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Analyses of *Phaseolus vulgaris* L. and *P. coccineus* Lam. hybrids by RFLP: preferential transmission of *P. vulgaris* alleles

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Summary. Restriction fragment length polymorphism (RFLP) was determined among *P. vulgaris* genotypes and *Phaseolus* species using 19 probes. The incidence of polymorphism was high (70–86%) between species, but relatively low (22–26%) between genotypes of *P. vulgaris*. Suitable probes were identified for the analysis of *P. vulgaris* and *P. coccineus* hybrids. The segregation pattern in F_2 populations was Mendelian for two probes (LHB and VEE20) and non-Mendelian for GS-g, CHS, and CHI. Statistical analyses indicated gametic selection with preferential transmission of the *P. vulgaris* alleles, which may account for the selective recovery of *P. vulgaris* progeny types observed earlier. The available hybrids of *P. vulgaris* and *P. coccineus* and the high degree of interspecific RFLP will facilitate the construction of a linkage map for *Phaseolus*.

Key words: *Phaseolus* interspecific hybrid – RFLP – Beans

Introduction

Interspecific hybridization of *Phaseolus* usually results in embryo abortion. We have studied the development of hybrid embryos in crosses of *P. vulgaris* to three other species – *P. lunatus*, *P. acutifolius*, and *P. coccineus*. The extent of hybrid embryo development ranges from the four-celled stage to maturity and depends on the parental species combination as well as on the direction of the cross (Mok et al. 1978a, b, 1986; Rabakoarihanta et al. 1979). The most advanced development occurs in crosses with *P. coccineus*: when *P. coccineus* is used as the female parent the embryos develop to the mid- to late-cotyledonary stage, while in the reciprocal cross embryos develop normally to maturity (Shii et al. 1982). Hybrid plants can be obtained from the abnormal embryos of *P. coccineus* × *P. vulgaris* with the aid of embryo culture techniques.

The combination between *P. vulgaris* and *P. coccineus* is of particular interest since, in addition to the dramatic difference in hybrid embryo development between reciprocal crosses, several other abnormalities can be detected following the initial hybridization (Shii et al. 1982). For instance, reciprocal hybrids differ in pollen viability. *P. vulgaris* × *P. coccineus* F_1 plants have 25% stainable pollen, whereas the reciprocal hybrids obtained from abnormal embryos have 80% stainable pollen. Reciprocal F_2 populations segregate into normal and two classes of abnormal embryos, the shrunken and underdeveloped types. Moreover, normal as well as non viable plants occur in the F_2. In later progeny populations (F_4 and F_5), the plants generally resemble one or the other parental type (Mok et al. 1987). This phenotypic reversion is accompanied by the recurrence of normal and abnormal embryos, varying degrees of pollen fertility, and differential viability of progeny plants. Although the causes of these complex developmental problems are yet unknown, simple-cytoplasmic effects cannot account for these phenomena, since the abnormalities occur in successive generations (at least to the F_4), regardless of the direction of the initial cross.

We observed that normal and abnormal embryos exhibited specific isozyme patterns resembling *P. vulgaris* and *P. coccineus* parents, respectively (Guo et al. 1989), which suggests that types of embryo development may be related to specific combinations of the parental genomes and/or to differential gene expression. We have begun to examine selected aspects of these problems, namely, the possibility of preferential transmission of specific linkage groups and occurrence of genetic combinations associated with embryo or plant viability. This paper describes the initial phase of the work using restriction fragments to obtain an estimate of inter- and intraspecific polymor-

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phism in *Phaseolus* and to determine if Mendelian or non-Mendelian segregation occurs in interspecific crosses.

Materials and methods

Plant materials and sources of DNA

To detect usable DNA probes and to estimate the degree of interspecific and intraspecific (within *P. vulgaris*) polymorphism, the following *Phaseolus* gnotypes were used: *P. vulgaris* L. cultivars Great Northern (GN), Tendergreen (TG), Improved Tendergreen (IT), Sanilac (SA), Contender (CO), *P. coccineus* Lam. cv Scarlet runner (SR), *P. lunatus* L. cv Kingston (K), and *P. acutifolius* A. Gray P.I. 321637 (AC2). *Vigna unguicularis* cv Purple Hull was used for intergeneric comparison. *P. vulgaris* GN and *P. coccineus* SR were used to generate F_1 and F_2 populations as reported earlier (Guo et al. 1989). Immature F_2 embryos were classified according to the type of development, normal or abnormal. Callus cultures were established from the cotyledons of individual embryos (Mok et al. 1978b) and plantlets were derived by culturing the embryonic axes (Shii et al. 1982).

DNA isolation

Plant (stem and/or leaf) and callus tissues were collected and immediately frozen under liquid nitrogen and then stored at −80 °C until DNA preparation. DNA from 5 g of sample was extracted and purified using the methods of Dellaporta et al. (1983), with the following modifications: 1.2 × DNA extraction buffer (DEB) was used for callus tissue. 2. Triton × 100 instead of SDS was used in DEB. 3. For plant tissue, DNA was extracted twice. The first grinding was done in mortar and pestle containing liquid nitrogen and the ground powder was then suspended in 15 ml DEB. After centrifugation, the precipitated cell debris was resuspended in DEB and homogenized with a polytron homogenizer. The first extraction yielded DNA with higher molecular weight (50–100 kbp). The second extraction with a polytron increased DNA recovery, but gave rise to lower molecular weight (10–25 kbp) fragments. Higher or lower MW DNA was used depending on the probes employed.

Southern hybridization

DNA samples (10 µg) were digested with restriction enzyme EcoRI (1 µg DNA/10–20 units enzyme) at 37 °C overnight. After electrophoresis in 1% agarose gels, DNA was transferred to IMMOBILON-N membrane, according to the manufacturer's instructions (Millipore). The filters were prehybridized at 65 °C for 2–4 h in solution containing 6 × SSC (1 × SSC consisted of 0.15 *M* NaCl and 0.15 *M* sodium citrate), 5 × Denhardt's solution, 0.5% SDS, 10 µg/ml salmon sperm DNA, and 5% dextran sulfate.

Hybridization with ^{32}P-labelled probes was carried out under the same conditions for 16–24 h. Genes of known function and random cDNA clones were used as probes. Probes were labelled using a multiprime DNA labelling system (Amersham) and purified by centrifugal dialysis in Centricon-10 by two washings (1 ml) of 2 × 0.2 *N* EDTA. Filters were washed at 65 °C with three changes of 2 × SSC and 0.1% SDS, and then autoradiographed at −80 °C using preflashed XAR-5 film in the presence of intensifying screens.

Cosntruction of cDNA libraries and isolation of cDNA clones

cDNA libraries were made from 5 µg poly(A)$^+$RNA isolated from GN or SR embryos of mixed developmental stages. The library constructions were as decribed by Lightfoot et al. (1988), except that vector pGEM-3Zf+ (Promega) was used.

Sources of DNA of known genes

Plasmid probes were maintained as frozen cell lines at −80 °C. Plasmid DNA was prepared by alkaline or boiling lysis and $CsCl_2$ or column purification (Stratagene). After restriction digestion, plasmid DNA was electrophoresed on a 1.5% ultrapure agarose gel. The appropriate bands were excised and the insert DNA was recovered using Glassmilk (Bio-101). Probes were obtained from the following sources:

CHI (chalcone isomerase): Mehdy and Lamb 1987.
CHS (chalcone synthase): Ryder et al. 1984.
CAB (chlorophyll a/b binding protein): Gallagher and Ellis 1982.
LHB (leghemoglobin), GS (glutamine synthetase): Bennett et al. 1989.
PAL (phenylalanine ammonia lyase): Edwards et al. 1985.
SSU (Rubisco small subunit): Bedbrook et al. 1980.
rRNA: gift, C. J. Rivin, Oregon State University
VML series (cDNA clones): Lightfoot et al. 1988.
VME series (cDNA clones): this work.
PIN (phaseolin): gift, S. Sung, University of Hawaii.
ACT (actin): D.A. Lightfoot (unpublished results).

Statistics

Segregation data obtained in F_2 populations were analyzed using the program LINKAGE.

Results

Embryo and plant development

F_2 embryos were recovered from pods formed on GN × SR F_1 plants. Of these embryos, 123 were classified as normal and 53 as abnormal. The ratio between normal and abnormal embryos did not differ significantly from 3:1 (Table 1). However, the abnormal embryo population was highly heterogeneous, precluding further conclusions concerning the precise mode of inheritance. Two main classes of abnormal embryos were observed, the underdeveloped and shrunken. The underdeveloped embryos were arrested at the early cotyledonary stage and most of them were surrounded by a thick layer of endosperm tissue. The strunken embryos had either

Table 1. Distribution of F_2 embryos and plants according to developmental classes

Embryo development			Plant development		
Normal	Abnormal		Normal	Abnormal	Lethal[a]
	Wrinkled	Underdeveloped			
123	28	25	43	29	66

[a] Included plantlets that perished before 4 weeks and axes that did not grow on culture medium

Table 2. RFLPs between genotypes of *P. vulgaris* and related species. Number outside of the parentheses denotes banding pattern. Numerals within the parentheses indicate the number of polymorphic bands in comparison to *P. vulgaris* cv GN

Probes	Bands in GN	*P. vulgaris* genotypes					Related species			
		GN	TG	IT	SA	CO	SR	K	AC2	VU
SSU	4	1 (0)	1 (0)	1 (0)	1 (0)		2 (4)	3 (4)	4 (4)	5 (4)
CHI	1	1 (0)	1 (0)	1 (0)	2 (1)	1 (0)	3 (1)	4 (1)	5 (1)	6 (1)
LHB	4	1 (0)	1 (0)	2 (1)	2 (1)	2 (1)	3 (4)	4 (4)	5 (4)	6 (4)
GS-a	2	1 (0)	1 (0)	1 (0)	2 (1)	1 (0)	3 (2)	4 (2)	5 (2)	6 (2)
GS-b	2	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	2 (2)	3 (2)	4 (2)
GS-g	3	1 (0)	2 (1)	2 (1)	1 (0)	2 (1)	3 (3)	4 (3)	5 (3)	6 (3)
GS-d	1	1 (0)	2 (1)	2 (1)	1 (0)	2 (1)	3 (1)	4 (1)	5 (1)	6 (1)
PIN	5	1 (0)	2 (1)	2 (1)	3 (1)	4 (1)	6 (5)	7 (5)	8 (5)	9 (5)
ACT	1	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	2 (1)	3 (1)	4 (1)
CHS	5	1 (0)	3 (2)	3 (2)	2 (2)	3 (2)	5 (1)	6 (5)	7 (5)	8 (5)
PAL	2	1 (0)	2 (1)	3 (1)	4 (1)	5 (1)	6 (2)	7 (2)	8 (2)	9 (2)
rRNA	3	1 (0)	2 (1)	2 (1)	2 (1)		3 (1)	4 (1)	5 (1)	6 (1)
VML22	5	1 (0)	2 (3)	3 (2)	4 (2)	5 (4)	6 (5)	7 (5)	8 (5)	9 (5)
VML23	2	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	2 (1)	2 (1)	2 (1)	2 (1)
VEE2	2	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	2 (1)	1 (0)
VEE3	2	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	2 (1)	3 (1)	4 (1)	5 (1)
VEE4	2	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	2 (1)	3 (1)	4 (1)	5 (1)
VEE6	2	1 (0)	1 (0)	1 (0)	1 (0)	3 (1)	4 (2)	5 (2)	6 (2)	2 (2)
VEE15	1	1 (0)	1 (0)	1 (0)	2 (1)	1 (0)	3 (1)	4 (1)	5 (1)	6 (1)
VEE16	3	1 (0)	2 (1)	2 (1)	2 (1)	2 (1)	3 (1)	4 (1)	5 (1)	6 (1)
VEE20	2	1 (0)	1 (0)	1 (0)	1 (0)	1 (0)	2 (1)	3 (1)	4 (1)	5 (1)
Polymorphic band (to GN)		(0)	(11)	(11)	(12)	(13)	(37)	(43)	(44)	(43)
% Polymorphism		0	22	20	24	26	70	82	86	84

Table 3. Distribution of GN × SR F_2 progeny according to genotypes of selected polymorphic probes and goodness of fit to the hypothesis of gametic selection

Probe	Progeny classes						*P*	Estimated allelic ratio		Goodness of fit for gametic selection
	Expected ratio			Observed F_2s						
	GN	F_1	SR	GN	F_1	SR		G	S	
LHB	1	2	1	44	79	38	0.77	0.52	0.48	NA
VEE20	1	2	1	35	73	45	0.44	0.50	0.50	NA
GS-g	1	2	1	68	76	20	10^{-7}	0.69	0.31	0.17
CHS	1	2	1	59	71	24	10^{-4}	0.61	0.39	0.94
CHI	1	2	1	81	47	19	10^{-7}	0.71	0.29	0.01

extremely or moderately wrinkled and soft cotyledons. Callus cultures were obtained from all embryos, but plants could be recovered from only a portion of the cultured embryo axes. About 48% of the plantlets obtained died before 4 weeks (Table 1). Another 21% had abnormal phenotypes, including spindly or dwarf growth habit, or wrinkled leaves and abnormal flowers, while the remaining plants (31%) were entirely normal. The abnormalities at later stages did not seem to be related to abnormal embryo development, since many normal embryos gave rise to spindly or excessively bushy plants.

Intra- and interspecific polymorphism and identification of usable gene probes

Restriction fragment length polymorphism was determined using a total of 19 probes and several genotypes of *P. vulgaris* and other legumes (Table 2). The incidence of RFLP was high between species (from 70–86%) but relatively low between genotypes of *P. vulgaris* (22–26%). Representative patterns of LHB, CHI, GS-g, and VEE20 are presented in Fig. 1. Probes that showed polymorphism between GN and SR were further used to

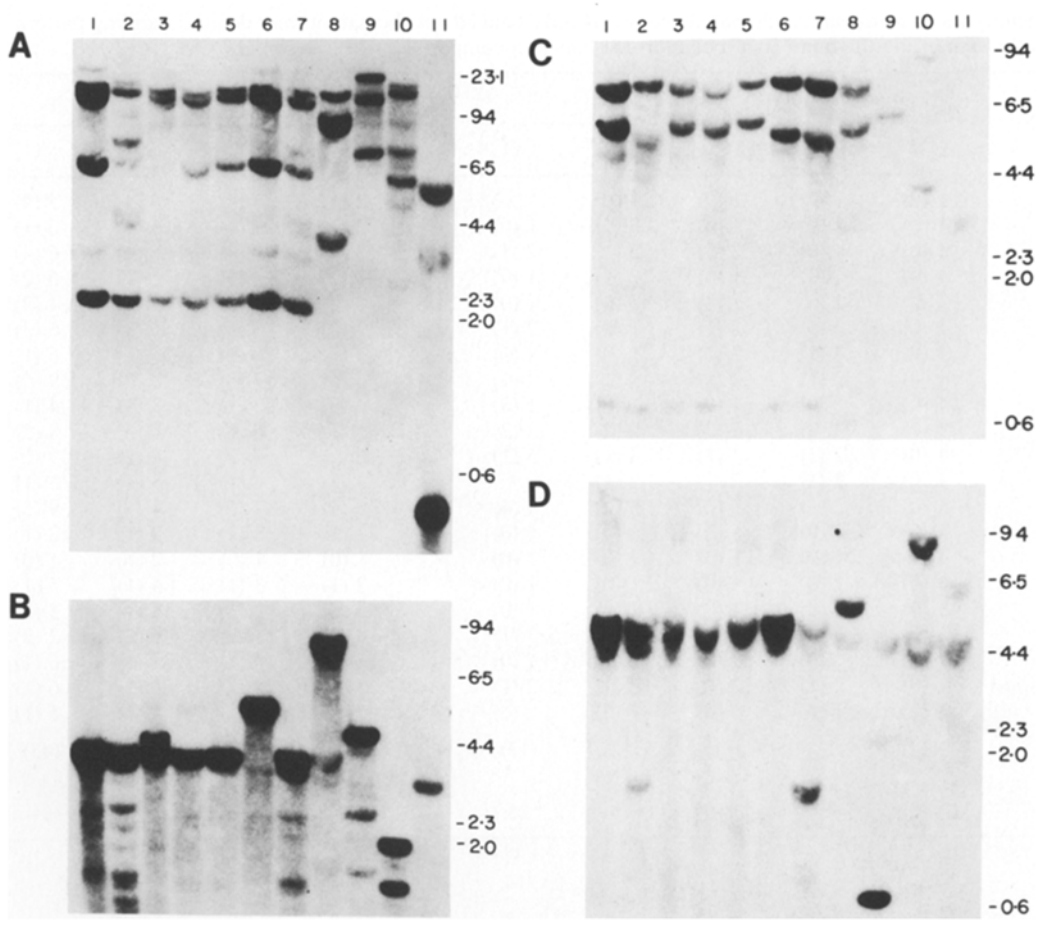

Fig. 1 A–D. Polymorphism of representative probes among genotypes of *P. vulgaris* and related species. **Panels A to D:** leghemoglobin (LHB), chalcone isomerase (CHI), glutamine synthetase-gamma (GS-g), and clone 20 (VEE20). *Lanes 1–7: P. vulgaris* genotypes (*1:* P725, *2:* P691, *3:* CO, *4:* IT, *5:* TG, *6:* SA, and *7:* GN). *Lanes 8–10: Phaseolus* species (*8: P. coccineus* SR, *9: P. lunatus* K, *10: P. acutifolius* AC2). *Lane 11: Vigna unguicularis*). Total genomic DNA was digested with EcoRI and separated by electrophoresis on 1% agarose gel. After Southern transfer to Immobilon-N, the filter was serially probed with DNA clones indicated

determine banding patterns in F_1s. Probes with bands that enabled unambiguous scoring of parental and F_1 types were selected to screen the F_2 populations. These included LHB, CHI, CHS, GS-g, and VEE20. DNA samples obtained from callus and plant parts of selected genotypes were compared and no difference in the restriction fragment patterns was found (data not shown).

Segregation analyses

Evaluation included more than 150 F_2s and the five selected probes. Representative RFLP patterns of F_2 are presented in Fig. 2. The distribution of progeny into genotypic classes (parental or F_1) is presented in Table 3. The segregation of LHB and VEE20 was Mendelian and did not deviate from the expected 1:2:1 ratio. However, the distribution of progeny for GS-g, CHS, and CHI differed significantly from the expected ratios. For all three probes, the SR class was underrepresented. In order to test the possible occurrence of gametic selection, the allelic ratio was calculated, and the expected number of progeny in each class was derived from these ratios (Table 3). Chi-square tests indicated that for GS-g and CHS, the expected and observed numbers of progeny were not significantly different, suggesting preferential transmission of GN allels. The significant deviation in

Table 4. Distribution of SR × GN F_2 progeny according to genotypes of selected polymorphic probes

Probe	Progeny classes						*P*
	Expected ratio			Observed F_2s			
	GN	F_1	SR	GN	F_1	SR	
LHB	1	2	1	13	24	8	0.52
VEE20	1	2	1	9	22	9	0.82
CHS	1	2	1	21	21	9	0.02
CHI	1	2	1	18	15	3	5×10^{-4}

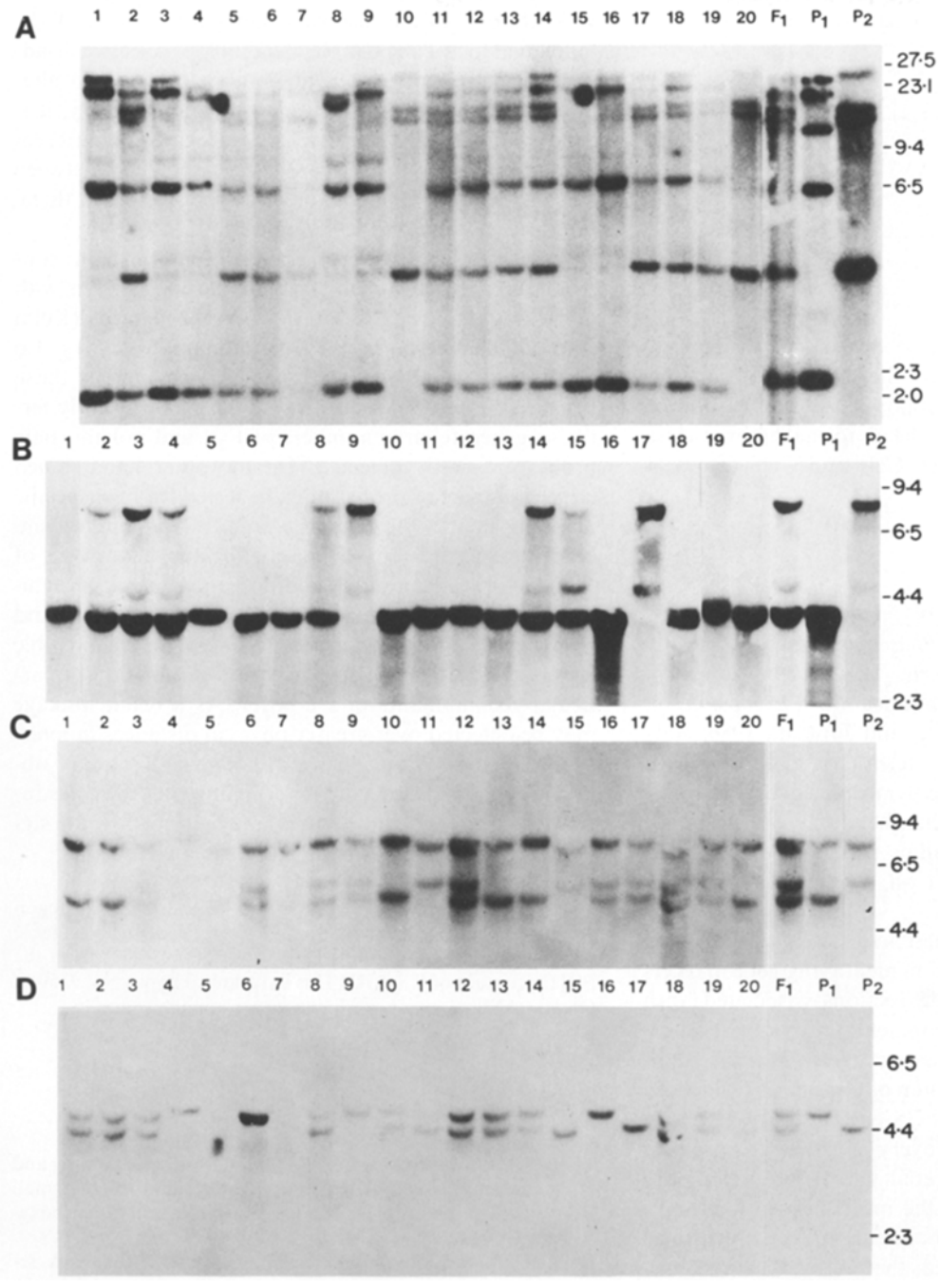

Fig. 2A–D. RFLP of *P. vulgaris* and *P. coccineus* parents, interspecific F_1, and representative F_2s. **Panels A to D:** leghemoglobin (LHB), chalcone isomerase (CHI), glutamine synthetase-gamma (GS-g), and clone 20 (VEE20). *Lanes 1–20:* F_2s; P_1: *P. vulgaris* cv GN; P_2: *P. coccineus* cv SR; F_1: GN × SR hybrid

the case of CHI indicates that mechanisms other than gametic selection must have contributed to the skewed distribution observed in the F_2 population.

Segregation ratios of probes in subpopulations classified according to embryo development, normal and abnormal, were similar to those in the complete population, with one exception. The F_2 ratio of CHS differed between subpopulations and was Mendelian in the subclass consisting of abnormal embryos.

A representative F_2 population obtained from the reciprocal cross (SR × GN) was also analyzed with four probes (LHB, CHI, CHS, and VEE20). The segregation patterns (Table 4) were essentially identical to those obtained for GN × SR progeny.

Table 5. Linkage detected between CHI and CHS probes

	Chi-square	*df*	*p*	*r*	SE
Total population	26.6	4	10^{-4}	0.29	0.034
Normal embryos	15.1	4	0.04	0.4	0.041
Abnormal embryos	13.6	4	0.009	0.27	0.058

Linkage analyses

Linkage could be detected only between two of the loci, CHI and CHS ($P=0.00002$) in the complete population (Table 5). Analyses of the subpopulations consisting of the normal embryo class and the abnormal embryo class also revealed linkage between CHI and CHS ($P=0.04$ and 0.009, respectively).

Discussion

RFLP between species of *Phaseolus* was substantially higher than within *P. vulgaris*. This observation is in agreement with results obtained for *Lycopersicum*, *Solanum*, and *Lens* (Bernatzky and Tanksley 1986; Harvey and Muehlbauer 1989; Helentjaris et al. 1986). In addition, the incidence of polymorphism between GN and SR was slightly lower than between GN and representative genotype of the other two *Phaseolus* species, confirming previous studies on evolutionary relationships of *Phaseolus* species.

Based on the results presented here, it seems that RFLP analyses will be useful in identifying gene markers and eventually chromosome sections associated with aberrations, including developmental abnormalities, resulting from interspecific crosses of *Phaseolus*. For example, the differential transmission of GN alleles (evidenced by particular probes such as GS-g, CHI, and CHS) may account for the selective recovery of *P. vulgaris* progeny types observed earlier (Mok et al. 1987). Our preliminary results suggest that one of the mechanisms of selective transmission could be gametic selection. As no reciprocal cross difference was detected, the preferential transmission does not appear to be affected by simple cytoplasmic sources. RFLP markers deviating from the expected F_2 ratios have been observed in interspecific crosses of *Lycopersicum* (Bernatzky and Tanksley 1986) and *Lens* (Harvey and Muehlbauer 1989). However, in these cases the majority of the markers examined segregated in Mendelian ratios, while in the GN × SR cross, non-Mendelian segregation seems to be a rather frequent event.

Loci detected by two of the probes, CHS and CHI, appeared to be linked. Although the map distance can be calculated (approximately 44 map units), it may not be accurate due to the distorted segregation ratios of the individual loci. The possible inviability or selective disadvantage of particular genetic combinations could alter the distribution of the alleles. Although less likely, it is conceivable that the two markers are located on different chromosomes, but that there is an interaction between the alleles of the two loci or other loci linked to them, resulting in more and less viable combinations.

Interspecific polymorphism has been utilized to construct linkage maps or to correlate particular traits with DNA markers using fertile interspecific hybrids (Keim et al. 1990; Paterson et al. 1990). Similar efforts may also be successful in *Phaseolus*, since hybrids such as those between *P. vulgaris* and *P. coccineus* are sufficiently fertile to generate large numbers of F_2s, and polymorphic probes are easily detected. On the other hand, much larger numbers of probes must be tested for intraspecific mapping due to the limited amount of polymorphism, but intraspecific crosses may offer the advantage of Mendelian segregation. In addition, map distances calculated from recombination frequencies in intraspecific and interspecific progenies may differ due to the possible lower incidence of crossing-over and elimination of particular recombinants in the latter. As a result, linkage may be detected over greater physical distances in interspecific combinations. Thus, although our primary objective is to study genetic abnormalities in *Phaseolus* interspecific crosses, these studies may also contribute significantly to the creation of a linkage map.

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