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Genetic validity of RAPD markers at the intra- and inter-specific level in wild *Brassica* species with n = 9

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Abstract The sequence homology of co-migrating RAPD markers within a genus, across species, and among populations of a species was investigated. DNA was isolated from ten wild *Brassica* species with n = 9 and the RAPD patterns were established using three random primers. Five RAPD markers which appeared to be characteristic for the n = 9 species (genus level), four markers which appeared to be species specific, and one population-specific marker were isolated from agarose gels and hybridized to the RAPD profiles of the ten *Brassica* species. Two RAPD markers were cloned for comparison with gel-isolated RAPD fragment probes in hybridization experiments. Non-specific and background hybridization, occurring when gel-isolated fragments were used as probes, disappeared when cloned fragments were used. A total of 250 RAPD-marker hybridizations were scored according to visual presence or absence in a gel lane. All except three markers hybridized as expected, resulting in an error rate of 1.2%. The deviating results included a lack of hybridization although a band was visible in the gel, a length polymorphism for one marker, and a dual hybridization signal for two single-band markers.

Key words RAPD markers · Marker reliability · RAPD fragment sequence homology · Co-dominant bands

Introduction

The estimation of genetic features in populations and at the individual level has been strongly influenced by polymerase chain reaction (PCR) technology. The development of random amplified polymorphic DNAs (RAPDs), which are specific fragment patterns generated from random oligomer primers, has permitted the genetic characterization of many materials which are difficult to treat using more traditional marker systems.

The data recovered using RAPDs are often assumed to have the same genetic basis and properties as the data acquired from the observation of other traits, such as isozyme alleles and RFLP profiles. Indeed, many studies support the genetic validity of RAPD patterns. Mendelian segregation and stable, dominant inheritance of RAPD markers have been observed in genetically diverse crop species, including soybeans, barley, and alfalfa (Williams et al. 1990; Echt et al. 1992; Tinker et al. 1993). In other applications, RAPD markers were able to verify and establish genetic relationships among species, crop cultivars, and isolates (Kresovich et al. 1992; Gonzalez et al. 1993; Morgan et al. 1993; dos Santos et al. 1994; Mailer et al. 1994).

However, discrepancies in both the RAPD pattern inheritance and the sequence identity of fragments have also been reported. In a mapping project for *Arabidopsis thaliana*, only 57% of the polymorphic RAPD markers were inherited in a Mendelian fashion (Reiter et al. 1992). Co-migrating RAPD markers present in different *Brassica* species were shown to have non-homologous DNA sequences in hybridization experiments (Thormann and Osborn 1992). In another study, a chromosome-specific RAPD fragment hybridized to each of a series of nullisomic wheat lines, including the line lacking the chromosome which the RAPD marker had been mapped to (Devos and Gale 1992).

The contradicting results concerning the reliability of RAPD markers indicate a need for further testing of particular RAPD polymorphic fragments before using them in genetic analyses. In the present study, a series of RAPD fragments from various accessions of *Brassica* species was tested by Southern hybridization for fragment homology at the population, species, and genus level. The *Brassica* species employed belong to the *B.*

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oleracea cytodeme (n = 9) and comprise ten wild species related to *B. oleracea*. Populations of the wild *B. oleracea* were analyzed using isozymes and RAPDs (Lannér-Herrera et al. 1996). This investigation of the genetic validity and homology of RAPD fragments was undertaken before extending a RAPD analysis to the species level.

Materials and methods

Plant material

Brassica species with the chromosome number n = 9 were acquired from the Genebank at Universidad Politecnica in Madrid, Spain (Table 1). A representative population was chosen for each species, except for *B. oleracea* and *B. cretica* which were represented by five and six populations, respectively, covering the natural range of the species in the wild. From 3 to 25 individual plants were available from each population.

RAPD analysis

DNA was isolated from leaf tissue according to Edwards et al. (1991). Primers were purchased from Operon Technologies (California, USA). The 100-bp ladder from Pharmacia (Stockholm, Sweden) was used as the molecular marker for agarose gels. PCR reactions were run as described by Lannér-Herrera et al. (1996). RAPD profiles were established for primers B-05, A-19, and F-06 using the same PCR reaction method. Bands characteristic for the different levels of discrimination (genus, species, population) were chosen for hybridization from a comparison of 3–5 individuals in each population sample.

Hybridization analysis

Individual RAPD fragments were cut from agarose gels and isolated using Prep-A-Gene DNA purification matrix (BioRad Laboratories Inc., California, USA). The isolated DNA fragments were further amplified by PCR and the resulting products were visualized via agarose-gel electrophoresis. If multiple fragments had been amplified, the desired fragment size was re-isolated from the agarose gel for use as a probe. DNA fragments were labelled using the Digoxigenin-11-dUTP labelling kit from Boehringer-Mannheim Biochemicals (Mannheim, Germany). DNA fragments from primer F-06 were also cloned into the *Eco*RI site of the Bluescript vector pBS-KS+ obtained from the Stratagene Company (California, USA). Capillary blots of PCR gels were carried out as described by Sambrook et al. (1989). Hybridizations were performed at 65 °C followed by washes at room temp in 2 × SSC, 0.1% SDS, 0.5 × SSC, 0.1% SDS for 15 min each and 0.1 × SSC, 0.1% SDS for 30 min at 65 °C. Chemiluminescent detection using Lumigen PPD (Boehringer-Mannheim) was carried out according to the manufacturer's recommendations except that buffer no. 1 was replaced by 0.1 M Tris pH 7.5, 0.15 M NaCl for the first wash. Films were exposed for 30–60 min.

Results

RAPD profile analysis

From each *Brassica* population, 3–5 individuals were used to screen the primers for diagnostic bands which could be considered as occurring in populations, species, or were common to all the *Brassica* samples included for testing.

The RAPD profile for primer A19 contained bands at 1920 bp and 420 bp which appeared to be common to all *Brassica* n = 9 species (genus characteristic) (Fig. 1a). Species-specific bands were found at 1500 bp, 650 bp, and 350 bp in the *B. cretica* sample, and at 600 bp in the *B. rupestris* sample. Although one of the *B. rupestris* individuals shown does not carry the A19: 600-bp band, it is present in all the other *B. rupestris* individuals tested but not in any of the other *Brassica* species.

The RAPD profile for primar B05 produced a band at 1850 bp which could be considered as genus characteristic (Fig. 2a). A 1420-bp band was genus characteristic to all the *Brassica* samples except for the *B. bourgeaui* population. The 1420-bp band was included to test whether the absence in the *B. bourgeaui* sample was real or whether the band was too faint to view. From the *B. insularis* sample, a 1200-bp band was chosen to test for species specificity. The smallest B05 fragment tested was

Table 1 Wild *Brassica* n = 9 species. Collection sites and original collection numbers are listed

Species	Origin	Pop. no.
B. bourgeaui	Canary Islands	CI120
B. cretica ssp. *aegaea*	Greece, Attica	Gr01
B. cretica ssp. *aegaea*	Greece, Evia	Gr25
B. cretica ssp. *aegaea*	Greece, Limnos	Gr44
B. cretica ssp. *laconica*	Greece, Lakonia	Gr14
B. cretica ssp. *cretica*	Greece, Achia	Gr20
B. cretica ssp. *cretica*	Greece, Crete	Gr41
B. hilarionis	Cyprus	Cy144
B. incana	Italy, Napoli	It65
B. insularis	Tunisia	Tn140
B. macrocarpa	Italy, Isole Egadi	It84
B. montana	France, Toulon	Fr115
B. oleracea	Spain, Guipuzcoa	Es210
B. oleracea	France, Charante Maritime	Fr211
B. oleracea	France, Seine Maritime	Fr224
B. oleracea	Great Britain, Kent	GB226
B. oleracea	Great Britain, Cornwall	GB237
B. rupestris var. *hispida*	Italy, Sicily	It80
B. villosa var. *villosa*	Italy, Sicily	It89

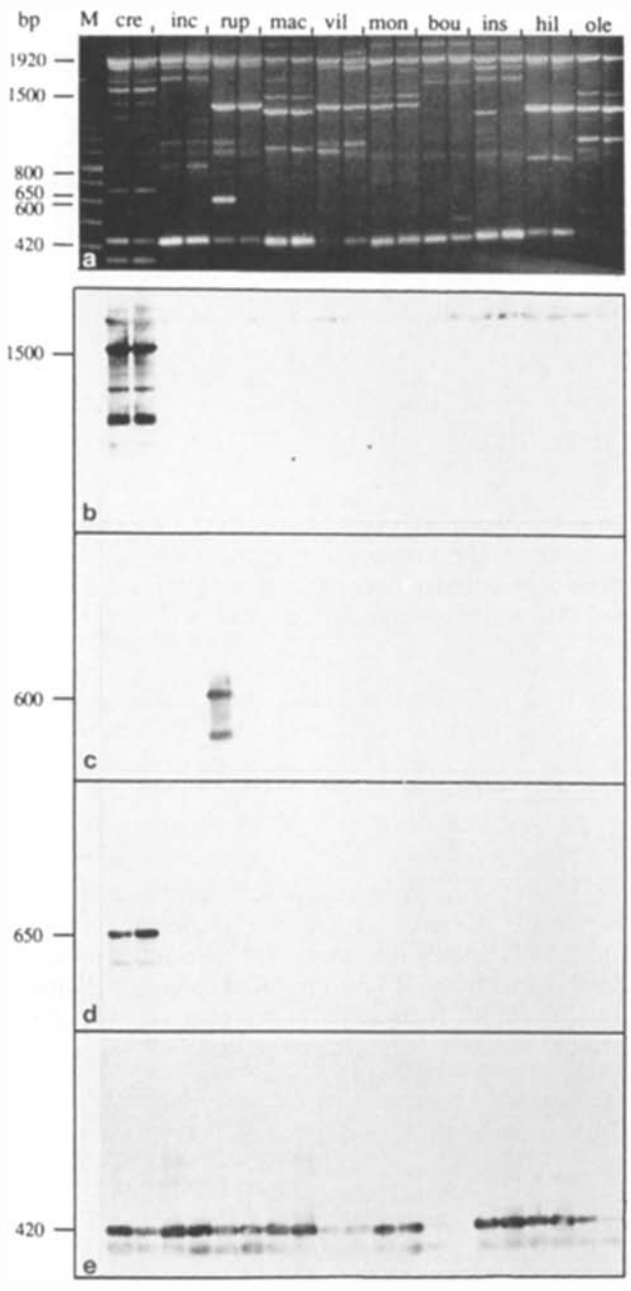

Fig. 1a–e Primer A19 RAPD profiles and hybridization results for A19 RAPD markers. **a** RAPD profiles on an agarose gel; lane order: *M* 100-bp ladder; *cre B. cretica*; *inc B. incana*; *rup B. rupestris*; *mac B. macrocarpa*; *vil B. villosa*; *mon B. montana*; *bou B. bourgeaui*; *ins B. insularis*; *hil B. hilarionis*; *ole B. oleracea*. **b** Southern hybridization of a A19: 1500-bp band. **c** Southern hybridization of a A19: 600-bp band. **d** Southern hybridization of a A19: 650-bp band. **e** Southern hybridization of a A19: 420-bp band

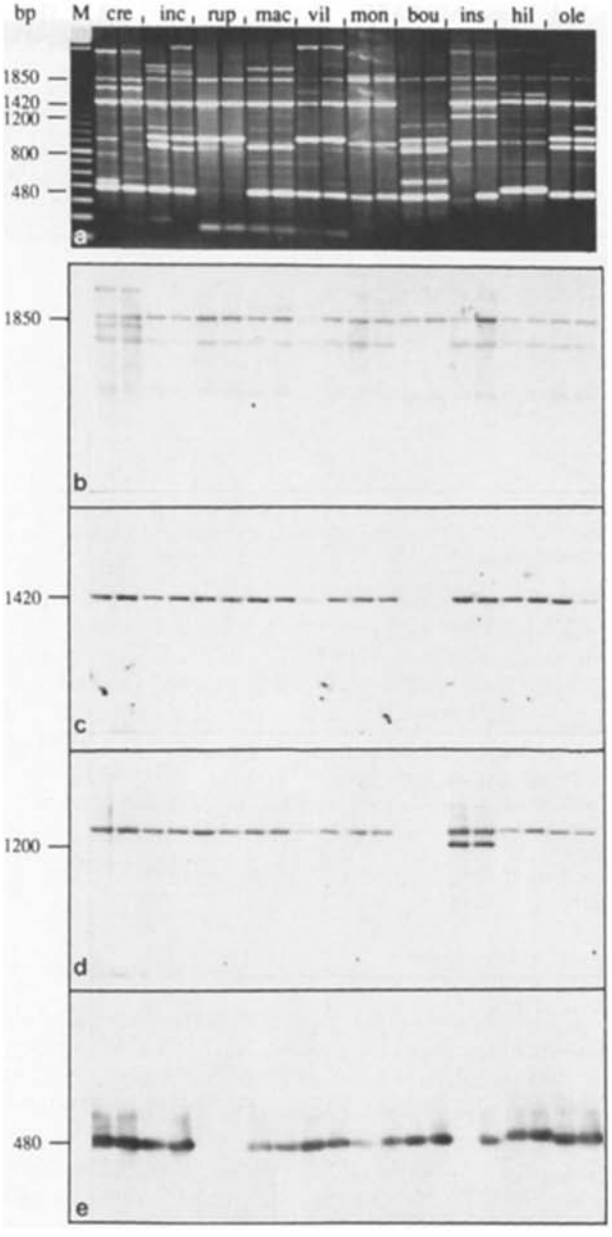

Fig. 2a–e Primer B05 RAPD profiles and hybridization results for B05 RAPD markers. **a** RAPD profiles on an agarose gel, lane order same as in Fig. 1. **b** Southern hybridization of a B05: 1850-bp band. **c** Southern hybridization of a 1420-bp band. **d** Southern hybridization of a B05: 1200-bp band. **e** Southern hybridization of a B05: 480-bp band

a 480-bp fragment, which appeared in most of the samples, except for *B. rupestris* and one *B. insularis* individual. In the *B. hilarionis* individuals, fragments migrating just above and below the 480-bp band were observed. In addition, a band apparently the same size as the *B. hilarionis* band just above the 480-bp position was present in one of the *B. cretica* individuals. The 480-bp fragment was included to test whether the slightly larger bands in the *B. hilarionis* and *B. cretica* populations were different and specific to those species and whether the absence in the *B. rupestris* and *B. insularis* samples was genuine.

Six *B. cretica* populations, Gr01, Gr14, Gr20, Gr41 and Gr44, were used to test the population specificity within a species of a 650-bp band from the RAPD profile of primer A19 (Fig. 3a). The 650-bp band appeared to be present only in the Gr14 population.

Primer F06 was used to investigate RAPD band homology among populations and the hybridization

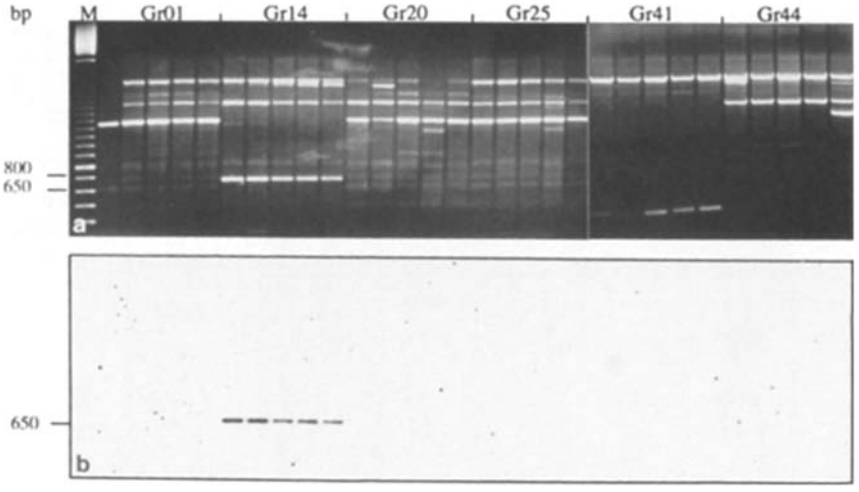

Fig. 3 a,b Primer A19 RAPD profiles for *B. cretica* populations and hybridization results for a A19: 650-bp marker. **a** RAPD profiles on an agarose gel; lane order: *M* 100-bp ladder; populations, Gr01, Gr14, Gr20, Gr25, Gr41, Gr44, five individuals each. **b** Southern hybridization of a A19: 650-bp band

fidelity of cloned RAPD bands in contrast to gel-isolated RAPD-band probes. When comparing five *B. oleracea* populations (GB237, GB226, Fr224, Fr211, Es210), primer F-06 produced a profile containing a band at 1300 bp which appeared to occur in the GB226, Fr224, and Es210 populations, and a band at 350 bp which was present in the Fr211 and Es210 populations (Fig. 4a). After cloning of the 1300-bp and 350-bp bands, inserts of the same size as the RAPD bands were isolated and used as probes in hybridization experiments.

Hybridization results

The RAPD bands chosen as genus characteristic for all members of the *Brassica* genus with $n = 9$ (A19: 1920 bp, A19: 420 bp, B05: 1850 bp, B05: 1420 bp, B05: 480 bp) hybridized accordingly (Fig. 1e, Fig. 2b,c,e; A19: 1920-bp data not shown). Despite some background, and the appearance of extra bands not visible on the agarose gels (A19: 420 bp, Fig. 1e), hybridization to the corresponding fragment size in all samples carrying the band was evident, thus demonstrating sequence homology among bands of identical size at the genus level. In the B05: 1850 bp hybridization, there was some hybridization to the B05: 1420-bp band and several bands in the *B. cretica* lanes (the B05: 1850-bp band was isolated from the *B. cretica* sample). However, there was no reciprocal hybridization in the B05: 1420-bp experiment, indicating that hybridization to the 1420-bp band was probably due to contamination of the 1850-bp band with the 1420-bp fragment. This is also probably the case for the extra hybridization bands in the *B. cretica* lanes. The absence of the B05: 1420-bp band in the *B. bourgeaui* gel lanes was supported by the absence of hybridization on the membrane (Fig. 2c). A corresponding absence on both the gel and the Southern was also shown by the B05: 480-bp band in the *B. rupestris* and *B. insularis* samples. The contrary situation was manifested by the A19: 420-bp band which seemed to be present in the *B. bourgeaui* RAPD pattern, but did not show a hybridization signal for the A19: 420-bp probe in one of the individuals.

The B05: 480-bp probe hybridized to all lanes where it was visible. Hybridization of the B05: 480-bp band to the slightly larger bands in the *B. hilarionis* sample was also evident, but not to the band in the *B. cretica* sample.

Species specificity of RAPD bands was demonstrated by the A19: 1500-bp and 650-bp bands in the species *B. cretica* (Fig.1b, d), the A19: 600-bp band in *B. rupestris* (Fig. 1b), and the B05: 1200-bp band in *B. insularis* (Fig. 2d). The fragments hybridized only to the species they were isolated from. In the experiments with the A19: 1500-bp, the A19: 600-bp, and B05: 1200-bp probes, hybridization was observed to other bands. The B05: 1200-bp probe hybridized most notably to the B05: 1420-bp band. This could be due to contamination of the B05: 1200-bp probe sample with material from the B05: 1420-bp band and not to any sequence homology between the two bands, since the B05: 1420-bp band showed no hybridization to the B05: 1200-bp band (Fig. 2c). A similar explanation may account for the extra bands in the A19: 1500-bp and 600-bp hybridizations.

Population specificity of RAPD markers was observed using the primer A19: 650-bp band. This band appeared exclusively in the *B. cretica* population Gr14 and hybridized only to the Gr14 individuals (Fig. 3a,b).

The F06 primer fragments of 400 bp and 1300 bp hybridized to the corresponding fragment size in the population samples scored as positive for the fragments (Fig. 4b–e). The hybridization results, however, displayed multiple bands in other lanes, plus significant background (Fig. 4b,d). This type of result was also observed using other gel-isolated RAPD fragments as probes. To examine whether the extra bands were due to sequence homology or to contamination of the F06: 400-bp and 1300-bp fragments with other fragments from the RAPD profile through fragment drag, both the F06: 400-bp and 1300-bp fragments were cloned and the

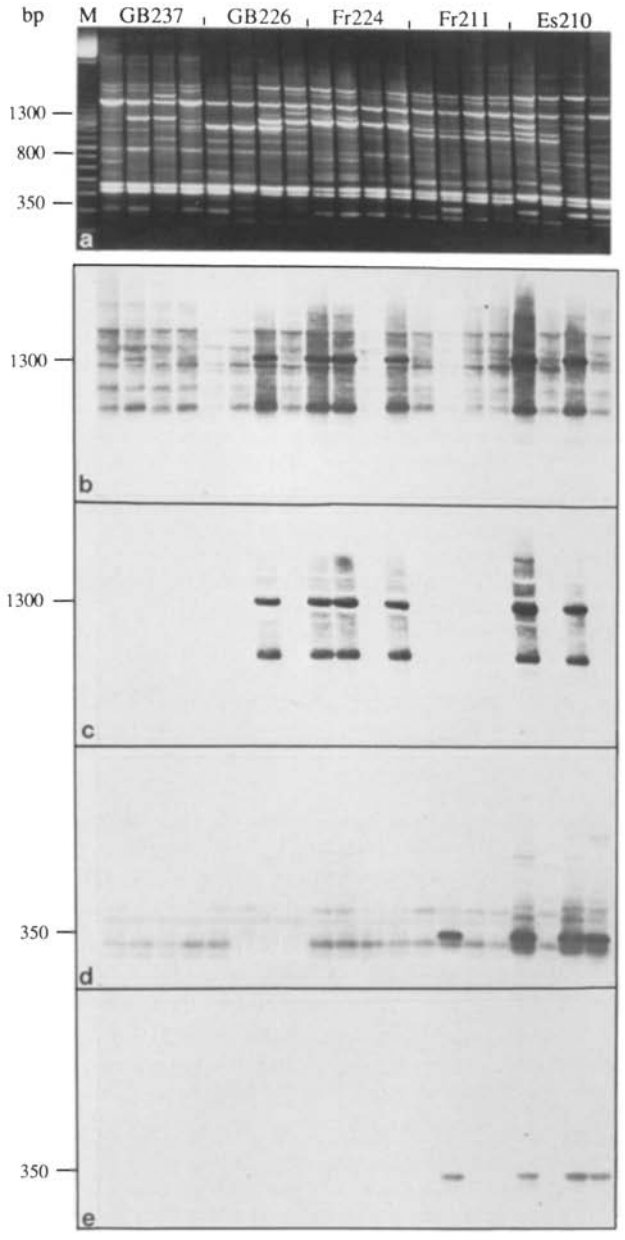

Fig. 4 a–e Primer F06 RAPD profiles for *B. oleracea* populations and hybridization results for F06 RAPD markers. **a** RAPD profiles on an agarose gel; lane order: *M* 100-bp ladder, populations, GB237, GB226, Fr224, Fr211, Es210, four individuals each. **b** Southern hybridization of a F06: 1300-bp gel-isolated band. **c** Southern hybridization of a F06: 1300-bp clone insert. **d** Southern hybridization of a F06: 400-bp gel-isolated band. **e** Southern hybridization of a F06: 400-bp clone insert

inserts were isolated for use as probes. In hybridization experiments with the two cloned fragments, all other background and non-specific bands disappeared (Fig. 4c,e). This indicates that the gel-isolated fragments were contaminated with other fragments, which upon amplification increased sufficiently in quantity to generate a non-specific background in hybridization. A similar explanation for the background and the multiple non-specific bands observed with other gel-isolated probes is probable.

Multiple bands other than those generated by non-specific background hybridization occurred in the experiments using the cloned F06: 1300-bp insert and the A19: 600-bp fragment as probes. The F06: 1300-bp fragment hybridized to an additional band at about 500 bp in all samples which carried the 1300-bp fragment, while the A19: 600-bp fragment hybridized to a fragment approximately 400 bp in size (Figs. 4c, 1c). The lack of non-specific hybridization and background in these instances indicates a true two-band hybridization pattern for these fragments.

Discussion

The fidelity of hybridization of RAPD fragments to identical-sized fragments in other RAPD profiles was high. Out of a total of 250 expected fragment hybridization patterns scored, 3 (1.2%) did not give the expected hybridization pattern based on a visual determination of the presence or absence of a band in an agarose gel. Therefore, the majority of PCR fragments migrating at the same position in a gel may be assumed to have homologous sequences and a high degree of genetic relatedness.

The exceptions to the expected hybridization patterns were of three kinds. In the first category, there was a presence-absence discrepancy for the primer A19: 420-bp fragment in the *B. bourgeaui* sample. The visual presence of a 420-bp band in the second individual was not confirmed by hybridization to the probe. This could be caused by a double band migrating at that position, only one representative of which was present in the second *B. bourgeaui* individual. Besides the scoring of a false positive from the agarose gel, this situation would result in failure to recognize two co-migrating RAPD fragments at that position. The effect on the data set is the loss of a RAPD locus and an incorrect interpretation of the RAPD marker pattern.

In the second category, RAPD fragment probes hybridized to multiple fragments in each sample which contained the fragment (F06: 1300 bp and A19: 600 bp). None of these additional hybridization bands were clearly visible on the agarose gels. Also, the multiple band patterns were identical in all samples which contained the RAPD fragment being tested, no segregation of bands among populations or species was observed. A similar, multiple band pattern, resulting from hybridization with a single fragment, was observed by Tinker et al. (1993) in inbred spring barley lines. They called the bands co-dominant and suggested that multiple priming sites within a fragment may have produced the extra hybridization bands. Internal priming sites could be weaker than external ones, since the shorter fragments are not produced in visible quantities like the larger, probe fragments. As amplification proceeds, the increased amount of template may permit fragments from

the weaker sites to be produced in quantities sufficient to detect via hybridization. The failure to score multiple weak bands, detectable only via hybridization, should not significantly affect the data provided by the RAPD marker. Since the bands all co-segregate with the visible fragment, they do not provide any new information. If the multiple bands are derived from weaker internal priming sites, their detection would only result in overscoring of the same locus.

A third category of possible RAPD marker irregularity is demonstrated by the B05: 480-bp fragment. This marker occurs in all species except *B. rupestris* and one *B. insularis* individual. In the *B. hilarionis* sample it is also absent and instead a strong band, slightly larger than 480 bp (about 550 bp), occurs. Although of a different size, the 550-bp band in *B. hilarionis* clearly hybridizes to the B05: 480-bp probe (Fig. 2e), making the 550-bp band in *B. hilarionis* a length polymorphism of the more common 480-bp band. In addition, a fragment of similar size (550 bp) occurs in one of the *B. cretica* individuals but does not hybridize to the 480-bp probe, which indicates that this fragment is a separate locus from the 480-bp fragment. Both 550-bp fragments would most likely have been scored as identical and as a separate locus from the 480-bp fragment in the *B. cretica* and the *B. hilarionis* samples, thereby generating a false score of relationship between these two species. This would, of course, seriously undermine the reliability of this RAPD marker for determining genetic relationship. However, this type of error occurred only twice among the 250 band hybridizations investigated, producing a low error rate of 0.8%.

The genetic homology of same-sized RAPD fragments in samples from different populations at the genus, species, and population level appears to be high, reliable, and repeatable. A RAPD fragment used as a probe seldom hybridized to unexpected differing positions in different samples. Sequence homology of comigrating RAPD fragments was also observed in a RAPD analysis of bark beetle species (Cognato et al. 1995). A lower level of homology was reported by Thormann and Osborn (1992). In their comparison of RAPD and RFLP markers in different accessions of *Brassica*, three RAPD fragments of 15 bp which were used as probes, did not hybridize to the same-sized fragments across accessions of different species. Homology of identical-sized bands was only observed within species or accessions. Whether this could be due to contamination of RAPD fragment probes with other fragments, is not possible to determine from the report.

In conclusion, the assumption of sequence homology, and therefore genetic relatedness, for identical-sized RAPD fragments at the genus level, among species, and within populations is generally valid. While discrepancies may occur, they are most often not likely to significantly affect the data set or even be detected via visual screening of RAPD profiles on agarose gels. However, if a particular band in a RAPD profile is to be used for discrimination of a particular genotype, it is still worthwhile to test for possible anomalies.

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