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Genetic linkage map in sour cherry using RFLP markers

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Abstract Restriction fragment length polymorphism (RFLP) linkage maps of two tetraploid sour cherry (*Prunus cerasus* L., 2n = 4*x* = 32) cultivars, Rheinische Schattenmorelle (RS) and Erdi Botermo (EB), were constructed from 86 progeny from the cross RS × EB. The RS linkage map consists of 126 single-dose restriction fragment (SDRF, Wu et al. 1992) markers assigned to 19 linkage groups covering 461.6 cM. The EB linkage map has 95 SDRF markers assigned to 16 linkage groups covering 279.2 cM. Fifty three markers mapped in both parents were used as bridges between both maps and 13 sets of homologous linkage groups were identified. Homoeologous relationships among the sour cherry linkage groups could not be determined because only 15 probes identified duplicate loci. Fifty nine of the markers on the linkage maps were detected with probes used in other *Prunus* genetic linkage maps. Four of the sour cherry linkage groups may be homologous with four of the eight genetic linkage groups identified in peach and almond. Twenty one fragments expected to segregate in a 1:1 ratio segregated in a 2:1 ratio. Three of these fragments were used in the final map construction because they all mapped to the same linkage group. Six fragments exhibited segregation consistent with the expectations of intergenomic pairing and/or recombination.

Key words *Prunus* · *Prunus cerasus* · Polyploid Linkage map

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Introduction

Marker-assisted selection and whole-genome backcross (BC) selection would be especially advantageous for sour cherry (*Prunus cerasus* L.) breeding. Sour 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 devoted to promising individuals. Additionally, in whole-genome BC selection using RFLP markers, it is estimated that the recurrent parent genotype could be reconstructed and the introduced gene maintained in three BC generations as opposed to the six BC generations required without RFLP genotyping (Tanksley et al. 1989). Map-based BC selection is especially attractive in sour cherry where a reduction in three BC generations could mean a saving of 9–15 years.

Despite the potential utility of a genetic linkage map in the tetraploid sour cherry, no linkage relationships have been reported. In *Prunus*, linkage maps are most advanced in the diploid species: peach (*P. persica*) (Chaparro et al. 1994; Rajapakse et al. 1995), almond (*P. amygdalus*, syn. *P. dulcis*) (Viruel et al. 1995), sweet cherry (*P. avium*) (Stockinger et al. 1996), sweet cherry × *P. incisa*, sweet cherry × *P. nipponica* (Bošković et al. 1997), peach × almond (Foolad et al. 1995) and peach × *P. davidiana* interspecific hybrid populations (Dirlewanger and Bodo 1994). For example, a map of about 800 cM with 107 markers has been constructed in a peach × almond cross (Foolad et al. 1995).

As in apple, the linkage mapping population in sour cherry is a 'pseudotestcross' in which informative markers are those that are heterozygous in one parent and homozygous recessive in the other parent and segregate 1:1 (Hemmat et al. 1994). However, in the

tetraploid sour cherry, if a band is present in one of the parents, the parental genotype can be either ++++, +++-, ++--, +-+-, or +---. Approximately 75 progeny are required to conclusively identify the informative Single Dose Restriction Fragment (SDFR, Wu et al. 1992) genotype (+---) based on 1:1 segregation.

A previous inheritance study with allozymes demonstrated that sour cherry exhibits disomic inheritance and is therefore an allotetraploid (Beaver and Iezzoni 1993). However, there was evidence of occasional intergenomic pairing and pre- or post-zygotic selection (an abundance of 2:1 ratios). Both these phenomena add to the complexity of linkage map construction in sour cherry.

The objectives of this study were to construct low-density RFLP linkage maps for two sour cherry cultivars and compare these maps to previously constructed *Prunus* RFLP maps. RFLP probes developed by other researchers were used to facilitate comparative mapping; specifically the alignment of sour cherry linkage groups with the eight linkage groups identified in peach and almond.

Materials and methods

Plant material and DNA isolation

The mapping population consists of 86 progeny from the cross between two sour cherry cultivars, Rheinische Schattenmorelle (RS) × Erdi Botermo (EB). RS and EB were chosen because they are from different geographic areas (Germany and Hungary, respectively) and differ for important horticultural traits such as bloom date, cold hardiness, fruit quality and percent fruit set. The parents and progeny population are maintained at the Michigan State University Clarksville Horticultural Experiment Station, Clarksville, Mich.

Young unfolded leaves were collected from 7-year-old trees and transported to the laboratory in coolers with dry ice. The leaf samples were frozen at −80°C overnight and then lyophilized for 48–72 h. DNA isolation for Southern analysis followed the procedure of Stockinger et al. (1996).

Source of DNA probes

DNA clones from the following sources were used to identify informative markers (Table 1): (1) Plum genomic and peach cDNA clones (F. Bliss and S. Arulsekar; University of California, Davis, Calif.); (2) peach genomic clones (S. Rajapakse and A. Abbott; Clemson Univ., Clemson, S.C.); (3) peach cDNA clones (A. Callahan; USDA, Kearneysville, W.Va.); (4) almond genomic and cDNA clones (P. Arús; IRTA, Barcelona, Spain); (5) *Pst*I genomic clones from the sweet cherry cultivar Emperor Francis; and (6) cDNA clones from a stylar cDNA library from the sweet cherry cultivar Emperor Francis.

Sweet cherry genomic clones

A genomic library was constructed using size-fractionated *Prunus avium* cv Emperor Francis DNA. Methylation-sensitive *Pst*I (Boehringer Mannheim, Indianapolis, Ind.) was used to digest genomic DNA which was isolated as described (Stockinger et al. 1996) except that an additional CTAB-chloroform extraction was done followed by ethanol-precipitation. The plasmid vector, pUC19, was cut with *Pst*I and de-phosphorylated with calf intestinal alkaline phosphatase (Gibco BRL, Gaithsburg, Md.). Size-selection of genomic DNA was done by fractionating the digested DNA on a 1% TAE agarose gel (Sambrook et al. 1989). Fragments of 500–2000 bp were isolated from the gel by placing a piece of DEAE NA45 membrane (Schleicher and Schuell, Keene, N.H.) into the gel and electrophoresing the appropriately sized DNA into the membrane. The membrane was prepared and the DNA was recovered according to the manufacturer (Schleicher and Schuell, Keene, N.H.). The size-selected DNA and pUC19 DNA were concentrated together in a Microcon concentrator (Amicon Inc., Beverly, Mass.), heated to 65°C for 5 min, then ligated in a 10-µl reaction with T4 DNA ligase (Boehringer Mannheim, Indianapolis, Ind.) as described by Sambrook et al. (1989). Recombinant plasmid DNA was then transformed by electroporation into *Escherichia coli* DH5-α electrocompetent cells using the manufacturer's protocol (Bio-Rad Laboratories, Hercules, Calif.). Individuals used in further analyses were white colonies picked from LB plates containing ampicillin (125 µg/ml), X-gal (40 µg/ml), and IPTG (0.95 µg.ml). To determine insert size and copy number, inserts were amplified by PCR using primers which flank the multiple cloning site of pUC19 (Promega, Madison, Wis.). The amplified insert DNA was checked for size on a 1% agarose gel and then used in dot blots by blotting approximately 100 ng of insert DNA onto a Zeta-Probe GT membrane (Bio-Rad Laboratories, Hercules, Calif.). Controls were DNAs which were known to be low-, medium-, and high-copy in the cherry genome. These probes are identified by "EF" referring to Emperor Francis and the probe number. For probe labeling, 1 µg of genomic DNA was labeled with ^{32}P dCTP using a nick translation kit (Boehringer Mannheim, Indianapolis, Ind.). Pre-hybridization and hybridization conditions were as described by Stockinger et al. (1996).

Sweet cherry cDNAs

RNA was isolated from approximately 1 gg of stylar tissue from the sweet cherry cultivar Emperor Francis by the method of Manning (1991) with the following modifications. Four phenol:chloroform:isoamylalcohol (25:24:1) extractions were performed and the [Na+] in the first butoxyethanol precipitation was adjusted to 100 mM. Stylar cDNA was prepared using a cDNA synthesis kit (Boehringer Mannheim, Indianapolis, Ind.) and a cDNA amplification protocol (Jepson et al. 1991). This stylar cDNA was subsequently used in a PCR reaction with two degenerate primers, ATNCA(T/C)GGN(C/T)TNTGGCC and (C/G)(A/T)(A/G)CANG TNCC(A/G)TG(T/C)TT, designed to amplify ribonuclease sequences. Primer design was based on conserved amino acids identified by aligning several S-allele and ribonuclease amino-acid sequences (T-H. Kao, personal communication). Four major bands resulting from amplification with the degenerate primers were isolated from a 5% native polyacrylamide gel (Sambrook et al. 1989). These fragments were then re-amplified, cloned into pUC118, and copy number determined as described above for the sweet cherry genomic clones. These probes were identified by "PS", for *Prunus* stylar tissue, and the clone number.

Southern analysis

DNA (6 µg) of both parents and 12 progeny was digested with 20–30 units of one of six restriction enzymes (*Bam*HI, *Dra*I, *Eco*RI, *Hin*dIII, *Pst*I, or *Xba*I; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and separated on a 0.9% agarose gel for 30 h at 23 V. Southern

Table 1 Probes from other *Prunus* research groups which were unlinked or mapped to one or more locations in sour cherry. RS and EB refer to the Rheinische Schattenmorelle and Erdi Botermo linkage groups, respectively

Probe[a]	Linkage group(s) in sour cherry map	References
AC1	Unlinked	Viruel et al. (1995)
AC6	Unlinked	
Pru2	RS 8	
AC27	RS 2, EB 2	
AG6	RS 12	
AG8	EB 13	
AG10	RS 7, EB 7	
AG21	RS 2	
AG40	RS 17, EB 17, RS 18, EB 18	
Ext1	RS 8, EB 8	
Ole1	RS 2	
B4G10	EB 6, RS 17	Rajapakse et al. (1995)
B6D1	Unlinked	
B7H2	RS 16	
B8A3	RS 19	
CPM2	RS 5, EB 5	F. Bliss (personal communication)
CPM6	RS 12	
CPM12	RS 1, EB 1	
CPM20	RS 5, EB 5, RS 5′, RS 6, EB 6	
CPM23	RS 6, EB 6, EB 14	
CPM30	RS5	
CPM39	RS 6, EB 6, RS 17, EB 17, RS 18, EB 18	
CPM48	RS 7, EB 7, EB 7′	
CPM53	RS 4, EB 4	
CPM57	RS 9, EB 9	
CPM58	RS 4, EB 4	
CPM59	RS 2	
CPM64	RS 7, EB 7	
CPM67	RS 7, EB 7	
CPM70	EB 5, RS 5′, RS 19	
CPM90	RS2	
CPM104	RS 6, RS 6′	
PLG10	Unlinked	
PLG86	RS 2, EB 2	
Hsp4	RS 2, EB 2	Callahan (personal communication)
pch108	Unlinked	
pch202	RS 5, EB 5	
pch205	RS 3	

[a] AC = almond cDNA clones, Pru2 = cDNA for the seed protein Prunin (P. Arús, personal communication), AG = almond genoic clones, Ext1 = cDNA for Extensine, Ole1 = cDNA for Oleosine, B- = peach genomic clones, CPM = peach mesocarp cDNA clones, PLG = plum genomic clones, Hsp4 = peach cDNA for a heat-shock protein, pch108 = peach cDNA for chlorophyll A/B-binding protein, pch202 = peach cDNA for a thioredoxin, pch205 = peach cDNA for a water-stress protein.

analysis was performed according to Stockinger et al. (1996) using Hybond-N+ membranes (Ambersham, Arlington Heights, Ill.).

Probe DNAs were prepared by PCR amplification of the inserts from pUC19 or pBluescript plasmids (Stratagene, La Jolla, Calif.) using a pair of primers flanking the cloning sites. Radiolabelling of probes with ^{32}P-dCTP (DuPont, Boston, Mass.) was done using the random primer hexamer-priming method of Feinberg and Vogelstein (1983). Those enzyme and probe combinations that identified useful polymorphisms from the two parents and 12 progeny were used to genotype the additional 74 progeny in the mapping population.

Inheritance and linkage analysis

Informative markers for a pseudotestcross mapping population are SDRFs that differ between both parents and segregate in a 1:1 (presence:absence) ratio, plus SDRFs present in both parents that segregate in a 3:1 ratio (Wu et al. 1992). Therefore, markers which differed between both parents were tested for fit to a 1:1 (presence:absence) ratio. Markers present in both parents were tested for fit to a 3:1 (presence:absence) ratio. Those markers which fit the appropriate ratios at the 5% level were used in the linkage analysis.

Markers present in one parent that did not fit to a 1:1 ratio were tested for fit to a 5:1 or 2:1 ratio. A 5:1 ratio would be expected for tetrasomic inheritance of a double-dose restriction fragment (DDRF, +-+-×---; Wu et al. 1992). A ratio of 2:1 would represent a skewed 1:1 ratio. Markers which fit a 2:1 ratio at the 5% level were included in an initial linkage analysis; however, only those 2:1 markers that exhibited linkage with each other were included in the final map.

Linkage analyses were done with JoinMap V2.0 (Stam 1993) using a minimum LOD score of 3.0 and a maximum recombination fraction of 0.35. Distances are presented in centi-Morgans calculated by the Kosambi function. Multiple loci detected using the same

probe were labeled with a letter after the probe designation (i.e., a, b, c, etc.). Where possible, linkage groups were numbered based upon suspected homology with previously constructed peach and almond linkage maps (Bliss, personal communication; Viruel et al. 1995).

Results and discussion

A total of 190 SDRFs were identified by 82 probes out of the 260 probes tested. Of this set, 110 SDRF markers fitting a 1:1 ratio were identified. RS and EB were heterozygous for 67 and 43 of these 1:1 markers, respectively. A total of 80 SDRF markers were present in both parents and fit a 3:1 ratio.

Twenty seven segregating fragments present in one parent and absent in the other parent (+---×----) did not fit a 1:1 ratio. Of these fragments, eight were present in RS and absent in EB and 19 were present in EB and absent in RS. All eight RS fragments fit a 2:1 ratio. Of the 19 EB fragments, 13 fragments fit a 2:1 ratio. Three other fragments fit a 5:1 (+, −) ratio which could result from tetrasomic inheritance of a DDRF (Wu et al. 1992). The other three fragments had presence:absence ratios of 79:6, 81:2 and 84:2, respectively. This could have resulted from loss of fixed heterozygosity due to occasional intergenomic recombination.

These results are consistent with previous allozyme inheritance and cytogenetic data in sour cherry suggesting that 2:1 ratios are prevalent along with occasional intergenomic recombination. A 2:1 ratio was accepted and the expected 3:1 ratio was rejected for three out of nine inheritance ratios for three unlinked allozyme loci (Beaver and Iezzoni 1993). This skewed segregation could result from gametophytic selection or zygotic lethals. Intergenomic recombination resulted in 15 out of 308 progeny exhibiting a loss of fixed heterozygosity for *6-Pgd-2* (Beaver and Iezzoni 1993). Cytogenetic studies support the theory that some of the segregation results are due to intergenomic recombination. Meiosis-I in sour cherry should result in the formation of 16 bivalents. However, quadrivalents occur at a low frequency and 14–15 bivalents and 2–4 univalents are also observed (Galletta 1959; Hruby 1939). Quadrivalent formation at meiosis-I was also observed for our mapping parents RS and EB (unpublished results).

Twenty six fragments that were present in both parents and segregating in the progeny did not fit a 3:1 ratio, which would have resulted from segregation of a SDRF in each parent (+---×+---). In these cases, it is possible that one or both of the parents was double dose for the scored fragment (+-+-×+--- or +-+-×+-+-). However, the progeny size of 86 was too small to conclusively distinguish between these various segregation hypotheses.

The RS linkage map consists of 126 markers assigned to 19 linkage groups covering 461.6 cM (Fig. 1). Seventeen markers remained unlinked. The EB linkage map possesses 95 markers assigned to 16 linkage groups covering 279.2 cM. Twenty three markers were unlinked. For both maps, a total of six fragments were removed from the data set because they were redundant, with two fragments identified with the same probe mapping to the same location. Thirteen EB linkage groups homologous to the 19 RS linkage groups were identified using 53 bridging markers heterozygous in both parents. EB counterparts to RS linkage groups 3, 12, 16, and 19, were not identified. Conversely, RS counterparts to EB linkage groups 13 and 14 were also not identified. Only three of the 21 fragments that fit a 2:1 ratio were included on the linkage maps. These three markers which mapped to EB Group 4 (EF156a, CPM53a, and EF182a) all had an over-abundance of the allele unique to EB, suggesting that these alleles may be preferentially selected.

Since sour cherry is a tetraploid with $x = 8$, the ultimate goal is to identify 16 linkage groups and the homoeologous relationships among these linkage groups. For example, Groups 17 and 18 may be homoeologous groups because markers identified with probes AG40 and CPM39 mapped an average of 18.2 and 14.4 cM apart in both linkage groups, respectively (Fig. 1). However, no other homoeologous segments could be identified with the set of probes used in this analysis. The ideal probe for identifying homoeologous linkage groups in a tetraploid is a probe that identifies two segregating bands which map to different linkage groups. Of the 82 probes that identified mapped fragments, only 15 met this criterion. Forty six probes identified one mapped fragment, and 36 probes identified more than two mapped fragments.

Fifty nine markers on the linkage maps were detected with probes placed on other *Prunus* linkage maps. Based on placement of these common probes, four of the sour cherry linkage groups may be homologous to previously identified *Prunus* linkage groups from an almond × peach map (Arús, personal communication) and a peach × almond map (Bliss, personal communication). Group-2 markers identified with the probes AG21 and Ole1 mapped 25.6 cM apart in RS (Fig. 1) and 24 cM apart in almond × peach (Arús, personal communication). Group-2 markers identified with the probes CPM90 and PLG86 mapped 11.1 cM apart in RS (Fig. 1) and 13.2 cM apart in peach × almond (Bliss, personal communication). However, Group-2 markers identified with the probes PLG86 and CPM 59 mapped 24.8 cM apart in RS (Fig. 1) and 48.2 cM apart in peach × almond (Bliss, personal communication). The sour cherry linkage Group 4 may be homologous to the peach × almond linkage Group 4. Markers identified with the probes CPM53 and CPM58 mapped 27.9 cM apart in peach × almond but just 1.5 cM and 3.7 cM apart in RS and EB, respectively. The sour cherry linkage Group 7 may be homologous to the peach × almond linkage Group 7. Markers identified

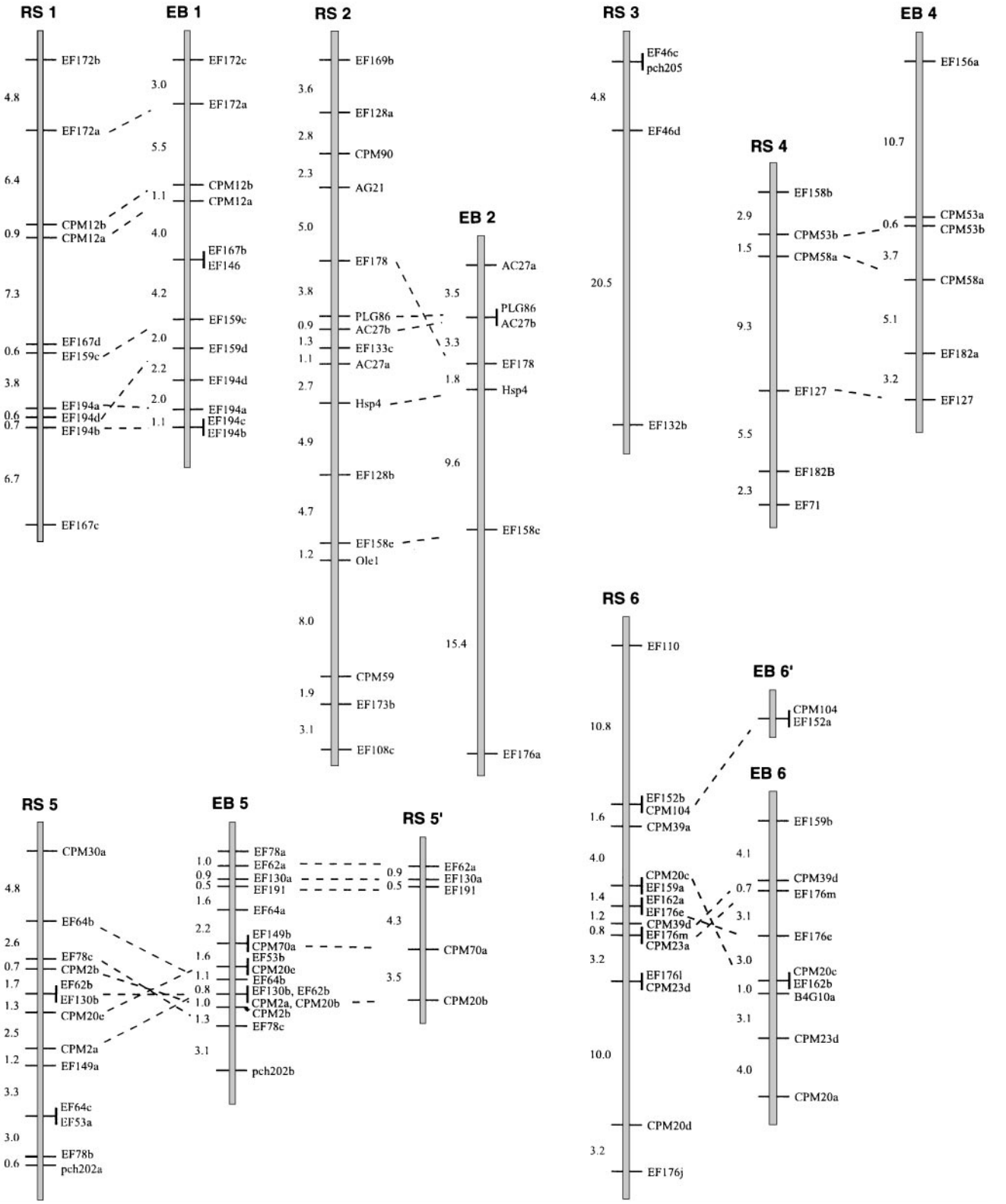

Fig. 1 Continued (see page 1223 for legend)

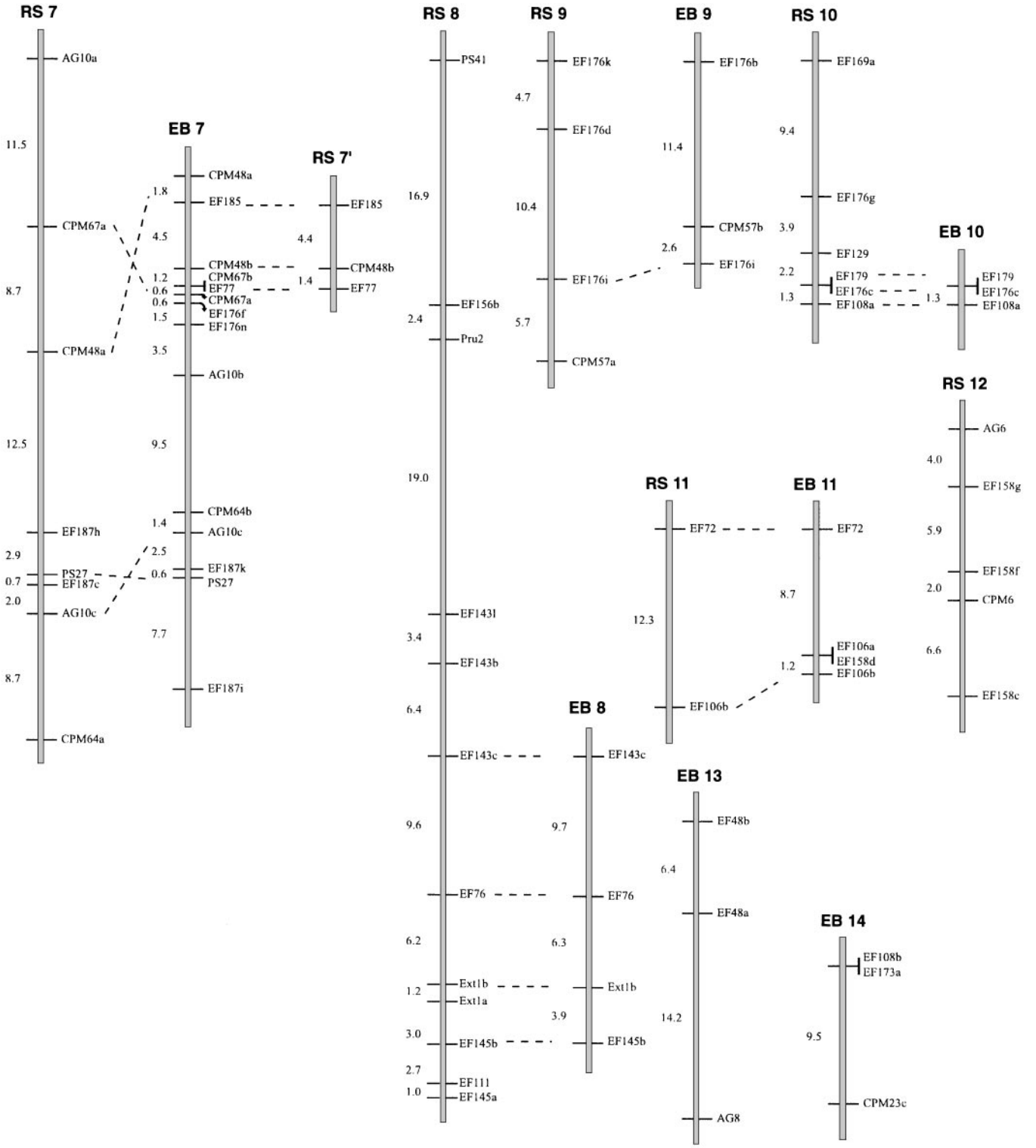

Fig. 1 Continued (see page 1223 for legend)

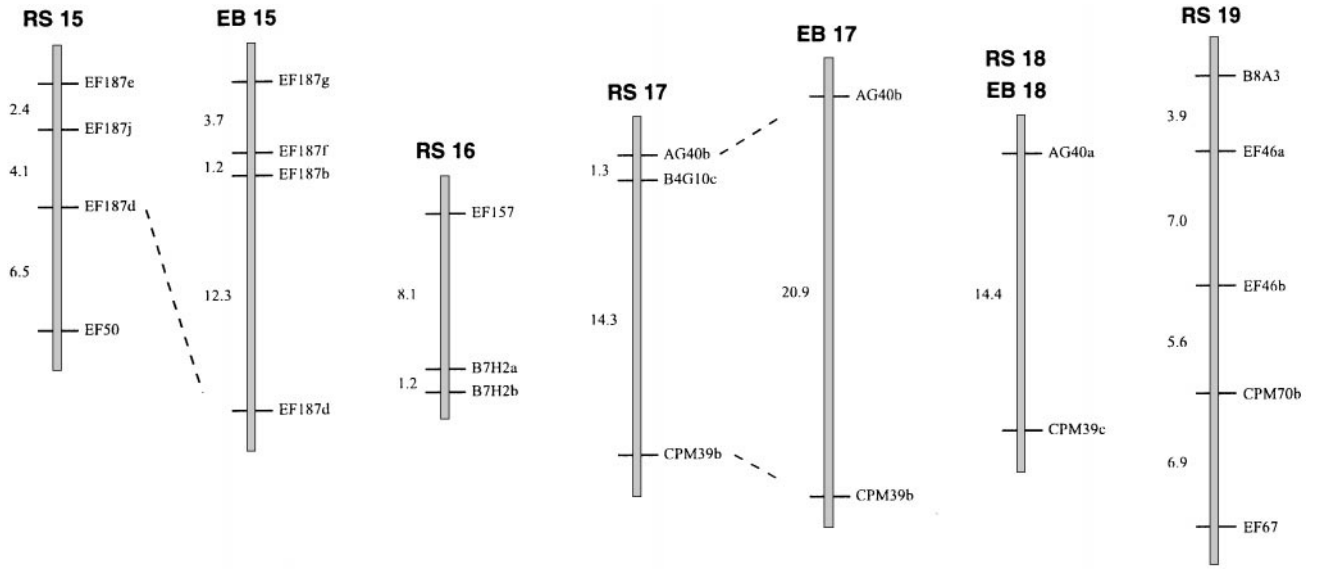

Fig. 1 RFLP maps for Rheinische Schattenmorelle (*RS*) and Erdi Botermo (*EB*). Markers shown on the rights are identified by the probe followed by a letter (i.e., *a*, *b*, *c*, etc.) when more than one marker is generated from a single probe. When two linkage groups from one parent align to one linkage group from the other parent, the smaller of the two linkage groups is identified with the same linkage group number followed by an apostrophe. Correspondences between anchor loci of RS and EB linkage groups are shown with *dashed lines*

with the probes CPM48, CPM64, and CPM67, all mapped to Group 7 on both the sour cherry and peach × almond linkage maps (Bliss, personal communication). The sour cherry linkage Group 8 may be homologous to the almond × peach linkage Group 8. Markers identified with Pru2 and Ext1 mapped 44.6 cM apart in RS and 52 cM apart in almond × peach (Arús, personal communication). These associations, however, are preliminary until more alignment comparisons can be made.

Sweet cherry, a diploid *Prunus*, is suspected to be an ancestral progenitor of sour cherry. Unfortunately, it is not possible to compare the sour cherry map with the previously published maps from sweet cherry, sweet cherry × *P. incisa*, and sweet cherry × *P. nipponica*, because these diploid maps consist exclusively of RAPD and isozyme markers (Stockinger et al. 1996; Bošković et al. 1997).

The longest *Prunus* linkage map published is a peach × almond map consisting of approximately 800 cM (Foolad et al. 1995). Given that sour cherry is a tetraploid, a map of comparable coverage should be 1500 cM. The requirements for an informative marker state in a tetraploid (i.e., +---×----, ----× +---, or +---× +---) dramatically reduce the likelihood of finding useful markers. In diploid *Prunus* species, for example, the criteria for informative markers for a pseudotestcross population are +-×--, --× +-, or +-× +-. A project to develop and map simple sequence repeat (SSR) loci is currently underway to determine if potentially higher levels of heterozygosity at SSR loci will increase the likelihood of identifying informative markers and mapping duplicate loci in sour cherry. Additionally, if SSRs are conserved among *Prunus* species, they would be excellent markers for comparative mapping.

Note The reported experiments comply with current U.S. law.

References

Beaver JA, Iezzoni AF (1993) Allozyme inheritance in tetraploid sour cherry (*Prunus cerasus* L.). J Am Soc Hort Sci 118: 873–877

Bošković R, Tobutt KR, Nicoll FJ (1997) Inheritance of isoenzymes and their linkage relationships in two interspecific cherry progenies. Euphytica 93: 129–143

Chaparro JS, Werner DJ, O'Malley D, Sederoff RR (1994) Targeted mapping and linkage analysis of morphological, isozyme, and RFLP markers in peach. Theor Appl Genet 87: 805–815

Dirlewanger E, Bodo C (1994) Molecular genetic map of peach. Euphytica 77: 101–103

Feinberg AD, Vogelstein G (1983) A technique for radiolabelling DNA restriction fragments to high specific activity. Anal Biochem 132: 6–13

Foolad MR, Arulsekar S., Becerra V, Bliss FA (1995) A genetic map of *Prunus* based on an interspecific cross between peach and almond. Theor Appl Genet 91: 262–269

Galletta GJ (1959) Comparative breeding, morphological and cytological studies of polyploid sweet cherries (*Prunus avium* L.). PhD thesis, University of California, Davis

Hemmat M, Weeden NF, Manganaris AG, Lawson DM (1994) Molecular-marker linkage map for apple. J Hered 85: 4–11

Hruby K (1939) The cytology of the Duke cherries and their derivatives. J Genet 38: 125–131

Jepson I, Bray J, Jenkins G, Schuch W, Edwards K (1991) A rapid procedure for the construction of PCR cDNA libraries for small amounts of plant tissue. Plant Mol Biol Rep 9: 131–133

Manning K (1991) Isolation of nucleic acids from plants by differential solvent precipitation. Anal Biochem 195: 45–50

Rajapakse S, Belthoff LE, He G, Estanger AE, Scorza R, Verde I, Ballard RE, Baird WV, Callahan A, Monet R, Abbott AG (1995) Genetic linkage mapping in peach using morphological, RFLP, and RAPD markers. Theor Appl Genet 90: 503–510

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Stam P (1993) Construction of integrated genetic linkage maps by means of a new computer package: JoinMap. Plant Jour 3: 739–744

Stockinger EJ, Mulinix CA, Long CM, Brettin TS, Iezzoni AF (1996) A linkage map of sweet cherry based on RAPD analysis of a microspore-derived callus culture population. J Hered 87: 214–218

Tanksley SD, Young ND, Paterson AH, Bonierbale, MW (1989) RFLP mapping in plant breeding: new tools for an old science. Bio/Technology 7: 257–264

Viruel MA, Messeguer R, de Vicente MC, Garcia-Mas J, Puidomenech P, Vargas F, Arús P (1995) A linkage map with RFLP and isozyme makers in almond. Theor Appl Genet 91: 964–971

Wu KK, Burnquist W, Sorrells ME, Tew TL, Moore PH, Tanksley SD (1992) The detection and estimation of linkage in polyploids using single-dose restriction fragments. Theor Appl Genet 83: 294–300