A genetic linkage map for hazelnut (*Corylus avellana* L.) based on RAPD and SSR markers

Shawn A. Mehlenbacher, Rebecca N. Brown, Eduardo R. Nouhra, Tufan Gökirmak, Nahla V. Bassil, and Thomas L. Kubisiak

Abstract: A linkage map for European hazelnut (*Corylus avellana* L.) was constructed using random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers and the 2-way pseudotestcross approach. A full-sib population of 144 seedlings from the cross OSU 252.146 × OSU 414.062 was used. RAPD markers in testcross configuration, segregating 1:1, were used to construct separate maps for each parent. Fifty additional RAPD loci were assigned to linkage groups as accessory markers whose exact location could not be determined. Markers in intercross configuration, segregating 3:1, were used to pair groups in one parent with their homologues in the other. Eleven groups were identified for each parent, corresponding to the haploid chromosome number of hazelnut ($n = x = 11$). Thirty of the 31 SSR loci were able to be assigned to a linkage group. The maternal map included 249 RAPD and 20 SSR markers and spanned a distance of 661 cM. The paternal map included 271 RAPD and 28 SSR markers and spanned a distance of 812 cM. The maps are quite dense, with an average of 2.6 cM between adjacent markers. The S-locus, which controls pollen–stigma incompatibility, was placed on chromosome 5S where 6 markers linked within a distance of 10 cM were identified. A locus for resistance to eastern filbert blight, caused by *Anisogramma anomala*, was placed on chromosome 6R for which two additional markers tightly linked to the dominant allele were identified and sequenced. These maps will serve as a starting point for future studies of the hazelnut genome, including map-based cloning of important genes. The inclusion of SSR loci on the map will make it useful in other populations.

Key words: Corylus avellana, hazelnut, filbert, linkage map, pseudotestcross, pollen–stigma incompatibility, random amplified polymorphic DNA, simple sequence repeat, microsatellite.

Résumé : Une carte génétique du noisetier (*Corylus avellana* L.) a été produite avec des marqueurs RAPD (ADN polymorphe amplifié au hasard) et microsatellites ainsi qu'une approche testcross double. Une population de plantes soeurs issues du croisement OSU 252.146 x OSU 414.062 a été employée. Les marqueurs RAPD montrant une ségrégation 1:1 ont été utilisés pour assembler des cartes distinctes pour chaque parent. Cinquante locus RAPD additionnels ont été assignés à des groupes de liaisons en tant que marqueurs accessoires dont la position exacte n'a pu être déterminée. Les marqueurs montrant une ségrégation 3:1 ont été employés pour établir la correspondance entre les groupes de liaison des deux cartes. Onze groupes de liaison ont été identifiés pour chaque parent, ce qui correspond au nombre haploïde de chromosomes chez le noisetier ($n = x = 11$). Les auteurs ont réussi à assigner 30 des 31 microsatellites à un groupe de liaison. La carte maternelle comprenait 249 RAPD et 20 microsatellites et s'étendait sur une distance génétique de 661 cM (Kosambi). La carte paternelle incluait 271 RAPD et 28 microsatellites pour une distance totale de 812 cM. Les cartes sont assez denses puisque la distance moyenne entre les marqueurs est de 2,6 cM. Le locus S qui contrôle l'incompatibilité pollen-stigmate a été situé sur le chromosome 5S et six marqueurs à moins de 10 cM ont été identifiés. Un locus conférant la résistance à la brûlure orientale du noisetier, causée par l'*Anisogramma anomala*, a été situé sur le chromosome 6R et deux marqueurs étroitement liés à l'allèle dominant ont été identifiés et séquencés. Ces cartes serviront de point de départ pour de futures études du génome du noisetier dont le clonage positionnel de gènes importants. L'inclusion de microsatellites sur la carte rendra celle-ci utile chez d'autres populations.

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Mots clés : Corylus avellana, noisetier, carte génétique, pseudo-testcross, incompatibilité pollen-stigmate, ADN polymorphe amplifié au hasard, microsatellite.

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Introduction

The European hazelnut (*Corylus avellana* L.) is an important commodity, fourth behind cashew (*Anacardium occidentale* L.), almond (*Prunus dulcis* (Miller) D.A. Webb), and Persian walnut (*Juglans regia* L.) (Food and Agriculture Organization 2003) among the tree nuts in terms of worldwide production. Turkey and Italy are major producers, with additional production located in the US, Spain, Azerbaijan, Georgia, France, and Greece. New cultivars from breeding programs, such as *Corylus avellana* L. 'Corabel' from Bordeaux, France, and *Corylus avellana* L. 'Lewis' from Oregon State University (OSU), Corvallis, Ore., are being planted, but the major cultivars in most production regions are selections from the local vegetation. The geographical distribution of *C. avellana* covers many different climatic zones and spans from the Mediterranean coast of North Africa and southern Spain to lat 68°N in Norway to the Ural Mountains of Russia, south through the Caucasus Mountains to the Talesh Mountains of northwestern Iran, and westward to Lebanon (Kasapligil 1964). Morphological and phenological traits show tremendous variability in the European hazelnut (Mehlenbacher 1991; Yao and Mehlenbacher 2000). Isozyme studies have also revealed a high level of heterozygosity and heterogeneity (Rovira et al. 1993; Cheng 1992). In spite of the crop's economic importance and abundant genetic diversity, genetic improvement efforts were begun only recently. The first breeding programs were initiated in the 1960's (Thompson et al. 1996).

Hazelnut is monoecious, dichogamous, and wind pollinated. Cross pollination is enforced by sporophytic incompatibility under the control of a single S locus with multiple alleles (Thompson 1979). Most cultivars are heterozygous at the S locus. Controlled pollinations followed by fluorescence microscopy can be used to identify the S alleles present in a cultivar (Thompson 1979; Mehlenbacher 1997*a*, 1997*b*). For each allele, a tester has been identified that expresses only that allele in its pollen. The stigmatic styles of heterozygotes express both alleles, but often only one allele is expressed in the pollen because of dominance (Mehlenbacher 1997*a*).

The objective of this study was to construct a genetic linkage map for hazelnut using random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers and the 2-way pseudotestcross approach. The first maps constructed using this approach and PCR-based markers were for *Eucalyptus* (Grattapaglia and Sederoff 1994) and apple (*Malus* spp.) (Hemmat et al. 1994). Additional examples among tree crops include European beech (*Fagus sylvatica* L.) (Scalfi et al. 2004), pedunculate oak (*Quercus robur* L.) (Barreneche et al. 1998), *Pinus* (Shepherd et al. 2003; Yin et al. 2003), *Populus* (Cervera et al. 2001), and rubber tree (*Hevea* spp.) (Lespinasse et al. 2000). Among the nut crops, maps have been constructed for European chestnut (*Castanea sativa* L.) (Casasoli et al. 2001) and *Macadamia* (Peace et al. 2003). Additional maps include those for olive (*Olea europaea* L.) (Wu et al. 2004), apple (Conner et al. 1997), apricot (*Prunus armeniaca* L.) (Lambert et al. 2004), and grape (*Vitis vinifera* L.) (Lodhi et al. 1995; Dalbo et al. 2000; Fischer et al. 2004). A screening of 1100 RAPD primers showed a high level of polymorphism in *C. avellana* and identified 20 markers linked in coupling to a dominant allele for resistance to eastern filbert blight caused by the pyrenomycete fungus *Anisogramma anomala* (Peck) E. Müller (Mehlenbacher et al. 2004). A preliminary map for hazelnut was presented by Mehlenbacher et al. (2005). This paper presents more complete maps for each parent, adding 82 RAPD markers and 113 cM to the maternal map and 102 RAPD markers and 198 cM to the paternal map. Thirty SSR loci were also placed on the map.

Materials and methods

Plant material

In 1993, a controlled cross of 2 heterozygous clones was made, generating a full-sib progeny of 144 seedlings designated 93001 (Fig. 1). The maternal parent, OSU 252.146, is susceptible to eastern filbert blight, while the paternal parent, OSU 414.062, is heterozygous at the resistance locus. The resulting seeds were stratified, sown in flats in the greenhouse as they sprouted, and the seedlings transplanted to 3.8 L pots when they had grown to a height of approximately 20 cm. They were grown in the greenhouse during the summer and transplanted to the field in October 1994 at a spacing of 0.9 m within the row and 2.7 m between rows.

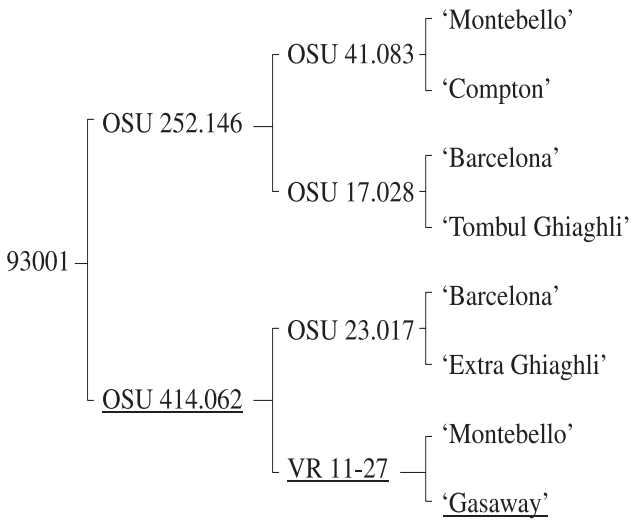
Identification of incompatibility alleles

Fluorescence microscopy, as described in detail by Mehlenbacher (1997*b*), was used to determine if S_3 or S_8 was present in the seedlings. The cross OSU 252.146 (S_3S_8) × OSU 414.062 (S_1S_1) would be expected to generate seedlings of two types (S_1S_3 and S_1S_8) in equal frequency. According to the dominance hierarchy of Mehlenbacher (1997*a*), only the second allele of these pairs would be expressed in the pollen. *Corylus avellana* L. 'Nonpareil' pollen was used as the S_3 tester and *Corylus avellana* L. 'Tombul Ghiaghli' pollen as the S_8 tester in pollinating female inflorescences collected from bagged branches of seedling trees. Pollen collected from seedlings was used to pollinate female inflorescences collected from numerous selections in the OSU hazelnut breeding program known to have either S_3 or S_8. An incompatible pollination, indicated by reduced germination and short pollen tubes that fail to penetrate the stigmatic surface, indicates that the same allele is expressed in both pollen and stigma. Indicator variables were created for S_3 and S_8, with 1 indicating presence of the allele and 0 indicating its absence.

DNA extraction

DNA was extracted in the spring from young leaves of field-planted trees using different methods in the two locations as

Fig. 1. Pedigree of the hazelnut mapping population. Maternal parents are listed on top. Genotypes heterozygous resistant to eastern filbert blight are underlined.



described by Mehlenbacher et al. (2004). In Corvallis, Ore., the method of Lunde et al. (2000) was used. In Saucier, Miss., a cetyltrimethylammonium bromide (CTAB)-based method and fresh young leaves sent from Corvallis were used (Wagner et al. 1987) with a proteinase K (0.5 mg) digestion performed subsequent to the addition of *N*-lauroylsarcosine. An additional chloroform–octanol (24:1) extraction was also performed and RNA was removed by incubation in the presence of RNAse A. DNA extracted in Corvallis and used to generate RAPD markers received no RNAse treatment; the treatment had no effect on PCR results.

RAPD primer screening

A total of 1420 primers were screened: kits A–AE from Operon Technologies (Alameda, Calif.) and primers 1–800 from the Michael Smith Lab at the University of British Columbia (Vancouver, B.C.). In Saucier, the PCRs were performed in a 24 μL volume containing 10 mmol/L Tris–HCl (pH 9.0), 50 mmol/L KCl, 0.10% Triton X-100, 1.5 mmol/L $MgCl_2$, 200 μmol/L each of dATP, dCTP, dGTP, and dTTP, 0.5 μmol/L of primer, 6.25 ng of template DNA, and 0.8 U *Taq* polymerase (Promega, Madison, Wis.). Reactions were loaded into flexible microtitre plates and overlaid with 25 μL of mineral oil. The plates were placed in a preheated (85 °C) programmable thermal cycler (PTC-100, MJ Research, Waltham, Mass.) and covered with mylar film. In Corvallis, the PCRs were performed in a volume of 15 μL containing 0.3 μmol/L of primer, 3–25 ng of template DNA, 0.4 U Biolase DNA polymerase (Biolase USA, Randolph, Mass.), and the ammonium-based buffer supplied by the manufacturer. Ninety-six reactions were run simultaneously in microtitre plates using a Geneamp® PCR System 9700 thermal cycler (Perkin-Elmer Corp., Foster City, Calif.). Both locations used the same thermal cycler program: 5 s at 95 °C; 1 min 55 s at 92 °C; 45 cycles of 5 s at 95 °C, 55 s at 92 °C, 1 min at 35 °C, and 2 min at 72 °C; 7 min at 72 °C; and ending with an indefinite hold at 4 °C. During the first 5 cycles the ramp time from 35 °C to 72 °C was reduced to 30% of the maximum to reduce non-specific binding of primers. Primer concentration was occasionally adjusted to improve repeatability of scoring. Amplification products were separated by electrophoresis on 2% *w*/*v* agarose gels, stained with ethidium bromide, and photographed using an imaging system (UVP, Upland, Calif.).

Eight genotypes were used in primer screening: the 2 parents, 3 resistant seedlings, and 3 susceptible seedlings. Primers that generated a band in one or both parents and that showed segregation among the 6 seedlings were investigated further. Primers that generated promising bands were scored on the whole population of 144 seedlings. For some primers, electrophoresis time was extended to allow separation of bands of similar size. The parental origin of each marker was noted and a level of confidence assigned to the scores.

SSR amplification and allele sizing

Primer pairs for 22 loci developed in Corvallis (CAC) (Bassil et al. 2005) and for 9 loci developed in Torino (CaT) (Boccacci et al. 2005) (Table 1) were used. Reverse primers were purchased from Operon Technologies (Qiagen, Valencia, Calif.). Forward primers fluorescently labeled with FAM and HEX were purchased from Operon Technologies and those labeled with NED were purchased from Applied Biosystems (Foster City, Calif.). PCRs were performed in a total volume of 10 μL and the reaction mixture contained 1× Biolase NH_4 reaction buffer, 2 mmol/L $MgCl_2$, 200 μmol/L each of dATP, dCTP, dGTP, and dTTP, 0.3 μmol/L each of forward and reverse primers, 0.25 U Biolase DNA polymerase (Bioline Inc., Randolph, Mass.), and 2.5 ng of template DNA. The PCR program consisted of 35 cycles of a 40 s denaturation step at 94 °C, a 40 s annealing step at the optimum annealing temperature (Table 1), and a 40 s extension step at 72 °C. The final step was 30 min at 72 °C to maximize non-templated adenosine addition to the 5′ ends and samples were then held at 4 °C until recovery. PCRs were run in Perkin-Elmer model 9700 thermocyclers (PE Applied Biosystems, Foster City, Calif.). PCR amplification and approximate fragment sizes were confirmed on 3% agarose gels using 4 μL aliquot and 5 μL loading dye (15% Ficoll® 400, 0.03% xylene cyanol FF, 0.4% orange G, 10mmol/L Tris–HCL pH 7.5, and 50 mmol/L EDTA). Gels were stained with ethidium bromide and photographed under UV light. Amplified PCR products were diluted 40 times with nanopure water and kept as stock for multiplexing. Stock solutions were further diluted 2–16 times (Table 1) and 1 μL of a mixture of 4 or 5 PCR products were separated on an ABI 3100 capillary electrophoresis instrument (Applied Biosystems, Foster City, Calif.) at the OSU Central Services Laboratory (CSL). DNA fragments were sized using GeneScan and Genotyper software.

Construction of a linkage map

RAPD markers were defined by the primer designation followed by the approximate size of the fragment in base pairs. Data for RAPD markers were entered into a spreadsheet as either 1 for marker present, 0 for marker absent, or n for unknown, and then recoded as h, a, and u, respectively, before exporting to a tab-delimited text file. Markers present in a ratio of approximately 1:1 (present in 40%–60% of the seedlings) were considered testcross markers, and markers originating in the susceptible maternal parent OSU 252.146 (S)

Table 1. Description of 31 hazelnut SSR loci: repeat motif, sequence of the fluorescent forward primer (FAM, NED, HEX) and the reverse primer (*R*), annealing temperature (T_m), and dilution factor in the multiplex.

SSR locus	Motif	Primers (5′→3′)	T_m (°C)	Dilution
CAC-A014a	$(CA)_{13}$	FAM-GGTTTGTTACAGAAATTCAGACG	60	1:640
		R-GCGTGTGGTTAATGTTTTCTTT		
CAC-A24b	$(GA)_{18}(AT)_7$	NED-CACAACATGCAACGTCTATGTA	62	1:120
		R-AGGTACGTATTGACAGGCTTTT		
CAC-A040	$(CA)_{13}$	NED-TGCTCAAGCAAATATTGCAC	62	1:213
		R-GTTTGGGATCCAATTAACCCTCT		
CAC-A102	$(AG)_{16}(AC)_{15}$	HEX-AAACTGTGACGAACGAAAACAC	62	1:80
		R-TTGCACTTCCATAACTGTCAAA		
CAC-B005	$(GA)_{22}$	FAM-CAAACTTATGATAGGCATGCAA	62	1:320
		R-TGTCACTTTGGAAGACAAGAGA		
CAC-B010	$(GA)_{16}$	FAM-AGCTTCCAAATCACACATTACC	62	1:320
		R-GAAGAGCATCCGTATGATTCAG		
CAC-B011	$(GA)_{11}$	NED-CACTGGTGATCTCACAGGTTTA	62	1:240
		R-GTCCTCAAAAGCTAAGCACAAG		
CAC-B020	$(GA)_{19}$	HEX-GGGAAAATACTCCAAATCGCT	60	1:240
		R-TCACCGAGCCGTCATAATC		
CAC-B028	$(AG)_{16}$	NED-ATGGACGAGGAATATTTCAGC	55	1:213
		R-CCTGTTTCTCTTTGTTTTCGAG		
CAC-B029b	$(GA)_{13}$	NED-CAATTTACACCTCAGGGAAGAG	58	1:160
		R-AAGTTCACCCAAGAAATCCAC		
CAC-B101	$(AG)_{14}$	HEX-GCAGACCAGAGTCTGTTATTCA	62	1:480
		R-AGACAATTTCGTGACTGGGTAT		
CAC-B105	$(GA)_{16}$	HEX-AAAGGAGCAAGCATGTTAGG	62	1:320
		R-GTTTGTACGGATGATCCACTGAG		
CAC-B109	$(GA)_{21}$	HEX-AATCCAAGCCTTTTCACTACC	58	1:320
		R-ACCCATCAAGTTCACCAATC		
CAC-B113	$(GA)_{14}$	HEX-TTGAGGAAGTCCAGGAAAAT	60	1:320
		R-GCCAGAGAGAGCAAGAGTTAG		
CAC-C001a	$(CACAGAG)_3$	FAM-CCCGTAACTAACCAATCACAAT	58	1:320
		R-TGGAGAAGAGGAGAGCTTAGTG		
CAC-C008	$(AAG)_{11}$	FAM-TTTCCGCAGATAATACAGGG	58	1:320
		R-TCCTTTGCTTTGGACCAG		
CAC-C028	$(GAA)_{10}$	NED-CTACCCCATCGCTTGACAC	60	1:213
		R-GGAGACTTGTTTGCCACAGA		
CAC-C040	$(GAA)_8(GGA)_5$	FAM-AGCCCCATTAGCCTTCTTAG	62	1:320
		R-GTTTCCAGATCTGCCTCCATATAAT		
CAC-C114	$(TTC)_6$	HEX-TCTCCCTCTCCCTCTCTTCTAC	60	1:400
		R-GAAAGGAAAAAGCACATAGCAA		
CAC-C115	$(TAA)_5(GAA)_{12}$	FAM-CATTTTCCGCAGATAATACAGG	60	1:320
		R-GTTTCCAGATCTGCCTCCATATAAT		
CAC-C118	$(AAG)^a$	HEX-AGCAACAGAGGTTAGGTGTG	60	1:320
		R-GCCCCATTAGCCTTCTTA		
CAC-C119	$(GA)_7,(GA)_9$	NED-CTCACCTTTACCCCTTCATTTT	62	1:213
		R-GTTTCCTCATCTTCTGAGAACCATC		
CaT-A114	$(TG)_{17}$	FAM-CGCCTTGATAGTATGTTCAAAC	60	1:320
		R-CGGCAGAATGTAGAAGTCCCC		
CaT-B106	$(AG)_{17}AA(AG)_6$	HEX-CCAATCGCCAATGAATCATC	60	1:320
		R-CCCTTTCCAAACTGGGCAT		

Table 1 (*concluded*).

SSR locus	Motif	Primers (5′→3′)	T_m (°C)	Dilution
CaT-B107	$(CT)_{14}$	NED-GTAGGTGCACTTGATGTGCTTTAC	58	1:160
		R-AACACCATATTGAGTCTTTCAAAGC		
CaT-B501	$(GA)_{21}$	NED-GAAATTCAATCACACCAATAAAGCA	64	1:160
		R-CCTCCCTTGTCCTCATCACTG		
CaT-B502	$(CT)^b$	FAM-CTCATGACTGCCCATTTCTCG	62	1:400
		R-AGGCATGCAGGCTTCACAC		
CaT-B504	$(CT)_{18}$	HEX-CGCCATCTCCATTTCCCAAC	60	1:400
		R-CGGAATGGTTTTCTGCTTCAG		
CaT-B507	$(GA)^c$	FAM-CTA AGCTCACCAAGAGGAAGTTGAT	62	1:400
		R-GCTTCTGGGTCTCCTGCTCA		
CaT-B509	$(GA)_{14}$	HEX-GTCTGGCATGGTTTTGAGAAGA	62	1:320
		R-CTTTCCCGCCCAAACCAC		
CaT-C502	$(CTT)^d$	HEX-GCATGCAAGGTGGTCGGT	62	1:320
		R-TTTGGCACCCAACAACTCTAGA		

[a] $(AAG)_3(GAA)_3(AAG)_8N_6(AAG)_4$
[b] $(GA)_1GC(GA)_2GC(GA)_{14}$
[c] $(CT)_{16}GCTTTTC(CT)_5$
[d] $(CTT)_1T_2(CTT)_{11}C_2T_4(CTT)_1$

were separated from those originating in the resistant paternal parent OSU 414.062 (R). The program JoinMap 3.0 (van Ooijen and Voorrips 2001) was used to construct linkage maps with the population type "BC_1", the default recombination frequency of 0.40, and very high LOD scores, generally 7.0, to minimize the merging of groups frequently observed at lower values. The Mapmaker/EXP 3.0 program was used to identify groups of markers and to construct maps in an initial group of about 250 markers, as well as to confirm the groupings in the final maps constructed by JoinMap. The first attempt to create a map using JoinMap for a selected group generated a list of markers followed by the error message "insufficient linkage to above group" and a second list of markers. For the second subgroup, linkage in repulsion rather than coupling was assumed. For these markers, "dummy variables" were created, in which presence of the marker was coded as 0 or *a*, and absence by 1 or *h*. This allowed the merging of coupling phase markers in the first subgroup with "dummies" of loci in the second subgroup, and construction of a single map for each chromosome in each parent. Dummy variables are indicated by a lower case letter *d* after the fragment size (Fig. 2). The goodness-of-fit of markers was indicated by a χ^2 test statistic, for which the degrees of freedom (df) were calculated by subtracting one less than the number of loci from the number of pairs used by JoinMap to place that locus on the map, as listed on the program output. Markers with large values were removed in stepwise fashion until all remaining markers had values less than an arbitrarily established value of 5. Occasionally an adjacent marker that had been scored with lower confidence was removed rather than the marker with the largest χ^2 value.

Markers present in both parents and about 75% of the seedlings were considered intercross markers and were placed in groups at LOD scores of 5.0 and 7.0. Maps were constructed for the intercross markers using JoinMap with the population type "F_2". The corresponding intercross markers were identified for each group of testcross markers by merging files of individual groups of testcross markers with the entire intercross data set, and the "group tree" command. The corresponding S and R groups of testcross markers were then identified as a result of grouping with the same set of intercross markers. Lastly, the population type cross pollinated (CP) was used to integrate the three maps, thereby allowing determination of the correct orientation of each pair of S and R maps as well as the approximate placement of the intercross markers on each. For this analysis, the segregation type code was lm×ll for markers from the susceptible parent and nn×np for markers from the resistant parent. Intercross markers were coded as h– for marker present and kk for marker absent and the segregation code was hk×hk.

Segregation at SSR loci

Four segregation patterns were expected for the SSR loci: 1:1 from the maternal parent, 1:1 from the paternal parent, and 1:2:1 or 1:1:1:1 from both parents (heterozygous in both parents). Observed segregation ratios were compared with the expected Mendelian segregation ratios using a χ^2 goodness-of-fit test. The appropriate df were calculated by subtracting one from the number of genotypic classes (Table 2). The Yates correction factor was not used.

Integration of SSR markers into a linkage map of hazelnut

SSR loci were placed on the RAPD marker based map using JoinMap. Indicator variables were created for each locus in each parent. The presence of an allele was scored as 1, the absence of an allele as 0, and unknown allele size as *n*. The indicator variables were pairs, as presence of one allele in a seedling also indicated the absence of the other allele from that parent at that locus. Two data sets were obtained, one for the susceptible maternal parent OSU 252.146 (S) and one for the resistant paternal parent OSU 414.062 (R). The marker data were then recoded as *h* for 1, *a* for 0 and *u* for

Fig. 2. Linkage groups in hazelnut (*Corylus avellana*). Groups are numbered from 1 to 11. Those from the maternal parent, susceptible to eastern filbert blight, are indicated with an S; those from the resistant paternal parent are indicated with an R. Intercross RAPD markers segregating 3:1 connect and orient the groups in the two parents. SSR markers are noted with an asterisk. The loci controlling pollen–stigma incompatibility and eastern filbert blight resistance are indicated by double asterisks.

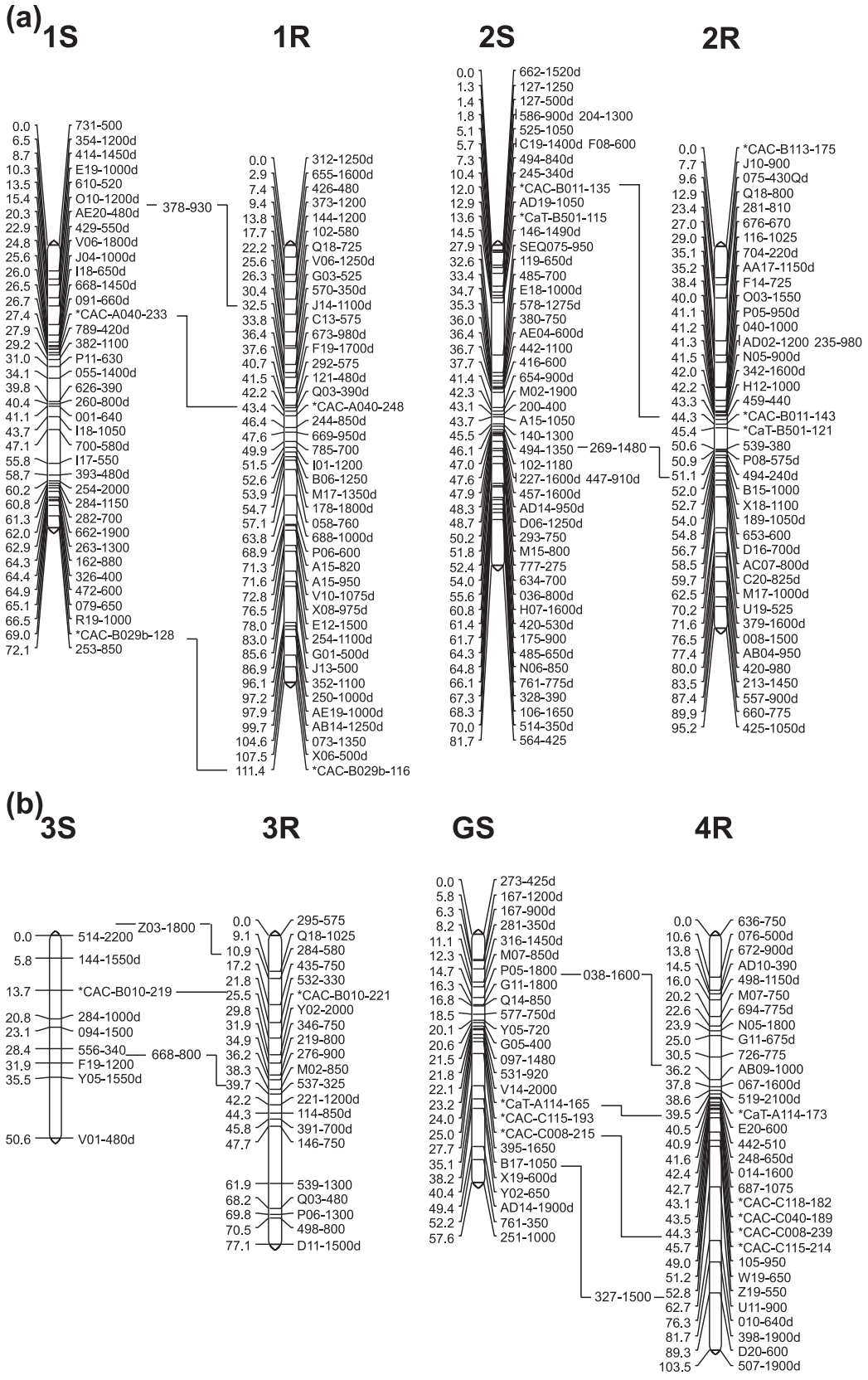


Fig. 2 (*continued*).

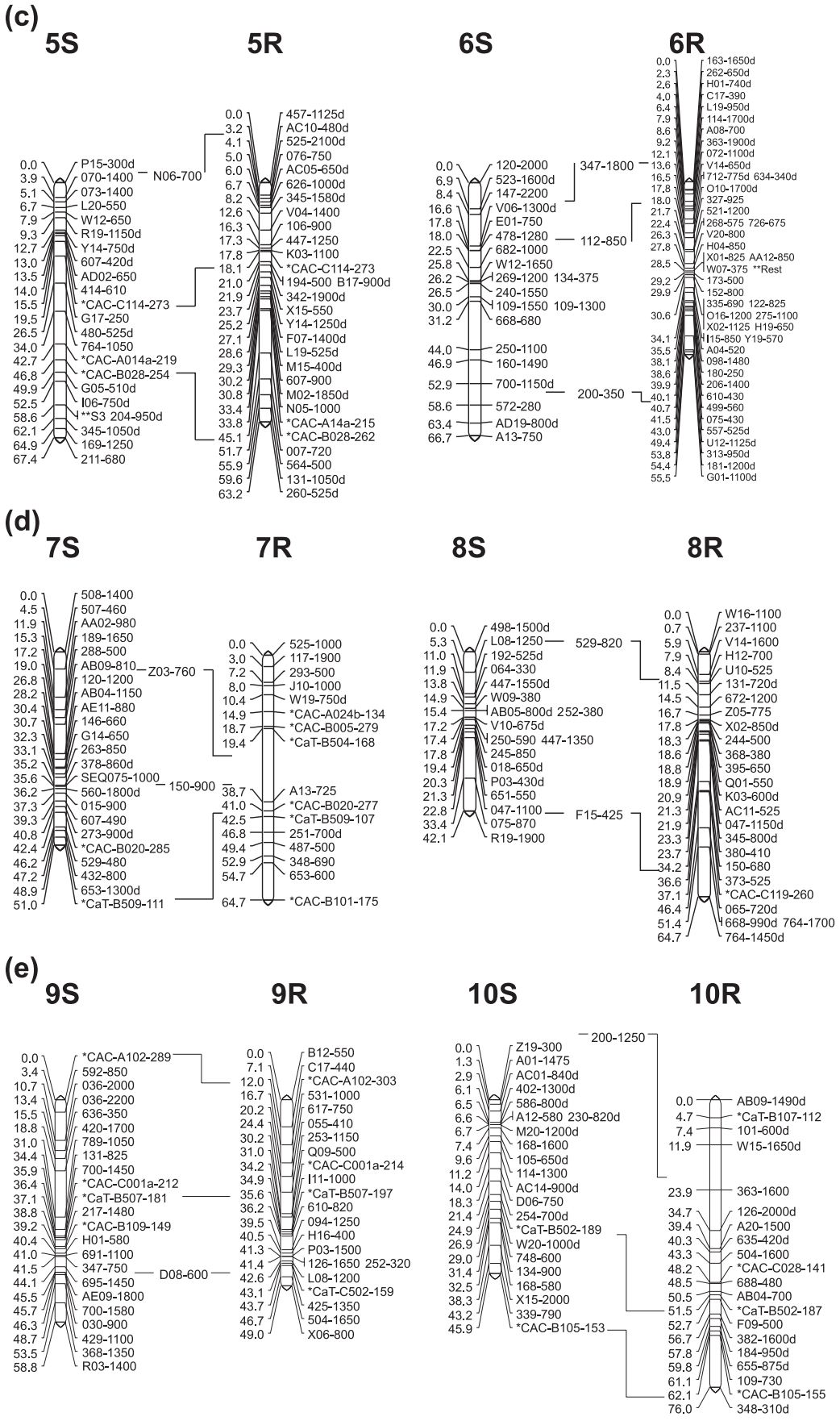


Fig. 2 (*concluded*).

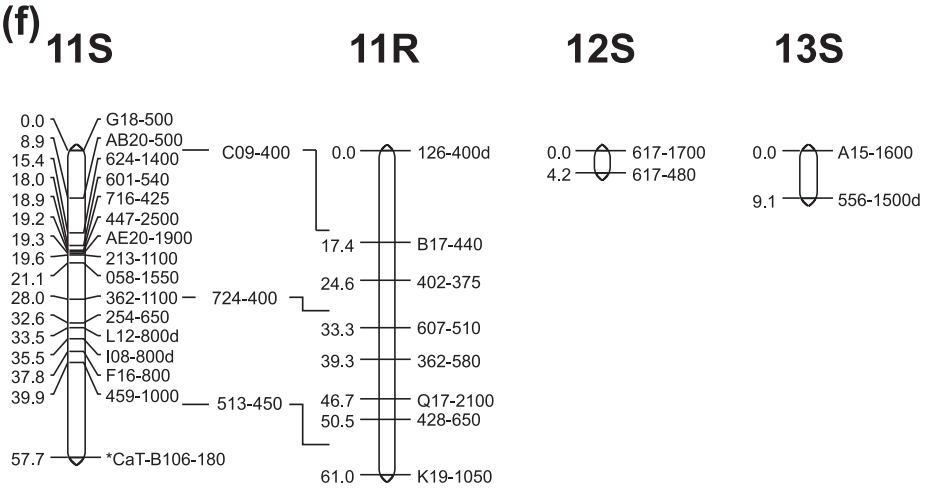


Table 2. Segregation of alleles at 31 hazelnut SSR loci and their linkage group assignments.

SSR locus	Allele sizes (♀ × ♂)	Expected ratio	Observed ratio	χ^2	df	*P*	Linkage group
CAC-A024b	(130/130) × (126/134)	1:1	73:71	0.028	1	0.87	7R
CAC-B005	(295/295) × (279/291)	1:1	70:74	0.444	1	0.51	7R
CAC-C028	(131/131) × (141/144)	1:1	63:81	2.25	1	0.13	10R
CAC-B109	(149/151) × (151/151)	1:1	70:74	0.111	1	0.749	9S
CAC-B113	(173/173) × (173/175)	1:1	59:85	4.694	1	0.03	2R
CAC-B101	(173/175) × (173/173)	1:1	69:75	0.25	1	0.62	Unlinked
CAC-C040	(186/186) × (186/189)	1:1	62:82	2.777	1	0.10	4R
CAC-C118	(179/179) × (179/182)	1:1	61:83	3.361	1	0.07	4R
CAC-C119	(258/258) × (260/264)	1:1	71:73	0.027	1	0.87	8R
CaT-C502	(155/155) × (155/159)	1:1	71:73	0.027	1	0.87	9R
CaT-B504	(158/158) × (168/182)	1:1	69:75	0.25	1	0.62	7R
CaT-B106	(168/180) × (180/180)	1:1	71:73	0.027	1	0.87	11S
CaT-B107	(112/112) × (112/128)	1:1	68:76	0.444	1	0.51	10R
CAC-A014a	(215/219) × (215/219)	1:2:1	27:80:37	1.917	2	0.38	5S, 5R
CAC-A102	(289/303) × (289/303)	1:2:1	41:73:30	1.708	2	0.43	9S, 9R
CAC-B028	(254/262) × (254/262)	1:2:1	36:81:27	3.375	2	0.19	5S, 5R
CAC-A040	(233/244) × (244/248)	1:1:1:1	27:33:40:44	4.722	3	0.19	1S, 1R
CAC-B010	(208/219) × (215/221)	1:1:1:1	30:38:41:35	1.833	3	0.61	3S, 3R
CAC-B011	(135/152) × (143/152)	1:1:1:1	38:30:43:33	2.722	3	0.44	2S, 2R
CAC-B020	(283/285) × (277/283)	1:1:1:1	38:31:41:34	1.611	3	0.66	7S, 7R
CAC-B029b	(116/128) × (116/122)	1:1:1:1	35:38:37:34	0.277	3	0.96	1S, 1R
CAC-B105	(153/155) × (155/159)	1:1:1:1	33:35:30:46	4.055	3	0.26	10S, 10R
CAC-C001a	(210/212) × (210/214)	1:1:1:1	35:40:39:30	1.722	3	0.63	9S, 9R
CAC-C008	(206/215) × (206/239)	1:1:1:1	32:45:29:38	4.166	3	0.24	4S. 4R
CAC-C114	(270/273) × (264/273)	1:1:1:1	39:24:42:39	5.5	3	0.14	5S, 5R
CAC-C115	(182/193) × (182/214)	1:1:1:1	33:46:29:36	4.388	3	0.22	4S, 4R
CaT-A114	(165/171) × (169/173)	1:1:1:1	30:37:31:46	4.5	3	0.21	4S, 4R
CaT-B501	(115/129) × (121/129)	1:1:1:1	37:31:40:36	1.166	3	0.76	2S, 2R
CaT-B502	(189/195) × (183/187)	1:1:1:1	40:30:42:32	2.888	3	0.41	10S, 10R
CaT-B507	(181/191) × (191/197)	1:1:1:1	38:31:34:41	1.611	3	0.66	9S, 9R
CaT-B509	(109/111) × (107/109)	1:1:1:1	34:36:43:31	2.166	3	0.54	7S, 7R

n, and imported into JoinMap 3.0. For the three loci segregating in a 1:2:1 ratio, the parental origin of alleles in heterozygous seedlings could not be determined. In this situation, the indicator variable for each allele in heterozygotes was coded as unknown.

A preliminary analysis was performed using a subset of about 200 RAPD markers for each parent to allow assignment of the SSR loci to a linkage group on the preliminary map. After linkage group assignment, the SSR marker data were appended to the RAPD marker data for that group, starting each SSR marker designation with an asterisk to clearly distinguish them. Linkage maps were constructed independently for each group in each parent as described above for RAPD marker data. The output of the first map-

ping attempt using a merged data set listed the RAPD markers and linked SSR markers, followed by the error message "insufficient linkage to above group" and a second list of SSR markers. The first list was of SSR alleles linked in coupling to the RAPD markers; the markers in the second group were alleles at the same loci linked in repulsion. The second group of alleles was removed, allowing JoinMap to create a map for each linkage group. Chi-square test statistics on each map were inspected and markers with large values were removed in stepwise fashion until all markers in each linkage group had a value of less than 6. The maps for each chromosome were again integrated using the CP function of JoinMap. SSR loci segregating 1:2:1 were coded hk×hk, loci segregating 1:1:1:1 with 3 alleles present were coded ef×eg, and loci segregating 1:1:1:1 with 4 alleles present were coded ab×cd. When an SSR locus could be placed in two R linkage groups, assignment for loci segregating 1:1:1:1 was determined based on presence of the same locus in the corresponding S group, while assignment for SSR loci segregating 1:1 was that which generated a lower χ^2 test statistic.

Marker cloning

Two markers closely linked to the eastern filbert blight resistance locus were excised from agarose gels, reamplified, and the fragment cloned using the TOPO TA Cloning® kit (Invitrogen, Carlsbad, Calif.) and introduced into One Shot® DH5aα-T1^R chemically competent *Escherichia coli* cells according to the supplier's instructions. Colonies were streaked on agar plates and cultured overnight at 37 °C. These colonies were then stabbed and amplified using PCR and the appropriate primer. Amplification products were run on 2% agarose gels, stained with ethidium bromide, and photographed.

Sequencing markers

Plasmid DNA from a putative transformant was sequenced in forward and reverse directions by the Central Services Laboratory of the OSU Center for Gene Research and Biotechnology using various primers (T3, T7, or SP6). The reverse complement of the reverse sequence was generated using the reverse complement program on the Web site of the University of Alberta (http://www.ualberta.ca/~stothard/javascript/rev_comp.html) and compared with the forward sequence using the program ClustalW (http://clustalw.genome.ad.jp/). Electropherograms were visually inspected to resolve discrepancies in the two sequences and a consensus sequence was determined.

Results

A total of 1420 decamer primers were screened using template DNA of 6 seedlings and the 2 parents. The RAPD primers revealed a high level of polymorphism. Primers that generated easily scored polymorphic bands were identified and used to amplify DNA of all seedlings in the population. Linkage maps were constructed using markers generated by 415 primers. Of the 415, 23 generated only intercross markers, while the remaining 393 primers generated testcross markers as well as additional intercross markers. A total of 710 markers were scored: 601 testcross markers and 109 intercross markers. Maps were initially constructed using testcross markers for each linkage group in each parent, using dummies for markers linked in repulsion. Linkage groups frequently merged at low LOD scores, resulting in very large groups for which maps could not be constructed, but the use of a higher LOD score for grouping minimized the excessive merging. Initially, JoinMap identified 11 linkage groups of RAPD markers in the susceptible parent, but only 10 in the resistant parent. The loci initially placed in the very large group 2R were clearly separated by MapMaker into a large (2R) and a small (7R) group. The newly created small group 7R was mapped at LOD 3.0 and grouped with the same intercross markers as the previously identified group 7S. The resulting number of pairs corresponds to the haploid chromosome number for hazelnut ($n = x = 11$) (Fig. 2). Thus, we believe that we have identified all 11 pairs of chromosomes. We numbered the pairs and presented them side-by-side, with S indicating groups from the susceptible maternal parent OSU 252.146 and R indicating groups from the resistant parent OSU 414.062. One hundred nine intercross markers were suitable for use in mapping. The approximate locations of a few of these are indicated to assure correct orientation. Two hundred forty-nine RAPD markers were placed on chromosomes of the susceptible parent, and 271 on those of the resistant parent. In addition, 50 RAPD markers were assigned to one of the 22 groups, but an exact location on the chromosome could not be assigned. These were considered accessory markers (Table 3). Most of these accessory markers had been initially placed on the map, but were then removed owing to high χ^2 values. The resulting chromosomes span 661 cM in the susceptible parent and 812 cM in the resistant parent, which translates to approximately one RAPD marker every 2.6 cM. Two additional pairs of loci from the susceptible parent (groups 12S and 13S) were identified. Only 20 RAPD markers, 9 from the susceptible parent and 11 from the resistant parent, remain unlinked (Table 3). Thirteen of the SSR loci displayed 1:1 segregation. The female parent was heterozygous at 3 of these loci, while the male parent was heterozygous at 10. All showed good fit to the expected ratio, with the exception of CAC-B113, which showed a slight deficiency of homozygotes. Three loci showed a segregation ratio of 1:2:1, and 15 loci showed a 1:1:1:1 ratio. A map location was determined for 30 of the 31 SSR loci, although CAC-B113 was placed with lower confidence than the others. We list locus CAC-B101 as unlinked. It was tentatively placed at the end of group 9S, 28 cM from CAC-A102, but such a large gap seems unlikely in a dense map.

The dominant S allele was determined in 105 seedlings. S_3 was present in 52 seedlings and S_8 in 53 seedlings, in agreement with the expected 1:1 ratio. The S locus controlling pollen–stigma incompatibility was placed on chromosome 5S and 6 markers linked at <10 cM were identified. Marker 204-950d cosegregates with allele S_3, indicating that 204-950 is tightly linked in coupling to S_8. Three additional alleles linked to S_8 were identified: 345-1050 on one side and I06-750 and G05-510 on the other. Markers 211-680 and 169-1250 are linked to S_3, both on the same side of the S locus.

A dominant allele for resistance to eastern filbert blight caused by *Anisogramma anomala* was placed on chromosome 6R for which two additional tightly linked markers (UBC335-690 and V20-800) were cloned and sequenced.

Table 3. Accessory and unlinked markers listed by group and parental origin.

Group	Susceptible	Resistant
1	I04-1100d	Y14-775d
	M04-700	G19-600
	217-750	426-1400d
		W03-450
2	I15-890d	028-440d
	668-350	K15-1700d
	K03-550	560-450
	293-750	A01-600
		051-1600
		AA04-400
		268-1150
3	081-900d	W12-1150
		AC06-1100d
4	AC06-325	R04-475
	W12-1000d	
	K02-1600	
5	None	425-600
6	080-550	124-1450
	559-600d	
	AC09-1100	
7	N20-1400d	None
8	763-1000	G19-440
	R19-1900	Y09-650
	075-870ubc	
9	392-1900	H16-700
	368-1350	N20-1100
		601-1000
10	140-1500	651-950
	199-1700d	
	254-700d	
	292-450	
11	AD20-500	354-1000
	G18-500	
	AB20-500	
Unlinked		
	205-460	102-1750
	211-990	129-900
	550-650	302-950
	615-800	345-1050
	A15-1600	579-800
	K15-400	634-400
	Q18-700	638-400
	V01-650	AA02-1250
	*CAC-B101	AA18-650
		G05-1100
		X10-825

Their sequences were submitted to GenBank and assigned accession numbers CZ257492 and CZ257493, respectively.

Discussion

The RAPD primers in this study revealed a high level of polymorphism, a result consistent with other measures of diversity in this outcrossing species that is clonally propagated for commercial production. We used a large number of primers and generated many markers, and chose for mapping only those that could be scored with confidence. In this manner, we avoided the problems with less robust RAPD markers encountered by many other researchers, including Pooler and Scorza (1995).

The 11 pairs of chromosomes presented in this study correspond to the haploid chromosome number of *Corylus*. The two additional pairs from the susceptible parent (12S and 13S) will probably merge with other groups as additional loci are scored. In several pairs, one group is shorter than its homologue. This map differs from the preliminary map of Mehlenbacher et al. (2005) in 6 important aspects. First, many testcross RAPD markers have been added and the maps extended. The map of the susceptible parent has 82 new testcross RAPD markers and has been extended by 113 cM. The map of the resistant parent has 102 new testcross RAPD markers and has been extended by 198 cM. Second, we present separate maps for the susceptible and resistant parents rather than a merged map. This clarifies the parental origin of the markers and makes the map more useful for other applications such as quantitative trait locus (QTL) analysis and identification of markers for marker-assisted selection. Third, we found that linkage groups 5 and 7 on the preliminary map were the same. After creating a set of dummy variables, we were therefore able to merge them. The merged group is now presented as No. 5. Fourth, the merged group presented as No. 2 on the preliminary map was created by merging one large group from the resistant parent, one large group of 3:1 markers, and two small groups from the susceptible parent. Analysis with MapMaker revealed that JoinMap had merged two linkage groups, which are now presented as separate linkage groups 2 and 7. Fifth, a new linkage group, 11R, was identified and is presented beside its homologue 11S, with common intercross markers indicated. The final difference is the addition of 30 SSR loci to the map.

Intercross RAPD markers are not efficient for mapping, as only 25% of the data points (absent, absent) are useful. Yet with the intercross RAPD markers alone we were able to pair all 11 linkage groups in the susceptible parent with their homologues in the resistant parent. We scored 109 intercross markers, 18 of which were scored with only moderate confidence. In Fig. 2, we include reliable intercross markers that clearly tie together the two homologues and clearly indicate their correct orientation. The addition of SSR markers segregating 1:2:1 and 1:1:1:1 confirmed that homologues had been correctly identified by the intercross RAPD markers.

Markers for the S locus

In this study, we identified 6 markers linked to the S locus. Pomper et al. (1998) identified RAPD markers J14-1700, linked to S_1 (7.6 cM), and I07-750, linked to S_2 (3.8 cM), and Bassil and Azarenko (2001) identified a third marker, N20 1300, linked to S_3 (3.75 cM). Thus, we now have a total of 9 RAPD markers for the S locus in hazelnut. If additional markers such as amplified fragment length polymorphism (AFLP) markers are identified and used to saturate the region around the S locus, map-based cloning of this important locus would be feasible. Sporophytic incompatibility has been studied extensively in *Brassica* (Fobis-Loisy et al. 2004), but hazelnut is taxonomically unrelated,

so it would be interesting to compare the two sequences. In apple and sweet cherry, differences in the sequences of alleles at the S locus have permitted the development of molecular typing systems to identify alleles in cultivars and selections. This would be useful in hazelnut as well, where more than 26 alleles have been identified (Mehlenbacher 1997*a* and unpublished data).

Extension of the map to other populations

A major disadvantage of a linkage map based solely on RAPD markers is that it is only useful for the population in which it was developed. The addition of 30 microsatellite loci to our map makes it universally useful in hazelnut seedling populations, since the locations of such "anchor loci" are highly conserved. Markers for the *Corylus avellana* L. 'Gasaway' resistance gene should be useful in other populations segregating for the same gene. An alternative way to make this map applicable to other populations would be to convert several RAPD markers to RFLP probes, although this would be quite laborious. Conversion of the RAPD markers to codominant CAPS markers is also possible and PCR amplification, followed by restriction enzyme digestion, is sufficiently easy for use in marker-assisted selection.

Applications for study of additional traits

In addition to the disease resistance gene from 'Gasaway', several other sources of resistance have been identified (Lunde et al. 2000; Chen et al. 2005) and are being used in breeding with the ultimate goal of assembling pyramids of resistance genes in a single cultivar with the expectation that the resistance would be more durable than that conferred by a single R allele. Study of the simply inherited traits nondormancy (Thompson et al. 1985) and contorted growth habit (Smith and Mehlenbacher 1996) would provide information useful for many plant species. The 30 SSR loci placed on this map will facilitate the assignment of these other loci to a linkage group.

Other considerations

Hazelnut has a small genome, estimated to be 0.48 pg per 1C nucleus, which is slightly smaller than that of rice (Bennett and Smith 1991; R. Meng personal communication). Salesses (1973) and Salesses and Bonnet (1988) reported the existence of reciprocal translocations in cultivars with reduced pollen viability. These cultivars include 'Barcelona', 'Tonda Gentile delle Langhe', 'Segorbe', and 'Tonda di Giffoni', which have been used as parents in breeding. This should be kept in mind as maps are created for different populations and as markers from this map (or those placed on this map in the future) are extended to other populations.

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