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Towards an integrated linkage map of common bean

1. Development of genomic DNA probes and levels of restriction fragment length polymorphism

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Summary. Two genomic libraries were established to provide markers to develop an integrated map combining molecular markers and genes for qualitative and quantitative morpho-agronomic traits in common bean. Contrasting characteristics were observed for the two libraries. While 89% of the *Pst*I clones were classified as single-copy sequences, only 21% of the *Eco*RI-*Bam*HI clones belonged in that category. Clones of these two libraries were hybridized against genomic DNA of nine genotypes chosen according to their divergent evolutionary origin and contrasting agronomic traits. Eight restriction enzymes were used in this study. *Pst*I clones revealed 80–90% polymorphism between the Andean and Middle American gene pools and 50–60% polymorphism within these gene pools. However, under the same conditions only 30% of the *Eco*RI-*Bam*HI clones showed polymorphism between the Middle American and Andean gene pools. Hybridization with *Pst*I clones to *Eco*RI-, *Eco*RV-, or *Hin*dIII-digested genomic DNA resulted in a cumulative frequency of polymorphism of approximately 80%. Hybridizations to *Bam*HI-, *Hae*III-, *Hin*fI-, *Pst*I-, and *Xba*I-digested genomic DNA detected no additional polymorphisms not revealed by the former three enzymes. In the *Pst*I library, a positive correlation was observed between the average size of hybridizing restriction fragments and the frequency of polymorphism detected by each restriction enzyme. This relationship is consistent with the higher proportion of insertion/deletion events compared with the frequency of nucleotide substitutions observed in that library.

Key words: *Phaseolus vulgaris* – RFLP – Genetic diversity

Introduction

Common bean (*Phaseolus vulgaris* L., $2n = 2\times = 22$) is grown worldwide on more than 12 million hectares (Singh 1989) and constitutes a major source of dietary protein in Third World countries (Pachico 1989). Most of the common bean production and consumption consists of dry bean, although green pods can also be important on a regional basis (Singh 1989). Recently, its health benefits (e.g., source of fibers, hypocholestesterolemic effect) have also received considerable attention (Andersen et al. 1984).

Although common bean has been used to identify or confirm important genetic concepts (e.g., segregation, Mendel 1865; genotype versus phenotype, Johannsen 1909; quantitative trait locus, Sax 1923), there have been fewer genetic studies conducted with common bean than with other crop plants such as maize, pea, or tomato. This has been due in part to the lack of available markers. This situation can be remedied using restriction fragment length polymorphism (RFLP) markers. Compared to traditional morphological and biochemical markers, the advantages of RFLPs include their high number, their higher levels of polymorphism, co-dominance, freedom from environmental, epistatic, or deleterious effects, and detectability in all tissues and developmental stages (Beckmann and Soller 1986; Tanksley 1983).

Our long-term goal is to establish an integrated linkage map of common bean, i.e., a linkage map including

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both molecular markers (particularly RFLPs) and genetic factors controlling agronomic traits. Because most of common bean breeding takes place within the primary gene pool *sensu* Harlan and de Wet (1971), polymorphisms at the molecular level should be identified within *P. vulgaris*. This, in turn, should increase the opportunities of applying the information form the linkage map to the development of improved bean cultivars by marker-assisted introduction of recessive or quantitative traits. Hence, a first objective of our mapping effort, detailed in this article, has been to identify bean genotype-DNA probe combinations that reveal high levels of intraspecific polymorphism. We describe here the establishment of two common bean genomic libraries and compare levels of polymorphisms between and within gene pools of *P. vulgaris*.

Material and methods

Plant material

In order to increase levels of polymorphism both at the molecular and agronomic levels, nine *P. vulgaris* genotypes were chosen according to two criteria: (1) divergent evolutionary origin; and (2) contrasting agronomic traits. BAT93 is a Middle American breeding line that is resistant to bean common mosaic virus (BCMV), rust (*Uromyces phaseoli*), common bacterial blight (*Xanthomonas phaseoli*), and anthracnose (*Colletotrichum lindemuthianum*), but is susceptible to angular leafspot (*Phaeoisariopsis angularis*). Jalo EEP558, an Andean landrace, exhibits opposite reactions to the same pathogens. 'Rio Tibagi', a small black-seeded Middle American cultivar, and ABA71, a large white-seeded Andean breeding stock, have distinct response to abiotic stress such as drought and low soil phosphorus levels. Midas, an Andean wax snap bean, and G12873, a Middle American wild accession, exhibit contrasting growth habits, pigmentation, pod fiber content, and photoperiod sensitivity.

Sierra, LEF2RB, and AC1028, all of Middle American origin, are breeding lines developed at Michigan State University and represent introgression between germ plasm from the arid upland Durango race with the humid lowland Mesoamerica race (Kelly et al. 1990; Singh et al. 1991 a).

DNA isolation, restriction enzyme digestion, and electrophoresis

Genomic DNA was isolated as described in Gepts and Clegg (1989) and Gepts et al. (1992). Seven to ten micrograms of DNA was digested at 37 °C for 4–5 h with different restriction enzymes (4–5 U/μg DNA) according to the recommendations of the manufacturers (BRL and New England Biolabs). Digested DNA was separated by electrophoresis on 0.8% agarose gels in TAE running buffer for 16–20 h at 1 V/cm of gel.

Southern blotting and hybridization

The Southern hybridization followed the Zetabind membrane protocol of AMF-CUNO (Meriden, Conn). Hybridizations with ^{32}P-labelled probes ($1-15 \times 10^8$ dpm) were performed at 42 °C in 50% formamide. Sequential washes were carried out for 60 min each in 2 × SSC, 0.1% SDS at room temperature, 0.1 × SSC, 0.1% SDS at room temperature, and 0.1 × SSC, 0.1% SDS at 60 °C. The washed nylon membranes were exposed to X-Omat X-ray film for 1 h to 5 days. Inspection of the autoradiograms revealed that under these experimental conditions most samples had been digested to completion as indicated by the detection of single bands by almost 90% of the probes. In addition, the banding patterns observed in these screening experiments were heritable (R. Nodari and P. Gepts unpublished observations).

Development of genomic libraries

Total plant DNA from *P. vulgaris* cv 'California Dark Red Kidney' was purified in a cesium chloride gradient (Sambrook et al. 1989). After digestion with either *Eco*RI and *Bam*HI or *Pst*I, fragments smaller than 4.0 kb were size selected on a 0.8% agarose gel for the *Eco*RI-*Bam*HI library or on a 10%–40% sucrose gradient for the *Pst*I library (Weis and Quertermous 1987). Subsequently, DNA was precipitated with 95% ethanol at −20 °C (overnight), rinsed with cold 70% ethanol, and resuspended in TE. The size-selected fragments were ligated into the pUC18 plasmid (Sambrook et al. 1989), and competent bacterial cells (DH5–α) were transformed with the ligated products. After selection on LB medium with ampicillin, X-gal, and IPTG (Sambrook et al. 1989), plasmids from white colonies were isolated through a minipreparation (Holmes and Quigley 1981).

Identification of single-copy genomic probes

Approximately 1 μg of plasmid DNA from clones of each library (around 500) was digested with either *Eco*RI and *Bam*HI or *Pst*I, size separated on a 0.8% agarose gel to verify the presence of an insert, and subsequently transferred to a Zetabind membrane, as described above. These blots were probed with 1 μg of total genomic DNA radio-labelled with α-^{32}PdCTP (Amersham) by nick translation (Rigby et al. 1977). Clones showing the absence or near-absence of signal on the X-ray film were selected as single-copy clones.

Probes

Single-copy sequence inserts were separated from the plasmid after restriction digestion and electrophoresis in low-melting-temperature agarose. Approximately 30 ng of DNA from each insert was radio-labeled with α-^{32}PdCTP (Amersham) by the random primer method (Feinberg and Vogelstein 1984).

Results

Comparison of the two genomic libraries

The two genomic libraries, one resulting from the digestion of genomic DNA with *Pst*I and the other with *Eco*RI-*Bam*HI, exhibited contrasting characteristics. Whereas 89% of 426 *Pst*I clones were classified as single-copy sequences, only 21% of 484 clones from the *Eco*RI-*Bam*HI library belonged to the same category.

The two libraries also exhibited different levels of polymorphism detected by the single-copy sequences. Eighty-two percent of 61 randomly chosen *Pst*I clones identified polymorphisms with at least one of eight restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Hin*fI, *Pst*I, and *Xba*I) in the BAT93 versus Jalo EEP558 comparison. A similar level was observed in a second comparison, 'Rio Tibagi' versus ABA71. This high level of polymorphism detected by the *Pst*I library was in marked contrast with lower levels of polymor-

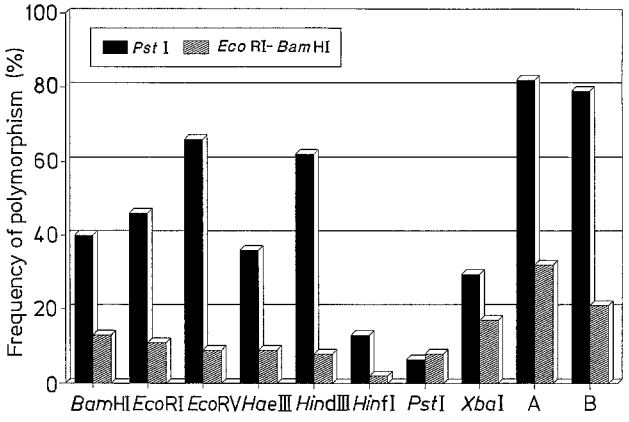

Fig. 1. Level of polymorphism detected by various restriction enzymes. *A* Cumulative polymorphism frequency detected by all eight restriction enzymes; *B* cumulative polymorphism frequency detected by *Eco*RI, *Eco*RV, and *Hin*dIII

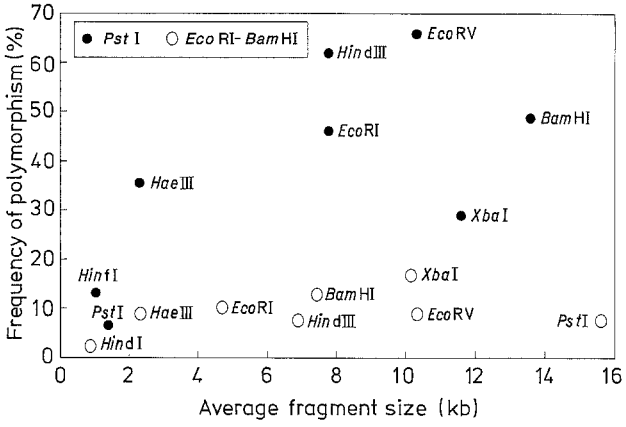

Fig. 2. Relationship between average fragment size and polymorphism level revealed by *Pst*I or *Eco*RI-*Bam*HI clones after restriction digestion of genomic DNA

phism (32%) detected by 57 *Eco*RI-*Bam*HI clones in the BAT93 versus Jalo EEP558 comparison using the same eight restriction enzymes.

When *Pst*I clones were used, we observed that the restriction enzymes used to digest parental DNA detected different levels of polymorphism. In the BAT93 versus Jalo EEP558 comparison, levels of polymorphism ranged between 6.5% (*Pst*I) and 66% (*Eco*RV) (Fig. 1). In the comparison involving 'Rio Tibagi' and ABA71, levels of polymorphism varied between 10% (*Hin*fI) and 67% (*Hin*dIII) (not shown). In both comparisons, high levels of polymorphisms (60–70%) were detected by *Eco*RV and *Hin*dIII, and intermediate levels were detected by *Eco*RI, *Hae*III, *Bam*HI, and *Xba*I. Any polymorphism seen with *Bam*HI, *Hae*III, *Hin*fI, *Pst*I, or *Xba*I, was also seen with *Eco*RI, *Eco*RV, or *Hin*dIII. Hence, the polymorphism observed with the latter three enzymes was as high as that observed with all eight restriction enzymes, i.e., approximately 80% (Fig. 1).

A different pattern was observed for the *Eco*RI-*Bam*HI clones. All of the enzymes detected similar low levels of polymorphism in the comparison BAT93 versus Jalo EEP558 (2–17%). Combined polymorphisms detected by these clones with the eight restriction enzymes amounted to 32%, whereas polymorphisms detected in *Eco*RI-, *Eco*RV-, or *Hin*dIII-digested genomic DNA amounted to 21% (Fig. 1).

What is the probability that an additional clone will reveal a polymorphism between two bean genotypes and, hence, can be mapped? Data from single comparisons – BAT93 versus Jalo EEP558 or Midas versus G12873 – indicated that approximately four-fifths of the *Pst*I clones were capable of detecting a polymorphism with the restriction enzymes *Eco*RI, *Eco*RV, or *Hin*dIII. When the data from two different comparisons were combined, 46 out of 61 clones (75%) detected polymorphisms in both comparisons; 12 out of 61 randomly chosen *Pst*I clones (20%) detected differences in one or the other comparison, and 3 out of 61 clones (5%) detected no polymorphism in either comparison. Hence, using two populations, it would be possible to map 95% of the *Pst*I clones.

Fragment size and frequency of polymorphism

An analysis involving 61 *Pst*I probes in the comparison BAT 93 versus Jalo EEP558 showed significant differences in the average size of the genomic fragments generated by different restriction enzymes (Table 1). Whereas *Hin*fI, *Pst*I, and *Hae*III produced small fragments of sizes 1.0, 1.4, and 2.3 kb, respectively, *Eco*RV, *Xba*I, and *Bam*HI generated fragments with an average size of 10.4, 11.6 and 13.5 kb, respectively. Between these extremes, two restriction enzymes, *Eco*RI and *Hin*dIII, generated fragments with average size of 7.7 and 7.8 kb, respectively. In general, the average fragment size generated by each restriction enzyme was positively correlated with the level of polymorphism revealed by that enzyme (Fig. 2). This association between fragment size and level of polymorphism was not observed for the *Eco*RI-*Bam*HI clones (Fig. 2).

Interversus Intra-gene pool polymorphism

The level of polymorphism was also affected by the degree of genetic similarity among genotypes chosen to identify polymorphisms. Results on levels of polymorphism presented in the previous two paragraphs involved comparisons between Mesoamerican and Andean genotypes. Comparisons within the Andean or Mesoamerican group yielded the following results. Of 57 *Pst*I clones 49% detected polymorphisms between the three cultivated genotypes from the Andean region (ABA71, Jalo EEP558, and Midas) (Table 2). Within the Middle American germ plasm (BAT93, G12873, AC1028, LEF2RB,

Table 1. Average fragment size generated by various restriction enzymes

Restriction enzyme	*Pst*I library			*Eco*RI-*Bam*HI library		
	Number of bands	Average size (kb)	Standard error	Number of bands	Average size (kb)	Standard error
*Hin*fI	50	1.0 a[a]	0.06	35	0.8 a	0.08
*Pst*I	49	1.4 b	0.15	33	15.6 f	1.24
*Hae*III	65	2.3 c	0.31	52	2.3 b	0.33
*Eco*RI	57	7.7 d	0.94	37	4.7 c	0.84
*Hin*dIII	65	7.8 d	0.82	40	6.8 cd	0.88
*Eco*RV	58	10.4 de	0.89	35	10.3 d	1.30
*Xba*I	48	11.6 e	1.21	38	10.2 d	1.26
*Bam*HI	52	13.5 e	1.25	32	7.4 cd	1.19

[a] Averages followed by the same letter within a column are significantly different according to pairwise *t*-tests (Steel and Torrie 1980)

Table 2. RFLP levels between and within the Mesoamerican and Andean cultivar groups

Comparison	Number of probes	Number of enzymes	RFLP level (%)
	Between Mesoamerican and Andean cultivar groups		
BAT93, Jalo EEP558	57	6[b]	86
	61	3[c]	82
Midas, G12873	61	3	89
	Within Mesoamerican or Andean cultivar groups		
Mesoamerican[a]	53	6	62
Andean[a]	57	6	49

[a] Mesoamerican: BAT93, G12873, Sierra, LEF2RB, AC1028; Andean: Jalo EEP558, ABA71, Midas
[b] *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Xba*I
[c] *Eco*RI, *Eco*RV, *Hin*dIII

Table 3. Frequency (in percentage) of site mutations and chromosomal rearrangements

Comparison	Number of probes	Frequency (%)		
		Site mutations	Insertions/deletions	Ambiguous
	*Pst*I library			
BAT93 versus Jalo EEP558	50	20	32	48
Rio Tibagi versus ABA71	23	13	35	52
	*Eco*RI-*Bam*HI library			
BAT93 versus Jalo EEP558	17	59	0	41

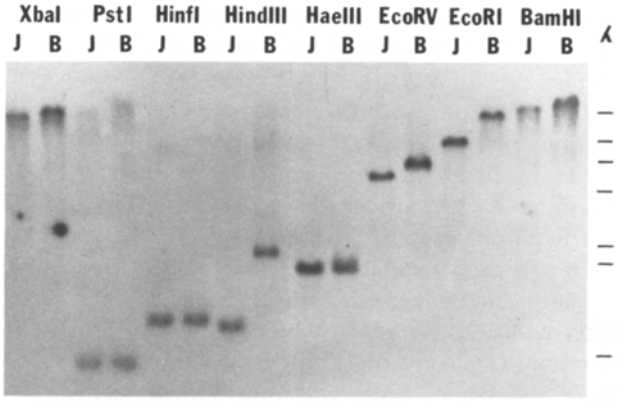

Fig. 3. Polymorphism detected by *Pst*I genomic clone GUC1049 between BAT93 (*B*) and Jalo EEP558 (*J*)

and Sierra), 62% of 53 *Pst*I clones revealed a polymorphism. As expected, therefore, comparisons within the Mesoamerican or Andean groups showed lower levels of polymorphism than between these two groups.

Type of polymorphisms

Polymorphisms can be generated either through nucleotide substitution or through insertions/deletions. If digestion with one or two of the eight restriction enzymes showed a polymorphism, then a nucleotide substitution at the specific recognition site was inferred. If a consistency in the polymorphism was observed among five or more restriction enzymes (e.g., one parent consistently exhibited a larger fragment, as illustrated in Fig. 3), the polymorphism was assumed to be due to an insertion/deletion (or chromosomal rearrangement). Intermediate cases were classified as ambiguous. Using these criteria, a majority of the polymorphisms revealed by *Pst*I clones appeared to be due to an insertion/deletion event, whereas *Eco*RI-*Bam*HI clones recognized mostly nucleotide substitutions (Table 3).

Discussion

Libraries enriched with single-copy sequences have been obtained in common bean (95% single-copy sequences; Chase et al. 1991) and in other species such as rice (85%; McCouch et al. 1988), maize (50.6%; Burr et al. 1988), tomato (92%; Miller and Tanksley 1990b), and barley (89%; Graner et al. 1990) when DNA was digested with *Pst*I, a methylation-sensitive enzyme (as opposed to *Bam*HI and *Eco*RI, two methylation-insensitive enzymes). Lack of methylation has been associated with gene activity in vertebrates and plants (e.g., Antequera and Bird 1988; Schwartz 1989; Monk 1990).

The relatively high level of polymorphism revealed by the *Pst*I library compared to that detected by the *Eco*-RI-*Bam*HI library (80% versus 32%) was unexpected. *Pst*I clones also exhibited high levels of polymorphisms in other species. In tomato, higher levels of polymorphism were observed with *Pst*I clones than with *Eco*RI clones (Miller and Tanksley 1990a). Of the *Pst*I probes 75% were polymorphic with three enzymes when tested in 38 2n potato lines (Gebhardt et al. 1989).

The wide range of polymorphisms revealed by different libraries illustrate the paramount importance of the restriction enzymes used to digest DNA for the establishment of a genomic library. The cloning procedure, such as the restriction enzyme and the fragment size, apparently induces a sampling bias which favors certain regions of the genome or specific sequences, depending on their base composition, copy number, or methylation level. *Pst*I will not cleave DNA at the recognition sequence (5′-CTGCAG-3′) if the cytosine at the 5′ end is methylated (Nelson and McClelland 1987; Gruenbaum et al. 1981). Our data suggest that DNA sequences homologous to the *Pst*I clones (or DNA sequences adjacent to them) are more variable than those homologous to the *Eco*RI-*Bam*HI clones. This variability revealed by *Pst*I clones appears to be due more often to small rearrangements than to nucleotide substitutions, although the latter could also play an important role. Variability revealed by *Eco*RI-*Bam*HI clones appeared to be due mostly to nucleotide substitutions. We are not aware of an explanation that could account for this difference in polymorphism levels detected with the two libraries.

Recently, two other groups have obtained estimates of RFLP levels in common bean. Chase et al. (1991) found that in a group of 14 Middle American and Andean accessions, 5 out of 18 (28%) random *Pst*I clones revealed polymorphisms in *Eco*RI digests of genomic DNA. For the *Eco*RV and *Hin*dIII digests, polymorphisms were observed with 5 out of 18 (28%) and 7 out of 18 (39%) of the clones, respectively. Within the Middle American group (excluding accession XR-235-1-1, which includes *P. coccineus* in its background), 5 out of 18 (28%) revealed polymorphisms whereas in the Andean gene pool 4 out of 18 (22%) revealed polymorphisms. The lower levels of polymorphisms observed by these authors can be attributed to differences in the plant material analyzed. For example, all Middle American accessions analyzed by Chase et al. (1991) belong to race Mesoamerica, whereas the accessions analyzed in our study belong to either race Mesoamerica or race Durango of the Middle American gene pool (Singh et al. 1991a).

Guo et al. (1991) observed only 22–26% polymorphisms within *P. vulgaris* but 70–80% between *P. vulgaris* and *P. coccineus*. The lower level of polymorphisms may be due to the use of a single restriction enzyme (*Eco*RI), to the expression of their results as the proportion of polymorphic bands to the total number of bands, and to different sources of probes (mostly anonymous cDNA probes and clones of genes of known function).

Although experimental conditions are not strictly comparable, the RFLP levels we have identified in common bean (around 80–90%) compare favorably with those observed in other species (Table 4), especially self-pollinated plants such as soybean (40–50%) and tomato (14%) (Keim et al. 1989; Helentjaris et al. 1985). Table 2 shows that while between gene pools we were able to detect 80–90% polymorphism, within gene pools we still detected 50–60% polymorphism. Hence, RFLP analysis confirmed the divergence between Mesoamerican and Andean gene pools detected previously with morphological, biochemical, and reproductive isolation data (Vanderborght 1987; Gepts et al. 1986; Gepts 1990; Gepts and Debouck 1991; Sprecher 1988; Khairallah et al. 1990; Koenig and Gepts 1989; Shii et al. 1980; Singh et al. 1991b, c). In order to increase levels of polymorphisms at the molecular level, parents should belong to contrasting gene pools, i.e., Mesoamerican versus Andean. However, the level of polymorphism within gene pools appears to be still high enough to develop efficient applications with these markers, such as marker-assisted selection or evolutionary studies.

Strong correlation have been found between the average size of the hybridizing restriction fragments and the frequency of polymorphisms generated by different restriction enzymes (McCouch et al. 1988; Miller and Tanksley 1990a). By testing 61 probes against BAT93 and Jalo EEP558 genomic DNA digested with eight restriction enzymes, we found similar results. This positive correlation is consistent with our finding that a majority of the RFLPs can be attributed to insertions/deletions.

From a practical point of view, mapping efficiency increases when the number of restriction enzymes used to digest DNA of parents and of segregating generations can be decreased without a significant reduction in polymorphism levels. Our results indicate that the level of polymorphism detected by the combined use of *Eco*RV, *Eco*RI, and *Hin*dIII is the same as the one detected by all

Table 4. Comparison of RFLP levels in common bean and other plants

Crop	Genotypes	Type of clone	Number of enzymes	RFLP (%)	Source
Arabidopsis	3 strains	Genomic: *Eco*RI	3	46	Chang et al. (1988)
Barley	48 cultivars	Genomic: *Pst*I	3	43	Graner et al. (1990)
	4 cultivars	Genomic: *Pst*I	5	54	Heun et al. (1991)
Brassica	3 species	Genomic: *Pst*I	1	95 between species	Figdore et al. (1988)
	(37 cultivars or genetic stocks)			80 between subspecies	Figdore et al. (1988)
				70 within subspecies	Figdore et al. (1988)
Common Bean	7 cultivars + 1 wild	Genomic: *Pst*I	3	89 between subspecies 62 within Mesoamerican 49 within Andean	Nodari et al. (present results)
Lentil	2 cultivars + 2 wild	Genomic: *Pst*I, *Eco*RI	4	38	Havey and Muehlbauer (1989)
		cDNA	4	64	
Lettuce	4 cultivars	Genomic: *Mbo*I	9	13	Landry et al. (1987)
		cDNA	9	25	Landry et al. (1987)
Maize	3 cultivars	cDNA	3	83	Helentjaris et al. (1985)
Potato	*phureja* × (*tuberosum* × *chacoense*)	Genomic: *Pst*I, *Eco*RI	11	60	Bonierbale et al. (1988)
		cDNA	11	51	Bonierbale et al. (1988)
Rice	*indica* × *javanica*	Genomic: *Pst*I	11	78	McCouch et al. (1988)
Soybean	5 cultivars	Genomic: *Sau*3AI, *Eco*RI	13	20	Apuya et al. (1980)
	58 wild + cultivated	Genomic: *Sau*3Ai	4	40–50	Keim et al. (1989)
Tomato	2 cultivars	cDNA	3	14	Helentjaris et al. (1985)
	esculentum-cheesmannii	cDNA	3	68	Helentjaris et al. (1985)
	esculentum-pennellii	cDNA	3	100	Helentjaris et al. (1985)

eight restriction enzymes together (around 80%). These three enzymes were also very efficient in detecting polymorphisms in lettuce (Landry et al. 1987) and potato (Bonierbale et al. 1988). When the data from two different pairwise genotypic comparisons were combined, the level of polymorphism was even higher than in a single comparison. This observation suggests that mapping in more than one population will lead to a denser map, in general, and will increase the probability of identifying polymorphic markers in a chromosome region of interest.

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