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Restriction fragment length polymorphisms as genetic markers in *Vicia*

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Genetic variability at the nucleic acid level has been examined in 16 *Vicia faba* accessions and in four *Vicia* species. Both six and four base-pair cutting restriction enzymes together with cDNA clones were used to assess the level of polymorphism detectable in *Vicia* section *Faba*. Nei's estimate of the number of shared fragments and principal co-ordinate analysis were used to examine and quantify the relationship between the *Vicia* accessions. The degree of polymorphism detected was considerably reduced when the four wild *Vicia* species were excluded from the analysis. One *Vicia faba* genotype, A × B × C, a three-way hybrid, was responsible for the majority of the RFLPs identified within the *Vicia faba* genotypes. The identification of hybridisable but diverse parents will improve the efficiency of creating a linkage map based on molecular markers. The nature of the polymorphism detected in *Vicia* is discussed together with the role of molecular markers in *Vicia faba* improvement programmes.

INTRODUCTION

Vicia faba L. ranks as the most important grain legume in western Europe on the basis of annual tonnage produced and fifth ranked on world basis (Hebblethwaite *et al.*, 1984). Despite its economic importance our knowledge of the basic genetics of this protein-rich diploid ($2n = 2x = 12$) organism is lacking. Ward and Chapman (1986) reported that only 27 nuclear loci had been identified in *V. faba.* These genetic loci consist primarily of morphological traits and their specific chromosomal location is largely unknown. Due to the pleiotropic effects associated with morphological mutant loci many of these markers are undesirable in a *Vicia faba* breeding programme. These factors together with the recessive nature of the mutant alleles have severly hindered the development and exploitation of genetic markers in this crop. Based on available morphological markers the construction of a genetic map of *Vicia faba* using conventional technology would be a slow process.

In order to expedite the production of linkage maps isozymes have been used in a range of crop plants (Tanksley and Orton, 1983). These biochemical markers are generally selectively neutral, well distributed over the genome and interact in a co-dominant manner. Protein or isozyme variants have been used in *Vicia* for evolutionary and taxonomic studies (Yamamoto, 1986; Kaser and Steiner, 1983), inbred line recognition and the determination of outcrossing frequencies (Gates and Boulter, 1979, 1980) and cultivar identification (Bassiri and Roumani, 1977). However, only a limited number of isozymes have been subjected to a rigorous genetical analysis (Peat and Adham, 1984, Mancini *et al.*, 1989). While it is anticipated that isozyme markers will contribute significantly to our understanding of *Vicia* genetics their relatively limited numbers will limit their usefulness in constructing a linkage map.

Molecular markers share a number of advantages with isozymes and have the potential to generate a large number of markers for use in genetical analyses (Michelmore and Hulbert, 1987). Linkage maps based on Restriction Fragment Length Polymorphisms (RFLPs) are being developed in several crops: tomato (Bernatzky and Tanskley, 1986), maize (Helentjaris *et al.*, 1986; Burr *et al.*, 1988), lettuce (Landry *et al.*, 1987) and potato (Bonierbale *et al.*, 1988; Gebhardt *et al.*, 1989). The potential usefulness of RFLPs is dependent on the degree of polymorphism existing among the organisms of interest. This feature of molecular markers is most evident when we consider the creation of a linkage map in tomato (Tanksley *et al.*, 1989). Both the

creation and exploitation of linkage maps in tomato have involved interspecific hybridisation between *Lycopersicon esculentum* and *L. chmielewskii* (Nienhuis *et al.*, 1987; Paterson *et al.*, 1988). Identifying the most genetically diverse parents for the creation of a segregating population is therefore a key feature for the development of a linkage map. Since interspecific hybrids invoving *V. faba* cannot be produced (Ramsay and Pickersgill, 1986) the identification of sexually hybridisable but diverse parents is obviously important. As a first step in the creation of a linkage map in *Vicia faba* we have assessed the level of polymorphism for molecular markers in *Vicia* genotypes. Parents exhibiting a high degree of polymorphism have been identified for the creation of a segregating mapping population.

MATERIALS AND METHODS

Plant material

Sixteen accessions of *Vicia faba* and four other species from *Vicia* section *Faba* were selected from the collection present at the Scottish Crop Research Institute. Accessions were chosen to represent the full spectrum of variation within *V. faba* and to include modern cultivars and related species. A list of the accessions of *V. faba* and other species is given in table 1.

For the wild species part of the seedcoat was removed to promote germination. Ten plants of each accession were grown in a greenhouse in 7 cm square pots filled with John Innes compost No. 2. After 3–10 weeks young leaves of each individual plant were harvested, freeze dried, ground in liquid nitrogen and stored at −20°C.

DNA isolation

Total genomic DNA was isolated by a modification of the procedure of Saghai-Maroof *et al.* (1984). Powdered freeze dried material was mixed with CTAB extraction buffer (100 mM TRIS *p*H 7·5, 1 per cent CTAB, 0·7 M NaCl, 10 mM EDTA, 1 per cent 2-mercaptoethanol) and incubated for one hour at 60°C. 9 ml of extraction buffer was used per 300–500 mg of tissue. The mixture was extracted with chloroform:isoamyl alcohol (24:1) and centrifuged at 3500 rpm for 20 min. The DNA containing aqueous phase was further purified with phenol and chloroform:IAA extractions prior to precipitation by addition of 0·6 vol of isopropanol. The precipitates were washed in 70 per cent ethanol and dissolved in an excess of TE buffer (10 mM TRIS-HCl, 1 mM EDTA, *p*H 8·0). Finally, the DNA preparations were treated with RNase A (10 μg/ml) for 15 mins at 68°C, reprecipitated with ethanol and dissolved in a small volume of TE at approximately 0·5 mg/ml. The DNA concentration was estimated by band-intensity on ethidium bromide stained gels.

Table 1 Plant material

Taxon	Accession	Origin	Description
1. *V. faba* var. *minor*	BPL 543	W. Pakistan	Inbred line
2. *minor*	BPL 822	Afghanistan	Inbred line
3. *minor*	IVS G	U.K.	Inbred, white flowered line
4. *equina*	H51/3	U.K.	Inbred line
5. *paucijuga*	172	Afghanistan	Short, highly branching, few fls/node, small seeds
6. *minor*	ETS 56/7/1	U.K.	Early maturing line
7. *minor*	ETS 137/2/1	U.K.	Early maturing line
8. *minor*	*cv.* Troy	FRG	Early maturing cultivar
9. *minor*	*cv.* Alfred	Netherlands	High yielding cultivar
10. *minor*	*cv.* Fritel	DDR	Determinate cultivar
11. *minor*	*cv.* Corton	U.K.	High yielding cultivar
12. *minor*	*cv.* Frinebo	DDR	Very high yielding cultivar
13. *major*	*cv.* Optica	Netherlands	White flowered, processing cultivar
14. *major*	*cv.* Masterpiece Green Longpod	U.K.	Garden cultivar, very large seeds
15. *minor*	Syn 3	U.K.	Synthetic of 6 ETS lines, including ETS 56/7/1
16. *minor*	A×B×C	France	Three way hybrid
17. *V. narbonensis*	557	Lebanon	
18. *V. johannis*	64	Turkey	
19. *V. galilaea*	44	Israel	
20. *V. hyaeniscyamus*	867152	Syria	

Restriction digests, electrophoresis and southern transfer

DNA from three plants of each line was combined and digested with the following restriction enzymes: *Bam*HI, *Eco*RI, *Eco*RV and *Hind*III, which recognise 6-base pair sequences. Digestion was carried out in a total volume of 100 μl using 10 μg of DNA, 50 units of enzyme, and 1× standard digestion buffer (0·033 M TRIS-Acetate, *p*H 7·8, 0·065 M potassium acetate, 10 mM magnesium acetate, 4 mM spermidine, 0·5 mM dithiothreitol) overnight. Digested DNA was precipitated by addition of 0·1 vol. 3 M Na Acetate and 2 vol. 100 per cent EtOH, washed in 70 per cent EtOH, dried and re-dissolved in TE. DNA fragments generated after digestion with 6 bp cutters were separated according to length on 1 per cent agarose gels with 1×TBE (0.089 M Tris, 0·089 M boric acid, 0·002 M EDTA) as running buffer. DNA was transferred to nylon membranes (either Zeta-probe (BIO-RAD) or Hybond N^+ (Amersham)) using the alkaline blotting procedure of Reed and Mann (1985). Alternatively, *Alu*I, *Hae*III, *Hpa*I, *Mbo*I, *Msp*I, *Pal*I, *Rsa*I or *Taq*I digested DNA was prepared and electrophoresed on 40 cm denaturing polyacrylamide gels as described by Gebhardt *et al.* (1989). DNA was transferred to the membrane electrophoretically in 0·4×TBE for 1 hour at 1 mA constant current in a Biorad electroblotting tank surrounded by iced water. DNA was bound to the membranes by baking at 80°C for 2 hours.

Library construction

RNA was isolated according to the method of Logemann *et al.* (1987) using 10 g of fresh leaves of *Vicia faba cv.* Optica. Double stranded cDNA was synthesised using a cDNA-synthesis-kit (Pharmacia) following the manufacturers instructions. The cDNA was ligated to *Eco*RI adaptors and cloned into the *Eco*RI site of pUC13. DH5α bacterial cells were transformed and recombinants identified as white colonies on ampicillin plates containing X-gal and IPTG (Maniatis *et al.*, 1982). Plasmids were isolated by the rapid alkaline SDS procedure of Silhavy *et al.* (1984). Clones containing inserts between 0·4 kb and 2·0 kb were used for probes.

Known function probes

DNA probes of known function and their sources are given in table 2.

Probe preparation

To separate inserts from vector, plasmid DNAs were digested with *Eco*RI and run on 5 per cent polyacrylamide gels (Maniatis *et al.*, 1982). Inserts were excised and isolated using a UEA electroelution apparatus (Undirectional Electroelutor Analytical, IBI) cleaned with phenol and chloroform: IAA extractions, precipitated with ethanol and dissolved in a small vol of TE. To estimate the concentration of the probe a small portion was run on an agarose gel alongside standards.

The probes were labelled with alpha [^{32}P]dCTP (specific activity 3000 Ci/mmol (Amersham)) using the random primer labelling method of Feinberg and Vogelstein (1984).

Hybridisation and authoradiography

The prehybridisation buffer contained: 5×SSPE, 0·5 per cent SDS, 5×Denhardts solution, 100 μg/ml sonicated salmon sperm DNA. The hybridisation buffer contained the same with the addition of Dextransulfate to 10 per cent (w/v). Membranes were pre-washed in 2×SSC and prehybridised in glass bottles for one hour to overnight at 65°C in a rotating oven (Hybaid). Labelled probe was denaturated by heating to 100°C for 5 min and added to approximately 20 ml of hybridisation buffer at 65°C. Hybridisation was for at least 12 hr at 65°C. Membranes were washed in 2×SSC, 0·5 per cent SDS at room temperature for 15 min; 2×SSC, 0·1 per cent SDS at room temperature for

Table 2 Clones of known function

Probe	Function	Source	Reference
pBG 35	rDNA repeat	Flax	Goldsbrough and Cullis (1981)
pRC 221	Vicillin	Pea	Lycett *et al.* (1984)
pAD 4·4	Legumin	Pea	Lycett *et al.* (1984)
pCW×27	Waxy	Maize	Rhode *et al.* (1987)
pBG 13	5S RNA repeats	Flax	Goldsbrough *et al.* (1981)
pGI-U2	Phosphoglucose isomerase	*Clarkia*	Tait *et al.* (1988)

15 min; 0·1 × SSC, 0·1 per cent SDS at room temperature for 15 min; 0·1 × SSC, 0·1 per cent SDS at 65°C for 15 min. The membranes were sealed in plastic bags and autoradiographed at −70°C for one to 7 days using Fuji NIF-RX X-ray film with 2 intensifying screens. The membranes were repeatedly stripped and re-probed (up to 10 times). To strip, boiling 0·5–0·7 per cent SDS was poured over the blots in a sandwich box and allowed to cool to room temperature. The membranes were autoradiographed overnight to check that the probe was removed.

RESULTS

Thirty-eight different clones randomly selected from a cDNA library (*ca.* 1.8×10^6 clones, average insert size 800 bp) constructed in pUC 13 were chosen as potential RFLP probes. These were used to sequentially screen filters to which DNA from the 16 *V. faba* accessions and four wild species had been transferred. The *Vicia* DNA was digested with four restriction enzymes: *Bam*HI, *Eco*RI, *Eco*RV and *Hin*dIII and examples of the level of polymorphism detected for two probe/enzyme combinations are given in fig. 1. The patterns detected by the cDNA probes could be divided into three groups. The first two of these were also observed in soybean (Apuya *et al.*, 1988). These were: (1) A simple pattern of hybridising fragments with one to three bands per track (fig. 2(a)); (2) More than three bands but a relatively simple pattern (fig. 2(b)); (3) Complex restriction fragment patterns. The first class accounted for the majority of the probes. The last class often consisted of a few intensely hybridising fragments which shared little variation and more polymorphic minor fragments (*e.g.*, fig. 5b). An explanation for the polymorphisms detected in the minor hybridising fragments has been suggested previously by Gebhardt *et al.* (1989) who obtained similar results in potato. They proposed that the complex patterns are most likely derived from multiple copy sequences containing homomorphic core fragments but differing in polymorphic borders according to the position of the lst restriction site in the flanking DNA. A similar explanation is consistent with the patterns obtained in *V. faba.*

Of the 38 probes, the hybridisation patterns from 14 were excluded from the results either because no hybridisation signal was detected or the signal was too weak to be useful. Of the remaining 24 probes, in 62 probe enzyme combinations, 20 detected at least one polymorphism. These results are summarised in table 3.

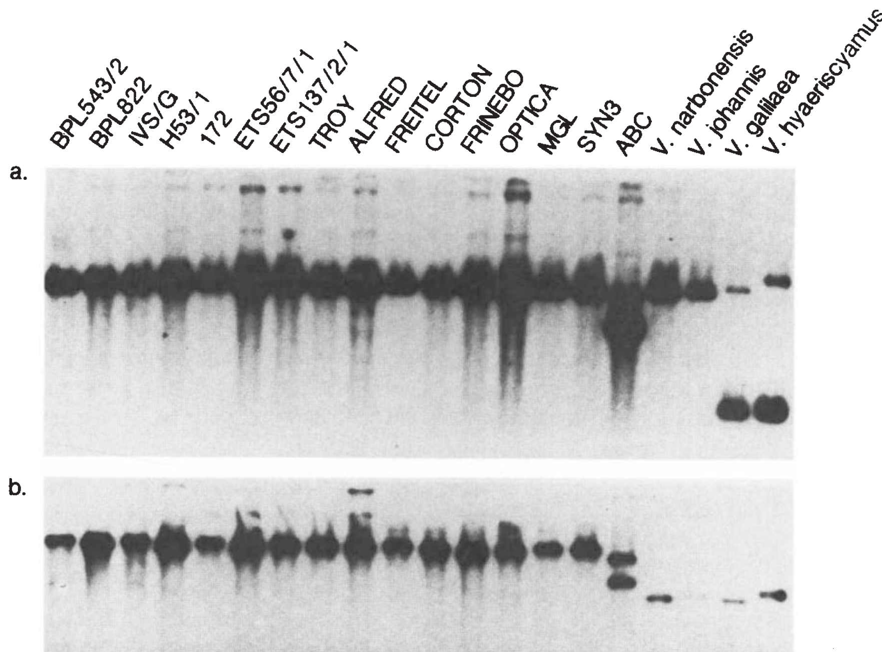

Figure 1 Polymorphism on the 20 *Vicia* accessions. (a) Restricted with enzyme *Hin*dIII and probed with cDNA probe 7·14. (b) Restricted with enzyme *Eco*RV and probed with cDNA probe 5·6.

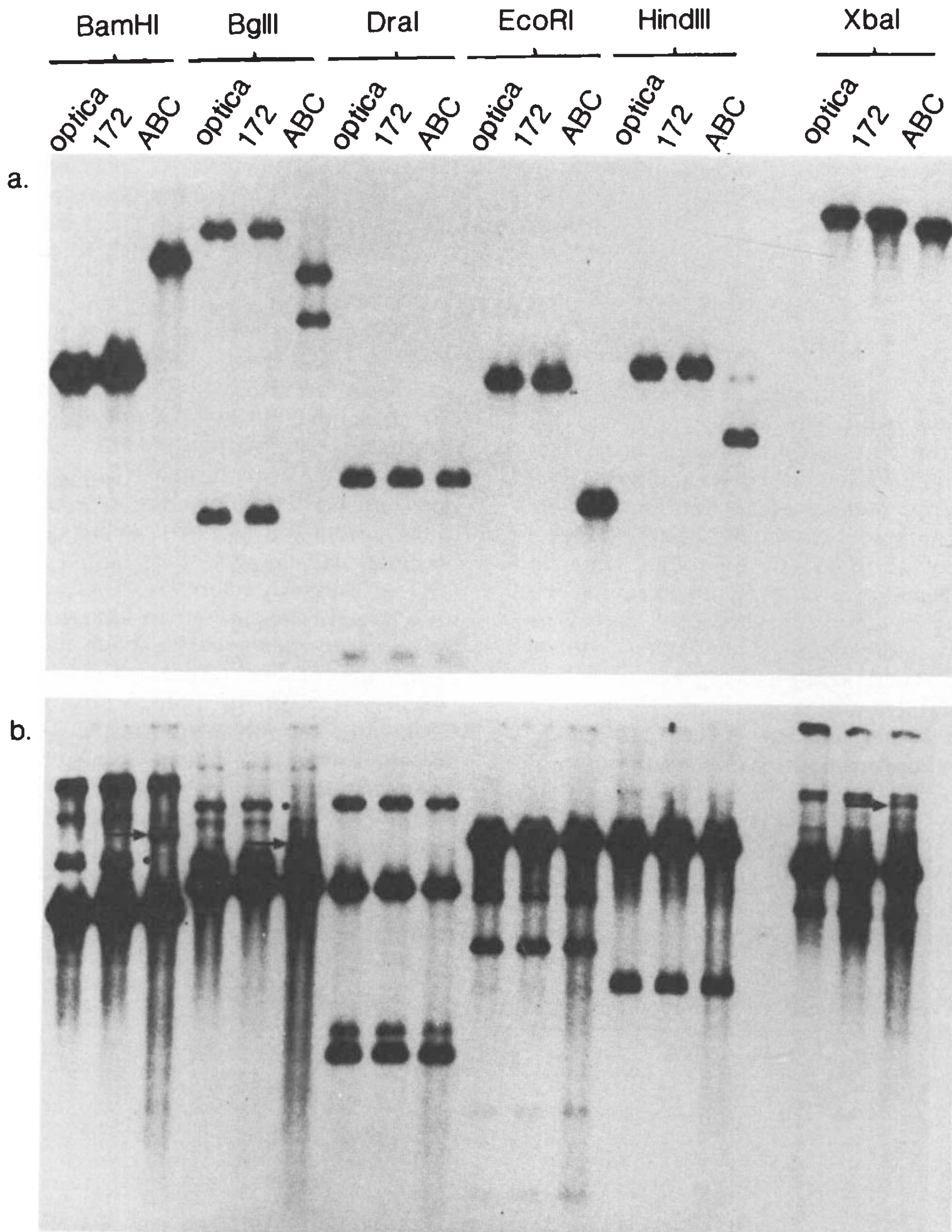

Figure 2 (a) A simple RFLP pattern of Optica, 172 and A × B × C probed with cDNA probe 7·14 and restricted with six six-base pair recognising enzymes. (b) Parental screening using cDNA probe 8·43. No polymorphism is detected between the Optica and 172. Polymorphism is shown between 172 and A × B × C with the restriction enzymes: *Bam*HI, *Bgl*II and *Xba*I. Arrows and dots indicate polymorphic bands.

The percentage of probes detecting polymorphism between all the *Vicia* genotypes examined varied from 75 per cent with *Eco*RV to 56·3 per cent with *Hin*dIII. However the proportion of polymorphic probes identified is reduced when the calculation is based on the 16 *V. faba* accessions. There are significant differences ($\chi^2_{(3)} =$ 14·5***) between restriction enzymes in terms of the number of probes detecting polymorphism. Most polymorphism is detected with *Eco*RV (57 per cent) and least with *Eco*RI (25 per cent).

The nature of the polymorphism detected for the 20 *Vicia* genotypes is presented in the form of a similarity matrix in table 4. Nei's estimate of

Table 3 Percentage probes detecting polymorphism between *Vicia* genotypes for four restriction enzymes

Enzyme	Recognition sequence	% Probes polymorphic between all genotypes	% Probes polymorphic within *Vicia faba* genotypes
*Eco*RI	GAATTC	60·9	25
*Eco*RV	GATATC	75·0	57·1
*Bam*HI	CGATCC	62·5	43·8
*Hin*dIII	AAGCTT	56·3	33
		$\chi^2_{(3)} = 3\cdot0$ ns	$\chi^2_{(3)} = 14\cdot5$***

*** $P > 0\cdot01$.

similarity based on the number of shared fragments (Nei and Lei, 1979) was used to generate the matrix. Reference to table 4 highlights the distinction between the *Vicia* wild species and the *V. faba* genotypes. Of greater significance is the relatively low level of similarity detected at the DNA level between genotype 16 (A × B × C) and the other 15 *Vicia faba* genotypes. In order to examine and quantify the relationship between the *Vicia* accessions in more detail principal co-ordinate analysis was undertaken. Fig. 3 represents the first two principal co-ordinates of the data that accounts for 70 per cent (54 per cent and 16 per cent) of the total variation. With the exception of A × B × C the 15 *V. faba* accessions dominate the bottom right quadrant of the graph. *V. johannis*, *V. galilaea*, and *V. hyaeniscyamus* are associated in the bottom left of the quadrant. Both *V. narbonensis* and A × B × C are clearly separated from the other accessions. The salient feature is the identification of A × B × C as being quite distinct from the other *Vicia faba* accessions. The identification of hybridisable but relatively diverse parents for the generation of a segregating population is highly desirable and represents a key aspect of the production of a genetic linkage map based on molecular markers.

The three-way hybrid A × B × C is responsible for a large proportion of the variation detected in the *Vicia faba* gene pool sampled. Information for 24 of the probes tested with four restriction enzymes is given in table 5. Twelve of the 24 detected useful polymorphisms between A × B × C and the remaining *V. faba* accessions. There are significant differences between restriction enzymes in the level of polymorphism detected. Of the 13 probes (27 probe/enzyme combination) detecting differences between A × B × C and the remaining accessions, 8 were detected with only a single restriction enzyme.

Table 4 Nei's estimate of genetic similarity based on the proportion of shared fragments 1–20, refer to the genotypes in Table 1

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	1·00																			
2	1·00	1·00																		
3	0·95	0·95	1·00																	
4	1·00	1·00	0·95	1·00																
5	0·95	0·95	0·90	0·95	1·00															
6	1·00	1·00	0·95	1·00	0·95	1·00														
7	1·00	1·00	0·95	1·00	0·95	1·00	1·00													
8	1·00	1·00	0·95	1·00	0·95	1·00	1·00	1·00												
9	1·00	1·00	0·95	1·00	0·95	1·00	1·00	1·00	1·00											
10	1·00	1·00	0·95	1·00	0·95	1·00	1·00	1·00	1·00	1·00										
11	0·97	0·97	0·92	0·97	0·92	0·97	0·97	0·97	0·97	0·97	1·00									
12	1·00	1·00	0·95	1·00	0·95	1·00	1·00	1·00	1·00	1·00	0·97	1·00								
13	0·97	0·97	0·92	0·97	0·97	0·97	0·97	0·97	0·97	0·97	0·95	0·97	1·00							
14	0·97	0·97	0·92	0·97	0·97	0·97	0·97	0·97	0·97	0·97	0·95	0·97	0·95	1·00						
15	1·00	1·00	0·95	1·00	0·95	1·00	1·00	1·00	1·00	1·00	0·97	1·00	0·97	0·97	1·00					
16	0·51	0·51	0·46	0·51	0·49	0·51	0·51	0·51	0·51	0·51	0·49	0·51	0·49	0·51	0·51	1·00				
17	0·32	0·32	0·32	0·32	0·32	0·32	0·32	0·32	0·32	0·32	0·29	0·32	0·32	0·32	0·32	0·13	1·00			
18	0·29	0·29	0·34	0·29	0·26	0·29	0·29	0·29	0·29	0·29	0·26	0·29	0·26	0·29	0·29	0·13	0·38	1·00		
19	0·29	0·29	0·32	0·29	0·26	0·29	0·29	0·29	0·29	0·29	0·26	0·29	0·26	0·29	0·29	0·13	0·30	0·76	1·00	
20	0·29	0·29	0·32	0·29	0·26	0·29	0·29	0·29	0·29	0·29	0·26	0·29	0·26	0·29	0·29	0·13	0·27	0·73	0·89	1·00

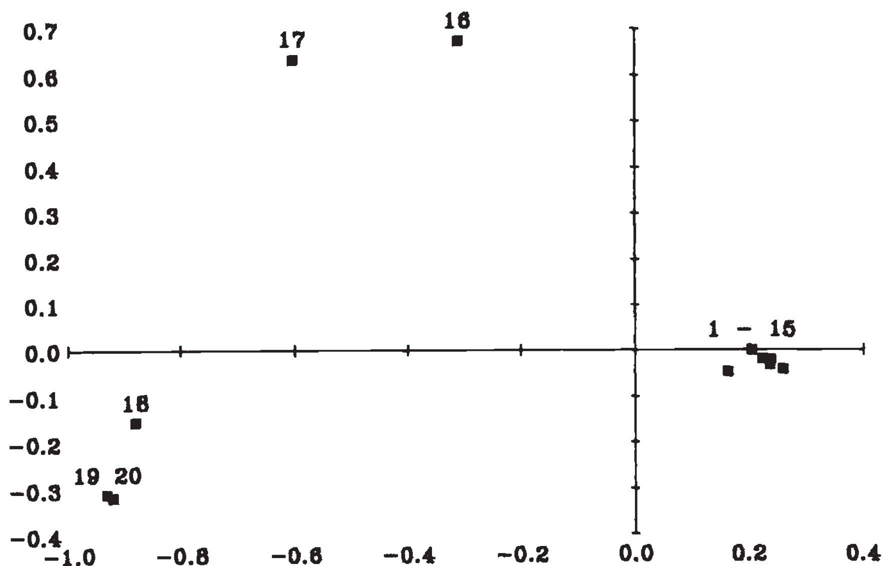

Figure 3 Principal co-ordinate analysis of the RFLP data for the 20 *Vicia* genotypes examined.

Table 5 Polymorphism attributable to A×B×C for 24 cDNA clones and four restriction enzymes

Probe	*Eco*RI	*Eco*RV	*Hin*dIII	*Bam*HI	Polymorphic enzymes
1·5	–	–	–	–	0
2·7	–	+	–	–	1
2·9	nd	+	+	nd	2
2·10	–	nd	nd	–	0
2·11	–	+	–	–	1
4·3	–	–	–	–	0
4·7	–	–	–	–	0
4·13	–	nd	–	–	0
5·6	+	+	+	+	4
5·15	nd	–	nd	nd	0
7·4	nd	nd	nd	–	0
7·8	–	–	nd	nd	0
7·14	+	nd	+	nd	2
7·15	nd	nd	–	–	0
7·17	–	+	nd	nd	1
7·18	–	–	–	–	0
7·19	+	–	nd	nd	1
7·28	–	+	–	+	2
8·3	nd	nd	+	+	2
8·5	nd	nd	–	+	1
8·13	–	+	nd	–	1
8·38	nd	nd	–	nd	0
8·42	–	nd		+	1
8·43	nd	nd	–	+	1
Polymorphic probes	3	7	4	6	$\chi^2_{(3)}=17{\cdot}6$***
Tested	16	14	16	16	
%	18·8	50	25	37·5	

+, polymorphism; –, non-polymorphic; nd, not determined.
*** $P > 0{\cdot}01$.

In addition to A×B×C, 172 and Optica were chosen as potential parents for the construction of a linkage map based on RFLPs. Table 6 summarises the results for the parental screening with seven 6 bp cutting restriction enzymes. Table 6(a) indicates the polymorphic probe/enzyme combination for A×B×C and 172 and table 6(b) for Optica and 172. Twenty-eight anonymous polymorphic probes were identified between A×B×C and 172. Clones of known function particularly

Table 6a Probes defining RFLP markers with different enzymes. Polymorphism shown between A×B×C and 172

Probe	Size (kb)	*Bam*HI	*Bgl*I	*Dra*I	*Eco*RI	*Eco*RV	*Hin*dIII	*Xba*I	Enzymes showing polymorphism
2·7	1·7	–	nd	nd	–	+	–	nd	1
2·9	0·6	+	nd	+	+	+	+	nd	5
2·11	1·1+0·6	nd	nd	nd	nd	+	nd	nd	1
2·12	1·2	+	nd	–	+	nd	+	+	4
2·16	0·85	+	nd	–	–	–	–	nd	1
5·6	0·6	+	nd	nd	+	+	+	nd	4
5·8	0·7	+	+	nd	nd	nd	+	+	4
6·3	0·75	+	+	+	+	nd	+	+	6
6·9	0·8	+	+	+	+	nd	+	+	6
7·4	0·67	–	–	–	–	nd	–	+	1
7·7	1·3	–	+	–	–	nd	–	–	1
7·8	0·75	+	+	–	+	+	–	+	5
7·14	0·9	+	+	–	+	nd	+	+	5
7·17	1·25	+	nd	+	–	+	–	nd	3
7·19	0·76	+	+	–	–	+	–	nd	3
7·28	0·75	+	+	nd	–	+	–	–	3
8·3	0·4	+	+	+	–	+	+	nd	5
8·4	0·6	+	+	+	+	+	+	nd	6
8·9	0·5	+	+	+	–	+	+	nd	5
8·13	1·3	+	+	–	+	+	+	–	5
8·38	1·0	+	nd	+	–	+	–	nd	3
8·42	0·63+0·2	+	+	nd	–	nd	–	+	3
8·43	0·85	+	+	–	–	nd	–	+	3
8·44	0·9	–	nd	+	–	–	–	nd	1
pBG 35		–	+	–	+	nd	–	–	2
pRC 221		+	nd	+	+	nd	+	nd	4
pAD 4·4		+	+	+	–	+	+	nd	5
pCW×27		+	+	+	+	+	+	+	7

nd, Not determined; +, Polymorphic; –, Not polymorphic.

Table 6b Polymorphism shown between Optica and 172

Probe	Size (kb)	*Bam*HI	*Bgl*I	*Dra*I	*Eco*RI	*Eco*RV	*Hin*dIII	*Xba*I	Enzymes showing polymorphism
2·12	1·2	–	nd	–	–	nd	–	+	1
6·3	0·75	–	–	+	–	nd	–	+	2
7·8	0·75	+	+	–	+	–	–	+	4
7·17	1·25	–	nd	+	–	–	–	nd	1
7·19	0·76	+	–	–	–	–	–	nd	1
8·4	0·6	+	+	+	+	+	–	nd	5
8·9	0·5	+	–	–	–	–	–	nd	1
8·38	1·0	–	nd	+	–	–	–	nd	1
8·42	0·63+0·2	–	–	nd	–	nd	–	–	0
8·44	0·9	–	nd	+	–	–	–	nd	1
pBG 35		+	–	–	–	nd	–	+	2
pRC 221		–	nd	–	–	nd	+	–	1
pAD 4·4		+	+	+	–	+	+	nd	5
pCW×27		+	+	+	+	+	+	+	7

pAD 44 and pCW×27 detected polymorphism with a wide range of restriction enzymes.

In order to improve the chances of detecting RFLPs eight 4 bp cutting enzymes were also used. The use of restriction enzymes specific for four nucleotide target sites should increase the probability of detecting polymorphism since the frequency of restriction sites in a given segment of DNA is increased (Kreitman and Aquade, 1986). A sample of 15 probes largely consisting of those not polymorphic for the 6 bp cutters was used in conjunction with the 4 bp cutters. An example of the polymorphism detected is given in fig. 4 for the probe 8·3. Although the resolution of the fragments is increased the frequency of polymorphism detected was not much greater than that detected with 6 bp cutting enzymes, with only one additional polymorphic probe detected using this approach. In contrast to the preliminary survey which was performed with only 4 restriction enzymes, examination of the polymorphisms detected between A×B×C and 172 with a wide range of probe×enzyme combinations indicates that only 4 of the 28 probes detect polymorphism with a single restriction enzyme. The lack of enzyme specificity associated with the detection of polymorphism suggests that alterations in the length of the fragments generated may be due to rearrangements of DNA by insertion or deletion rather than simple point mutations. This is similar to the situation found in soybean (Apuya *et al.*, 1988) and rice (McCouch *et al.*, 1989) where a significant proportion of the RFLPs are due to genomic rearrangements.

The partially allogamous breeding system of *Vicia faba* is also of relevance and the degree of

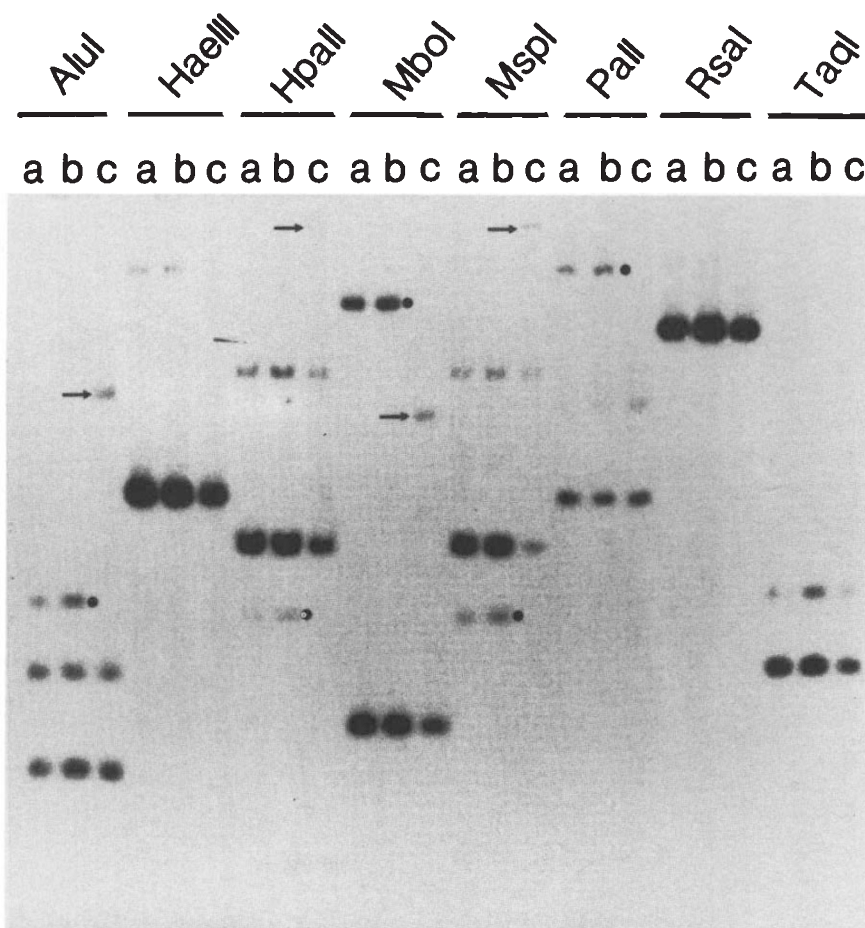

Figure 4 RFLP pattern of Optica, 172 and A×B×C probed with cDNA probe 8·3 and restricted with eight four-base-pair recognising enzymes. Dots and arrows indicate the polymorphic bands.

heterogeneity was assessed on ten individual plants from five of the cultivars used in our initial survey (Optica, A × B × C, 172, H51/3, Fritel). Two probes in eight probe/enzyme combinations were tested and no within cultivar variability was detected for Optica, H51/3 and 172. Between plant variability was detected for Fritel, and A × B × C. The within cultivar variability detected for Fritel is illustrated in fig. 5. A total of six probes in 22 probe/enzyme combinations were examined and the information is given in table 7. All the probes examined were polymorphic with at least one restriction enzyme. For the ten individual plants of Fritel, nine unique RFLP patterns were found. For A × B × C three probes were screened in 10 probe/enzyme combinations (table 8). Polymorphism was detected in two plants (3 and 5) with probe 8·38 in combination with *Eco*RI and *Bam*HI.

It is important to note that the polymorphism detected within the *Vicia faba* cultivars is different from that observed between cultivars and accessions. The polymorphism within cultivars was in minor bands as opposed to the polymorphism in the major hybridising fragments detected between cultivars. Thus the creation of a linkage map based on the RFLP markers identified here

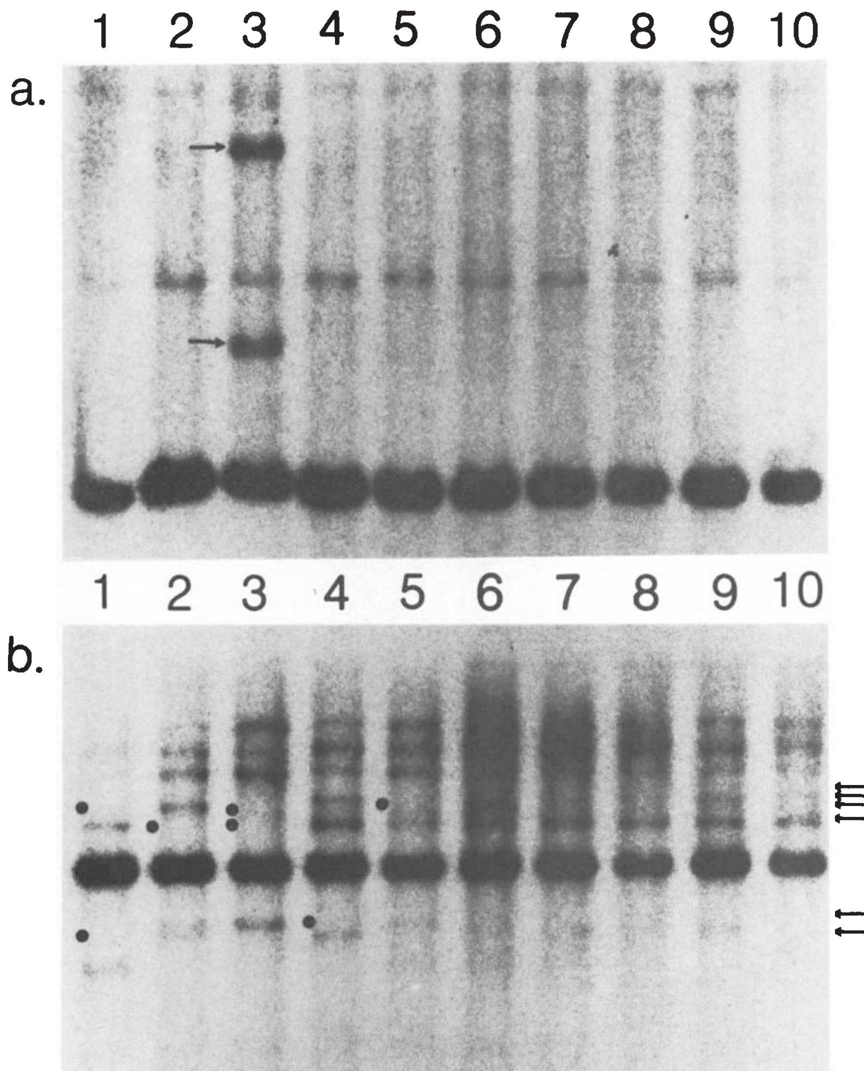

Figure 5 Variation within ten individual Fritel plants probed with cDNA probe 8·4 and restricted with *Bam*HI (a): (b), *Eco*RI polymorphism is only detected on minor bands. Arrows and dots indicate polymorphic minor bands.

Table 7 Polymorphism within *Vicia faba cv.* Fritel

Probe	Size (kb)	Enzyme	Fritel plants										Number of phenotypes
			1	2	3	4	5	6	7	8	9	10	
1·5	0·5	*Eco*RI	a	a	a	b	a	a	b	a	a	a	2
		*Eco*RV	a	a	a	a	a	a	a	a	a	a	1
		*Bam*HI	a	b	a	a	a	a	b	b	b	b	2
		*Hin*dIII	a	a	a	a	a	a	a	a	a	a	1
4·3	1·5	*Eco*RI	a	a	a	b	a	a	b	a	a	a	2
		*Eco*RV	a	a	a	a	a	a	a	a	a	a	1
		*Bam*HI	a	b	a	a	a	a	c	b	b	b	2
		*Hin*dIII	a	a	a	a	a	a	a	a	a	a	1
5·6	0·6	*Eco*RI	a	a	a	a	a	a	a	a	a	a	1
		*Eco*RV	a	a	a	a	a	a	a	a	a	a	1
		*Bam*HI	a	b	a	a	a	a	c	b	b	b	3
		*Hin*dIII	a	a	a	a	a	a	a	a	a	a	1
7·18	0·3	*Eco*RI	a	a	a	b	a	a	b	a	a	a	2
		*Bam*HI	a	b	a	a	a	a	c	b	b	b	3
8·4	0·6	*Eco*RI	a	b	c	d	e	d	e	e	d	e	5
		*Eco*RV	a	b	b	b	a	a	a	a	a	a	2
		*Bam*HI	a	a	b	a	a	a	a	a	a	a	2
		*Hin*dIII	a	b	c	a	a	d	a	a	a	a	4
8·44	0·9	*Eco*RI	a	a	a	a	a	a	a	a	a	a	1
		*Eco*RV	a	a	a	a	a	a	a	a	a	a	1
		*Bam*HI	a	b	a	a	a	a	a	b	b	b	2
		*Hin*dIII	a	a	a	a	a	a	a	a	a	a	1

a, b, c, d, e, different banding patterns.

should not be impeded by the heterogenetiy within a given *Vicia* accession.

DISCUSSION

The present manuscript represents the first report to our knowledge of the examination of RFLPs in *Vicia*. The objectives were to assess the level of polymorphism detectable at the nucleic acid level and identify suitable parents for use in the creation of a linkage map. The choice of parents for genetic mapping studies is of crucial importance. For example, in tomato an interspecific cross had to be analysed to ensure sufficient polymorphism (Tanksley *et al.*, 1989). Interspecific crosses involving *V. faba* cannot be produced and the selection of the most diverse parents for subsequent crossing

Table 8 Polymorphism within *Vicia faba cv.* A × B × C

Probe	Size (kb)	Enzyme	A × B × C plants										Number of phenotypes
			1	2	3	4	5	6	7	8	9	10	
5·6	0·6	*Eco*RI	a	a	a	a	a	a	a	a	a	–	1
		*Hin*dIII	a	a	a	a	a	a	a	a	a	–	1
8·38	1·0	*Eco*RI	a	a	a	a	b	a	a	a	a	–	2
		*Eco*RV	a	a	a	a	a	a	a	a	a	–	1
		*Bam*HI	a	a	b	a	b	a	a	a	a	–	2
		*Hin*dIII	a	a	a	a	a	a	a	a	a	–	1
pAD4·4	1·1	*Eco*RI	a	a	a	a	a	a	a	a	a	–	1
		*Eco*RV	a	a	a	a	b	a	a	a	a	–	2
		*Bam*HI	a	a	a	a	a	a	a	a	a	–	1
		*Hin*dIII	a	a	a	a	a	a	a	a	a	–	1

a, b, different banding patterns.

is a prerequisite for the creation of a detailed linkage map based on molecular markers. The present study identified the genotype A × B × C as a suitable parent due to the relatively large numbers of probes detecting polymorphism with this genotype. It may be relevant to consider the breeding history of A × B × C. This three way hybrid was created with the objective of maximising the level of heterosis. For this purpose diverse *V. faba* var. *equina* and var. *major* accessions from the U.K., Germany and The Netherlands were hybridised with the aid of cytoplasmic male sterility (Duc, personal communication). To our knowledge mutagenesis was not used in the creation of the male sterile stocks but mutagenesis may have a role to play in inducing a higher level of RFLPs (Maluszynski, 1989). It is generally difficult to determine the types of mutation causing the polymorphism detected in RFLP analysis (Landry *et al.*, 1987). However, the polymorphisms associated with A × B × C did not appear to be specific to certain restriction enzymes which would suggest that genomic rearrangements may be responsible for the differentiation of A × B × C from the other *Vicia* accessions.

The nature of the polymorphisms identified in *Vicia* has parallels with that reported for soybean (Keim *et al.*, 1989). In a survey of 58 soybean genotypes the majority of RFLP loci had only two alleles per locus and for some loci one allele was very rare. This is similar to our findings in *Vicia* where the majority of the polymorphisms are characterised by the presence of 2-3 hybridising bands. Isozyme studies in *Vicia* (Mancini *et al.*, 1989) have also indicated that multiple alleles are relatively rare. Glutamate oxaloacetate transaminase (GOT) isozymes examined by Mancini *et al.* at two loci, Got-3 and Got-2, were shown to be under the control of two and three alleles respectively. Superoxide dismutase (SOD) loci were also shown to be under the control of two alleles per locus.

The availability of molecular markers will allow the phylogenetic affinity between different *Vicia* accessions and species to be examined. The evolution and taxonomy of *Vicia* species has previously been studied by examining nuclear DNA content (Raina and Rees, 1983; Raina and Narayan, 1984; Brace, 1985; Youssef and Heseman, 1985), the chloroplast (Ko *et al.*, 1987; Ko and Straus, 1986) and the mitochondrial genome (Negruk, 1986). Detailed studies of the nuclear genome have been largely restricted to highly repeated (Kato *et al.*, 1984; Lamppa *et al.*, 1984; Yakura *et al.*, 1984; Rogers and Benedict, 1987) or highly expressed (Wobus *et al.*, 1984) DNA sequences. The use of low copy cDNA clones identified in the present study will provide another approach to examining DNA diversity in *Vicia*.

The availability of a linkage map based on molecular markers offers new opportunities for applications in genetics and breeding (Tanksley *et al.*, 1989). In particular the potential of establishing linkage between a diagnostic marker and important agronomic traits is feasible. Future research will focus on the segregation of alleles at RFLP loci in segregating progeny of an A × B × C × 172 cross. Currently we have identified a number of polymorphic clones and these will be used for the creation of a linkage map. Primary trisomics can be produced in *Vicia faba* (Cabrera and Martin, 1989) and these can be used to assign probes rapidly to specific chromosomes via dosage effects as described by Young *et al.* (1987). In this way we aim to generate a linkage map with genetic markers distributed across the *Vicia faba* genome. We anticipate that this map will provide a powerful tool for monitoring recombination between specific loci and enable the potential variation available in *Vicia faba* to be manipulated with greater precision.

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