

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.

Received 5 April 2005. Accepted 29 August 2005. Published on the NRC Research Press Web site at <http://genome.nrc.ca> on 3 February 2006.

Corresponding Editor: J.B. Bell.

S.A. Mehlenbacher,¹ R.N. Brown,² E.R. Nouhra,³ T. Gökirmak,⁴ and N.V. Bassil.⁵ Department of Horticulture, Oregon State University, 4017 Agricultural and Life Sciences Building, Corvallis, OR 97331-7304, USA.

T.L. Kubisiak. USDA Forest Service, Southern Institute of Forest Genetics, 23332 Highway 67, Saucier, MS 39574-9344, USA.

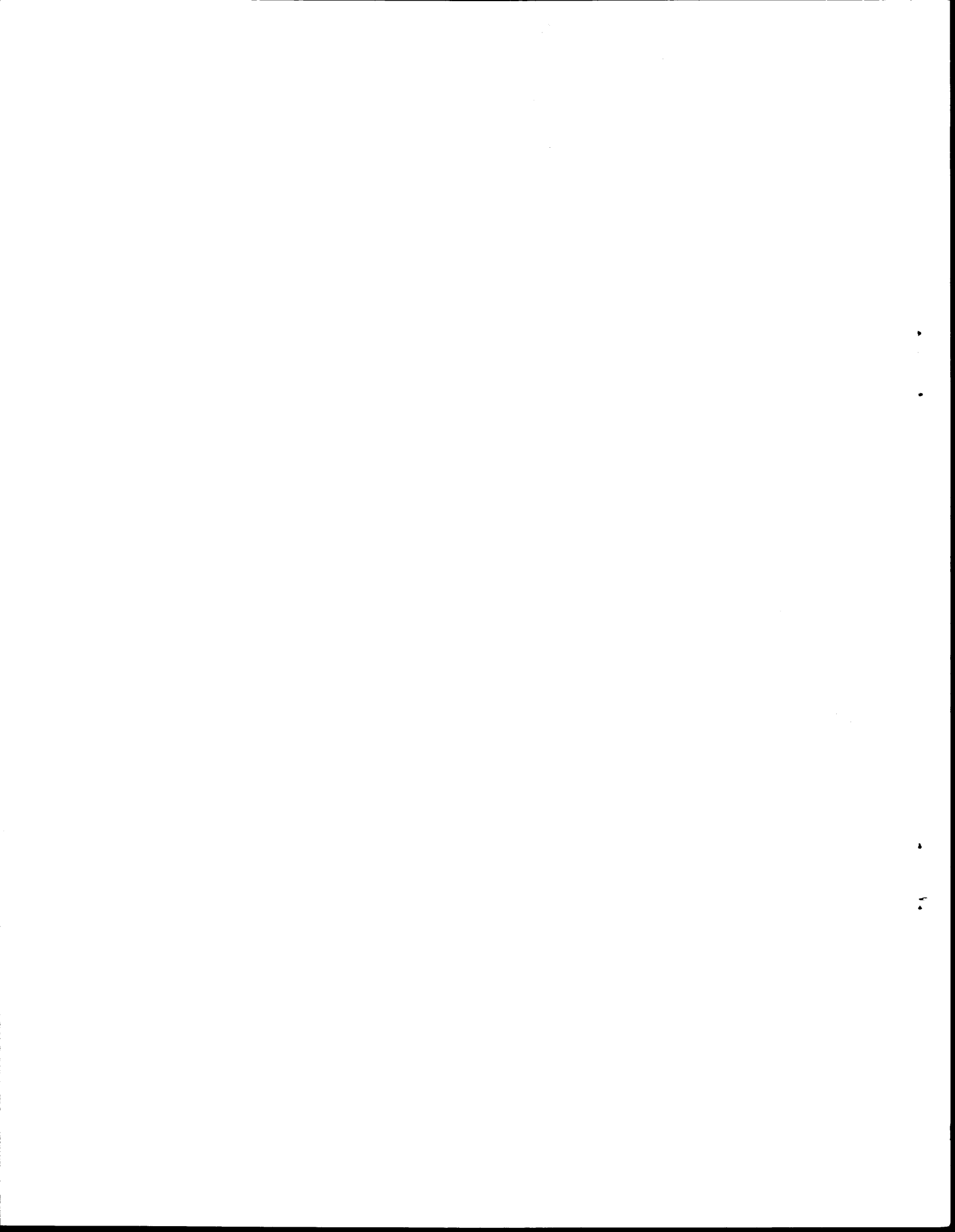
¹Corresponding author (e-mail: mehlenbs@hort.oregonstate.edu).

²Present address: Department of Plant Science and Entomology, Woodward Hall, Alumni Avenue, University of Rhode Island, Kingston, RI 02881, USA.

³Present address: Instituto Multidisciplinario de Biología Vegetal, Universidad Nacional de Córdoba, CC495, Córdoba 5000, Argentina.

⁴Present address: Department of Horticultural Sciences, University of Florida, Box 110690, Gainesville, FL 32611-0690, USA.

⁵Present address: USDA-ARS National Clonal Germplasm Repository, 33447 Peoria Rd., Corvallis, OR 97333, USA.



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

[Traduit par la Rédaction]

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 (Kasapliligil 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 1997a, 1997b). 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 1997a).

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.

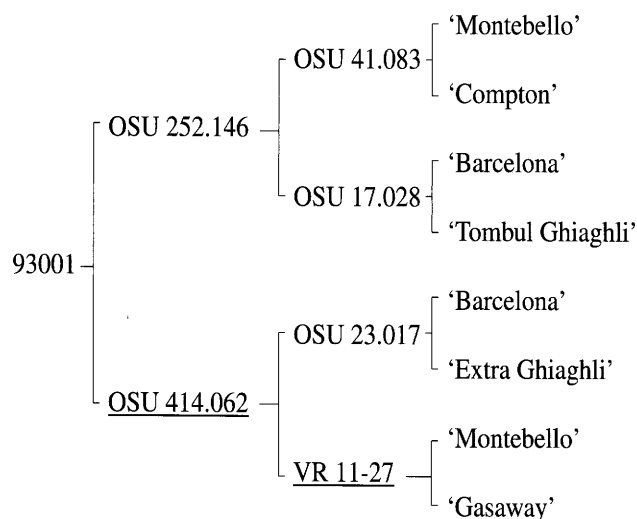
Identification of incompatibility alleles

Fluorescence microscopy, as described in detail by Mehlenbacher (1997b), was used to determine if S₃ or S₈ was present in the seedlings. The cross OSU 252.146 (S₃S₈) × OSU 414.062 (S₁S₁) would be expected to generate seedlings of two types (S₁S₃ and S₁S₈) in equal frequency. According to the dominance hierarchy of Mehlenbacher (1997a), only the second allele of these pairs would be expressed in the pollen. *Corylus avellana* L. 'Nonpareil' pollen was used as the S₃ tester and *Corylus avellana* L. 'Tombul Ghiaghli' pollen as the S₈ 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 S₃ or S₈. 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 S₃ and S₈, 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 μ L volume containing 10 mmol/L Tris–HCl (pH 9.0), 50 mmol/L KCl, 0.10% Triton X-100, 1.5 mmol/L MgCl₂, 200 μ mol/L each of dATP, dCTP, dGTP, and dTTP, 0.5 μ mol/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 μ L 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 μ L containing 0.3 μ mol/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) (Bocacci 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 μ L and the reaction mixture contained 1 \times Biolase NH₄ reaction buffer, 2 mmol/L MgCl₂, 200 μ mol/L each of dATP, dCTP, dGTP, and dTTP, 0.3 μ mol/L each of forward and reverse primers, 0.25 U Biolase DNA polymerase (Biolase 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 μ L aliquot and 5 μ L 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 μ L 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-GGTTTGTACAGAAATTCAGACG R-GCGTGTGGTTAATGTTTTCTTT	60	1:640
CAC-A24b	(GA) ₁₈ (AT) ₇	NED-CACAACATGCAACGTCTATGTA R-AGGTACGTATTGACAGGCTTTT	62	1:120
CAC-A040	(CA) ₁₃	NED-TGCTCAAGCAAATATTGCAC R-GTTTGGGATCCAATTAACCCTCT	62	1:213
CAC-A102	(AG) ₁₆ (AC) ₁₅	HEX-AAACTGTGACGAACGAAAACAC R-TTGCACCTCCATAACTGTCAAAA	62	1:80
CAC-B005	(GA) ₂₂	FAM-CAAACCTTATGATAGGCATGCAA R-TGTCACCTTTGGAAGACAAGAGA	62	1:320
CAC-B010	(GA) ₁₆	FAM-AGCTTCCAAATCACACATTACC R-GAAGAGCATCCGTATGATTCAG	62	1:320
CAC-B011	(GA) ₁₁	NED-CACTGGTGATCTCACAGGTTTA R-GTCCTCAAAAAGCTAAGCACAAAG	62	1:240
CAC-B020	(GA) ₁₉	HEX-GGGAAAATACTCCAAATCGCT R-TCACCGAGCCGTCATAATC	60	1:240
CAC-B028	(AG) ₁₆	NED-ATGGACGAGGAATATTTTCAGC R-CCTGTTTCTCTTTGTTTTTCGAG	55	1:213
CAC-B029b	(GA) ₁₃	NED-CAATTTACACCTCAGGGAAGAG R-AAGTTCACCCAAGAAATCCAC	58	1:160
CAC-B101	(AG) ₁₄	HEX-GCAGACCAGAGTCTGTTATTCA R-AGACAATTTTCGTGACTGGGTAT	62	1:480
CAC-B105	(GA) ₁₆	HEX-AAAGGAGCAAGCATGTTAGG R-GTTTGTACGGATGATCCACTGAG	62	1:320
CAC-B109	(GA) ₂₁	HEX-AATCCAAGCCTTTTCACTACC R-ACCCATCAAGTTCACCAATC	58	1:320
CAC-B113	(GA) ₁₄	HEX-TTGAGGAAGTCCAGGAAAAT R-GCCAGAGAGAGCAAGAGTTAG	60	1:320
CAC-C001a	(CACAGAG) ₃	FAM-CCCGTAACTAACCAATCACAAT R-TGGAGAAGAGGAGAGCTTAGTG	58	1:320
CAC-C008	(AAG) ₁₁	FAM-TTTCCGCAGATAATACAGGG R-TCCTTTGCTTTGGACCAG	58	1:320
CAC-C028	(GAA) ₁₀	NED-CTACCCCATCGCTTGACAC R-GGAGACTTGTTTGCCACAGA	60	1:213
CAC-C040	(GAA) ₈ (GGA) ₅	FAM-AGCCCCATTAGCCTTCTTAG R-GTTTCCAGATCTGCCTCCATATAAT	62	1:320
CAC-C114	(TTC) ₆	HEX-TCTCCCTCTCCCTCTCTTCTAC R-GAAAGGAAAAAGCACATAGCAA	60	1:400
CAC-C115	(TAA) ₅ (GAA) ₁₂	FAM-CATTTTCCGCAGATAATACAGG R-GTTTCCAGATCTGCCTCCATATAAT	60	1:320
CAC-C118	(AAG) ₆	HEX-AGCAACAGAGGTTAGGTGTG R-GCCCCATTAGCCTTCTTA	60	1:320
CAC-C119	(GA) ₇ (GA) ₉	NED-CTCACCTTTACCCCTTCATTTT R-GTTTCTCATCTTCTGAGAACCATC	62	1:213
CaT-A114	(TG) ₁₇	FAM-CGCCTTGATAGTATGTTCAAAC R-CGGCAGAATGTAGAAGTCCCC	60	1:320
CaT-B106	(AG) ₁₇ AA(AG) ₆	HEX-CCAATCGCCAATGAATCATC R-CCCTTTCCAACTGGGCAT	60	1:320