Theor Appl Genet (1995) 90: 1068–1073

H. Benet · R. P. Guries · S. Boury
E. B. Smalley

Identification of RAPD markers linked to a black leaf spot resistance gene in Chinese elm

Received: 7 September 1994 / Accepted: 22 November 1994

Abstract Black leaf spot (*Stegophora ulmea*) is a common foliage disease on Chinese (*Ulmus parvifolia*) and Siberian elms (*U. pumila*), two species which have been widely used as sources of Dutch-elm disease-resistance genes for interspecific elm hybrids. A dominant gene controlling resistance to black leaf spot was identified in a population derived from self-pollination of a single *U. parvifolia* tree. Using RAPD markers, in combination with bulked segregant analysis, we have identified three markers linked to this resistance gene. A survey of Chinese-elm hybrids revealed that the same gene is likely to confer a high level of resistance to black leaf spot in interspecific elm hybrids, although other genetic factors may also be involved in the determination of a disease phenotype.

Key words Anthracnose · Elm black leaf spot · Disease-resistance breeding · Random amplified polymorphic DNA · Bulked segregant analysis

Introduction

Black leaf spot of elm [*Stegophora ulmea* (Schw.: Fries) Sydow & Sydow (syn. *Gnomonia ulmea*)] is one of a number of common foliar diseases of woody plants caused by ascomycetous fungi. Such diseases, often called anthracnoses, induce symptoms which range from innocuous leaf spots to severe blights of leaves and shoots to cankers and dieback of twigs and branches (Sinclair et al. 1987). The biology of *S. ulmea* and the effects of environmental factors upon its development are well known, as is the variation in susceptibility among elm species (McGranahan and Smalley 1984a, b; Sinclair et al. 1987). As with other anthracnoses, high humidity and cool temperatures enhance disease incidence, and a severe infestation may cause complete defoliation in especially susceptible individuals. Repeated defoliation may eventually result in the death of affected individuals.

Black leaf spot has not been a major concern to elm breeders, whose primary objective has been the development of trees resistant to Dutch-elm disease (DED; *Ophiostoma ulmi* and *O. novo-ulmi)* (Burdekin 1983; Smalley and Guries 1993; Sticklen and Sherald 1993). Eurasian elms, especially Siberian elm (*Ulmus pumila* L.), and more recently Chinese elm (*U. parvifolia* Jacq.), have served as major sources of genes for the development of interspecific elm hybrids resistant to DED (Townsend and Schreiber 1976; Smalley and Guries 1993). These two species have also been considered resistant to black leaf spot (McGranahan and Smalley 1984a). However, recent severe damage caused by black leaf spot to some accessions of *U. parvifolia*, *U. pumila*, and various interspecific hybrids, following cool, moist Springs, has raised questions about the general resistance of these species to the disease, as well as the possible development of new or more virulent races of the pathogen. Thus, it is particularly important to identify sources of anthracnose resistance among Eurasian elms in order to avoid the inadvertent introduction of susceptibility to anthracnose into new interspecific hybrids. If anthracnose resistance is controlled by one (or a few) major genes, as is the case for beans (Pastor-Corrales and Tu 1989) or maize (Cowen et al. 1991), then the "tagging" of these genes with molecular markers would enable breeders to track them during successive generations of selection and breeding.

In this study, we describe the use of RAPD markers (Williams et al. 1990), in combination with the method of

Communicated by G. Wenzel

H. Benet
Centre d'Etudes Océanographiques et de Biologie Marine, CNRS, UPR 4601, F-29680 Roscoff, France

R. P. Guries (✉)
Department of Forestry, University of Wisconsin-Madison, 1630 Linden Drive, Madison, WI 53706-1598, USA

S. Boury
Groupement d' Intérêt Publique Prince de Bretagne Biotechnologie, Penn Ar Prat, F-29250 Saint Pol de Léon, France

E. B. Smalley
Department of Plant Pathology, University of Wisconsin-Madison, 1630 Linden Drive, Madison, WI 53706-1598, USA

"bulked segregant analysis" (BSA; Michelmore et al. 1991; Paran and Michelmore 1993), to identify flanking markers linked to a putative dominant gene (herein referred to as *Bls*; further screening in other families is needed to adequately characterize this gene) controlling resistance to black leaf spot in Chinese elm. In addition, we demonstrate a strong association between these flanking markers linked to *Bls* and black leaf spot resistance in interspecific elm hybrids derived from matings using a mother tree heterozygous for *Bls*.

Materials and methods

Plant populations

As part of a continuing effort to evaluate exotic germplasm for use in our elm breeding program, self-pollinations were completed using a single *Ulmus parvifolia* tree (W114-1), which originated from a collection made in 1958 by the National Forest Experiment Station, Seoul, Korea. In 1992, a total of 822 progenies derived from the self-pollination of W114-1 were planted at the Arlington Research Station (Columbia Co, Wis.) at 0.7-m spacing in rows, on the leeward side of an older *U. pumila* shelterbelt. Spring 1993 was particularly wet and cool, and a natural inoculum of *S. ulmea* originating in the shelterbelt resulted in a severe infestation in the 1992 trials. Two phenotypes could be distinguished easily among selfed progeny, a resistant phenotype with essentially no evidence of black leaf spot, and a susceptible one showing a severe defoliation of infected individuals. Resistant and susceptible individuals were observed to be randomly distributed in the field, and a chi-square test confirmed a 3:1 segregation of resistant to susceptible individuals (597 R:225 S; χ^2=2.5; P=0.12).

Table 1 Phenotype/genotype of 24 interspecific Chinese-elm hybrids surveyed for co-segregation of black leaf spot disease phenotytpe and RAPD markers (+ = marker present; – = marker absent)

Hybrid plant accession	Male parent	Disease phenotype	Primer $OPP17_{1630}$	Primer $OPC20_{900}$
HR1	*U. americana*	1	+	+
HR2	*U. americana*	1	+	–
HR3	*U. carpinifolia*	1	+	+
HR4	*U. americana*	1	+	+
HR5	*U. carpinifolia*	1	+	+
HR6	*U. americana*	1	+	+
HR7	*U. americana*	1	+	–
HR8	*U. americana*	1	+	+
HR9	*U. carpinifolia*	2	–	–
HR10	*U. carpinifolia*	2	–	+
HR11	*U. carpinifolia*	2	–	–
HRS1	*U. carpinifolia*	3	+	+
HRS2	*U. carpinifolia*	3	–	–
HS1	*U. americana*	4	–	–
HS2	*U. americana*	4	–	–
HS3	*U. americana*	4	–	–
HS4	*U. carpinifolia*	4	–	+
HS5	*U. americana*	4	–	–
HS6	*U. americana*	4	–	–
HS7	*U. americana*	4	–	–
HS8	*U. carpinifolia*	4	–	–
HS9	*U. carpinifolia*	5	–	–
HS10	*U. carpinifolia*	5	–	–
HS11	*U. americana*	5	+	–

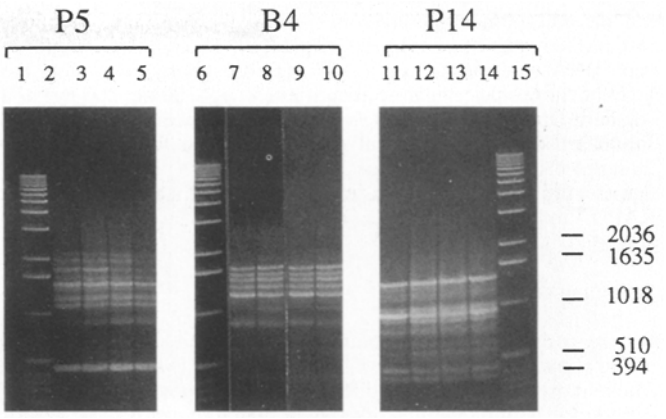

Fig. 1 Patterns of amplified DNA fragments obtained from Chinese-elm progenies with primers P5 (*lanes 2–5*), B4 (*lanes 7–10*), and P14 (*lanes 11–14*). The DNA bulks R2 (*lanes 2, 4, 7, 9, 11 and 13*) and S2 (*lanes 3, 5, 8, 10, 12 and 14*) were used as template DNA. Two independent amplifications were performed for each template DNA/primer in order to evaluate the reproducibility of amplifications. Molecular-weight markers (1-kb ladder) are present in *lanes 1, 6, and 15*

Planted in the same field as the selfed progeny were various interspecific hybrids (approximately 300 individuals) obtained by cross-pollination of *U. parvifolia* W-114-1 with selected American (*U. americana* L.) and smooth-leaved (*U. carpinifolia* Gled.) elm males. Unlike the selfed progenies, the phenotypes of these hybrids varied in a more-or-less continuous fashion in terms of black leaf spot injury. Phenotypes were scored for black leaf spot damage on a scale ranging from at least 75% defoliation (scored as a 1) to a virtual absence of symptoms (scored as a 5), with 2–4 corresponding to < 10%, 11–25%, and 25–75% defoliation, respectively. Twenty-four hybrid progeny were subsequently sampled for RAPD analysis to examine the co-occurrence of markers linked to the *Bls* gene and the phenotypes of selected interspecific hybrids (phenotypic data shown in Table 1).

Efficient screening of RAPD markers segregating in the selfed progeny required the existence of an adequate level of polymorphism. Preliminary study of a subset of progenies from W114-1 using 113 RAPD markers identified 29 (26%) polymorphic DNA regions, suggesting that *U. parvifolia*W114-1 might be highly heterozygous. A further sample of 109 selfed progenies (76 resistant and 33 susceptible phenotypes not included in any subsequent bulked-DNA samples) was randomly chosen from the 822 available plants for segregation analysis of the putatively linked RAPD markers. Defoliated plants produced sufficient new foliage by early August to permit leaf sampling for DNA extraction.

Total DNA isolation

DNA was extracted from 0.5 g of new foliage (2–3 weeks old) according to the procedure of Bousquet et al. (1990) with the following modifications: leaves were deep-frozen at –80°C, followed by grinding in Eppendorf tubes with plastic autoclaved grinders and glass powder. When tissue was reduced to fine powder, 600 µl of extraction buffer (2% CTAB, 1.5 M NaCl, 50 mM EDTA pH 8.0, 100 mM Tris pH 7.5, 50 mM DTT) was added and tubes were agitated for 5 s. Extraction proceeded at room temperature for 15 min, with regular gentle shaking of the tubes. The homogenate was emulsified with 1 vol of chloroform-isoamylic alcohol (24:1 v/v) and centrifuged (30 min; 5000 g, 20°C). The supernatant was recovered, transferred to clean tubes and the DNA precipitated with 0.6 vol of isopropanol. After 1 h incubation at 4°C, the tubes were centrifuged (10 min; 8000 g, 20°C). DNA pellets were washed with 70% etha-

nol, dried at room temperature and re-dissolved in 200 µl TE buffer (10 mM Tris pH 7.5; 1 mM EDTA pH 8.0). The relative amount of each DNA was checked on a 0.8% agarose gel by comparing the level of fluorescence against quantities (50 ng, 100 ng, 200 ng) of a standard DNA of known concentration. The concentration of DNA samples used for RAPD analysis was adjusted to approximately 20 ng/µl prior to bulking. This simple procedure provided an average of 4 mg DNA, allowing us to obtain consistent and reproducible RAPD amplifications (Fig. 1).

Screening of RAPD markers

In order to determine the optimum number of DNA samples per bulk, and to allow for the detection of "false positives" (Martin et al. 1991; Michelmore et al. 1991), two different DNA composites (bulks) were created for both phenotypic classes of plants (bulks R1 and R2 for the resistant phenotype, and S1 and S2 for the susceptible), containing respectively 10 (S1 and R1) and 20 (S2 and R2) samples of individual plant DNA (20 µl were drawn from DNA aliquots adjusted to 20 ng/µl). A total of 220 10-base primers (kits B, C, D, E, F, O, P, R, S, T and U; Operon Technologies, Alameda, Calif.) was screened for polymorphisms within the two pairs of bulks. Polymorphism between bulks was confirmed by repeating the amplification twice.

RAPD procedure

Each analysis was performed as a 12-µl reaction using 15–20 ng of genomic DNA template, 0.75 U of *Taq* polymerase (Promega), 20 ng of 10-base primers (Operon Technologies), 200 µM of each dNTP (Gibco BRL), 2.5 mM $MgCl_2$ and 1 × reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100) provided with the enzyme. These reactions were overlaid with one drop of mineral oil (Sigma). Amplifications were carried out in a Perkin-Elmer Cetus 480 thermal cycler with amplification conditions adapted from Williams et al. (1990), following Yu and Pauls (1992). After an initial denaturation of 3 min at 94°C, samples were subjected to 45 cycles of 15 s 94°C, 30 s 35°C, 1 min 30 s 72°C, and a final extension of 10 min at 72°C. RAPD fragments were size-fractionated in 1.4% agarose gels in TAE buffer, with a 1-kb ladder molecular-weight marker (Gibco BRL) included in each run. Gels were stained in an ethidium bromide solution (1 µg/ml) for 30 min followed by a water rinse for 10 min to enhance contrast, and then photographed.

Marker analysis with selfed progenies

The putatively linked markers were screened using the 109 randomly chosen progenies to confirm their linkage to the *Bls* locus. Chi-square tests were used to test for departures from the expected 3:1 ratio for markers with a dominant mode of inheritance, as well as to test for linkage between markers and the *Bls* gene. Linkage analysis was completed using the Macintosh 2.0 version of MAPMAKER (Lander et al. 1987). The Kosambi mapping function (Kosambi 1944) was used to convert the recombination fraction to approximate map distances between the markers and the *Bls* gene. Nomenclature for the markers follows the convention of primer designation followed by the fragment size in basepairs.

Genotyping of hybrid progenies

In order to determine whether the *Bls* gene conferred black leaf spot resistance to interspecific hybrids, a set of *U. parvifolia* interspecific hybrids was analyzed to determine the presence/absence of the flanking RAPD markers most tightly linked to the *Bls* gene. The sample of 24 hybrid progenies was divided into two groups according to the symptoms observed; plants scored as 1 or 2 were classified as "resistant", whereas those scored as 4 or 5 were considered "susceptible" (Table 1). Individuals scored as 3 were excluded to reduce the chance of misclassification. Chi-square tests were performed to estimate the likelihood of independence between the occurrence of flanking markers and black leaf spot resistant and susceptible phenotypes.

Results

Identification and linkage analysis of RAPD markers

Of the 220 10-base primers screened, 199 (90.5%) provided 1210 fragments ranging in size from 200 to 3000 kb, while the remaining 21 did not provide reproducible amplified fragments. Ten primers (5% of the 199 useful) provided 11 polymorphic RAPD fragments within pairs of DNA bulks. Four of these proved to be "false positives" as segregation tests showed that they were not linked to the disease phenotype. Two of these fragments were present in the R2 bulk only, while one was present in all bulks except S1. Four other markers were weakly linked to the disease phenotype (>20 cM), whereas the final three (0.2% of the 1210 scored) proved to be tightly linked. The sequences

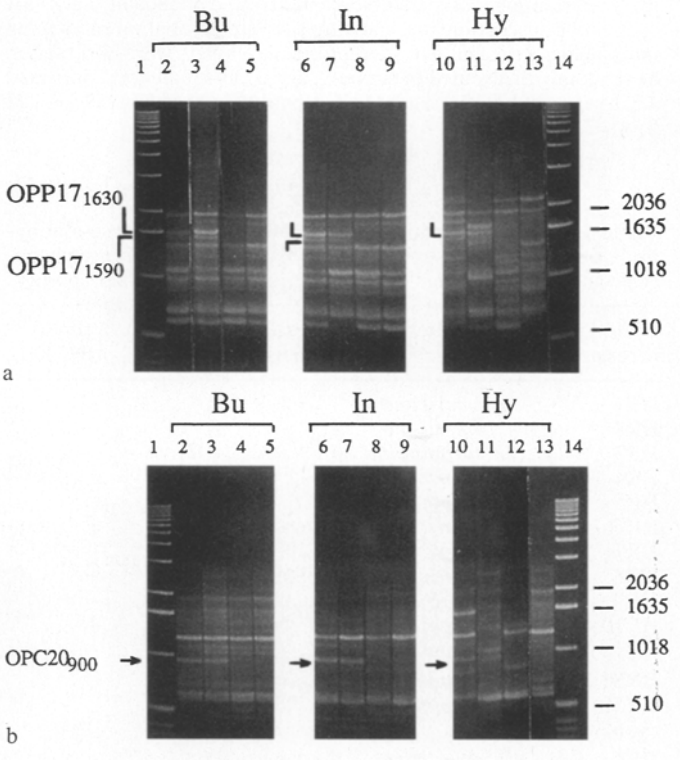

Fig. 2a, b Patterns of amplified DNA fragments obtained from Chinese-elm progenies with primers OPP17 (**a**) and OPC20 (**b**). The first group of DNA templates (*Bu*) contains the four DNA bulks R1 (*lane 2*), R2 (*lane 3*), S1 (*lane 4*) and S2 (*lane 5*). The second group (*In*) is composed of two selfed progenies presenting a resistant phenotype (*lanes 6 and 7*) and two others presenting a susceptible phenotype (*lanes 8 and 9*). The third group (*Hy*) contains templates from the hybrid individuals HR1 (*lane 10*), HR3 (*lane 11*), HS1 (*lane 12*) and HS2 (*lane 13*). Molecular-weight markers (1-kb ladder) are present in *lanes 1 and 14*

Table 2 Phenotype frequencies and χ^2 values for goodness of fit to 3:1 (χ^2A; χ^2B) and to 9:3:3:1 (χ^2AB) expected segregation ratios for 109 selfed Chinese-elm progenies

Locus		Phenotypes (a)				Chi-square value		
A	B	AB	Ab	aB	ab	χ^2A	χ^2B	χ^2AB
Bls	$OPC20_{900}$	72	4	1	32	1.77	4	121.7**
Bls	$OPP17_{1630}$	71	5	4	29		2.4	94.5**
Bls	$OPP17_{1590}$	65	7	4	29		4	91.2**
$OPC20_{900}$	$OPP17_{1630}$	67	6	8	28			80.4**
$OPC20_{900}$	$OPP17_{1590}$	66	7	7	29			91.2**
$OPP17_{1630}$	$OPP17_{1590}$	65	10	8	26			64.0**

** Significant at the 0.01 probability level

[a] Phenotypes: AB: anthracnose-resistant and RAPD markers present; Ab: anthracnose-resistant and RAPD markers absent; aB: anthracnose-susceptible and RAPD markers present; ab: anthracnose-susceptible and RAPD markers absent

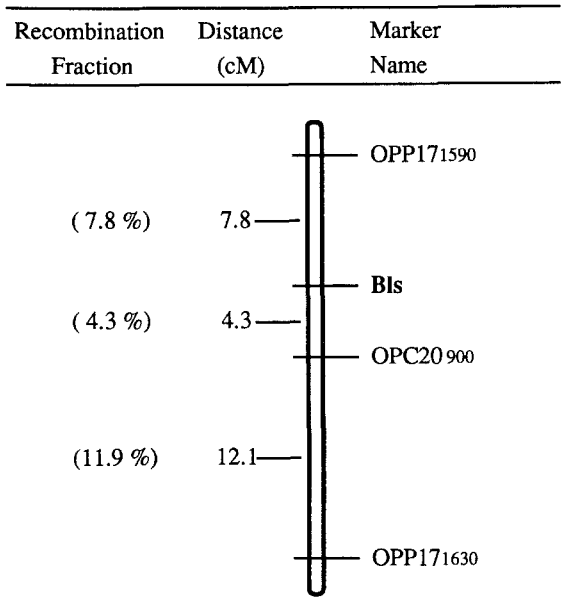

Fig. 3 Genetic map of the region of Chinese-elm genome containing the *Bls* gene. Genetic distances have been computed using the Kosambi mapping function of MAPMAKER

of the two primers providing these three markers are

5′TGACCCGCCT3′ (OPP17); and
5′ACTTCGCCAC3′ (OPC20).

For each case, a clear bright fragment was amplified in both resistant bulks while it was absent in both susceptible bulks (Fig. 2). However, by repeating the analysis with primer OPP17, the fragment $OPP17_{1630}$ was slightly amplified in the S1 bulk in one experiment. After decomposing this bulk in order to analyze separately each of ten individuals, we found that 3 of 10 plants were actually recombinant.

Scoring of the tightly linked markers using the 109 selfed progeny suggested that resistance to black leaf spot in *U. parvifolia* conforms to a single-gene model with resistance dominant to susceptibility (Table 2). Fortuitously, all tightly-linked markers were in coupling-phase to one another and to the *Bls* gene. The resistant bulks were composed of both homozygous- and heterozygous-resistant individuals, preventing the tagging of the susceptibility allele. The distances between *Bls* and the flanking markers $OPC20_{900}$, $OPP17_{1630}$, and $OPP17_{1590}$ ranged from 4.3 to 12.1 (Fig. 3).

Segregation analysis within the hybrid progeny

Analysis of the hybrid progeny (Table 1) revealed that phenotypes scored as 1, 4 or 5 had a marker genotype consistent with the hypothesis of a major effect of the *Bls* gene on the control of resistance. However, two of the three plants scored as 2 did not possess the RAPD markers although they exhibited limited disease symptoms. Considering the remaining 22 individuals scored as 1, 2, 4, or 5, the probability of independence between the *Bls* gene and disease resistance is not significant (χ^2=25.5 for $OPP17_{2000}$ and χ^2=26.3 for $OPC20_{900}$). We conclude that the *Bls*gene is likely to be the principal factor responsible for the "resistant" phenotype observed among the interspecific hybrid progeny.

Discussion

Using RAPD techniques in combination with bulked segregant analysis, we were able to identify and map three RAPD markers linked to a dominant gene conferring resistance to black leaf spot in *U. parvifolia*. From a total of 1210 fragments, only three (0.2%) were linked to the *Bls* gene. This proportion of linked markers is about one-half the number detected in tagging downy mildew resistance genes in lettuce (Michelmore et al. 1991), a result we attribute to our use of selfed progeny from a wild accession rather than an F_2 population. The expected level of polymorphism was much lower in our case, even though the *U. parvifolia* parent tree was apparently highly heterozygous.

Working with two different bulks of resistant and susceptible phenotypes proved to be useful as only the RAPD markers were strongly amplified in both resistant bulks and absent in both susceptible bulks, thus enabling the detection of most false positives and weakly linked markers. As an example, the marker $OPP17_{1630}$ was slightly amplified in the S1 bulk because it contained, by chance, three recombinant plants.

In the case of black leaf spot of elm, even though the *Bls* gene does not provide absolute resistance for the interspecific hybrids studied here, selection of hybrid individuals possessing the two flanking markers will aid in the selection of a high level of resistance. Such marker-aided selection (MAS) has been proposed to facilitate early selection in woody plants, where breeding is hampered by long generation intervals, strong environmental effects upon economically important traits, and imperfect juvenile-mature

correlations (Bernatsky and Mulcahy 1992; Williams and Neale 1992), but successful application of MAS in woody plants is uncommon. For elms, especially American elm (a tetraploid, 2n=4x=56) in combination with other diploid species, observed inheritance patterns are likely to be complicated by variation in ploidy level. No genetic maps yet exist for any elm species, so the impact of this variation on MAS is uncertain. However, these interspecific hybrids between American and Chinese elm also present a range of phenotypes for resistance to Dutch-elm disease (Smalley et al. 1993), suggesting that bulked segregant analysis might offer a means to identify genes controlling resistance to DED as well.

The scoring of interspecific hybrid-elm phenotypes showed a pattern very different from the selfed progenies, since resistance appears to segregate as a quantitative trait. This may result from the existence of other genes of smaller effect introduced into the progeny by the *U. carpinifolia* and *U. americana* parents. The presence of other genetic factors influencing resistance might explain the apparent resistance among the hybrids scored as a 2 (Table 1) but which did not possess the *Bls* gene. Phylogenetic studies of elm species have shown that the three species *U. parvifolia*, *U. carpinifolia* and *U. americana* are not closely related (Wiegrefe 1992), and it is possible that genetic control of resistance to black leaf spot is different in each species.

Previous studies of resistance to foliage anthracnose in different crops often show a simple inheritance as described here, although the pathogen involved varies from crop to crop. Resistance to bean anthracnose (Pastor-Corrales and Tu 1989) has proven to be controlled by several single dominant genes, each being efficient for a specific race of the pathogen. Recently, anthracnose stalk rot resistance in maize was found to be controlled by two closely linked genes (Cowen et al. 1991). However, an attempt at mapping resistance to anthracnose (*Colletotrichum gloeosporioides*) in *Stylosanthes* failed because the F_2 population studied displayed a complex segregation pattern (Kazan et al. 1992).

RAPD markers have already proved useful for genetic analysis in trees such as aspen (Liu and Furnier 1993), peach (Chaparro et al. 1994), cacao (Wilde et al. 1992), and several conifers (Carlson et al. 1991), and were successfully used for the genome mapping of pines (Nelson et al. 1993) and eucalyptus (Grattapaglia and Sederoff 1994). The procedure of RAPD/BSA appears particularly valuable for woody plants for which no genome map is available, pedigrees are rare, and creation of near-isogenic lines would be extremely time consuming or impossible. For these species, the tagging of resistance genes may enable breeders to select putatively resistant individuals during early phases of germplasm evaluation. In addition, phenotypic diagnosis of resistance to diseases like anthracnose typically requires screening of plants in the greenhouse, or under controlled environment conditions. Given the cost and time involved in disease resistance screening, marker-aided selection could be employed to screen hybrids created using *U. parvifolia*, *U. pumila*, and others, by using RAPD markers to identify other genes involved in black leaf spot resistance. This method could be extended to other cases of pest resistance in trees, including oligogenic resistance, provided that adequate segregating populations exist or can be created.

Acknowledgements This work was supported by the School of Natural Resources, College of Agricultural and Life Sciences, University of Wisconsin-Madison, and by support from the McIntire-Stennis Cooperative Forestry Research Program, project 142-2968.

References

Bernatsky R, Mulcahy DL (1992) Marker-aided selection in a backcross breeding program for resistance to chestnut blight in the American chestnut. Can J For Res 22:1031–1035

Bousquet J, Simon L, Lalonde M (1990) DNA amplification from vegetative and sexual tissues of trees using polymerase chain reaction. Can J For Res 20:254–257

Burdekin DA (ed) (1983) Research on Dutch-elm disease in Europe. Forest Comm Bull No 60, HMSO London

Carlson JE, Tulseriam LK, Glaubitz JC, Luk VWK, Kauffeld C, Rutledge R (1991) Segregation of random amplified DNA markers in F_1 progeny of conifers. Theor Appl Genet 83:194–200

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

Cowen NM, Woosley A, Skokut M, Armonstrong K, Toman J, White DG (1991) Mapping anthracnose stalk rot resistance in maize using RFLP analysis. Proc 33rd Ann Maize Genet Conf Abstract 29

Grattapaglia DP, Sederoff R (1994) Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: mapping strategy and RAPD markers. Genetics 137:1121–1137

Kazan K, Manners JM, Cameron DF (1993) Inheritance of random amplified polymorphic DNA markers in an interspecific cross in the genus *Stylosanthes*. Genome 36:50–55

Kosambi D (1944) The estimation of map distances from recombination values. Ann Eugen 12:172–175

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Linclon SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Liu Z, Furnier GR (1993) Comparison of allozyme, RFLP, and RAPD markers for revealing genetic variation within and between trembling aspen and bigtooth aspen. Theor Appl Genet 87:97–105

Martin GB, Williams JGK, Tanksley, SD (1991) Rapid identification of markers linked to a *Pseudomonas* resistance gene by using random primers and near-isogenic lines. Proc Natl Acad Sci USA 88:2336–2340

McGranahan GH, Smalley EB (1984a) Influence of moisture, temperature, leaf maturity and host genotype on infection of elms by *Stegophora ulmea*. Phytopathology 74:1296–1300

McGranahan GH, Smalley EB (1984b) Conidial morphology, axenic growth and sporulation of *Stegophora ulmea*. Phytopathology 74:1300–1303

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Nelson CD, Nance WL, Doudrick RL (1993) A partial genetic linkage map of a slash pine (*Pinus elliottii* Englm. var. *elliottii*) clone based on random amplified polymorphic DNAs. Theor Appl Genet 87:145–151

Paran I, Michelmore RW (1993) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor Appl Genet 85:985–993

Pastor-Corrales MA, Tu JC (1989) Anthracnose. In: Schwartz HF, Pastor-Corrales MA (eds) Bean production problems in the tropics, 2nd edn. CIAT, Columbia, pp 77–104

Sinclair WA, Lyon HH, Johnson WT (1987) Diseases of trees and shrubs. Cornell University Press, Ithaca, New York

Smalley EB, Guries RP (1993) Breeding elms for resistance to Dutch-elm disease. Annu Rev Phytopathol 31:325–52

Smalley EB, Guries RP, Lester DT (1993) American Liberty elms and beyond: going from the impossible to the difficult. In: Sticklen MB, Sherald J (eds) Dutch-elm disease: cellular and molecular approaches, pp 26–45, Springer-Verlag, New York

Sticklen MB, Sherald J (eds) (1993) Dutch-elm disease: cellular and molecular approaches. Springer-Verlag, New York

Townsend AM, Schreiber LR (1976) Resistance of hybrid elm progenies to *Ceratocystis ulmi*. Phytopathology 66:1107–1110

Wiegrefe SJ (1992) Molecular genetic variation in the Ulmaceae: phylogenetic implications. PhD dissertation, University of Wisconsin, Madison, USA

Wilde J, Waugh R, Powell W (1992) Genetic fingerprinting of *Theobroma* clones using random amplified DNA markers. Theor Appl Genet 83:871–877

Williams CG, Neale DB (1992) Conifer wood quality and marker-aided selection: a case study. Can J For Res 22:1009–1016

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535

Yu K, Pauls KP (1992) Optimization of the PCR program for RAPD analysis. Nucleic Acids Res 20:2606