymerase chain reaction (PCR)-based molecular markers (Grattapaglia and Sederoff 1994); (2) to compare random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers as tools for establishing a preliminary framework of linkage maps. To our knowledge, within the *Fagaceae* family two genetic linkage maps have already been published: a F_2 progeny derived from a cross between *Castanea dentata* and *C. mollissima* was used to construct a genetic linkage map in the genus *Castanea* based on isozyme, restriction fragment length polymorphism (RFLP) and RAPD markers (Kubisiak et al. 1997); an integrated molecular map including RAPD, sequence-characterized amplified region (SCAR), microsatellite, minisatellite, isozyme and 5srRNA markers was constructed using a F_1 progeny of *Quercus robur* L. (Barreneche et al. 1998). Our chestnut map is the first reported linkage map for the European chestnut (*Castanea sativa* Mill.). Its availability would greatly contribute to a further understanding of the chestnut genome structure, evolution and function.

Material and methods

Plant material

A controlled cross was performed in 1998 in the experimental field of the Agroforestry Institute (National Research Council, Porano, Italy) between two 9-year-old trees, chosen on the basis of their heterozygosity at the isozyme level and their high degree of variation for physiological traits. The female parent and the male parent originated from the Bursa and Hopa Turkish regions, respectively. A F_1 full-sib family of 186 offspring was obtained. A small piece of cotyledon tissue was cut out for isozyme analysis and DNA extraction; the remaining seed was sown; after germination the seedlings were maintained in the greenhouse. A total of 96 F_1 individuals were used to construct two separate genetic linkage maps: a female or Bursa map and a male or Hopa map.

DNA isolation

Total genomic DNA was isolated from frozen cotyledon tissue and from fresh leaves using the Doyle and Doyle (1987) protocol. DNA quality was checked by gel electrophoresis. The purified DNA was quantified at 260 nm using a spectrophotometer, diluted to a working concentration of 10 ng/µl in sterile water and the stock stored at –20°C.

Isozyme analysis

Isozyme analysis was performed using cotyledon tissue as described in Villani et al. (1991) in order to: (1) detect any possible pollen contamination in the F_1 segregating progeny; (2) select informative polymorphic loci to be integrated into the map. Eighteen isozyme loci were tested: esterase (*Est*, E.C.3.1.1.2), leucine aminopeptidase (*Lap-1,- 2, -3*, E.C.3.4.11.1), peroxidase (*Prx*, E.C.1.11.1.7), glucose phosphate isomerase (*Gpi 2*, E.C.5.3.1.9), alcohol dehydrogenase (*Adh*, E.C.1.1.1.1), diaphorase (*Dia-1,- 2*, E.C.1.6.4.3), phosphoglucomutase (*Pgm*, E.C.2.7.5.1), glutamate oxaloacetate transaminase (*Got-1, -2, -3, -5*, E.C.2.6.1.1), superoxide dismutase (*Sod*, E.C.1.15.1.1), isocitrate dehydrogenase (*Idh-1,- 2*, E.C.1.1.1.42), 6-phosphogluconate dehydrogenase (6*Pgdh*, E.C.1.1.1.49).

RAPD analysis

A total of 340 ten-base oligonucleotides primers of arbitrary sequence (Operon Technologies, Alemada, Calif.) were used for RAPD analysis (Williams et al. 1990). The reaction mixture (12.5 µl) was prepared as follows: 10 m*M* Tris HCl pH 8.3, 50 m*M* KCl, 1.5 m*M*, $MgCl_2$, 200 µ*M* each of dATP, dCTP, dGTP and dTTP, 0.4 µ*M* primer, 100 µg/ml BSA, 50 ng genomic DNA, and 0.4 U *Taq* polymerase (Boehringer Mannheim). Amplifications were run in a DNA Thermal Cycler 480 (Perkin Elmer Cetus); after an initial denaturation (94°C, 3 min), the samples were amplified for 45 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C, followed by a final extension of 10 min at 72°C. Amplification products were analysed for band presence and absence after electrophoretic separation on 1.4% agarose gel and staining with ethidium bromide. RAPD fragments were identified by the Operon primer code and the fragment size in base pairs: RAPD marker D13–287 corresponds to a RAPD fragment amplified by Operon primer OPD13 with a size of 287 bp.

ISSR analysis

One hundred ISSR primers (Zietkiewicz et al. 1994) from the University of British Columbia Biotechnology Laboratory (UBCBL) primer set no. 9 were tested. The reaction mixture (12.5 µl) was prepared as follows: 10 m*M* Tris-HCl pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 µ*M* each of dATP, dCTP, dGTP, dTTP, 0.4 µ*M* primer, 100 µg/ml BSA, 2.5% formamide, 20 ng genomic DNA, 0.4 U *Taq* polymerase (Boehringer Mannheim). Amplifications were run in a DNA Thermal Cycler 480 (Perkin Elmer Cetus); an initial denaturation of 7 min at 94°C was followed by 45 cycles of 30 s at 94°C, 45 s at the annealing temperature (Table 2), 2 min at 72°C, and a final 7 min extension at 72°C (Tsumura et al. 1996). Amplification products were analysed for band presence and absence after electrophoretic separation on 2% agarose gel and staining with ethidium bromide. Each ISSR marker was designated using the UBC primer identification number and the fragment size in base pairs: ISSR marker I814–230 corresponds to an ISSR fragment amplified by primer 841 with the size 230 bp.

Segregation analysis and map construction

Three different types of segregation models were classified: testcross markers (Grattapaglia and Sederoff 1994) segregating 1:1 and intercross markers (Verhaegen and Plomion 1996) segregating 3:1 or 1:2:1. Each marker was tested for Mendelian segregation by the chi-square test (α=0.05). According to the two-way pseudo-testcross strategy (Grattapaglia and Sederoff 1994) two separate data sets were organized, one for each parent, using testcross markers. The linkage analysis was performed using a combination of MAPMAKER 2.0 (Lander et al. 1987) for Macintosh and JOINMAP (Stam 1993) software. In both cases recombination fractions were transformed into genetic distances in centiMorgans by the Kosambi mapping function (Kosambi 1944). Using MAPMAKER software, we constructed two separate (maternal and paternal) linkage maps. The raw data files were duplicated, and each testcross marker was re-coded to allow MAPMAKER to detect both coupling and repulsion phases according to Nelson et al. (1993). The framework map was constructed as follows: a minimum LOD score of 4.0 for statistical acceptance of linkage and a maximum recombination fraction of 0.40 were chosen for grouping the markers; the marker order within each linkage group was optimized using “first order” and “ripple” (LOD>2) commands to

compare all three-locus permutations of the framework order. The markers that could not be ordered with equal confidence were placed as accessory markers and located to the closest framework marker using the "near" command. The "error" detection function of MAPMAKER (Lincoln and Lander 1992) was employed to check potential genotyping errors in the framework markers. The JOINMAP software was finally used to map the intercross loci in order to find homologies between maternal and paternal linkage groups. Genome map sizes were estimated following method "3" of Chakravarti et al. (1991): $G_{(Z)}=2MX_{(Z)}/K_{(Z)}$ where $G_{(Z)}$ is the genome length estimate in centiMorgans; M=n(n-1)/2 is the number of informative meiosis; $X_{(Z)}$ is the maximum observed map distance among marker pairs above a given threshold LOD, Z; $K_{(Z)}$ is the number of locus pairs linked with a LOD value at or above Z. Using the "LODs" function of MAPMAKER, we estimated the total length map for both parental maps at three different LOD values (LOD 3, 4, 5) and chose the mean value as the genome length estimate. These estimations were calculated by taking into consideration only pairwise comparisons between framework markers.

Results

Marker analysis

RAPD and ISSR

A total number of 340 RAPD primers were screened against the two parents (Bursa and Hopa) and six F_1 individuals; 198 (58%) of these showed at least one segregating band, thereby enabling the classification of 450 polymorphic markers; 120 primers, detecting 311 RAPD markers, were selected for further genotyping work on the basis of the repeatability of the amplification pattern and the number of polymorphic bands per primer. The number of informative bands per primer was 2.6. The 311 RAPD markers were classified into two different groups on the basis of the segregation model (Table 1): (1) loci in a testcross configuration segregating 1:1 that were heterozygous in one parent and homozygous null in the other; a total of 271 testcross markers were identified (143 for the female parent and 128 for the male parent); (2) loci in an intercross configuration that were heterozygous in both parents and segregated in a 3:1 ratio; 40 intercross markers were identified.

Out of 100 ISSR primers examined, 50 (50%) gave a clear banding pattern: 6 of these did not show any distinct polymorphism while 44 enabled 122 segregating fragments to be classified. We selected 22 ISSR primers to genotype all 96 F_1 individuals. These 22 primers produced clear profiles and were characterized by a high polymorphic bands/primer ratio. Sixty-five ISSR fragments were scored for segregation: 60 were in testcross configuration and five in intercross configuration (Table 1). As also found by Fang et al. (1997) and Wang et al. (1998) all $(AT)_n$ primers failed to give amplification products. Table 2 shows the 22 ISSR primers used,

Table 1 Number and segregation types of markers used for chestnut map construction

	Testcross (segregation 1:1) female	Testcross (segregation 1:1) male	Intercross (segregation 3:1 or 1:2:1[a])	Total
RAPD	143	128	40	311
ISSR	41	19	5	65
Isozymes	3	1	1[a]	5
Total	**187**	**148**	**46**	**381**
Distorted (α=0.05)	18 (9.6%)	15 (10.1%)	5 (10.9%)	38 (10.0%)

Table 2 ISSR primers selected for genotyping

Primer code[a]	Sequence (5′→3′)[b]	Annealing temperature (°C)	Polymorphic bands/primer
810	GAGAGAGAGAGAGAGAT	50	5
814	CTCTCTCTCTCTCTCTA	50	4
815	CTCTCTCTCTCTCTCTG	52	3
818	CACACACACACACACAG	52	4
820	GTGTGTGTGTGTGTGTC	52	2
823	TCTCTCTCTCTCTCTCC	52	2
824	TCTCTCTCTCTCTCTCG	52	2
825	ACACACACACACACACT	50	2
828	TGTGTGTGTGTGTGTGA	50	3
834	AGAGAGAGAGAGAGAGYT	52	3
836	AGAGAGAGAGAGAGAGYA	52	4
841	GAGAGAGAGAGAGAGAYC	52	3
843	CTCTCTCTCTCTCTCTRA	52	5
844	CTCTCTCTCTCTCTCTRC	52	5
845	CTCTCTCTCTCTCTCTRG	52	2
848	CACACACACACACACARG	52	3
852	TCTCTCTCTCTCTCTCRA	52	2
858	TGTGTGTGTGTGTGTGRT	52	4
873	GACAGACAGACAGACA	48	3
879	CTTCACTTCACTTCA	38	1
880	GGAGAGGAGAGGAGA	48	1
881	GGGTGGGGTGGGGTG	54	2

[a] Primers were obtained from the University of British Columbia, set number 9
[b] Y=C, T; R=A, G

Table 3 Comparison of RAPD and ISSR markers

	Total number of primers used for genotyping	Polymorphic loci mapped	Polymorphic bands/primer	Bands in testcross configuration	Bands in intercross configuration
RAPD	120	311	2.6	271 (87%)	40 (13%)
ISSR	22	65	3.0	60 (92%)	5 (8%)

the annealing temperature and the number of polymorphic bands detected per primer.

The polymorphism level revealed by ISSR primers was higher than the polymorphism detected by RAPD primers: the preliminary analysis showed a ratio of 1.3 polymorphic fragments/primer for RAPD against 2.8 polymorphic fragments/primer for ISSR; a similar situation was observed for the primers selected for mapping (Table 3). The high degree of polymorphism shown by ISSR markers compared to RAPD markers has been observed previously by Tsumura et al. (1996) and Nagaoka and Ogihara (1997).

Isozymes

Segregation analysis on each chestnut seed with 18 isozyme loci did not detect any contamination from foreign pollen. Out of 18 isozyme loci tested, five (*Dia-1*, *Prx*, *Gpi-2*, *Got-2*, 6*Pgdh*) revealed polymorphism in the female and male parents. Mendelian segregation was also verified in the mapping population. Based on Jacobs et al. (1995) we re-coded the segregating data for isozymes following the coding scheme used by MAPMAKER and JOINMAP software. *Dia-1*, *Prx*, *Gpi-2* (female parent) and *Got-2* (male parent) loci were in the testcross configuration while 6*Pgdh* was in the intercross configuration and segregated in a 1:2:1 ratio. Isozyme markers were integrated into the map. Three isozyme loci (*Gpi-2*, *Prx*, 6*Pgdh*) were linked in the same linkage group, LG1 (see Fig.1); the *Dia-1* locus mapped on LG2 and the *Got-2* locus on LG12.

Map construction

Our mapping strategy was subdivided into four main steps: (1) Mendelian segregation test; (2) marker grouping; (3) marker ordering within each linkage group (framework map construction); (4) homology search between parental maps.

A total number of 381 markers were used to construct the chestnut map (Table 1). In the first step of our analysis we tested each marker for departure from the 1:1 (testcross loci) or 3:1, 1:2:1 (intercross loci) Mendelian segregation ratio (α=0.05). Approximately 10% of the markers analysed showed segregation distortion. These markers were excluded from the preliminary linkage analysis.

Two separate maternal and paternal linkage maps were established using 187 and 148 testcross markers, respectively. Marker grouping and ordering (steps 2 and 3) were performed using the MAPMAKER software. We preliminarily tested different LOD thresholds (from LOD 3 to LOD 6) to group the markers: both female and male markers appeared to be organized in 12 linkage groups based on the haploid number of chestnut, *n*=12 (Jaynes 1962). A minimum LOD score of 4.0 and a maximum recombination fraction θ of 0.40 were then chosen as a good compromise between a reliable statistical criterion and the maximum number of linked markers. The introduction of distorted markers did not affect the result of marker grouping. Correspondence between the number of linkage groups and the chestnut haploid number of chromosomes as well as the low number of unlinked markers (0 in the female map and 10 in the male map) suggests a good level of saturation of the chestnut genome.

As recently pointed out by Remington et al. (1999), the marker ordering within each linkage group is a persistent problem in genetic mapping, especially when the number of loci increase. Furthermore, genotyping errors can influence both locus ordering and the total genetic length of the map (Lincoln and Lander 1992). In order to obtain a sufficiently rigorous marker order we first optimized the framework map using the "ripple" command of MAPMAKER as described in the Materials and methods; then we performed a comparative analysis using JOINMAP software (Messmer et al. 1999). In most cases no order ambiguities were observed for the framework markers optimized with MAPMAKER. When some incongruities were noticed the error detection function of MAPMAKER was used to display potential genotyping errors, the gel pictures were re-checked and the ordering analysis performed again. The ordering analysis was first performed considering non-distorted markers. The distorted markers were added in a second step and integrated into the map framework whenever their introduction did not affect the statistical confidence of marker order previously optimized, even though Kuang et al. (1999) showed that frequency recombination is not affected by segregation distortion caused by a single viability gene or sampling errors. The female framework map contains 92 markers (49% of the testcross loci) covering 720 cM, while the male framework map contains 95 markers (64% of the testcross loci) covering 721 cM. The size of the female linkage groups range from 10 to 108 cM with an average of 60±28 cM; a similar situation was observed for the male linkage groups, ranging from 15 to 95 cM with an average of 60±22 cM (Table 4). The average distance between two framework markers was 9.0 cM for the female map and 8.7 cM for the male map.

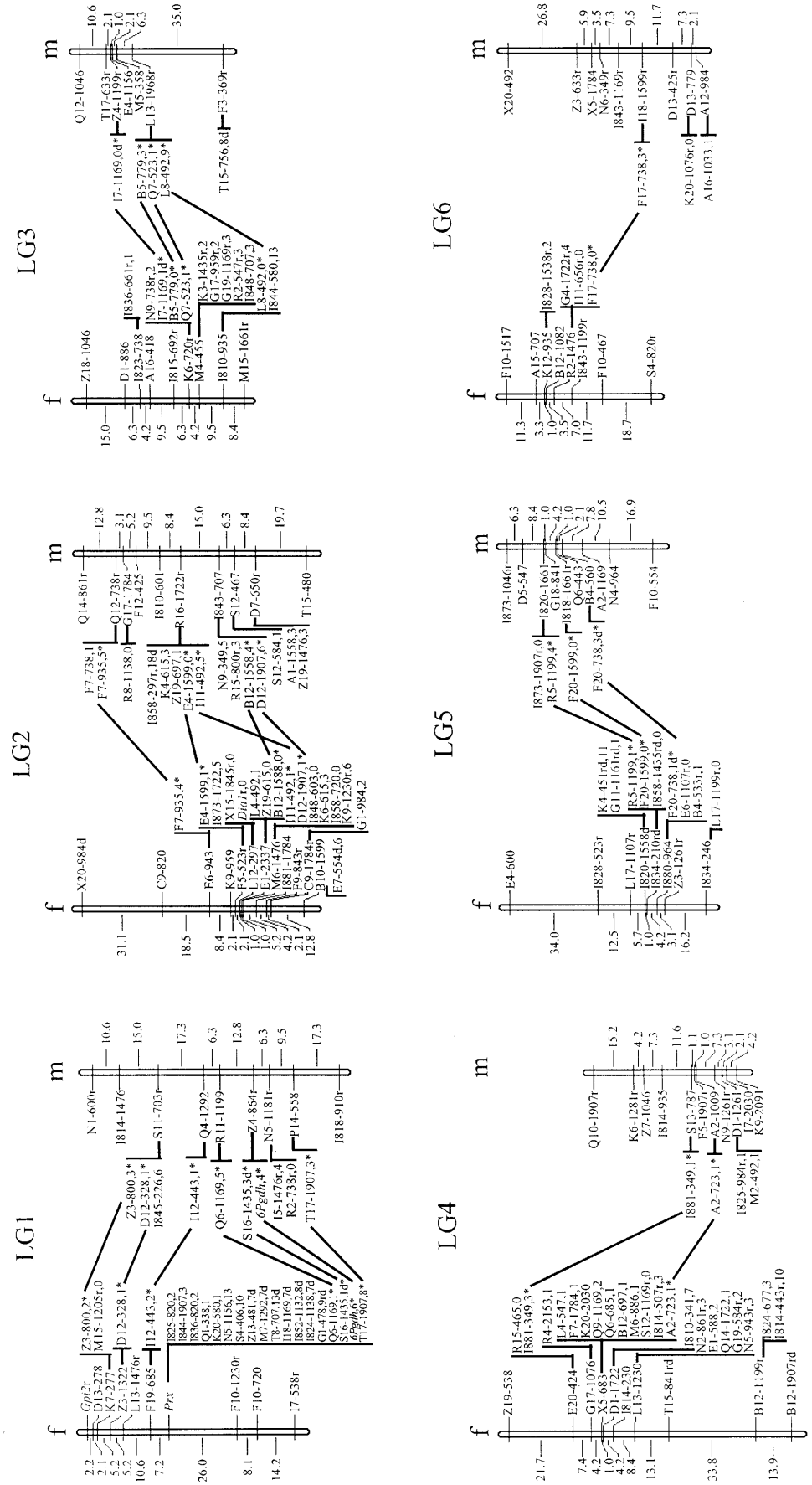

Fig. 1a, b Genetic linkage map of European chestnut (*Castanea sativa* Mill.); female, or Bursa (*f*), map on the *left* and male, or Hopa (*m*), map on the *right*. The map framework is organized in 12 linkage groups (*LG1–12*). Accessory markers are listed beside their closest framework marker. Distorted markers are indicated by *d*. Intercross markers (*) were used to find homologies between parental maps: 11 groups could be joined; bridge markers are interconnected with *bold lines*. Marker names ending with *r* are in reverse linkage phase to those not so designated

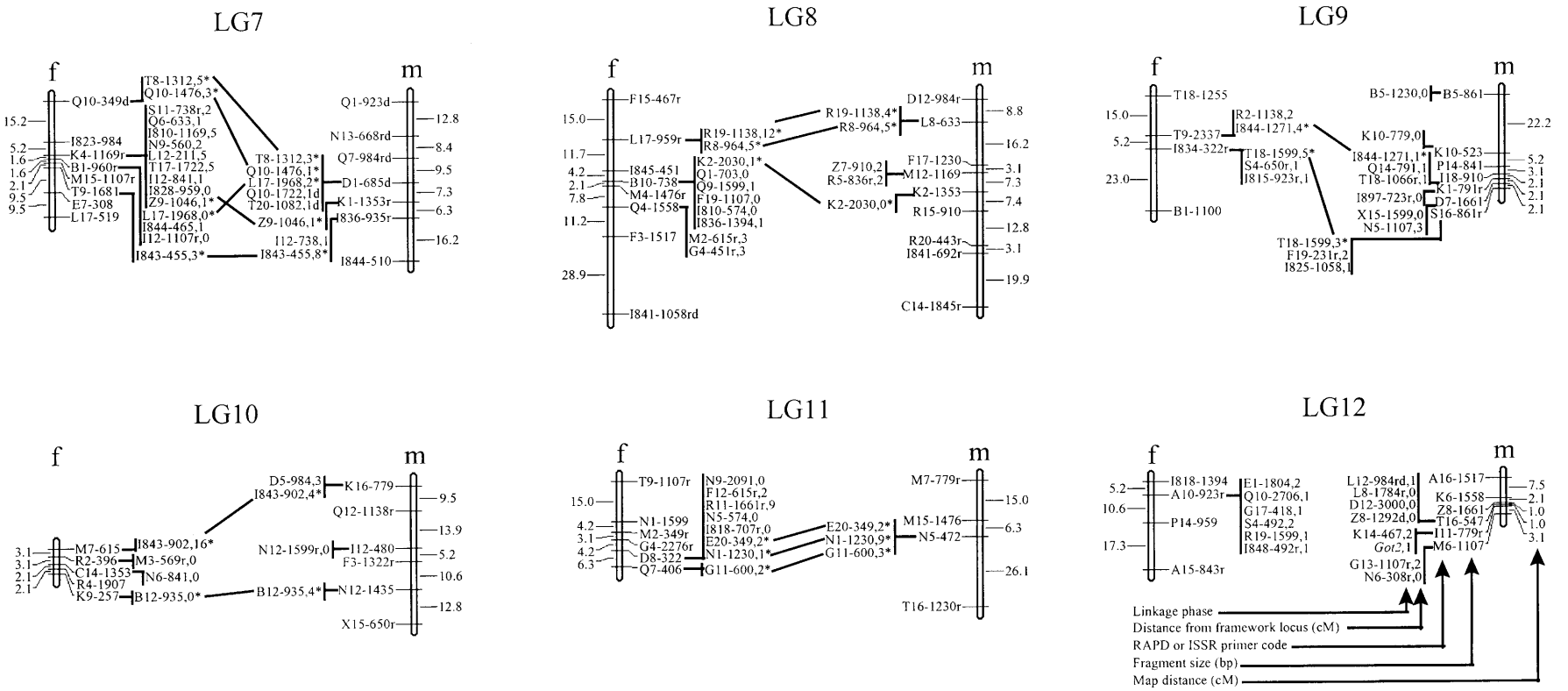
LG7
f
m
Q10-349d
T8-1312,5*
Q10-1476,3*
S11-738r,2
Q6-633,1
I810-1169,5
N9-560,2
L12-211,5
T17-1722,5
I12-841,1
I828-959,0
Z9-1046,1*
L17-1968,0*
I844-465,1
I12-1107r,0
I843-455,3*
T8-1312,3*
Q10-1476,1*
L17-1968,2*
Q10-1722,1d
T20-1082,1d
Z9-1046,1*
I843-455,8*
I12-738,1
I823-984
K4-1169r
B1-960r
M15-1107r
T9-1681
E7-308
L17-519
I844-510
I836-935r
K1-1353r
D1-685d
Q7-984rd
N13-668rd
Q1-923d
15.2
5.2
1.6
1.6
2.1
9.5
9.5
12.8
8.4
9.5
7.3
6.3
16.2
LG8
f
m
F15-467r
L17-959r
I845-451
B10-738
M4-1476r
Q4-1558
F3-1517
I841-1058rd
R19-1138,12*
R8-964,5*
K2-2030,1*
Q1-703,0
Q9-1599,1
F19-1107,0
I810-574,0
I836-1394,1
M2-615r,3
G4-451r,3
R19-1138,4*
R8-964,5*
L8-633
Z7-910,2
R5-836r,2
M12-1169
K2-1353
K2-2030,0*
R15-910
R20-443r
I841-692r
C14-1845r
D12-984r
F17-1230
15.0
11.7
4.2
2.1
7.8
11.2
28.9
8.8
16.2
3.1
7.3
7.4
12.8
3.1
19.9
LG9
f
m
T18-1255
T9-2337
I834-322r
B1-1100
R2-1138,2
I844-1271,4*
T18-1599,5*
S4-650r,1
I815-923r,1
T18-1599,3*
F19-231r,2
I825-1058,1
B5-1230,0
B5-861
K10-779,0
I844-1271,1*
Q14-791,1
T18-1066r,1
I897-723r,0
X15-1599,0
N5-1107,3
K10-523
P14-841
I18-910
K1-791r
D7-1661
S16-861r
15.0
5.2
23.0
22.2
5.2
3.1
2.1
2.1
2.1
LG10
f
m
M7-615
R2-396
C14-1353
R4-1907
K9-257
I843-902,16*
M3-569r,0
N6-841,0
B12-935,0*
D5-984,3
I843-902,4*
K16-779
Q12-1138r
N12-1599r,0
I12-480
F3-1322r
B12-935,4*
N12-1435
X15-650r
3.1
3.1
2.1
2.1
9.5
13.9
5.2
10.6
12.8
LG11
f
m
T9-1107r
N1-1599
M2-349r
G4-2276r
D8-322
Q7-406
N9-2091,0
F12-615r,2
R11-1661r,9
N5-574,0
I818-707r,0
E20-349,2*
N1-1230,1*
G11-600,2*
E20-349,2*
N1-1230,9*
G11-600,3*
N5-472
M7-779r
M15-1476
T16-1230r
15.0
4.2
3.1
4.2
6.3
15.0
6.3
26.1
LG12
f
m
I818-1394
A10-923r
P14-959
A15-843r
E1-1804,2
Q10-2706,1
G17-418,1
S4-492,2
R19-1599,1
I848-492r,1
L12-984rd,1
L8-1784r,0
D12-3000,0
Z8-1292d,0
K14-467,2
Got2,1
G13-1107r,2
N6-308r,0
A16-1517
K6-1558
Z8-1661
T16-547
I11-779r
M6-1107
5.2
10.6
17.3
7.5
2.1
1.0
1.0
3.1
Linkage phase
Distance from framework locus (cM)
RAPD or ISSR primer code
Fragment size (bp)
Map distance (cM)

Table 4 Descriptive data of female (Bursa) and male (Hopa) maps

	Female (Bursa)	Male (Hopa)		Female (Bursa)	Male (Hopa)
Number of markers	233 (187 testcrosses)	194 (148 testcrosses)	Estimated genome length (Chakravarti)	947	1,054
Linkage groups (*n*=12)	12	12	Genome coverage	76%	68%
Number of framework markers	92	95	Average distance (centiMorgans) between two framework markers and variation range	9.0 (1.0–34.2)	8.7 (1.0–35.0)
Total genome length covered (Kosambi)	720	721	Variation range (centiMorgans) of chromosome length	10–108 (60±28)	15–95 (60±22)

Out of 381 markers 46 (12%) were in intercross configuration (Table 1). Following Verhaegen and Plomion (1996), these markers were used to identify homologies between the female and male linkage groups using JOINMAP software that accepts mixed segregation data (i.e. testcross and intercross loci). Because of the low information content between testcross and intercross marker pairs (Ritter et al. 1990) only those intercross markers with at least three links to markers within a particular group were mapped, as suggested by Debener and Mattiesch (1999). Following this approach we were able to map 37 intercross markers with high confidence. Of the 12 linkage groups (LG) for each parent, 11 could be joined. For one linkage group (LG12) no common intercross loci could be detected. Dominant markers segregating 3:1 can be considered good joining points between parental maps, particularly when several common markers can be clearly placed on both homologous linkage groups. A more reliable merging of female and male maps requires codominant and locus-specific markers such as isozymes, microsatellites and RFLPs which represent preferential anchor points for performing comparative mapping studies (Maliepaard et al. 1998).

Genome coverage and marker distribution

The length of the chestnut genome was estimated from linkage data according to Chakravarti et al. (1991) as described in the Materials and methods section. The length of the female genome was estimated to be 947 cM (mean out of three values – 1,037, 931 and 874 cM – calculated at LOD 3, 4 and 5, respectively), while the male genome amounted to 1,054 cM (mean out of three values – 1,117, 1,049 and 995 cM – calculated at LOD 3, 4 and 5, respectively). Considering that the 12 linkage groups obtained span 720 and 721 cM in the female and male maps, respectively, our framework map covers 76% of the female genome and 68% of the male genome. Because genetic linkage maps with a high level of confidence in locus order are suitable for QTL analysis, gene location and comparative mapping studies (Remington et al. 1999), our strategy was mainly focused on finding a good compromise between the maximum genome coverage and the more reliable locus order. Nevertheless, using a more relaxed LOD criterion (LOD≥3) for the grouping step and taking into consideration the first optimization of locus order carried out by MAPMAKER, we found that genome coverage amounted to 84% and 75% for the female and male maps, respectively (data not shown).

Markers on most chestnut linkage groups (see Fig.1) showed a clustering area along the chromosome. The average distance between two framework markers (Table 4) was 9.0 and 8.7 cM for the female and male maps, respectively; nevertheless, in both cases the variation range of gaps between consecutive markers was really quite wide: 1.0–34.2 cM (female map) and 1.0–35.0 cM (male map). Both results suggest a nonrandom distribution of molecular markers mapped throughout the genome. As suggested by Thuriaux (1977) the recombination is concentrated on structural genes, while a suppression of recombination rate is observed around the centromeric and/or telomeric regions (Tanksley et al. 1992). Without any cytological information about the centromere localization on chestnut chromosomes the marker clustering observed can be due both to centromere effect and to a tendency of some molecular markers to map in clusters (Nilsson et al. 1997; Alonso-Blanco et al. 1998; Vuylsteke et al. 1999).

Discussion

The present study is the first reported genetic map of *Castanea sativa.* Integration of RAPD, ISSR and isozyme markers allowed us to reach a good saturation of the genome. RAPD and ISSR markers proved to be simple and efficient methods to generate, in a short time, a large number of molecular markers suitable for establishing preliminary genetic maps for previously unstudied species. Although in previous papers (Tsumura et al. 1996; Fang et al. 1997; Nagaoka and Ogihara 1997) ISSR markers were considered to be more repeatable and reliable than RAPD markers, in the present study we observed a good and comparable reliability for both of them. Both techniques demonstrated a high repeatability and reliability across the different experimental tests

used in our genotyping work where three steps were performed: six F_1 individuals were scored at the beginning during the primer screening phase; the mapping population was then divided in two sets of 45 individuals each; for each subgroup of samples DNA extraction, DNA amplification and genotype scoring were performed independently allowing us to verify the high reproducibility both for RAPD and ISSR amplification products.

An important advantage of composite maps is the better coverage of different genome regions, probably due to distinct target areas of different molecular markers on the genome (Davila et al. 1999). For that purpose we introduced ISSR and isozymes markers into the chestnut map.

To our knowledge, ISSR markers have been mapped on plant chromosomes in only a few cases (Dirlewanger et al. 1998; Kojima et al. 1998; Davila et al. 1999; Arcade et al. 2000). The high polymorphism revealed by ISSR markers, the tendency to be distributed throughout the chromosomes, based on a widespread distribution of SSR markers (Kojima et al. 1998), and the very simple technique, similar to the RAPD procedure, are interesting characteristics of ISSR markers for mapping purposes. Two main features can be noticed from ISSR introduction on chestnut map. (1) A stretching of the map was induced after the ISSR markers were added: a total genetic distance of 111 cM was added by the introduction of 16 ISSR loci in the female framework and 74 cM were added by 12 ISSR loci in the male framework. (2) The 65 ISSR loci were distributed throughout the chestnut chromosomes. At least one ISSR marker was present in each of the 12 female linkage groups; three male linkage groups (LG3, LG11, LG12) did not include any ISSR marker, nevertheless only 19 ISSR markers were integrated in the male map. No conclusion can be drawn on a possible differential distribution of ISSR markers in comparison with RAPD markers. The distribution of ISSR markers (i.e. number and spacing of markers per chromosome) was compared with that of the RAPD markers. No significant differences between RAPD and ISSR markers were noticed when the number of markers was regressed on the genome length covered (data not shown). A more complete genome coverage could be useful to compare the target regions on the genome of RAPD and ISSR markers. On the other hand, the wide distribution of ISSR markers throughout the chestnut genome is in agreement with the results obtained by Kojima et al. (1998).

The importance of having included isozymes in the linkage groups can be summarized in the following two points. (1) As pointed out by Binelli and Bucci (1994), a genetic linkage map can be useful in population genetics studies by allowing the choice of unlinked markers to achieve an unbiased estimate of population genetics parameters. Among different markers, isozymes have been the most widely employed in population genetics studies on European chestnut (Villani et al. 1991, 1994, 1999); therefore the knowledge of their chromosome location could highly contribute to the selection of unlinked isozyme loci particularly suitable for population genetics. (2) Despite their limited number, isozyme markers are excellent anchor points for establishing a reliable correspondence between genetic maps derived from different progenies or as bridge markers for comparative mapping studies.

Approximately 10% (38) of the molecular markers showed a segregation distortion (Table 1). Of the 381 markers, 19 were expected to be distorted ($P<0.05$) by chance. Segregation distortion can be due to different reasons: statistical bias, genotyping and scoring errors (Plomion et al. 1995) and biological reasons like chromosome loss, viability or lethal genes, genetic isolating mechanisms and genetic load (Bradshaw and Stettler 1994). Marker distortion has been reported in numerous cases in plants, including forest trees (Kuang et al. 1999 and references therein), and genetic load has been proposed as a probable cause of segregation distortion in trees (Strauss and Conkle 1986). With respect to the distribution of distorted markers on the chestnut map two observations can be made: (1) a clustering of distorted markers was identified in LG1, LG5 (female linkage groups) and LG7 (male linkage group); (2) most of distorted markers mapped on regions of marker clustering or at the end of linkage groups. Clustering of distorted markers can be due to lethal or incompatibility genes; chromosome arrangements or non-homologous pairing (Lefebvre et al. 1995). Among the three linkage groups mainly implied in Mendelian segregation distortion, for LG7 we can suppose a biological basis as the possible explanation for that distortion clustering, such as the presence of a lethal gene or a conflict between genetic isolating mechanisms evolved in the parental populations (Bradshaw and Stettler 1994). About 50% of the distorted markers, linked to the male map, were located in this linkage group, covering a genetic distance of 31 cM; in addition, the female homologous linkage group contained a distorted marker at the same end of the linkage group. On the other hand, the reduced recombination rate around centromeres as well as some telomeric effect could be a possible explanation of distortion in these areas (Debener and Mattiesh 1999). A better understanding of the phenomenon will require more detailed analysis such as the genotyping of a large number of individuals to avoid statistical bias, and the mapping of the same distorted area in other populations.

The present genetic map permits further investigations focused on the structure, evolution and function of the chestnut genome.

1) Genome structure: a complete saturation of chestnut map could be obtained in a near future by adding AFLP markers. As recently reported (Marques et al. 1998; Vuylsteke et al. 1999; Costa et al. 2000) the AFLP technique (Vos et al. 1995) is one of the most powerful technologies for fingerprinting studies on plant species. A growing number of genetic maps based on different kinds of molecular markers now include AFLP, thereby showing their efficiency for mapping purposes

(Dirlewanger et al. 1998; Arcade et al. 2000; Costa et al. 2000; Lespinasse et al. 2000; Saliba-Colombani et al. 2000; Sebastian et al. 2000).

2) Genome evolution: by introducing locus-specific and codominant markers as isozymes, microsatellites and RFLPs, comparative mapping studies can be planned among our chestnut map and the previously reported maps on oak and American chestnut (Barreneche et al. 1998; Kubisiak et al. 1997). Successful amplification of 47% of oak microsatellite loci (Steinkellner et al. 1997) was observed in *Castanea*, a promising result on which to base in the near future a comparative mapping between oak and chestnut genomes based on SSR markers.

3) Genome function. The advent of genetic mapping projects has made it feasible to map and characterize polygenes underlying quantitative traits in natural populations (Paterson et al. 1988; Lander and Botstein 1989). More recently plant functional genomics has demonstrated a progression toward a comprehensive understanding of the genetic mechanisms that control plant growth, development and responses to the environment (Somerville and Somerville 1999). Many QTLs map close to candidate genes, and there is growing evidence from synteny studies of corresponding chromosome regions carrying similar QTL in different species (Kearsey and Farquhar 1998). The identification of QTLs related to drought tolerance and phenological traits will enable a better understanding of the genetic and molecular basis of adaptation of chestnut (and other forest tree species) to contrasting environments and will provide both important information for the conservation of adaptive potential of the species and a set of molecular tools useful in markers-assisted breeding.

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