A Linkage Map of Sweet Cherry Based on RAPD Analysis of a Microspore-Derived Callus Culture Population

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A partial linkage map was constructed for the sweet cherry (Prunus avium L.) cultivar Emperor Francis from a population of 56 microspore-derived callus culture individuals. The callus cultures were genotyped for two allozymes and 90 random amplified polymorphic DNA (RAPD) markers using 79 random decanucleotide DNA primers and the polymerase chain reaction (PCR). Eighty-nine markers mapped to 10 linkage groups totaling 503.3 cM. DNA blot and hybridization analysis using five cloned RAPDs as probes demonstrated that one of the decanucleotide primers amplified a region of the Emperor Francis genome containing a unique sequence, whereas the other four decanucleotide primers amplified regions of the Emperor Francis genome containing repeated sequences. The five cloned RAPD probes also recognized putative homologous regions in ground cherry, P. fruticosa Pall., and sour cherry, P. cerasus L., a naturally occurring allopolyploid between P. fruticosa and P. avium.

Marker-assisted selection would be especially advantageous for sweet and sour cherry (Prunus avium L. and P. cerasus L., respectively) breeding. Cherry seedlings require a minimum of 3-5 years of growth before they flower and fruit. If prior knowledge of linkage relationships between marker loci and important flower and fruit characteristics were available, undesirable individuals could be eliminated from progeny populations and more resources (i.e., planting spaces, evaluation time, etc.) could be devoted to the promising individuals.

Despite the potential utility of a genetic linkage map for sweet and sour cherry, linkage map development in cherry is behind other Prunus mapping efforts. In Prunus, linkage maps are the most advanced in the diploid species: peach (P. persica) and almond (P. amygdalus, syn. P. dulcis) and peach × almond and peach × P. davidiana interspecific hybrid populations (Arós et al. 1994; Chaparro et al. 1994; Dirlewanger E, personal communication; Dirlewanger et al. 1995; Foolad et al. 1995; Rajapakse et al. 1994, 1995; Viruel et al. 1995). For example, a 107 marker genetic linkage map covering about 800 cM has been constructed for peach × almond (Foolad et al. 1995). In contrast, only 26 isozyme loci and one morphological trait have been mapped in the diploid sweet cherry (n = 8) (Tobutt and Nicoll 1992; Tobutt K, personal communication). In the tetraploid sour cherry (n = 16), linkage map construction has not begun.

Linkage map development in sour cherry is complicated due to the species polyploid origin. The presumed progenitor species of the tetraploid sour cherry are the diploid sweet cherry and the tetraploid ground cherry (P. fruticosa) (Olden and Nybom 1968). Although sour cherry exhibits disomic inheritance, there is evidence for occasional intergenomic pairing characteristic of a segmental allopolyploid (Beaver and lezzoni 1993). Toward the goal of constructing a linkage map of a segmental allopolyploid, we chose for simplicity, to construct a linkage map in sweet cherry. This would be essentially equivalent to mapping one genome in sour cherry.

Our objective was to construct a linkage map in sweet cherry using a microspore-derived callus culture mapping population and RAPDs (Welsh and McClelland 1990; Williams et al. 1990) as the markers. RAPD markers were chosen because they have been shown to be abundant, highly polymorphic, and require less DNA than restriction fragment length polymorphisms (RFLPs). A microspore-derived callus culture population was used because a segregating population for sweet cherry was not available. Additionally, because the tissue to be screened would be haploid,
heterozygous loci that cannot be scored in a majority of the random primer amplifications would not occur, increasing the efficiency of the linkage analysis. This haplotyping strategy, which had originally been developed for sperm typing (Li et al. 1988), has accelerated linkage map development in many previously difficult to map species. For example, extensive linkage maps have been constructed using RAPD markers and haploid populations of megagametophytes in Gymnosperm tree crops (Tulsieram et al. 1992) and embryos in zebrafish (Danio rerio) (Postlethwait et al. 1994). Finally, associating horticulturally important traits with molecular markers, not possible without regenerating plants from the callus cultures, was not an objective. Instead, our future goal is to use the markers mapped in sweet cherry as possible single dose restriction fragments (Wu et al. 1992) to construct a linkage map in sour cherry. As a result, our second objective was to determine if cloned RAPDs that mapped in sweet cherry would recognize putative homologous regions in sour and ground cherry.

Materials and Methods
Plant Material
Fifty-six microspore-derived callus cultures from the sweet cherry cultivar Emperor Francis were used for linkage mapping. Production of the microspore-derived callus cultures used as the mapping population is described elsewhere (Long et al. 1994). To eliminate maternal and multiple microspore-derived callus cultures, each culture was previously screened for two allozymes heterozygous in Emperor Francis, 6-phosphogluconate dehydrogenase locus 1 (6-Pgd-1) and glucose phosphate isomerase locus 2 (Pgi-2) (Long et al. 1994). To minimize variation due to somatic mutation while in culture, each individual callus was subdivided into and maintained as two distinct subcultures at the second transfer. After this subdivision, ~10 transfers were performed prior to DNA extraction. The DNAs from each of the two subcultures were isolated and served as replicates through the RAPD analyses.

All field-grown plant material used in this study was maintained at the Clarksville Horticultural Experiment Station, Clarksville, Michigan, as part of the Prunus germplasm collection at Michigan State University (MSU). The following selections were used: Emperor Francis sweet cherry, clone 323-2 ground cherry (2n = 32), and Rheinische Schattenmorelle and Erdi Botermo sour cherry (2n = 32).

DNA Isolation
Genomic DNA was isolated from leaf and callus tissues using a modification of the method of Murray and Thompson (1980). Callus tissue (0.1-0.3 g fresh weight) was lyophilized and then ground to a fine powder in a microfuge tube using a 1 ml pipette tip with a flame-sealed end. After 10-20 s of vigorous grinding, 400 ml of extraction buffer (0.2 M Tris pH 8.5, 5 mM EDTA, 0.7 M NaCl, 0.25% CTAB, 125 mM sorbitol, and 2% 2-mercaptoethanol) was added. Once the tissue was completely dispersed in the buffer, samples were incubated at 60°C for 20-30 min. One volume of chloroform/isooamyl alcohol (24:1) was added, and the samples were gently inverted until complete emulsification was achieved. After centrifugation (18,000 x g), the aqueous phase was removed to a new tube. One milliliter of 5% CTAB was added, the samples were inverted several times, and then they were centrifuged at 2,000 x g for 5 min. The supernatant was discarded, and the precipitate was resuspended in 400 ml TNE (10 mM Tris pH 8.0, 0.7 M NaCl, 0.5 mM EDTA). Two volumes of 100% ethanol were added, the samples were inverted several times, and then they were centrifuged at 18,000 x g for 2 min. The DNA pellet was washed twice with 70% ethanol, dried, and resuspended in 600 ml of TE (10 mM Tris pH 8.0, 0.5 mM EDTA). The resulting DNA concentrations were ~10 ng/ml.

Isolation of Prunus spp. genomic DNA from leaf tissue for DNA blot analysis followed the procedure described above but was scaled up 40-fold using 50-ml Oak Ridge tubes and a Sorval SS34 rotor.

Polymerase Chain Reaction
The PCR reactions were carried out in 25-ml reaction volumes using ~25 ng target DNA, 16.5 ng decanucleotide primer DNA, 10 mM Tris pH 8.5, 50 mM KCl, 0.2 mM of each dNTP, 3 mM MgCl2, 0.13 U Taq DNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN) overlaid with a drop of mineral oil. A 7-min denaturation at 94°C was followed by 47 cycles using the conditions established by Williams et al. (1990): 94°C 1 min, 36°C 1 min, and 72°C 2 min. A 5-min incubation at 72°C followed the final cycle. RW1 primer was synthesized at the MSU Macromolecular Structure Facility and has the sequence 5' GTGCCGAAGAC-3'. All other 10-mer oligonucleotides were obtained from Operon Technologies (Alameda, CA). and are designated by the k letter and primer number. Amplifications were performed on a Perkin Elmer Cetus (Emeryville, CA) Thermo Cycler model 480. Amplified products were loaded onto 1.0-1.2% agarose gels and electrophoresed in TAE buffer (40 mM Tris-acetate pH 8.0, 1 mM EDTA) from 500 to 650 volt hours. Bands stained with ethidium bromide were scored based on fragment presence or absence.

All primers were initially scored on Emperor Francis leaf tissue and a small test population consisting of eight microspore-derived individuals (two subcultures per individual). Any amplified band present in Emperor Francis leaf tissue that segregated in the test population was considered polymorphic, heterozygous in Emperor Francis, and was subsequently used to score the 48 remaining individuals. If the two replicates (subcultures) in the 48 remaining individuals did not agree, that individual was not scored for the band in question.

Linkage Analysis
The RAPD fragments and allozymes were tested for fit to 1:1, 2:1, or 1:2 ratios using x2 tests. Those fragments with an x2 value >0.05 for a 1:1, 2:1, or 1:2 ratio were assumed to represent Mendelian genetic loci and were included in the linkage analysis using the IBM version of MAPMAKER (Lander et al. 1987). Our intent was to reject for linkage analysis any marker that did not fit a 1:1, 2:1, or 1:2 ratio, because higher ratios could be explained not only by segregation distortion, but by band co-migration of unlinked amplification fragments. For example, a 3:1 (+/-) ratio could be explained by co-migration of two unlinked PCR products. The data was entered using a modified backcross data file permitting the identification of both coupling (+/-/-) and repulsion (+/-/-) phase linkages (Nelson et al. 1993). Linkage groups were identified by three point analysis with a LOD score of ≥3.0 and the Kosambi mapping function. Assignment of linkage order and map distance was based on the highest log-likelihood score. RAPD markers are identified by the primer used and the molecular weight of the polymorphic band scored.

RAPD Cloning and DNA Blot Analysis
Five PCR fragments which were scored as RAPD markers were cloned into vector pUC118 for RFLP analysis (OPE16-700, RW1-900, OPE12-440, OPE1003-840, and OPE20-1400). PCR products from Emperor
Figure 1. RAPD analysis with primers that identify the heterozygous condition in Emperor Francis. Operon Primers OPAC-20 and OPAE-04 amplified RAPD bands with molecular weights of 1,450 and 680 bp, respectively. Template DNA in lane 1 is Emperor Francis leaf DNA; lanes 2–9 are eight microspore-derived callus individuals. Arrows denote the polymorphic bands.

Francis leaf tissue and a null haploid individual were electrophoresed in adjacent lanes through low-melt agarose. The polymorphic PCR product of interest was confirmed by its absence in the null haploid individual and subsequently dissected from the Emperor Francis lane of the gel. Purification from the agarose followed the CTAB extraction procedure by Langridge et al. (1980). After purification, the fragment was reamplified using the PCR conditions described above. Following reamplification, the volume was adjusted to 150 µl with TE and subsequently removed from the mineral oil to a new microfuge tube. The PCR products were then treated with proteinase-K (50 ng/ml; 37°C; 2 h) followed by phenol/chloroform/isoamyl (25:1.8

Figure 2. RAPD map of sweet cherry cv. Emperor Francis. Markers shown on the right are identified by the Operon Primer used and the molecular weight of the polymorphic band scored. Markers that did not segregate 1:1, but did segregate 2:1 or 1:2, are identified by * and **, respectively (P < .05, 1 df). Two allozymes, 6-Pgd-1 and Pgi-2, are also mapped. Bold type is used to indicate phase relationships between linked markers, i.e., adjacent markers both in bold or non-bold are in coupling (−/++ or ++/−).
Results and Discussion

Each callus culture exhibited a subset of the bands present in Emperor Francis leaf DNA (Figure 1). Disagreement between the two replicates (subcultures) occurred <10 times for the entire mapping experiment. The discrepancies between the two replicates could have been due to somatic mutation or error in RAPD reproducibility or scoring. Variation between replicates for RAPDs has been reported to be 0–7%, depending on the fragment being analyzed (Weeden et al. 1992).

Nineteen percent of the 403 primers screened identified a RAPD. Of this 19%, only 11% of these primers amplified more than one RAPD. This low level of RAPD polymorphism is similar to peach, where 16% of 522 primers tested yielded polymorphic fragments and successful primers yielded only one polymorphic band (Chaparro et al. 1994). Low levels of isozyme polymorphism have also been reported in sweet cherry (Weeden et al. 1992), rice (Guiderdoni 1991), and maize (Cowen et al. 1992).

Segregation distortion for unknown reasons is also a common occurrence among gametes and progeny individuals in the absence of a tissue culture phase. Segregation distortion occurred among megagametophytes and in F2 progeny from sexual crosses. In conifers, 27 out of 69 primers (39%) revealed segregating bands that did not fit a 1:1 segregation ratio (Tulsieram et al. 1992). In diploid alfalfa, nine out of 37 RAPD loci (24%) had a significant deviation from the expected $\chi^2$ value (Echt et al. 1992). In a segregating peach F2 population, 13 of 48 (27%) of the RAPD markers mapped deviated significantly from the expected 1:2:1 ratio (Beaver and Iezzoni 1993). Although Fooled et al. (1995) screened their peach × almond mapping population for Pgi-2 and 6-Pgd-1, and the isozymes were not linked and mapped to groups 1 and 2, respectively. This agrees with previous results in sour cherry where 238 self-pollinated progeny of the sour cherry cultivar Montmorency were screened for Pgi-2 and 6-Pgd-1, and the isozymes were not linked (Beaver and Iezzoni 1993).

Sixty-eight (76%) of the markers mapped fit a 1:1 ratio. The remaining 21 markers fit either a 2:1 (−,+)- or a 1:2 (−,−) ratio. Markers that segregated 2:1 or 1:2 (−,+) tended to either cluster together (Figure 2: linkage groups 3 and 4) or reside on the most terminal portion of a linkage group (Figure 2: linkage groups 2, 5, and 10). The tendency for the skewed loci to be linked, suggests that the segregation distortions may be due to selection pressure in culture. Differential transmission of specific chromosome regions associated with the production of microspore-derived haploids has been reported in barley (Thompson et al. 1991; Zivy et al. 1992), rice (Guiderdoni 1991), and maize (Cowen et al. 1992).
from the expected Mendelian ratios (Dirlewanger and Bodo 1994). In a peach × almond population, 48 of 118 loci (41%) deviated significantly from the expected Mendelian ratios (Foolad et al. 1995). These levels of distortion are similar to or greater than the 24% observed in our analysis. Irrespective of the individual Mendelian ratios, it is important that our linkage map be verified in a sexual cross. In sugarcane (Saccharum spontaneum), linkage relationships were inconsistent for three out of five linkage groups constructed from a haploid population versus a population derived from a sexual cross (da Silva et al. 1993).

Plasmid DNAs were radiolabeled and hybridized to filters containing digested genomic DNAs from sweet, sour, and ground cherry. Hybridization patterns were similar in all three Prunus spp. examined. Of the five amplified probes tested, one hybridized to low copy DNA (POPB12-440), three hybridized to middle-repetitive DNA (POPE16-700, pRW1-900, pOPE20-1400), and one hybridized to highly repetitive DNA (POPD03-840). Hybridization patterns with POPB12-440 and POPE16-700 are shown in Figure 3. Between the two sour cherry cultivars, Rhenishen Schottenmelle and Erdi Botermo, one and 12 polymorphisms (presence/absence) were identified with POPB12-440 and POPE16-700, respectively.

Only one of the five RAPD fragments cloned is suitable as a single copy RFLP probe. The four other amplified probes hybridized to repetitive DNA. Cloned RAPD fragments hybridizing to repetitive DNA is not unusual. In soybean (Glycine max), of 11 amplified RAPD probes tested, six hybridized to single-copy DNA, three hybridized to middle-repetitive DNA, and two hybridized to highly repetitive DNA (Williams et al. 1990). However, these amplification products would potentially provide DNA markers in genomic regions not accessible to RFLP analysis due to the presence of repetitive DNA sequences. Erdi Botermo and Rhenishen Schottenmelle are the parents of the MSU sour cherry mapping population. Polymorphic clones identified with the single copy and middle-repetitive RAPD probes will be tested for 1:1 segregation among the sour cherry progeny.

Despite the effort required to obtain single copy probes from cloned RAPD markers, these probes can be very useful. Along with the allostomes, single copy clones can be used in comparative mapping to identify homologous segments with other Prunus species and within cherry as genetic linkage maps from other populations are generated.

References


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