New Simple Sequence Repeat Markers on Linkage Groups 2 and 7, and Investigation of New Sources of Eastern Filbert Blight Resistance in Hazelnut

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ABSTRACT. Eastern filbert blight (EFB), caused by *Anisogramma anomala*, is a fungal disease threatening the European hazelnut (*Corylus avellana*) industry in the Willamette Valley of Oregon. The pathogen is endemic to the eastern United States where it causes little damage to the wild *Corylus americana* but causes severe cankers on most cultivars of the commercially important European hazelnut. The host genetic resistance in ‘Gasaway’ is conferred by a dominant allele at a single locus on linkage group 6 (LG6), and resistance from several other sources has been mapped to the same region. Some fungal isolates can overcome ‘Gasaway’ resistance, prompting a search for other sources of resistance. Resistance from other sources has been mapped to LG2 and LG7, for which additional simple sequence repeat (SSR) markers would facilitate marker-assisted selection (MAS). In this study, an *in silico* approach was used to develop new polymorphic SSR markers in the EFB resistance regions on LG2 and LG7. Starting with a search of 17 contigs of the ‘Jefferson’ genome sequence, 45 new polymorphic SSR markers were developed, characterized, and placed on the linkage map. The new SSR markers had an average of 10.18 alleles per locus, and average values for expected heterozygosity, observed heterozygosity, polymorphism information content, and frequency of null alleles of 0.72, 0.65, 0.68, and 0.068, respectively. Of the 42 new polymorphic SSRs segregating in the mapping population, 24 were on LG2, 12 were on LG7, and six were placed on other LGs. The new and previously developed SSR markers were used to study six new sources of EFB resistance, four from Russia and two from Crimea. Six resistant selections were crossed with susceptible selections, resulting in 7 progenies. Phenotyping for disease response revealed that segregation in progenies of the two Moscow selections (#2 and #27), one Russian selection (OSU 1187.101), and one Crimean selection (H3R12P62) fit the 1:1 segregation ratio expected for control of resistance by a dominant allele at a single locus; but in progenies of the other Russian selection (OSU 1166.123) and the other Crimean selection (H3R07P11), there was an excess of resistant seedlings. Correlation of disease scores and alleles at SSR loci indicated that resistance from three Russian selections (Moscow selections #2 and #27 and OSU 1166.123) and the Crimean selection H3R12P62 was on LG7, while resistance from Russian selection OSU 1187.101 was on LG2. Resistance from Crimean selection H3R07P11 was not correlated with markers on LG6, or LG2, or LG7. These sources and new SSR markers will be useful in MAS and the pyramiding of resistance genes in the breeding of new EFB-resistant cultivars.

European hazelnut (*Corylus avellana*), also known as filbert, is an economically important nut crop. Hazelnuts are consumed as whole kernels or used as ingredients by the food industry. Hazelnut oil can also be used as cooking oil and for biofuel.

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Hazelnuts are highly nutritious and provide unsaturated fatty acids, diet-friendly fiber, protein, vitamins, and antioxidants. Additionally, a balanced intake of hazelnuts reduces the risk of cardiovascular disease (Ramalhosa et al., 2011). Ninety-nine percent of the U.S. hazelnut production is in the Willamette Valley of Oregon [U.S. Department of Agriculture (USDA)–National Agricultural Statistics Service (NASS), 2018]. The acreage has increased rapidly in the past decade as orchards of new cultivars from Oregon State University (OSU) have been planted.

Eastern filbert blight (EFB), caused by the pathogen *Anisogramma anomala*, is the most destructive disease of hazelnut in Oregon. The pathogen is endemic to eastern North America, where it occasionally causes small cankers on selections of the native *C. americana* (Capik and Molnar, 2012). However, the fungus causes severe infections on most cultivars of European hazelnut (Pinkerton et al., 1993), and it was first reported in the Pacific Northwest United States in 1970 (Davison and Davidson, 1973). The causal agent is an obligate, biotrophic ascomycete with a 2-year life cycle. The disease cycle and symptoms were described by Stone et al. (1992), Pinkerton et al. (1992, 1995, 1998, 2001), and Johnson et al. (1994, 1996). The cycle begins when stromata in cankers become wet in the spring, and ascospores are disseminated by wind and rain. These spores infect

young shoots and breaking buds (Pscheidt, 2010). Cankers with stromata become visible 12 to 16 months after infection. The cankers expand each year, producing new stromata, ultimately causing dieback and death of susceptible plants in 5 to 12 years in the absence of fungicides (Pinkerton et al., 1993). EFB control measures include scouting for cankers, pruning of infected wood, and regular fungicide applications (Johnson et al., 1994; Pscheidt, 2010). These cultural practices delay disease spread but are expensive. The development and planting of EFB-resistant cultivars is considered the most cost-effective and environmentally friendly control method (Mehlenbacher, 1995).

There are two classes of host genetic resistance. Quantitative resistance reduces the severity of the disease, while qualitative resistance is usually a high level of resistance conferred by a dominant allele at a single locus. Most qualitative resistance genes (R-genes) follow the gene-for-gene model first proposed by Flor (1942). Resistance genes in various plants have been placed in categories based on their similar DNA sequences. The most represented group of R-genes has a nucleotide-binding domain (NB) and a leucine-rich repeat (LRR) domain (Hulbert et al., 2001; McHale et al., 2006). In hazelnut, 115 putative NB-LRR proteins were annotated in the version 1 (V1) genome sequence of 'Jefferson' produced by sequencing using Illumina (San Diego, CA) technology (Rowley et al., 2018).

The hazelnut breeding programs at OSU and Rutgers University have identified more than 150 accessions that exhibit a very high level of EFB resistance (S.A. Mehlenbacher and T.J. Molnar, unpublished data). 'Gasaway', a late-blooming pollinizer, was the first *C. avellana* identified with very high resistance to EFB (Cameron, 1976). The resistance of 'Gasaway' is controlled by a dominant allele at a single locus (Mehlenbacher et al., 1991) that was placed on linkage group 6 [LG6 (Mehlenbacher et al., 2006)]. Grower selection 'Zimmerman' also has a high level of resistance (Lunde et al., 2006). Simple sequence repeat (SSR) markers, also known as microsatellites, indicate that 'Zimmerman' is from a cross of 'Barcelona' and 'Gasaway' (Gökirmak et al., 2009) and thus is the same source of resistance. The OSU hazelnut breeding program has extensively used 'Gasaway' resistance and recent releases, including 'Yamhill', 'Jefferson', 'Dorris', 'Wepster', 'McDonald', and 'PollyO' (Mehlenbacher et al., 2009, 2011, 2013, 2014, 2016, 2019); and the pollinizers 'Gamma', 'Epsilon', 'Theta', 'York', and 'Felix' (Mehlenbacher and Smith, 2004; Mehlenbacher et al., 2012, 2018) carry this resistance. Sathuvalli et al. (2017) fine mapped and predicted genes in the EFB resistance region in 'Jefferson'.

Additional sources of EFB resistance have been identified and assigned to LGs. Resistance in OSU 408.040 from Minnesota (Sathuvalli et al., 2012), OSU 495.072 from Russia, 'Culpla' from Spain, and 'Crvenje' and 'Uebov' from Serbia (Bhattarai et al., 2017a; Colburn et al., 2015) was assigned to LG6. Resistance in 'Ratoli' from Spain (Sathuvalli et al., 2011a), *C. americana* 'Rush', and interspecific hybrid 'Yoder #5' (Bhattarai et al., 2017b) was assigned to LG7. Resistance from OSU 759.010 from the Republic of Georgia and Rutgers University selection H3R07P25 from Holmskij, Russia was assigned to LG2 (Honig et al., 2019; Sathuvalli et al., 2011b). Sathuvalli et al. (2010) reported that Moscow selections #2 and #27 were EFB resistant following greenhouse inoculation. Additional resistant selections have been identified in germplasm collected in Russia, Crimea, and Poland (Capik et al., 2013; Molnar et al., 2007). Two selections from seeds purchased in Crimea,

H3R7P11 and H3R12P62, were included in the germplasm characterization study of Muehlbauer et al. (2014). These two Rutgers University selections were inoculated with the EFB pathogen in the greenhouse and field and found to be resistant (Capik et al., 2013; Molnar et al., 2007). At OSU, potted trees of two additional selections of Russian origin, OSU 1166.123 from seeds collected in Sochi and OSU 1187.101 from seeds purchased in a market in Holmskij, remained free of EFB following exposure under a structure topped with diseased wood in 2014 and 2013, respectively. Subsequent greenhouse inoculation at OSU confirmed their resistance. In New Jersey, OSU 1166.123 remained free of disease signs and symptoms (Leadbetter et al., 2016).

The long-term durability of a single source of EFB resistance is a concern of the Oregon hazelnut industry (Lunde et al., 2006; Molnar et al., 2010a), especially as cultivars with Gasaway resistance have been extensively planted in the Willamette Valley in the past decade. Small cankers have been observed on OSU releases, including 'Jefferson' and 'McDonald', under high disease pressure (Pscheidt and Ocamb, 2020). Molnar et al. (2010b) and Capik and Molnar (2012) demonstrated pathogenic variation in *A. anomala*, with isolates from New Jersey and Michigan able to infect 'Gasaway' and its offspring. The use of multiple sources of resistance and the pyramiding of two or more R-genes in a single host genotype are approaches to reduce the likelihood of the pathogen overcoming resistance.

Mehlenbacher et al. (2006) constructed the first genetic linkage map for hazelnut using random amplified polymorphic DNA (RAPD) and SSR markers. More than 700 polymorphic SSR markers have been developed in *C. avellana* (Bassil et al., 2005a, 2005b, 2013; Bhattarai and Mehlenbacher, 2017, 2018; Boccacci et al., 2005, 2015; Colburn et al., 2017; Gürcan and Mehlenbacher, 2010a; Gürcan et al., 2010), of which ≈ 450 have been placed on the reference linkage map (Mehlenbacher and Bhattarai, 2018). The reference map revealed segregation distortion at loci on LG2 and LG7 and the merger of the two linkage groups in the map of the male parent (Mehlenbacher and Bhattarai, 2018; Mehlenbacher et al., 2006). This was attributed to a reciprocal translocation involving LG2 and LG7 (Komaie Koma, 2020; Salesses, 1973; Salesses and Bonnet, 1988). Komaie Koma (2020) used bioinformatic methods to disentangle the "pseudo-linkage" of markers on LG2 and LG7 in the male parent (OSU 414.062).

Rowley et al. (2018) used Illumina short-read sequencing to generate the first genome sequence ($93\times$) for Jefferson hazelnut, which was chosen as the reference cultivar. More recently, the 'Jefferson' genome was sequenced using long reads from the Pacific Biosciences (PacBio, Menlo Park, CA) RSII sequencer providing $\approx 49\times$ coverage (Snelling et al., 2018). The resulting contigs were error-corrected using the Illumina reads of Rowley et al. (2018) and constituted the version 2 (V2) reference genome for 'Jefferson'. The error-corrected PacBio reads were assembled into chromosome-level scaffolds using Hi-C proximity ligation procedures (Dovetail Genomics, Scotts Valley, CA). The resulting 11 scaffolds correspond to the haploid chromosome number of hazelnut ($2n = 2x = 22$) and represent the V3 genome sequence of 'Jefferson'. The V3 genome (V3) shows two separate scaffolds for LG2 and LG7. The coordinates of SSR markers on the two scaffolds have been identified.

Table 1. Hazelnut accessions used for characterization of 45 new polymorphic simple sequence repeat markers from genome sequences. Accessions 1 to 24 were used to screen for polymorphism on agarose gels, while all 50 accessions were used for characterization of the new markers. The country and region of origin, as well as the source of scions or seed, are shown for each. Some accessions were imported as scions, and others as seed lots from which individuals were selected. Plant Inventory (PI) numbers are provided for accessions from the USDA–ARS National Clonal Germplasm Repository collection.

No.	Accession	Inventory no.	Origin	Source
1	OSU 495.049	PI 557421	Russia, southern	southern Russia (seeds)
2	Albania 55	PI 617207	Albania	Cajup, Albania (seeds)
3	Fusco Rubra	PI 557047	Germany	Morton Arboretum, Lisle, IL, USA (scions)
4	Ala Kieri (CCOR 187)	PI 557080	Finland	Lappa, Finland (seeds)
5	Pendula	PI 557048	France	Arnold Arboretum, Boston, MA, USA (scions)
6	Hall's Giant	PI 557027	Germany/France	OSU Entomology Farm, OR, USA (scions)
7	Gasaway	PI 557042	USA, Washington	Orchard in Washington, USA (scions)
8	Rode Zeller	PI 271280	Netherlands	Q.B. Zielinski from the Netherlands (scions)
9	Cosford	PI 557039	England, Reading	Cornell Univ., Geneva, NY, USA (scions)
10	Du Chilly	PI 557099	England	OSU Entomology Farm, OR, USA (scions)
11	Palaz	PI 304632	Turkey, Ordu	Q.B. Zielinski from Greece (scions)
12	Pellicule Rogue	PI 271110	France	Q.B. Zielinski from France (scions)
13	Imperiale de Trebizonde	PI 271105	Turkey	Q.B. Zielinski from France (scions)
14	Tombul Ghiaghli	PI 304634	Turkey	Q.B. Zielinski from Greece (scions)
15	Tonda Bianca	PI 296206	Italy, Campania	Q.B. Zielinski from Italy (scions)
16	Negret	PI 270340	Spain, Tarragona	Q.B. Zielinski from Spain (scions)
17	Tonda Gentile delle Langhe	PI 557075	Italy, Piemonte	Univ. di Torino, Italy (scions)
18	Tonda Romana	PI 557025	Italy, Lazio	ISF Rome, Italy (scions)
19	Römische Nuss	PI 557171	Unknown	Hermansverk, Norway (scions)
20	Casina	PI 557033	Spain, Asturias	Q.B. Zielinski from Asturias, Spain (scions)
21	Ratoli	PI 557167	Spain, Tarragona	IRTA Mas Bove, Reus, Spain (scions)
22	Mortarella	PI 339723	Italy, Campania	Q.B. Zielinski from Italy (scions)
23	Tonda di Giffoni	PI 296207	Italy, Campania	Q.B. Zielinski from Italy (scions)
24	Barcelona	PI 557156	Spain	Oregon nursery, USA (tree)
25	Cutleaf	PI 557306	England	Arnold Arboretum, Boston, MA, USA (scions)
26	OSU 681.078	PI 634203	Russia, Moscow	Moscow, Russia (seeds) collected by J. Henkin
27	Barcelloner Zellernuss	PI 557156	Spain?	Faversham, England, UK (scions)
28	Aurea	PI 557050	France	Morton Arboretum, Lisle, IL, USA (scions)
29	OSU 408.040	PI 617266	Univ. Minnesota	Univ. Minnesota research farm (seeds)
30	Des Anglais	PI 557423	Unknown	INRA, Bordeaux, France (scions)
31	OSU 26.072	PI 323961	Russia, North Caucasus	North Caucasus, Russia (seeds) collected by H. Brooks
32	Bergeri	PI 557114	Belgium, Luttich	ISF Rome, Italy (scions)
33	Alli	PI 686862	Estonia	Polli, Estonia (scions)
34	Kadetten	PI 557090	Germany	Faversham, England, UK (scions)
35	OSU 759.010	–	Georgia	L. Lazareishvili, Tbilisi, GA (scions)
36	Contorta	PI 557049	England	Arnold Arboretum, Boston, MA, USA (scions)
37	OSU 556.027	PI 617269	Turkey, Istanbul	Istanbul market, Turkey (seeds)
38	B-3	PI 557122	Macedonia	Skopje, Macedonia (scions)
39	OSU 54.039	PI 557060	Turkey, Giresun/Ordu	Giresun, Turkey (seeds) collected by M.M. Thompson
40	Gunslebert	PI 557191	Germany, Gunsleben	INRA Bordeaux, France (scions)
41	Sant Jaume	PI 557103	Spain, Tarragona	IRTA Mas Bove, Reus, Spain (scions)
42	Iannusa Racinante	PI 557183	Italy, Sicily	Univ. di Torino, Italy (scions)
43	Gem	PI 557029	Washington	Orchard in Oregon, USA (scions)
44	Artellet	PI 557108	Spain, Tarragona	IRTA Mas Bove, Reus, Spain (scions)
45	Simon	PI 557166	Spain, Tarragona	IRTA Mas Bove, Reus, Spain (scions)
46	Gustav's Zellernuss	PI 557085	Landsberg	Faversham, England, UK (scions)
47	Buttner's Zeller	PI 557094	Germany, Landsberg	Faversham, England, UK (scions)
48	Tapparona di San Colombano Cortemoli	PI 617239	Italy, Liguria	Univ. di Torino, Italy (scions)
49	OSU 252.146	–	Oregon State Univ.	Parent of mapping population 93001
50	OSU 414.062	–	Oregon State Univ.	Parent of mapping population 93001

The objectives of this study were to develop new SSR markers in the EFB resistance regions on LG2 and LG7 and to investigate EFB resistance from six new sources, four of Russian and two of Crimean origin.

Materials and Methods

PLANT MATERIALS FOR MARKER DEVELOPMENT. To validate polymorphism on agarose gels, a set of 24 diverse european

hazelnut accessions was used (Table 1). These 24, an additional 24 hazelnut accessions, and the two parents of the reference mapping population (OSU 252.146 and OSU 414.062) were used to characterize the new SSR markers (Table 1). These accessions were investigated by Gökirmak et al. (2009) and chosen to represent the genetic diversity in the *C. avellana* germplasm collection. This set of 50 diverse accessions was previously used in numerous SSR marker characterization studies (Bhattarai and Mehlenbacher, 2017, 2018; Colburn et al., 2017; Gürcan and Mehlenbacher, 2010a; Gürcan et al., 2010). To map the SSR markers, we used 138 seedlings of the mapping population. While the female parent (OSU 252.146) is susceptible, the male parent (OSU 414.062) is resistant to EFB.

PLANT MATERIALS FOR DISEASE RESISTANCE STUDIES. Six EFB-resistant selections were crossed with EFB-susceptible selections over a 4-year period (2011–14), generating seven progenies that segregated for disease response and a total of 545 seedlings. Their pedigrees are shown in Supplemental Table 1. Selections Moscow #2 and Moscow #27 were imported as scions from the Russian Research Institute of Forestry and Mechanization in Pushkino, Moscow Province, Russian Federation; and four selections originated from imported seed lots. OSU 1187.101 was from seeds purchased in an outdoor market in the village of Holmskij, Russia (near Krasnodar); the lot was designated RUS-10 by Molnar et al. (2007). OSU 1166.123 was from seeds collected at the Institute of Floriculture and Subtropical Cultures in Sochi, Russia; and two Rutgers University selections (H3R7P11 and H3R12P62) were from seeds purchased from roadside vendors near Simferopol, Crimea. The two Rutgers University selections were from seed lots designated RUS-26 and RUS-23, respectively, by Molnar et al. (2007) and Capik et al. (2013). The selection OSU 1166.123 was crossed in 2013 with susceptible parents OSU 1235.078 and OSU 1236.134, generating progenies 13030 and 13031, respectively.

DNA EXTRACTION. For SSR marker development and characterization, young leaves were collected from trees growing in the field at OSU's Smith Horticulture Research Farm and the USDA–Agricultural Research Service National Clonal Germplasm

Repository (NCGR) in Corvallis, OR. Total DNA was extracted from two to four fresh young leaves, as described by Lunde et al. (2000), without RNAase treatment. The quality and quantity of the DNA samples were assessed using a microplate reader (Synergy2; BioTek Instruments, Winooski, VT) paired with Gen5 data analysis software and adjusted to a concentration of 20 ng·μL⁻¹. For EFB resistance mapping, DNA was extracted from the parents and seedlings of the five progenies inoculated under the structure (10015, 11024, 12030, 14031, and 14035) and the two progenies inoculated in the field (14030 and 14031). Fresh young leaves were collected from seedlings in the EFB nursery or seedling rows at the Smith Horticultural Research Farm in Corvallis, OR. DNA extraction procedures for the seedlings in progenies segregating for EFB response, and the 138 seedlings used for mapping, were the same as for the 50 accessions used for characterization.

MICROSATELLITE MARKER IDENTIFICATION. The DNA sequences of a set of previously developed SSR markers on LG2 and LG7 (Table 2) were aligned with the 'Jefferson' genome (V2) assembled from PacBio sequences (Snelling et al., 2018). In addition, 28 double digest restriction site associated DNA sequencing (ddRAD-Seq) markers with positions from 2.57 to 24.76 cM on LG2 were aligned with the V2 'Jefferson' reference genome, of which RUCaSl_18603 and RUCaSl_14004 flanked the EFB resistance quantitative trait locus (QTL) in Rutgers University selection H3R07P25 from Holmskij, Russia (Honig et al., 2019). The alignment of the marker sequences with the V2 genome sequence identified contigs associated with the EFB resistance regions on these two LGs, and the contigs were mined for SSR motifs > 16 bp using the GMATo program (Wang et al., 2013). The SSRs were placed in separate folders according to the length of the repeat motif: di-, tri-, tetra-, penta-, and hexa-nucleotide repeats. Of these, di-nucleotide repeats with at least eight repeat units were pursued for marker development. SSRs that contained only As and Ts were removed, because such markers were previously found to be difficult to score. The sequences were trimmed using Samtools (Li et al., 2009) to 500 bp with the SSR in the center. The genomes of seven other *C. avellana* accessions ('Barcelona', 'Ratoli', 'Tonda Gentile delle Langhe', 'Tonda di

Table 2. Contigs in the genome sequence (V2) of 'Jefferson' hazelnut pursued for simple sequence repeat marker development and the markers used to identify them.

Linkage group	Contig	Markers used for identification
LG2	026F	GB829, B005, B504
	046F	B509
	107F	KG852
	113F	B501
	121F	BR230
	140F	KG846, A024b
	229F	GB329
	230F	GB609
	266F	KG852
	284F	GB329, GB609
LG7	061F	BR238
	046F	GB467, B733, KG830, B753, KG830, B733, B509
	074F	B730
	076F	GB822, B020
	126F	B751
	170F	GB482, GB372
	294F	GB822
	535F	B730

Giffoni', 'Daviana', 'Hall's Giant', and 'Tombul') resequenced using Illumina HiSeq 2000 (Rowley et al., 2018) at ≈20× coverage were aligned with the V2 'Jefferson' sequence, and the aligned reads were visualized using Tablet software (Milne et al., 2010). Sequences with variation in the number of repeats (but with conserved flanking regions) were identified, and only SSRs that showed ≥ three alleles were chosen for further study. All sequences were analyzed for redundancy using the ClustalW program (Thompson et al., 2002), and duplicates were removed. Then, a BLAST search (Ye et al., 2006) was performed to identify and eliminate sequences containing SSRs that had been previously developed. Nonfluorescent forward and reverse primers were designed using Primer 3 software (Untergasser et al., 2012)

with the following criteria: 1) expected length of the amplified fragment between 90 and 315 bp; 2) primer length between 19 and 23 bases; and 3) annealing temperature 60 °C. Primers were ordered from Integrated DNA Technologies (Coralville, IA).

INITIAL SCREENING OF MARKERS. Primer pairs were screened for polymorphism using a set of 24 *C. avellana* accessions (Table 1). PCRs were performed in 10 µL final volumes with a mixture of 20 ng DNA, 1 × NH₄ reaction buffer, 2.5 mM mix dNTP, 2 mM MgCl₂, 0.3 µL of each forward and reverse primer (10 µM), and 0.25 units of DNA polymerase (Biolase; Bioline USA, Taunton, MA). PCRs were performed in 96-well plates on thermal cyclers (GeneAmp PCR System 9700; Applied Biosystems, Foster City, CA). The amplification program consisted of an initial denaturation for 5 min at 94 °C, followed by 40 cycles of 40 s at 94 °C, 40 s at the annealing temperature (60 or 62 °C), 40 s for elongation at 72 °C, a final extension step of 7 min at 72 °C, and then an infinite hold at 4 °C. The PCR products were separated by electrophoresis on 3% agarose gels in TBE buffer at 90 V for 3.5 h, stained with ethidium bromide, destained in distilled water, and photographed under ultraviolet light using a BioDoc-It Imaging System (UVP, Upland, CA). The gel images were inspected for size polymorphism among the 24 accessions.

GENOTYPING WITH POLYMORPHIC SSRs. For each SSR primer pair showing clear polymorphism on agarose gels, a forward primer labeled at its 5' end with fluorescent dye FAM or HEX was ordered from Integrated DNA Technologies. DNA from 50 diverse accessions (Table 1) was amplified with the fluorescent forward primers and nonfluorescent reverse primers with the same PCR cocktail ingredients and amplification program used in the initial screening. Post-PCR multiplexing of products with different sizes and fluorescent tags allowed up to seven SSRs to be simultaneously separated by capillary electrophoresis. After PCR, products were multiplexed by mixing 2 µL of each PCR product in water to a final volume of 200 µL, and an aliquot (1.8 µL) was submitted to the Core Laboratories of OSU's Center for Genome Research and Biocomputing (CGRB) for fragment sizing by capillary electrophoresis on an ABI 3730 instrument using ROX-500 as the size standard. Allele sizes were visualized and scored with ABI GeneMapper software (Life Technologies, Carlsbad, CA), and sizes were recorded in a spreadsheet. Ease of scoring was also noted. If amplification failed, or the capillary electrophoresis output could not be scored with confidence, the PCR and sizing were repeated. Allele sizes in the seedling populations were determined in the same manner.

MARKER CHARACTERIZATION. After capillary electrophoresis sizing, the data were used to calculate measures of genetic diversity. The number of alleles per locus, observed heterozygosity (H_o), expected heterozygosity (H_e), and the polymorphism information content (PIC) were estimated for each marker using PowerMarker software (Liu and Muse, 2005). Cervus software (Field Genetics, London, UK) was used to estimate null allele frequency [$F(\text{null})$], according to the maximum likelihood procedure of Kalinowski and Taper (2006). A null allele at an SSR locus consistently fails to amplify to detectable levels by PCR.

MAPPING OF POLYMORPHIC SSRs. Newly developed SSR markers were placed on the linkage map using 138 seedlings of the reference mapping population from the cross OSU 252.146 × OSU 414.062 (Mehlenbacher et al., 2006). A two-way pseudo-testcross approach (Grattapaglia and Sederoff, 1994) and JoinMap 5 (Kyzama, Wageningen, The Netherlands) were used

with “BC1” as the population type, Kosambi as the mapping function, and a logarithm of the odds (LOD) threshold ≥ 6 for grouping. Because of the merger of LG2 and LG7 in the previous maps of the male parent, only SSR markers were used for mapping; and the scaffolds and coordinates of new and previously developed SSR markers in the ‘Jefferson’ genome (V3) were consulted in map construction. Each SSR allele size was scored as present or absent in each seedling. Allele scores for the new SSR markers were combined with those for the previously mapped SSR markers on the two V3 genome scaffolds and imported into JoinMap 5 software. Linkage map construction was as described by Bhattarai and Mehlenbacher (2017), with a LOD score of 6 for grouping. Markers segregating in a ratio of 1:1 or 1:1:1:1 were scored as “h,” “a,” or “u” for presence, absence, or unknown status, respectively, for each allele in each seedling. Similarly, markers segregating in a ratio of 1:2:1 were scored as “h” if present in homozygous state, “a” if absent (homozygous), and “u” if heterozygous or having an unknown status. At loci segregating in a 1:2:1 ratio, heterozygotes provide no information useful for mapping. Markers that clustered loosely with the others and fell out at LOD scores < 6 were removed. Markers present in the repulsion phase were included in the map by creating “dummy variables,” the use of which allowed the merger of the repulsion phase and coupling phase markers, and generation of a single map for each LG in each parent. The JoinMap output was inspected for “Fit and Stress,” and markers were removed in stepwise manner until the “Nearest Neighbor Stress” value for all markers was less than an arbitrarily set value of 6.60. The “Nearest Neighbor Fit (cM)” values were also inspected, as high values indicate blocks of markers that fit poorly with adjacent markers. In addition, the length of the gaps between markers was inspected, with gaps of > 20 cM considered suspicious. Maps that included previously mapped RAPD markers were also constructed for LG2 and LG7 in the female parent for comparison with maps with only SSR markers. For marker loci not placed on LG2 or LG7, their scores were merged with those of previously mapped RAPD and SSR markers and maps constructed as described by Mehlenbacher and Bhattarai (2018).

STRUCTURE INOCULATIONS. Hybrid seeds from the crosses of the two Moscow selections, two Rutgers University selections, and OSU 1187.101 were harvested in late August, stratified, and planted in flats in the greenhouse as they germinated. When ≈ 30 cm tall, they were transplanted to 3-L pots and grown in the greenhouse during the summer. Potted seedlings were placed under the EFB inoculation structure at the OSU Smith Horticulture Research Farm in the spring, about 2 years after the crosses were made. In this technique, diseased branches with stromata were placed on top of the wire mesh platform held over the seedlings, as described by Pinkerton et al. (1993). Cultivars with known levels of susceptibility were included as controls: Ennis (highly susceptible), Barcelona (intermediate), Lewis (moderate resistance), Sacajawea (high quantitative resistance), and Tonda di Giffoni (high quantitative resistance). After the EFB exposure, the trees were transplanted to nursery rows, and disease phenotypes were scored 18 months after inoculation. Seedlings were rated on a scale of 0 to 5, with 0 being absence of disease symptoms and 5 being severe disease symptoms [1 = if sunken area without any stromata is smaller than 10 cm; 2 = small cankers (< 5 cm) with few stromata; 3 = cankers with mature stromata; 4 = pustules are very common, covering up to 50% of the branch length; 5 = pustules

are also common over the tree, and shoot tips are dead]. For correlation and segregation analyses, plants scoring 0–2 were considered resistant, and 3–5 were considered susceptible.

FIELD INOCULATIONS. For progenies 13030 and 13031 segregating for resistance from OSU 1166.123, the hybrid seeds and seedlings were handled as described previously for structure inoculation, but they were planted in the field without EFB inoculation in Oct. 2016 at a spacing of 0.9 m apart in rows 3.0 m apart. A total of 156 seedlings were planted in the field, where they were exposed to EFB spores from a nearby orchard. By Jan. 2018, cankers were noted on some seedlings, and those without cankers were pruned and given supplemental inoculation. If the seedling showed no disease, the small suckers at its base were removed with loppers or pruning shears, leaving ≈ 5 cm at the base, while the main stem and two largest suckers remained uncut. The trimming of suckers was intended to stimulate new growth at the base and increase the likelihood of EFB infection in susceptible seedlings. Diseased branches with mature cankers were collected in Dec. 2017 from the OSU Smith Horticulture Research farm and stored in polyethylene bags at -20°C . In Mar. 2018, a single diseased branch ≈ 15 cm long was tied in the upper part of the tree before leaf budbreak, as described by Molnar et al. (2007), except that no wax was used to seal the ends of the twigs. Disease was scored in these seedlings as present or absent in Apr. 2019, and scores were verified in Aug. 2019.

DATA ANALYSIS FOR DISEASE RESPONSE. Chi-square tests were performed to assess goodness-of-fit to a 1:1 segregation ratio in all progenies. For the two progenies of OSU 1166.123, a

homogeneity chi-square test was used to determine if the data could be pooled.

SSR MARKER SCORING AND CORRELATION ANALYSIS. For correlation analysis, the disease response and alleles at SSR markers were scored in a set of 32 seedlings in progenies 10015, 11024, and 12030, while 46 seedlings and the two parents were used for the remaining four progenies (13030, 13031, 14031, and 14036). The DNA was amplified using PCR and primers for ten SSR markers previously placed on the linkage map (Table 3) in regions where EFB resistance had been mapped. Of the ten, three were on LG6 in the ‘Gasaway’ resistance region, four were on LG2 in the Georgian resistance region, and three were on LG7 in the ‘Ratoli’ resistance region. Disease ratings and allele sizes at each SSR marker for each seedling in each progeny were entered in a spreadsheet. Resistant seedlings were scored as 1, and susceptible seedlings were scored as 0. Presence or absence of a marker allele was scored as 1 or 0, respectively. Pearson product-moment correlation coefficients were calculated for scores of disease and presence of each SSR allele. If the absolute value of the correlation coefficient was >0.5 , additional seedlings in the population and additional SSR markers on that LG were pursued.

MAPPING RESISTANCE LOCI. After the linkage group had been identified, primers of several SSR markers previously assigned to that linkage group were used to amplify the parents and seedlings of each progeny. The selected markers were easy to score and were located within 20 cM of a previously mapped resistance locus. All seedlings were scored at SSR marker loci segregating on that LG. The SSR alleles of the seedlings were

Table 3. Correlation coefficients for eastern filbert blight disease scores and simple sequence repeat markers on three linkage groups in seven hazelnut progenies. New markers on linkage groups 2 and 7 have an MS prefix. Resistant parents are shown in bold.

Linkage group		Progeny						
		10015	11024	12030	13030	13031	14031	14035
		Parents						
		OSU 1187.101 × OSU 1155.049	OSU 814.073 × Moscow #2	OSU 1269.020 × Moscow #27	OSU 1166.123 × OSU 1235.078	OSU 1166.123 × OSU 1236.134	Sacajawea × H3R07P11	H3R12P62 × OSU 1378.046
		Seedlings (no.)						
	Marker	32	32	32	46	46	46	46
6	GK6.63	—	—	—	—	—	—	0.11
6	GK6.81	—	—	—	—	—	0.16	0.15
6	LG682	—	—	—	—	—	0.03	0.11
2	B504	0.76	0.11	0.09	—	—	—	—
2	B509	—	—	0.15	—	—	—	—
2	B758	0.52	—	—	0.18	0.09	—	0.02
2	MS0026.04	—	0.05	—	—	—	—	—
2	MS0026.08	0.75	0.08	—	0.02	0.04	0.06	0.30
2	MS0026.10	0.72	—	—	—	—	—	—
2	MS0026.11	0.71	0.11	—	—	—	—	—
2	MS0026.14	—	—	—	0.09	—	—	0.24
2	MS0113.04	0.62	—	—	0.21	0.14	0.11	—
7	B020	—	0.88	0.78	—	—	—	—
7	B751	—	0.50	0.76	—	—	—	—
7	B753	—	0.90	—	—	—	—	—
7	GB319	—	0.85	—	—	—	—	—
7	GB372	—	0.87	0.88	—	—	—	—
7	GB482	—	—	0.84	—	—	—	—
7	KG817	—	—	0.73	—	—	—	—
7	MS0046.01	—	0.60	0.71	—	—	—	—
7	MS0046.02	—	—	0.81	0.52	0.68	0.31	0.68
7	MS0061.02	—	—	—	0.47	0.68	0.22	—
7	MS0170.02	—	0.87	1.00	—	—	—	0.75

compared with those of the parents to identify possible off-type seedlings. SSR markers segregating in the progenies were scored as “h,” “a,” or “u” for presence, absence, or unknown, respectively for each allele. Similarly, disease response was scored as “h” for resistant or “a” for susceptible. Mapping was performed using JoinMap 5 (Kyazma), with “BC1” as the population type, Kosambi as the mapping function, and a LOD threshold of ≥ 6 for grouping. After the initial mapping, scores for markers and disease response in the spreadsheet were placed in columns in order by position in the reference mapping population and inspected for conflicts. The genome coordinates of the markers were also consulted. If the scores for disease response and presence of the flanking markers were in conflict, the disease response was rescored as “u” for unknown and a new map was drawn. Initially, separate maps were constructed for progenies 13030 and 13031 segregating for resistance from Sochi selection OSU 1166.123, and then a single map was constructed for this parent from the combined data.

Results

Alignment of the sequences of SSR and ddRAD-Seq markers with the genome sequence of ‘Jefferson’ (V2) identified 17 PacBio contigs for development of additional SSR markers. A total of 5455 SSR loci with repeat motifs of 2 to 6 bp were found in these PacBio contigs. The number of SSRs by repeat length are 3008 di-nucleotide, 723 tri-nucleotide, 737 tetra-nucleotide, 223 penta-nucleotide, and 764 hexa-nucleotide repeats (Table 4). AT-rich repeats were the most abundant, accounting for 3273 (60%) of the total. Di-nucleotide repeats were the most common motifs, and only di-nucleotide repeats were pursued. After removal of the 1870 sequences with only AT repeats, 1138 di-nucleotide repeats remained. Visual screening of aligned sequences with Tablet software identified 373 SSRs as clearly polymorphic, showing variation in the number of repeats but conserved flanking regions and ≥ 3 alleles. Alignment of the 373 sequences with the ClustalW tool indicated that 22 were duplicates. A BLAST search against previously developed SSRs in the National Center for Biotechnology Information (NCBI, Bethesda, MD) GenBank indicated that 341 of the 351 nonredundant sequences had not previously been used for marker development. Nonfluorescent primers were designed from the flanking regions for 96 of them using Primer 3 software and used to amplify DNA of 24 hazelnut accessions. Of these, 45 loci were clearly polymorphic on agarose gels and had one or two bands of the expected size. The other 49 primer pairs were not investigated further because they appeared to be monomorphic on agarose gels or failed to amplify any products. Then, forward primers with a fluorescent tag were ordered, 26 with FAM and 19 with HEX (Supplemental Table 2). In the set of 45 new SSRs, the most common motifs are AG (26.6%) and CT (26.6%). The new SSRs were characterized (Table 5) using 50 hazelnut accessions. A total of 458 alleles were amplified, with an average of 10.18 per marker (range 2–23). Expected heterozygosity (H_e) averaged 0.72 and ranged from 0.374 to 0.927. Observed heterozygosity (H_o) averaged 0.64 and ranged from 0.16 to 0.94. PIC values averaged 0.68 and ranged from 0.345 to 0.912. The most informative markers were MS0046.01, MS0026.08, and MS0113.03, with PIC values of 0.91, 0.89, and 0.86, respectively. The least informative markers were MS0074.08, MS0076.08, MS0074.6, and MS0061.05, with PIC values < 0.40 . These four markers

Table 4. Number of simple sequence repeats by motif length detected in 17 contigs of the genome sequence (V2) of ‘Jefferson’ hazelnut. For each motif length, the number of sequences that only contain bases A & T and those with other motifs.

Motif length	Simple sequence repeats (no.)		
	Total	Only A & T	Other motifs
Di-nucleotide	3008	1870	1138
Tri-nucleotide	723	512	211
Tetra-nucleotide	737	522	215
Penta-nucleotide	223	109	114
Hexa-nucleotide	764	260	504
Total	5455	3273	2182

also had low observed heterozygosity ($H_o < 0.5$) and fewer than six alleles. The average frequency of null alleles in the 45 SSRs was 0.068, and the range was from -0.09 to 0.47 . The frequency of null alleles was > 0.20 for eight markers (Table 5).

Primer pairs for the 45 new SSRs were used to amplify the 138 seedlings and the parents of the reference mapping population. Of the 42 markers that segregated, 25 showed a 1:1 ratio, 13 showed a 1:1:1:1 ratio, and 4 showed a 1:2:1 ratio (Table 6). Segregation distortion was observed at marker MS0107.05 on LG2, preventing JoinMap from placing it on the map; but two other markers from the same contig mapped to LG2. The three SSRs that failed to segregate in the mapping population were assigned to a linkage group based on other SSRs developed from the same V2 contig (MS0026.07 and MS0107.04 to LG2, and MS0046.05 to LG7), which was consistent with their coordinates in the V3 genome. Of the 45 markers, 24 were assigned to LG2 and 15 to LG 7, while SSRs developed from contigs 74, 294, and 535 were placed on LGs 10, 9, and 10 respectively (Table 6). Maps are presented for LG2 (Fig. 1) and LG7 (Fig. 2). Although all segregating markers were assigned to a LG, the step-by-step procedure for map construction removed a few markers because they fell out at $LOD < 6$, and other markers were removed from the maps of one parent because of poor fit. The positions of the same markers in the maps of the female and male parent were in general agreement but showed slight shifts. A notable exception were the markers with prefix MS0113 that were not placed on LG2F because of very large gaps from the other markers, and in spite of their good fit on the map of LG2M.

DISEASE SCORES, SEGREGATION RATIOS, AND CORRELATION WITH SSR MARKERS. Segregation ratios for disease response (Table 7) fit the expected 1:1 ratio in four progenies (10015, 11024, 12030, and 14035), but ratios showed a deficiency of susceptible seedlings in three progenies (13030, 13031, and 14031). In both field-inoculated progenies of OSU 1166.123 progenies (13030 and 13031), around 63% of the seedlings were scored as resistant, and segregation in neither progeny fit a 1:1 or 3:1 ratio. Homogeneity chi-square tests showed that the data from these two progenies could be pooled ($\chi^2 = 0.002$, $P = 0.97$). In progeny 14035, segregating for resistance from H3R12P62, about 63% of the seedlings were resistant and did not fit either a 1:1 or 3:1 segregation ratio. Disease scores in seedlings of OSU 1187.101 were correlated with scores for markers on LG2; while for Moscow #2, Moscow #27, OSU 1166.123, and H3R12P62 disease scores were correlated with scores for markers on LG7 (Table 3). Progeny 14031, segregating for resistance from Rutgers University selection H3R07P11, has $\approx 63\%$ resistant seedlings. The

Table 5. Characteristics of 45 new simple sequence repeat markers developed from the genome sequence (V2) of ‘Jefferson’ hazelnut. The sequences are available from the National Center for Biotechnology Information under the listed GenBank numbers.

Marker	GenBank no.	Repeat motif	Linkage group	Alleles (no.)	Expected heterozygosity	Observed heterozygosity	PIC	Frequency of null alleles
MS0026.03	MT181628	(CT)16	2	15	0.86	0.92	0.83	-0.04
MS0026.04	MT181629	(TC)18	2	11	0.78	0.42	0.75	0.30
MS0026.07	MT181630	(AC)11	2	10	0.77	0.71	0.73	0.04
MS0026.08	MT181631	(GA)17	2	22	0.91	0.92	0.89	-0.01
MS0026.10	MT181632	(AG)10	2	6	0.47	0.42	0.43	0.07
MS0026.11	MT181633	(CT)15	2	10	0.77	0.40	0.73	0.32
MS0026.13	MT181634	(TC)17	2	10	0.80	0.33	0.77	0.42
MS0026.14	MT181635	(AG)10	2	13	0.88	0.82	0.86	0.03
MS0107.02	MT181651	(TC)18	2	9	0.82	0.76	0.78	0.04
MS0107.03	MT181652	(CT)16	2	12	0.82	0.86	0.78	-0.03
MS0107.04	MT181653	(CT)8	2	5	0.42	0.16	0.39	0.47
MS0107.05	MT181654	(AG)11	2	10	0.84	0.90	0.81	-0.04
MS0113.01	MT181655	(TC)19	2	9	0.79	0.80	0.75	-0.02
MS0113.02	MT181656	(TC)11	2	13	0.82	0.31	0.79	0.46
MS0113.03	MT181657	(AG)16	2	20	0.88	0.90	0.86	-0.02
MS0113.04	MT181658	(AG)16	2	12	0.79	0.92	0.75	-0.09
MS0113.06	MT181659	(GA)18	2	10	0.76	0.87	0.72	-0.08
MS0121.02	MT181661	(TC)16	2	11	0.81	0.80	0.77	0.00
MS0121.04	MW396735	(GA)16	2	15	0.81	0.71	0.78	0.06
MS0140.02	MT181663	(CT)14	2	7	0.67	0.74	0.60	-0.08
MS0140.04	MT181664	(CT)20	2	3	0.55	0.51	0.47	0.01
MS0140.05	MT181665	(AG)13	2	5	0.60	0.67	0.53	-0.07
MS0229.02	MT181667	(CT)12	2	16	0.81	0.70	0.79	0.07
MS0284.01	MT181670	(AG)11	2	8	0.73	0.56	0.68	0.13
MS0046.01	MT181636	(TC)22	7	23	0.93	0.94	0.91	-0.01
MS0046.02	MT181637	(CT)18	7	11	0.80	0.71	0.76	0.06
MS0046.05	MT181638	(TC)9	7	10	0.57	0.45	0.53	0.12
MS0046.07	MT181639	(GA)13	7	9	0.63	0.60	0.61	-0.01
MS0061.02	MT181640	(AC)10	7	8	0.45	0.28	0.43	0.22
MS0061.03	MT181641	(CT)12	7	9	0.70	0.62	0.65	0.08
MS0061.04	MT181642	(GA)12	7	4	0.68	0.66	0.62	0.02
MS0061.05	MT181643	(AG)15	7	4	0.46	0.49	0.37	-0.04
MS0076.03	MT181648	(GA)12	7	2	0.50	0.42	0.37	0.09
MS0076.04	MT181649	(CT)17	7	10	0.75	0.60	0.71	0.11
MS0076.08	MT181650	(AG)9	7	2	0.50	0.32	0.37	0.21
MS0126.04	MT181662	(TC)16	7	21	0.87	0.80	0.85	0.04
MS0170.02	MT181666	(AG)12	7	12	0.72	0.72	0.68	0.00
MS0230.01	MT181668	(CT)15	7	12	0.86	0.88	0.83	-0.02
MS0230.03	MT181669	(GA)16	7	15	0.83	0.80	0.80	0.02
MS0294.03	MT181671	(AC)8	9	11	0.86	0.76	0.83	0.06
MS0074.02	MT181644	(TC)15	10	12	0.78	0.81	0.74	-0.03
MS0074.03	MT181645	(AG)15	10	10	0.80	0.82	0.76	-0.02
MS0074.06	MT181646	(CT)10	10	2	0.50	0.28	0.37	0.27
MS0074.08	MT181647	(AG)11	10	5	0.37	0.40	0.35	-0.07
MS0535.01	MT181672	(TC)12	10	4	0.73	0.69	0.68	0.02
Mean				10.2	0.72	0.65	0.68	0.07

PIC = polymorphism information content.

disease response ratio did not fit either a 1:1 or 3:1 segregation ratio [1:1 ratio: $\chi^2 = 4.26$, $P = 0.038$; and 3:1 ratio: $\chi^2 = 4.35$, $P = 0.037$ (Table 7)]. Disease response in this progeny was not correlated with marker scores for SSRs on LG6, LG2, or LG7 ($r < 5$), indicating that resistance may be on one of the other eight chromosomes.

MAPPING OF EFB RESISTANCE. After the linkage group had been identified, the newly developed SSR markers and several

easy-to-score SSR markers previously assigned to that LG and located within 20 cM of a previously mapped resistance locus were used to amplify the parents and seedlings. Maps were constructed for six of the seven progenies, all except for H3R07P11. All seedlings in each progeny were genotyped with the selected SSR markers. In progeny 13031 (OSU 1166.123 $\times$ OSU 1236.134), comparison of SSR marker scores in the seedlings and parents indicated that 17 of the 94 seedlings were not from

Table 6. Segregation at 42 new simple sequence repeat marker loci in the hazelnut (*Corylus avellana*) reference mapping population (OSU 252.146 x OSU 414.062) and linkage group assignment.

Marker	Parent alleles	Segregation ratio		χ^2			Allele and linkage group
		Expected	Observed	df	Value	P	
MS0026.03	294/294 × 281/296	1:1	65:62	1	0.071	0.790	296 on 2M
MS0026.04	205/205 × 195/205	1:1	69:76	1	0.338	0.561	195d on 2M (poor fit)
MS0026.08	277/287 × 271/283	1:1:1:1	26:38:41:28	3	4.895	0.180	277 on 2F, 271 on 2M
MS0026.10	251/255 × 251/257	1:1:1:1	38:29:34:37	3	1.420	0.701	255d on 2F, 257 on 2M
MS0026.11	281/293 × 295/null	1:1:1:1	31:36:41:29	3	2.533	0.469	281 on 2F, 295 on 2M
MS0026.13	242/260 × 260/260	1:1	72:66	1	0.261	0.609	242 on 2F
MS0026.14	260/262 × 270/270	1:1	67:71	1	0.116	0.733	260 on 2F
MS0107.02	162/162 × 166/170	1:1	72:64	1	0.471	0.493	170 on 2M
MS0107.03	216/216 × 203/209	1:1	72:64	1	0.471	0.493	209 on 2M
MS0107.05	208/208 × 210/212	1:1	90:48	1	12.783	0.000	unmapped (distorted)
MS0113.01	277/277 × 273/288	1:1	63:70	1	0.368	0.544	288 on 2M
MS0113.02	264/264 × 264/272	1:1	59:69	1	0.781	0.377	272 on 2M
MS0113.03	193/213 × 210/210	1:1	64:66	1	0.031	0.861	193 on 2F
MS0113.04	199/211 × 201/203	1:1:1:1	34:33:41:33	3	1.270	0.736	212 on 2F
MS0113.06	313/315 × 313/315	1:2:1	30:76:23	2	4.860	0.088	312hk on 2F (poor fit)
MS0121.02	259/262 × 269/272	1:1:1:1	39:36:27:33	3	2.333	0.506	262 on 2F, 272 on 2M
MS0121.04	250/255 × 257/257	1:1	61:70	1	0.618	0.432	250 on 2F
MS0140.02	259/259 × 259/261	1:1	72:68	1	0.114	0.735	261 on 2M
MS0140.04	132/132 × 130/136	1:1	72:66	1	0.261	0.610	136 on 2M
MS0140.05	280/280 × 278/284	1:1	71:66	1	0.182	0.669	284 on 2M
MS0229.02	287/287 × 275/null	1:1	63:60	1	0.073	0.787	null on 2M
MS0230.01	195/195 × 208/210	1:1	65:72	1	0.358	0.550	208 on 2M
MS0230.03	266/266 × 260/268	1:1	70:61	1	0.618	0.432	268 on 2M
MS0284.01	259/259 × 253/259	1:1	70:69	1	0.007	0.932	253d on 2M
MS0046.01	278/291 × 270/270	1:1	57:66	1	0.659	0.417	291 on 7F
MS0046.02	171/187 × 179/183	1:1:1:1	32:34:33:39	3	0.841	0.840	187 on 7F, 183 on 7M
MS0046.07	139/139 × 139/149	1:1	68:77	1	0.694	0.405	149 on 7M
MS0061.02	176/182 × 182/187	1:1:1:1	32:44:34:35	3	2.338	0.505	176 on 7F, 187 on 7M
MS0061.03	85/89 × 85/91	1:1:1:1	34:36:32:44	3	2.274	0.518	89 on 7F, 91 on 7M
MS0061.04	178/180 × 178/180	1:2:1	34:69:42	2	1.221	0.543	180 hk on 7F with MS0061.02-176F
MS0061.05	259/259 × 259/261	1:1	65:79	1	1.361	0.243	261 on 7M
MS0076.03	169/171 × 169/171	1:2:1	32:71:41	2	1.153	0.562	171hk on 7F with GB467
MS0076.04	284/291 × 284/291	1:2:1	42:72:32	2	1.397	0.497	284hk on 7F, 180hk on 7M
MS0076.08	172/172 × 168/172	1:1	76:68	1	0.444	0.505	168 on 7M
MS0126.04	278/291 × 289/null	1:1:1:1	32:31:40:32	3	1.563	0.668	291 on 7F, 289 on 7M
MS0170.02	139/141 × 139/148	1:1:1:1	35:36:34:41	3	0.795	0.851	141 on 7F, 184 7M
MS0074.02	255/255 × 255/268	1:1	59:80	1	3.173	0.075	268d on 10M near KG826
MS0074.03	189/201 × 201/209	1:1:1:1	37:33:27:48	3	6.476	0.091	189d on 10F near GB949, 209d on 10M near KG826
MS0074.06	205/205 × 203/205	1:1	81:63	1	2.250	0.134	203d on 10M near GB724
MS0074.08	154/156 × 154/154	1:1	70:74	1	0.111	0.739	156 on 10F near AC01-840d
MS0294.03	203/204 × 199/203	1:1:1:1	35:33:34:42	3	1.389	0.708	204 on 9F near 636-350, 199 on 9M near GB725
MS0535.01	244/267 × 240/243	1:1:1:1	37:31:41:29	3	2.638	0.451	267 on 10F near AC01-840d, 243 on 10M near 363-1600

those parents. Hence, they were removed, and a new map constructed for the progeny. After the initial mapping, comparison of disease scores revealed a few conflicts with the scores of the flanking SSR markers. Some seedlings lacked cankers, but the SSR marker data indicated susceptibility. We considered them to have escaped infection, changed the disease phenotype from “resistant” to “unknown,” and then redrew the map. A few seedlings were scored as susceptible while marker data indicated resistance. This type of conflict may have resulted from phenotyping errors or recombination between the marker and

disease resistance loci. An additional possibility is that resistant seedlings occasionally develop small cankers (Lunde et al., 2006); and in fact, trees of ‘Jefferson’ and ‘McDonald’ with ‘Gasaway’ resistance in orchards under high disease pressure trees occasionally develop cankers (Pscheidt and Ocamb, 2020). In these situations, the disease score was changed to “unknown,” and the map redrawn. *A. anomala* has a 2-year life cycle with a 15-month latent period; and although the April assessment of disease response was only 13 months after the inoculation, the August assessment was 17 months after inoculation. The map

2F

2M

Fig. 1. Maps of hazelnut linkage group 2 (LG2) in female parent OSU 252.146 and male parent OSU 414.062 in the reference mapping populations constructed using simple sequence repeat markers. New markers are indicated with an asterisk (*), and markers common to the maps of the parents are underlined.

for progeny 10015 placed resistance from OSU 1187.101 at the proximal end of LG2 closest to marker B504 (Fig. 3). The map for progeny 11024 (Fig. 4) placed resistance from Moscow #2 on LG7 between markers B020 and MS0046.01 on one side, and B751 on the other side. The map for progeny 12030 (Fig. 4) placed resistance from Moscow #27 on LG7 between markers B020 and GB482. The map for progenies 13030 and 13031 (Fig. 4) segregating for resistance from OSU 1166.123 placed resistance on LG7 between markers B509 and B753. The map for progeny 14035 (Fig. 4) placed resistance from Rutgers University selection H3R12P62 on LG7, co-segregating with markers B020, B753, and MS0046.07.

Discussion

In silico approaches for developing polymorphic SSR markers have gained in popularity because of their efficiency and low cost compared with traditional methods (Hoffman and Nichols, 2011; Vukosavljev et al., 2015; Zhang et al., 2013). In silico SSR detection has been reported in different crop species including tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), and rice (*Oryza sativa*) (Tang et al., 2008), *Typha minima* (Csencsics et al., 2010), peanut [*Arachis hypogaea*

(Shirasawa et al., 2012)], and pistachio [*Pistacia vera* (Kho-daeiaminjan et al., 2018)]. In hazelnut, Boccacci et al. (2015), Colburn et al. (2017), and Bhattarai and Mehlenbacher (2017, 2018) used in silico mining to develop SSRs from expressed sequence tags (ESTs), transcriptome, and genomic sequences, respectively. In this study, genomic SSRs were developed in two regions where EFB resistance had previously been mapped. In 17 PacBio sequence contigs of 'Jefferson', a total of 5455 microsatellites were detected, and primers designed for a subset of dinucleotide repeats. Additional ones could be pursued in the future. Many of those considered "not clearly polymorphic" on agarose gels would likely show polymorphism if scored by capillary electrophoresis. For the markers that were difficult to score, new primers could be designed or pig-tailing pursued (Brownstein et al., 1996). Only di-nucleotide repeats were pursued because they tend to have higher polymorphism and because SSRs with longer repeat motifs had been developed by Bhattarai and Mehlenbacher (2017, 2018) and Colburn et al. (2017). Based on the results of Gürcan and Mehlenbacher (2010b) and Boccacci et al. (2015), we expect many of these SSRs to be useful in other species of *Corylus* and the related genera *Betula* and *Alnus*.

Of the 45 new SSR marker loci, segregation in the mapping population was observed at 42 of them. On LG2 (Scaffold 1), 24

7F

7M

Fig. 2. Maps of hazelnut linkage group 7 (LG7) in female parent OSU 252.146 and male parent OSU 414.062 in the reference mapping populations constructed using simple sequence repeat markers. New markers are indicated with an asterisk (*), and markers common to the maps of the parents are underlined.

SSRs were developed from 7 contigs, while 15 SSRs developed from 6 contigs were placed on LG7 (Scaffold 7). The coordinates of the new SSR markers were identified in the V2 and V3 'Jefferson' genomes (Supplemental Table 3). The markers on LG2 are on scaffold 1 in positions from 47,025 bp to 17,687,764 bp. The markers on LG7 are on scaffold 7 in positions from 1,191,371 to 17,584,843. On the linkage maps, as in the V3 genome, the new SSR markers cover large distances. Of the newly developed SSR loci, the most useful are easily scored markers

with high PIC values and low frequency of null alleles. On LG2 (Fig. 1), MS0026.08, MS0026.10, MS0026.11, and MS0026.14 are four that meet these criteria and are in the region where EFB resistance from Georgian selection OSU 759.010 (Sathuvalli et al., 2011b) and H3R07P25 from Holmskij, Russia (Honig et al., 2019) was mapped. On LG7 (Fig. 2), MS0046.02, MS0046.07, MS0061.02, MS0061.03, MS0061.05, MS0076.08, and MS0170.02 meet these criteria and are in the region where

Table 7. Segregation for eastern filbert blight resistance in seven hazelnut (*Corylus avellana*) progenies.

Progeny	Parents (female × male)	Origin of resistance	Inoculation method	Plants (no.)		Expected ratio	Chi-squared ^z	
				Resistant	Susceptible		Value	P
10015	OSU 1187.101 × OSU 1155.049	Holmskij, Russia (RUS-10)	Structure	67	52	1:1	1.891	0.169
11024	OSU 814.073 × Moscow #2	Moscow, Russia	Structure	46	40	1:1	0.419	0.517
12030	OSU 1269.020 × Moscow #27	Moscow, Russia	Structure	39	31	1:1	0.914	0.339
13030	OSU 1166.123 × OSU 1235.078	Sochi, Russia	Field	50	29	1:1	5.582	0.018
13031	OSU 1166.123 × OSU 1236.134	Sochi, Russia	Field	49	28	1:1	5.727	0.017
14031	Sacajawea × H3R07P11	Crimea (RUS-26)	Structure	38	22	1:1	4.266	0.039
14035	H3R12P62 × OSU 1378.046	Crimea (RUS-23)	Structure	34	20	1:1	3.629	0.056

^zOne degree of freedom for all Chi-squared tests.

LG2-Holmskij

Fig. 3. Map of linkage group 2 (LG2) in hazelnut progeny 10015 segregating for OSU 1187.101 resistance from Holmskij, Russia.

populations without a translocation involving LG2 and LG7 would clarify the marker positions.

Resistance was expected to be controlled by a single locus and that each resistant parent was heterozygous. However, deviation from the expected 1:1 segregation ratio (resistance: susceptible) was seen in four progenies in this study. In several previous studies, segregation distortion for EFB response was observed for 'Culpla', 'Crvenje', Russian OSU 495.072 (Colburn et al., 2015), 'Uebov' (Bhattarai et al., 2017a), and 'Zimmerman' (Lunde et al., 2006). In some progenies, there is an excess of resistant seedlings; while in others, there is an excess of susceptible seedlings. Segregation distortion has been documented in various plant species for several traits and is attributed to a diverse array of mechanisms. Previous mapping revealed segregation distortion at loci on

EFB resistance from 'Ratoli' was mapped (Sathuvalli et al., 2011a). These new SSR markers will be useful in future studies of EFB resistance, cultivar fingerprinting, diversity studies, and marker-assisted selection, including the pyramiding of resistance genes. Future mapping efforts in

LG2 and LG7, and the merger of the two linkage groups in the map of the male parent (Mehlenbacher and Bhattarai, 2018; Mehlenbacher et al., 2006). This was attributed to a reciprocal translocation involving LG2 and LG7 (Komaie Koma, 2020; Salesses, 1973; Salesses and Bonnet, 1988). Komaie Koma (2020) used

LG7-Moscow#2

LG7-Moscow#27

LG7-Sochi

LG7-Crimea

Fig. 4. Maps of linkage group 7 (LG7) in hazelnut progenies segregating for resistance from four sources: Moscow #2 (progeny 11024), Moscow #27 (progeny 12030), OSU 1166.123 (progenies 13030 and 13031), and Rutgers University selection H3R12P62 (progeny 14035).

bioinformatic methods to disentangle the “pseudo-linkage” of markers on LG2 and LG7 in the male parent (OSU 414.062). The use of populations segregating for disease response and without a translocation involving LG2 and LG7 would clarify the positions of the resistance loci and markers.

In this study, resistance from Moscow selections #2 and #27, OSU 1166.123, and H3R12P62 was placed on LG7; and resistance from OSU 1187.101 was placed on LG2. All five are different from the ‘Gasaway’ resistance (LG6) that has been extensively used in the OSU hazelnut breeding program. The use of common SSR markers in mapping allows alignment of the various maps of the same LG for different progenies. These new resistance sources and linked SSR markers will allow MAS, including the pyramiding of resistance genes, and facilitate the development of new cultivars with durable EFB resistance.

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Supplemental Table 1. Pedigrees of seven hazelnut progenies segregating for resistance to eastern filbert blight. Resistant selections are underlined; all others are susceptible.

10015 = OSU 1187.101 x OSU 1155.049
OSU 1187.101 from seeds purchased in Holmskij, Russia (RUS- #2)
 OSU 1155.049 = OSU 517.030 x OSU 556.046
 OSU 517.030 = OSU 255.003 x Tonda di Giffoni
 OSU 255.003 = OSU 43.098 x Casina
 OSU 43.098 = Montebello x Casina
 OSU 556.046 = Römische Nuss x OSU 54.039
 OSU 54.039 from seeds collected in Giresun, Turkey

11024 = OSU 814.073 x Moscow #2
 OSU 814.073 = OSU 486.001 x Tonda Pacifica
 OSU 486.001 = OSU 225.077 x Brixnut
 OSU 225.077 = Tombul Ghiaghli x OSU 42.103
 OSU 42.103 = Montebello x Compton

12030 = OSU 1269.020 x Moscow #27
 OSU 1269.020 = OSU 593.071 x OSU 806.021
 OSU 593.071 = Segorbe x OSU 265.139
 OSU 265.139 = Tombul (Extra Ghiaghli) x Willamette
 OSU 806.021 = Waterloo x OSU 384.095
 OSU 384.095 = Casina x OSU 55.129
 OSU 55.129 = Tonda Gentile delle Langhe x Tombul (Extra Ghiaghli)

13030 = OSU 1166.123 x OSU 1235.078
OSU 1166.123 from seeds collected in Sochi, Russia
 OSU 1235.078 = OSU 539.031 x OSU 772.046
 OSU 539.031 = OSU 275.031 x Sant Pere
 OSU 275.031 = Montebello x OSU 74.37
 OSU 74.037 = 14.084 x OSU 17.068
 OSU 14.084 = Barcelona x Daviana
 OSU 17.068 = Barcelona x Tombul Ghiaghli
 OSU 772.046 = OSU 444.067 x OSU 383.087
 OSU 444.067 = Willamette x OSU 54.021
 OSU 54.021 from seeds collected in Giresun, Turkey
 OSU 387.087 = Casina x OSU 55.129
 OSU 55.129 = Tonda Gentile delle Langhe x Tombul (Extra Ghiaghli)

13031 = OSU 1166.123 x OSU 1236.134
 OSU 1236.134 = OSU 593.071 x OSU 776.095
 OSU 593.071 = Segorbe x 265.139
 OSU 265.139 = Tombul (Extra Ghiaghli) x Willamette
 OSU 776.095 = Tonda Pacifica x OSU 443.129
 OSU 443.129 = OSU 183.060 x OSU 54.056
 OSU 183.060 = Montebello x OSU 14.084
 OSU 14.084 = Barcelona x Daviana
 OSU 54.056 from seeds collected in Giresun, Turkey

14031 = Sacajawea x Rutgers H3R07P11
Rutgers H3R07P11 from seeds purchased from a roadside vendor near Simferopol, Crimea

14035 = Rutgers H3R12P62 x OSU 1378.046
Rutgers H3R12P62 from seeds purchased from a roadside vendor near Simferopol, Crimea
 OSU 1378.046 = OSU 943.020 x OSU 885.056
 OSU 943.020 = OSU 617.015 x OSU 495.045
 OSU 617.015 = OSU 311.046 x Clark
 OSU 311.046 = OSU 23.017 x Tonda Gentile delle Langhe
 OSU 23.017 = Barcelona x Tombul (Extra Ghiaghli)
 OSU 495.045 from seeds received from southern Russia
 OSU 885.056 = OSU 539.044 x Tonda Pacifica
 OSU 539.044 = OSU 275.031 x Sant Pere
 OSU 275.031 = Montebello x OSU 74.37
 OSU 74.037 = 14.084 x OSU 17.068
 OSU 14.084 = Barcelona x Daviana
 OSU 17.068 = Barcelona x Tombul Ghiaghli

Supplemental Table 2. Primer sequences of 45 polymorphic simple sequence repeat markers in hazelnut.

Marker	LG ²	Primer sequence		Size (bp) ³
		Forward	Reverse	
MS0026.03	2	TACCGTCGAAAGCTGACTC	CGTGCCAAACATACAAGCAG	295
MS0026.04	2	TGAGCCAACAGAGTCCCTA	TTTCAGCCCTTCCGATAGAG	206
MS0026.07	2	CTTTTCTCCCTGGGTCCAT	ATCCCAATTCCTGCATCAAC	287
MS0026.08	2	TAACCAAACGGGTGGAGAGA	TGCCTTTGATTTCCGATCT	288
MS0026.10	2	TTAATGCTGCTCCGACAA	GTATGCGGCTGCTGTATGGT	257
MS0026.11	2	TTAACTGAACCGAGCCGAAC	TAAGCCACCGACATGGTAG	295
MS0026.13	2	CCCTTCAAACACCCATTG	TTCATGGTGTAAGCCGAGA	261
MS0026.14	2	GGTCTGTTGATTTGAGCAA	AAATGGGCTTTGAGTCATGG	262
MS0107.02	2	TGCTTGTAACGACTCAATACA	TGCAGCCTAAAGTCTTTTCC	172
MS0107.03	2	CACACGCAATTCTCACCATT	CCTGCATAGGAATCCCCTTT	209
MS0107.05	2	GTGGGCATCAGATCACAAA	TCGACCTGGAAGAGATGACC	207
MS0113.01	2	TCCCTCACCCACCATTTTA	CCTTCGCAAAATCACAAACA	287
MS0113.02	2	TGGGATTCCACCTATCAG	CACCTTTAACTGACGGAATGAGA	271
MS0113.03	2	CCCCTATTTTGCCATTTT	TGCCTGAAAACTCTGCATT	210
MS0113.04	2	CCACTCTTCTTGCTTGCT	CTTCTGCCGCTAGAGTGCTT	214
MS0113.06	2	TCAGGCAATCAAGTGGGTTA	AAGAGGTTTTCGGTTACCA	315
MS0121.02	2	AAGCAAAGAAAGGGCGAGAT	AGGACTCCAAGCGTGGAT	272
MS0121.04	2	CAAGATGGCCTGCAATAAGC	CTGCCGATCTCTCTCTC	255
MS0140.02	2	AACTGAGCAAACCCACAA	TGCGTCAGGCTGTAGTTGTT	264
MS0140.04	2	GGAGTTGCTTGCCTAATCG	CTCTCCCTCAACCAATCAG	136
MS0140.05	2	TCTGCTCTGGCTTGGCTAAA	AAGCAAAAACGAGGGAAA	285
MS0229.02	2	AGAAACAATGCAATCCCAGA	TGTACGGAGATGACAAGAGGTG	285
MS0230.01	2	CCGAGTACATCTCCATGCAA	CCCTTCTCCCACTTTTCTT	209
MS0230.03	2	CCATCACCCAGATCAAATCC	ACCCTCACGACAGCTACC	263
MS0284.01	2	GATCAAATCCACACCAAGC	AAGAAGCAAACCTCACAGC	258
MS0046.01	7	CAGTCGAGGACCCATCTCTC	GGGGCTTATCAGATTGCTGT	296
MS0046.02	7	GAAAACGATCCCTCTCGTCA	GGTCCGAGGATCAGGAATT	188
MS0046.07	7	AGGCAAAATGCACATCACAA	CCAAGGAACGAAAGCAAGAG	143
MS0061.02	7	TCACATCATCACCTCCAAAGTC	GGAGAGGAGGAGACCAAGAA	183
MS0061.03	7	CCCAGGTCTCTCCCTCTCTC	TGCCTTTTGTGTCATGAA	92
MS0061.04	7	GTTGTGGTGCCAAAGAATCC	AGCAAAATCCCTCTCTCC	181
MS0061.05	7	GCACTACGTACCCACTTCA	TTGTTGCACAGGAGATGG	256
MS0076.03	7	GAGCCGACACCTTCTAGGC	TGACCAATCCCTCTCAA	172
MS0076.04	7	AACCATTTCTGAAGCAAGC	GCTAGCGGTTACCATGTTT	285
MS0076.08	7	AGAGCAAGAGCAACGGAGAG	CGGGTTGCGGTATAAGAAT	170
MS0126.04	7	CCGGAAAATAGGGCACTTCT	CCTGGCAAGATGACCAGTTT	290
MS0170.02	7	CCAGGACACAAATCTCAAACC	GCACCAGAGCTTTGAATGG	144
MS0074.02	10	CCCACAAATTCAGCTTGAG	CGGTGGAGGAGTTGATGAGT	265
MS0074.03	10	AACACCATGTAAAGCAACTG	GCAAGTGTCTGCCCATTTC	205
MS0074.06	10	TCTGCCACAACATTGAAAGC	CTCTGACGTTCTTGTTGGT	205
MS0074.08	10	GCCCAACCAATTTCACT	GCCTCAGCAGTACCTCTTC	153
MS0294.03	3	CCCATCAAAATCCCATACC	TGTTGCTTCACATCATCCAA	207
MS0535.01	10	GCGTCAAAATCGCTCTCTCT	GCCACCCGAGTAAGAATTA	266

² Linkage group based on genetic mapping and scaffold in the 'Jefferson' genome sequence (V3).

³ Expected size based on the contig sequence in the 'Jefferson' genome (V2) used for primer design.

Supplemental Table 3. Coordinates of 45 new simple sequence repeat markers in the genome sequence of 'Jefferson' hazelnut (V2 and V3). Also shown are the V3 coordinates of simple sequence repeats previously placed on the maps of linkage groups 2 and 7 in both parents.

LG	Marker	Coordinates (V2)			Coordinates (V3)		
		Contig	Start	End	Scaffold	Start	End
2	MS0230.03	230	481968	482499	1	47010	47542
2	MS0230.01	230	44477	45006	1	484503	485033
2	GB722				1	807056	807585
2	MS0284.01	284	20442	20963	1	877686	878208
2	MS0229.02	229	81021	81544	1	1402327	1402851
2	MS0107.05	107	639095	639616	1	2467432	2467954
2	MS0107.03	107	373893	374424	1	2732624	2733156
2	MS0107.02	107	243481	244016	1	2863032	2863568
2	MS0140.05	140	724995	725520	1	3922023	3922533
2	MS0140.04	140	545159	545698	1	4360840	4361380
2	MS0140.02	140	210151	210678	1	4695860	4696388
2	MS0026.03	26	530036	530567	1	6191882	6192414
2	MS0026.04	26	739774	740309	1	6401620	6402156
2	MS0026.07	26	1311185	1311706	1	6973031	6973553
2	MS0026.08	26	1526448	1526981	1	7188294	7188828
2	MS0026.10	26	1909574	1910093	1	7571420	7571940
2	MS0026.11	26	2151015	2151544	1	7812861	7813391
2	MS0026.13	26	2507884	2508417	1	8169730	8170264
2	MS0026.14	26	2752198	2752717	1	8414044	8414564
2	GB827				1	13471867	13472343
2	MS0113.01	113	51754	52291	1	15009616	15010154
2	MS0113.02	113	259277	259798	1	15217139	15217661
2	MS0113.03	113	503338	503869	1	15461200	15461732
2	B501				1	15543392	15543579
2	MS0113.04	113	658259	658790	1	15616121	15616653
2	MS0113.06	113	862705	863240	1	15820567	15821103
2	B011				1	16174207	16174783
2	GB889				1	16305669	16306107
2	MS0121.02	121	274642	275173	1	17076258	17076790
2	MS0121.04	121	885620	886151	1	17687236	17687768
7	GB307				7	1107239	1107929
7	MS0170.02	170	229380	229903	7	1191369	1191893
7	GB372				7	1228690	1229210
7	MS0076.08	76	1452827	1453344	7	4533752	4534270
7	GB319				7	4774917	4775312
7	MS0076.04	76	694194	694727	7	5292369	5292903
7	MS0076.03	76	469409	469932	7	5517164	5517688
7	GB822				7	5561744	5562253
7	B020				7	5989286	5989874
7	B753				7	7146391	7146801
7	MS0046.07	46	1435084	1435609		7720735	7721261
7	B733				7	8051584	8051958
7	MS0046.02	46	233137	233672	7	8922672	8923208
7	MS0046.01	46	112816	113359	7	9042985	9043529
7	B751				7	14541791	14542211
7	MS0126.04	126	857591	858122	7	15166514	15167046
7	MS0061.02	61	301442	301961	7	16276619	16277139
7	MS0061.03	61	556683	557206	7	16531860	16532384
7	MS0061.04	61	1202097	1202620	7	17177274	17177798
7	BR238				7	17178112	17178711
7	MS0061.05	61	1609138	1609667	7	17584315	17584845
7	KG840				7	20781605	20782387