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Spatial Patterns of Diversity at the Putative Recognition Domain of Resistance Gene Candidates in Wild Bean Populations

J. de Meaux, C. Neema

Laboratoire de Pathologie Végétale, INRA INA P-G, 16 rue Claude Bernard, 75231 Paris Cedex 05, France

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Abstract. Leucine Rich Repeats (LRR) domains have been identified on most known plant resistance genes and appear to be involved in the specific recognition of pathogen strains. Here we explore the processes which may drive the evolution of this putative recognition domain. We developed AFLP markers specifically situated in the LRR domain of members of the PRLJ1 complex Resistance Gene Candidate (RGC) family identified in common bean (*Phaseolus vulgaris*). Diversity for these markers was assessed in ten wild populations of *P. vulgaris* and compared to locally co-occurring pathogen populations of *Colletotrichum lindemuthianum*. Nine PRLJ1 LRR specific markers were obtained. Marker sequences revealed that RGC diversity at PRLJ1 is similar to that at other complex R-loci. Wild bean populations showed contrasting levels of PRLJ1 LRR diversity and were all significantly differentiated. We could not detect an effect of local *C. lindemuthianum* population diversity on the spatial distribution of *P. vulgaris* PRLJ1 diversity. However, host populations have been previously assessed for neutral (RAPD) markers and for resistance phenotypes to six strains of *C. lindemuthianum* isolated from cultivated bean fields. A comparative analysis of PRLJ1 LRR diversity and host diversity for resistance phenotypes indicated that evolutionary processes related to the antagonistic *C. lindemuthianum*/*P. vulgaris* interaction are likely to have shaped molecular diversity of the putative recognition domains of the PRLJ1 RGC family members.

Correspondence to: Claire Neema; *email:* neema@inapg.inra.fr

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Introduction

In plant–pathogen interactions, host resistance can be expressed in response to specific pathogen strains. This specific type of resistance is governed by major genes in both host and pathogen, and results from the simultaneous presence of a resistance allele in the host and an avirulence allele in the pathogen (Flor 1956). Over the last ten years, the identification and cloning of over 20 resistance genes has revealed that resistance genes share sequence similarities and can be categorized into several R-gene classes.

The genomic organization of resistance genes ranges from clusters of multigenic families to isolated single copy genes (reviewed in Hammond-Kosack and Jones 1997). The detailed analysis of diversity at complex R-loci has identified several molecular mechanisms by which R-genes evolve (reviewed in Ellis et al. 2000; Young 2000). Intragenic recombination and point mutations appear to be the major evolutionary mechanisms generating novelty. In addition, unequal intergenic crossing-overs can also lead to the generation of new paralogs, although these events seem to be rare enough to prevent concerted evolution. Thus resistance genes are hypothesized to follow a birth-and-death model (Michelmore

and Meyers 1998) in which the generation and maintenance of duplicated loci results in the complex organization of resistance loci.

The most common functional domain found in resistance genes is the C-terminal Leucine Rich Repeat (LRR) domain. This domain was first characterized in the porcine ribonuclease inhibitor protein and was shown to be involved in protein/protein interactions (Kobe and Kajava 2001). It consists of several repetitions of the xx(L)x(L)xx motif separated by approximately 20 amino acid residues. Crystallisation of the protein has shown that xx(L)x(L)xx repetitions fold into a β-turn-β-strand structure, where variable residues (x) are solvent exposed. Resistance genes could therefore interact through their LRR domain with a protein or protein complex resulting from the presence of a pathogenic strain. Although evidence for a direct interaction remains scarce (Jia et al. 2000), some experimental data support this hypothesis (Young 2000). Indeed, the LRR domain has been shown to be highly variable, with the hallmark of diversifying selection detected on the solvent exposed residues of many R-genes (Botella et al. 1998; Ellis et al. 1999; McDowell et al. 1998; Meyers et al. 1998; Parniske et al. 1997). Thus, LRR diversity may be primarily shaped by pathogen diversity (Michelmore and Meyers 1998).

To date, empirical studies have been based on only a few genotypes in a small number of species and remain disconnected from the ecological context in which diversity at complex R-loci has evolved (Bergelson et al. 2001). The processes which drive the evolution of the LRR domain in nature remain to be studied.

The common bean (*Phaseolus vulgaris*) and the pathogenic fungus *Colletotrichum lindemuthianum* are a valuable model to assess the evolutionary processes driving LRR diversity at a complex resistance locus because: (i) the interaction occurs in the wild, (ii) the interaction is hypothesized to have a gene-for-gene determinism (Kelly and Young 1996), and (iii) a complex family of Resistance Gene Candidates (RGCs) has been identified in *P. vulgaris* and maps together with eight resistance specificities to *C. lindemuthianum* (Geffroy et al. 1999). The members of this RGC family belong to the major class of R-genes (Young 2000) and show an NBS (Nucleotide Binding Site) domain 5′ from an LRR domain.

The common bean is a highly inbred diploid found in small wild populations in which host-parasite interactions have little anthropogenic influence (Ibarra-Perez et al. 1997). Populations are distributed along a 5000-km north-south axis in Latin and South America. Based on both molecular and phenotypic markers, major centers of diversity were identified in Meso-America and in the Southern Andes (Velasquez and Gepts 1994). The fungus *Colletotrichum lindemuthianum* is the causal agent of anthracnosis. This disease is one of the most prevalent in both cultivated fields and wild populations and impacts host fitness (J. Capelle, personal communication). No sexual form of the fungus has been observed in natural epidemics (Bryson et al. 1992). Fungal spores comigrate mainly with the short-distance dispersing seeds of the host (Tu 1992) and cross-inoculation experiments have shown that pathogen strains are generally adapted to host plants from the same origin (Geffroy et al. 1999).

An RFLP study of molecular diversity at the PRLJ1 locus in wild bean populations indicates that selection may have acted upon this locus, although this effect could not be detected at all spatial scales (de Meaux et al. 2003; Neema et al. 2001). However, the effect of locally co-occurring pathogen populations on RGC diversity has not been investigated. Here, we focus specifically on molecular diversity in the LRR domain of the PRLJ1 RGC family in wild bean populations for which *C. lindemuthianum* diversity was also assessed. We ask whether LRR variation is influenced by ecological factors related to the interaction or whether it is predominantly governed by genome-wide evolutionary processes resulting from host population dynamics.

For this purpose we developed PRLJ1 LRR markers using a modified AFLP technique. Diversity for these markers was assessed in ten wild *P. vulgaris* populations from Mexico. The diversity of these host populations has been previously assessed using neutral markers (RAPD) and phenotypic tests for resistance to *C. lindemuthianum* (Cattan-Toupance 1997). Diversity for the pathogen populations associated with seven of these host populations, in which disease was most severe, has been previously characterized both for neutral markers (RAPD) and for virulence phenotypes (Sicard et al. 1997). We compare PRLJ1 LRR diversity to both host and pathogen diversity obtained for neutral markers as well as phenotypic markers involved in the interaction, and discuss the relative impact of distinct evolutionary processes (e.g., gene flow, pathogen populations) on the shaping of LRR diversity at the PRLJ1 locus.

Material and Methods

Plant Material

Plants were collected from ten populations distributed across three states in Mexico. Populations M1 to M3 were collected in Morelos, population M4 in Michoacan and populations M7 to M14 in Jalisco (Fig. 1). These wild populations, with an average size of 20 individuals, belong to the Meso-American diversity centre of *P. vulgaris*. Approximately 50% of the total population (91 plants) was sampled in 1994 at each location. Seeds of each plant were increased in INIFAP (Mexico) for further characterization. Forty-one strains of *Colletotrichum lindemuthianum* were isolated from

Fig. 1. Wild bean diversity was assessed at ten sites collected in different states of Mexico. At seven of these sites (M1, M2, M7, M8, M10, M12, and M14), disease due to *Colletotrichum lindemuthianum* was most severe and pathogen population diversity was assessed.

infected plants. Severity of disease was evaluated in each population as the frequency of diseased plants which varied from 20% (degree 1) to 50% (degree 2) and 90% (degree 3) (Table 1).

Determination of the LRR Specific Primers

The PRLJ1 RGC family was identified by a candidate gene strategy using degenerated NBS specific primers (Geffroy et al. 1999). The numerous members (or paralogs) of the gene family, over 15, showed all the characteristics of the large NBS-LRR class of resistance genes (Ferrier-Cana et al. 2003). Using recombinant inbred lines (RILs) derived from a cross between two cultivars, all members of the PRLJ1 family were mapped at the tip of linkage group 4 on the common bean genetic map, and were found to cosegregate with the genetic determinants of eight resistance specificities to *C. lindemuthianum*, and thus constitute a complex R-locus. The parent lines used for these crosses are representative of the two major gene pools of bean crops resulting from two independent domestication events in Meso-America and Southern-Andes (Geffroy et al. 1999; Velasquez and Gepts 1994). Four full-length RGCs were isolated from a cDNA bank and their sequences were aligned (B8, B11, J71, and J78 corresponding to GenBank accessions AF306503 to AF306506; Ferrier-Cana et al. 2003). We defined a LRR-specific primer (BJ5; 5′-GAGCAACTCTTCGTTTGC-3′) on a consensus zone among these four full-length RGCs, which precedes LRR repeat 11 and was designed to allow amplification downstream from this LRR.

Polymorphism for the LRR of the PRLJ1 RGC Family

A subsample of 68 plants collected from the ten Mexican populations was analyzed for AFLP polymorphisms specifically situated in the LRR domains of the PRLJ1 RGCs. We used a typical AFLP primer (the primer specific for *Mse*I restriction sites Mse00–5′-GACGATGAGTCCTGAGTAA-3′), following Vos et al. (1995) in conjunction with the PRLJ1 LRR-specific primer described above. AFLP profiles were independently replicated three times for each individual.

DNA Extraction

DNA was extracted from leaves of 15-day-old seedlings. Fresh leaves were plunged into liquid nitrogen and stored at −80°C. Frozen leaves were ground with a mortar and pestle with liquid nitrogen. Next, 10 mL extraction buffer (Tris-HCl 100 mM, NaCl 100 mM, EDTA 10 mM, Sarcosyl 3%, pH 8.5) and 10 mL phenol-chloroform (1 vol water saturated phenol/1 vol chloroform, qui-

Table 1. Nei diversity indices calculated for both *P. vulgaris* and *C. lindemuthianum* populations in each sampled site; the number of individuals analyzed in each population is also indicated

	Host populations						Pathogen populations			
	PRLJ1 LRR markers		RAPD markers		Resistance phenotype to *C. lindemuthianum* strains				RAPD diversity	Diversity for virulence
Sampled site	H Nei	Number of plants	H Nei	Number of plants	H Nei	Number of plants	Disease severity[a]	Number of strains	H Nei	H Nei
M1	0.21	7	0.04	7	0.10	5	2	6	0.11	0.10
M2	0.16	6	0.05	6	0.18	4	2	7	0.15	0.13
M3	0.03	7	0.02	7	0.31	4	1	2	(nd)	(nd)
M4	0.11	9	0.02	9	(nd)	1	1	2	(nd)	(nd)
M7	0.00	7	0.02	7	(nd)	1	3	6	0.07	0.09
M8	0.09	7	0.16	7	0.20	3	2	4	0.11	0.07
M9	0.05	6	0.04	6	(nd)	1	1	1	(nd)	(nd)
M10	0.00	5	0.00	5	0.05	4	2	4	0.14	0.05
M12	0.00	7	0.00	7	0.09	5	2	4	0.11	0.08
M14	0.00	7	0.04	7	0.30	5	2	5	0.10	0.09

[a] The disease severity is an estimation of the prevalence of disease at sampled sites at the time of collection.
(nd) non determined.

noleine 0.1%, pH 8) were added. Centrifugation (25 min, 12,000*g*) was followed by 15 min shaking on ice. The supernatant was collected and 10 mL phenol-chloroform was added. A second centrifugation was performed (25 min, 3000 rpm) followed by 20 min shaking at room temperature. Supernatant was collected and DNA precipitated with 10 mL isopropanol and 1.25 mL NaAc 3M. DNA was collected with a curved Pasteur pipette and transferred to an Eppendorf tube. DNA was then rinsed twice with 800 μL ethanol 70% and once with 800 μL absolute ethanol. DNA was dried for 10 min under vacuum, resuspended in 150 μL of 0.1×T.E (Tris-HCl 10 mM, EDTA 0.1 mM, pH 8), and stored at 4°C after addition of RNAse (50 μg/mL final concentration).

Specific AFLP Protocol, Cloning, and Sequencing of Amplification Products

Purified DNA (0.5 μg) was digested at 37°C for 1 h simultaneously with the enzymes *Mse*I and *Eco*RI (Life Technologies, 5 u each) in 40 μL RL Buffer (final concentrations 10 mM Tris-HAc, pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 50 ng·μL^{-1} BSA). Then 10 mL of RL Buffer with 5 pmol of EcoRI adaptator, 50 pmol of *Mse*I adaptator, 1.2 μL ATP (10 mM), and T4-DNA ligase (1 u Euromedex) were added to the restriction solution and ligation was performed for 3 h at 37°C. We used the adaptators designed by Vos et al. (1995). Both amplifications were performed using the *Mse*I adaptator-specific Mse00 primer and the BJ5 primer, without additional selective nucleotides. The *Mse*I/BJ5 primer was chosen thereafter. The first amplification was carried out in 50 μL containing 5 μL of the digestion-ligation solution (1.5 μL $MgCl_2$ 50 mM; 1.5 μL BJ5 primer 50 ng·μL^{-1}; 1.5 μL primer Mse-00 50 ng·μL^{-1}; 1 μL dNTP 10 mM; Gibco BRL Taq polymerase 1 u; 5 μL of the PCR buffer provided by Taq manufacturer). Amplification cycles were as follows: 35 cycles of [30 s at 94°C; 30 s at 54°C; 60 s at 72°C]. The PCR products were then diluted 20 times in distilled water, and a second amplification was carried out in 20 μL containing 5 μL of the dilution plus 0.6 μL $MgCl_2$ 50 mM, 0.5 μL BJ5 primer 50 ng·μL^{-1}, 0.6 μL primer Mse-00 50 ng·μL^{-1}, 0.4 μL dNTP 10 mM, Gibco BRL Taq polymerase 1 u and 2 μL of the PCR buffer provided by Taq manufacturer. The amplification conditions were as follows: 12 cycles of [30 s at 94°C; 30 s at 63°C with 0.7°C decrease per cycle; 60 s at 72°C] followed by 24 cycles of [30 s at 94°C; 30 s at 54°C; 60 s at 72°C]. Final amplification products were separated by electrophoresis in 38- × 50-cm 5.2% polyacrylamide denaturing gel (Sequigen Biorad, Power Pac 3000). Electrophoresis was run between 50 and 55°C for 100 min at 95 W. One of the glass plates was treated with γ-metacryloxypropyltrimethoxysilane in order to fix the gel to the glass plate for subsequent staining. Amplification products were revealed by silver nitrate staining (Bassam et al. 1991) after successive treatments with acetic acid 10%, silver nitrate solution (Prolabo 99.8% purity R.P. Normapur, 3 g·L^{-1}) and sodium carbonate (Prolabo, 99.8% purity, 60 g·L^{-1}). All bands were manually scored. In order to confirm that the amplified fragments belong to the PRLJ1 RGC family, polymorphic differently sized bands were separately reamplified from one individual as described in Chalhoub et al. (1997). Bands were subsequently cloned using the T-easy cloning kit (PROMEGA) and the size of the cloned fragment was checked. Corrected single strand sequencing of one clone of each differently sized band was performed by MWG-Biotech (Germany).

Sequence Analysis

Computer-aided sequence similarity searches were made using the BLAST program (Altschul et al. 1997). Multiple alignments were performed with the CLUSTALW service provided by INFOBIOGEN (htttp://www.infobiogen.fr) and were further corrected by eye. Divergence among markers was evaluated for each pair of markers by calculating the rate of synonymous mutation (Ks). The calculation was performed by the DnaSP version 3 software (Rozas and Rozas 1999). To estimate divergence levels among the PRLJ1 LRR markers, we calculated a Ks* averaging Ks over all possible pairs of markers following Bergelson et al. (2001).

Data Scoring and Statistical Analysis

Each band showing a given size was considered as a locus with two alleles, and individuals were scored for presence (1) and absence (0) of each of these bands. As *P. vulgaris* is predominantly selfing, plants were assumed to be homozygous and each profile was recorded as a haplotype. The same hypothesis was made for resistance phenotypes and for RAPD data, which are described in the following sections (Cattan-Toupance 1997).

Diversity was quantified by Nei's unbiased diversity index (Nei 1978). The amount of genetic divergence among all populations was calculated for each marker or overall by Weir and Cockerham's θ, which is analogous to Wright's Fst (Wright 1965; Weir and Cockerham 1984), using the FSTAT software (Goudet 1999). This software also provided confidence intervals by bootstraping over loci.

Genetic distances among pairs of populations was evaluated using the AMOVA software. This software computes a Φst matrix, which is a multilocus estimation of the correlation of haplotypic diversity among each possible pair of populations (Excoffier et al. 1992). Correlation between these Φst matrices was tested using the Mantel procedure, which allows one to evaluate and test the degree of similarity between two variables without assuming normality and independence. This procedure was provided by the Genetix 3.3 software (Belkhir et al. 1998) and 5000 permutations were used to test the correlation.

Statistical associations between markers were analyzed by calculating linkage disequilibria that were tested by a Linkdis permutation procedure (Genetix 3.3 software). For multiple permutation tests, no Bonferroni test was used as null hypotheses were different (Lemaire et al. 2000).

Host Diversity for RAPD Markers

The 68 plants analyzed for LRR diversity were previously characterized for RAPD diversity. DNA amplification with three primer pairs gave 32 amplified products, among which 13 were polymorphic (Cattan-Toupance 1997).

Host Diversity for Resistance at the Phenotypic Level

A subsample of 33 plants were previously characterized for resistance polymorphism by testing their resistance or susceptibility to six strains of *C. lindemuthianum* isolated from cultivated beans of different origins: strains 80, 3616, 100, 2, 9, and 21 (Cattan-Toupance 1997). The genetic determinant of three of these six resistance specificities has been mapped using RILs originating from the cultivated parents mentioned above (Geffroy et al. 1999, 2000). The resistance to strain 80 was mapped at the PRLJ1 locus. The resistance to strain 3616 was shown to be governed by two genes, only one of which was located at the PRLJ1 locus. The resistance to strain 100 was mapped elsewhere on the genome on a distinct linkage group. We established population structure for resistance phenotypes by computing the pairwise Φst matrix among seven of the ten populations (i.e., populations in which the amount of individuals was too low were excluded; see Table 1).

The genetic determinism of resistance specificities to seven additional *C. lindemuthianum* strains had also been localized on the PRLJ1 locus (Geffroy et al. 1999, 2000). We further examined a

subsample of the 33 plants for diversity for these seven additional resistance phenotypes (Table 2). The plants were inoculated with these seven strains as described in Cattan-Toupance et al. (1998), and inoculation reactions were scored. Given the restricted number of individuals in the subsamples, the structure of host population for these seven additional resistance phenotypes could not be assessed (Table 2). Patterns of linkage disequilibria with the PRLJ1 LRR markers were nonetheless examined.

Comparison to Previously Assessed Pathogen Diversity

Forty-one strains of *Colletotrichum lindemuthianum* had been isolated in the same host populations in 1994, and their diversity had been previously evaluated using 33 polymorphic RAPD markers obtained with four primer pairs (Sicard et al. 1997). Virulence polymorphism had also been assessed by testing strains against a set of 12 differential cultivars proposed by CIAT (Pastor-Corrales 1988). We established population structure for pathogen populations by computing the pairwise Φst matrix among seven populations (M1, M2, M7, M8, M10, M12, and M14), that showed the highest levels of disease severity (Table 1).

Results

Characterization of LRR Diversity at the PRLJ1 RGC Locus

A total of 68 plants distributed across ten populations were analyzed for diversity specifically situated in the LRR domain of PRLJ1 RGC family members using an AFLP-derived technique. Figure 2 shows the profiles that were typically obtained. Altogether, 27 fully reproducible bands were obtained, whereas three bands were inconsistently observed. Among these 27 bands, six were monomorphic. Band sizes varied from 100 to 420 bp. Plants showed from 11 to 18 bands, with an average of 15.6 bands per individual ($\sigma = 4.31$).

Among the 21 polymorphic markers, 14 were cloned and sequenced. Blast analysis revealed no significant homology to any known gene for five of the markers. The other nine markers could be aligned together with three of the four full-length PRLJ1 RGCs isolated from bean cultivars (Fig. 3). We therefore restricted the polymorphism analysis to these nine bands (Table 3; GenBank accession numbers AY136945 to AY136953). Each band was considered to have originated from a distinct paralog of the PRLJ1 RGC family (see discussion below). By comparison with the sequence of the full-length PRLJ1 RGC J78, it was possible to identify insertion-deletion (indel) events that all seem to have affected the zone preceding LRR 13 (Fig. 3). Patterns of conservation in flanking sequences indicate that some deletion events are shared by two PRLJ1 LRR markers or with complete RGC clones B8 and B11, suggesting coancestry by descent. Putative solvent-exposed residues seem to be associated to a higher level of diversity (Fig. 3). Therefore the polymorphism observed is due to both point mutations and/or insertion/deletion events.

In order to evaluate divergence levels among members of the PRLJ1 family, we calculated an average rate of synonymous mutation among pairs of PRLJ1 LRR markers. On average Ks* was 0.0837 ($s = 0.08$). This high value is mainly due to marker 5-181, which is highly divergent from other markers in the region following the deletion zone. Pairwise Ks between 5-181 and the other markers range from 0.18 to 0.30. When this marker is excluded, average Ks* reaches 0.040 ($s = 0.04$), with pairwise Ks values ranging from 0 to 0.12. Most polymorphic sites were singletons and thus no phylogenetic tree could be resolved.

Distribution of PRLJ1 LRR Marker Diversity

The distribution of variation at the nine focal markers differed among populations. Frequencies of allele presence ranged from 0.02 to 0.82. Some populations were monomorphic whereas others could show up to four different haplotypes with five polymorphic markers (Table 3). The spatial distribution of markers was heterogeneous across populations. All markers, except marker 5-19, showed significant differentiation among the populations (Table 4). Single marker population differentiation, as estimated by Weir and Cockerham's θ, varied from 0.517 ($\sigma = 0.216$) for marker 5-16, to 1.00 for within population monomorphic markers (e.g., markers 5-25, $\sigma = 0.00$; 5-22, $\sigma = 0.9$; and 5-181, $\sigma = 0.9$). Overall differentiation reached 0.77 [0.66; 0.89] (Table 5).

Host Diversity for RAPD and Phenotypic Resistance Markers

The diversity of the 68 plant sample was assessed for molecular markers using a RAPD procedure. Thirteen polymorphic markers were obtained, with frequencies of allele presence ranging from 0.07 to 0.88. Two populations were monomorphic and the others showed a low diversity index (Table 1). RAPD markers were heterogeneously distributed across populations with overall differentiation reaching 0.87 [0.76; 0.9] (Table 5).

Resistance or susceptibility of the plants was assessed against six pathogen strains isolated from cultivated beans. Ten multilocus resistance phenotypes were identified in the ten populations studied. These phenotypes showed between two and six resistance specificities against the six strains tested. These specificities were distributed heterogeneously across the populations and overall differentiation reached 0.62 [0.49; 0.74] (Table 6).

Table 2. Analysis of linkage disequilibrium between PRLJ1 LRR markers and resistance phenotypes with mapped genetic determinism

		Resistance phenotypes governed by genes mapped at the PRLJ1 locus									
		38	CRC531	E33C	E42B	M126	T134	CLM7[a]	80	3616[b]	100[c]
	Number of plants analyzed	14	17	17	16	14	16	25	33	33	33
	Proportion of plants showing a resistant phenotype	0.5	0.82	0.94	0.75	0.71	0.93	0.52	0.94	0.45	0.66
PRLJ1 LRR markers	5-15	0.35	0.23	0.23	0.26	0.09	0.22	1	0.49	0.46	0.25
	5-151	0.2	0.74	0.91	<0.01	0.94	0.87	0.03	0.15	0.14	0.46
	5-16	0.68	0.43	0.14	0.59	0.17	0.44	0.18	1	0.01	0.42
	5-181	0.7	0.75	0.91	0.7	0.78	0.89	1	1	0.03	0.27
	5-19	—	0.87	0.7	0.73	0.66	0.71	1	1	1	1
	5-20	0.79	0.75	0.48	0.06	0.5	0.62	0.64	1	1	0.04
	5-21	0.05	0.02	0.79	<0.01	0.07	1	1	0.10	1	0.04
	5-22	0.62	0.78	0.95	0.72	0.85	0.88	1	1	0.03	0.27
	5-25	0.67	0.58	0.06	0.35	0.67	0.67	0.19	1	1	0.01

P-values associated with linkage disequilibria are indicated here. Significant linkage disequilibrium was determined by a *p*-value inferior to 0.05 (underlined).
The number of wild plants analyzed for their inoculation reaction to each strain is indicated in the top line of the table together with the proportion of resistance reactions observed.
[a] A QTL explaining 80% of the resistance phenotype to CLM7 was mapped at the PRLJ1 RGC locus.
[b] The resistance phenotype to strain 3616 is governed by two genes, one of which was mapped at the PRLJ1 RGC locus, the other one mapping at another linkage group.
[c] The resistance phenotype to strain 100 is determined by one gene that does not belong to the PRLJ1 RGC locus, but instead to a distinct linkage group. The remaining seven resistance phenotypes are governed by genes mapping at the PRLJ1 locus.

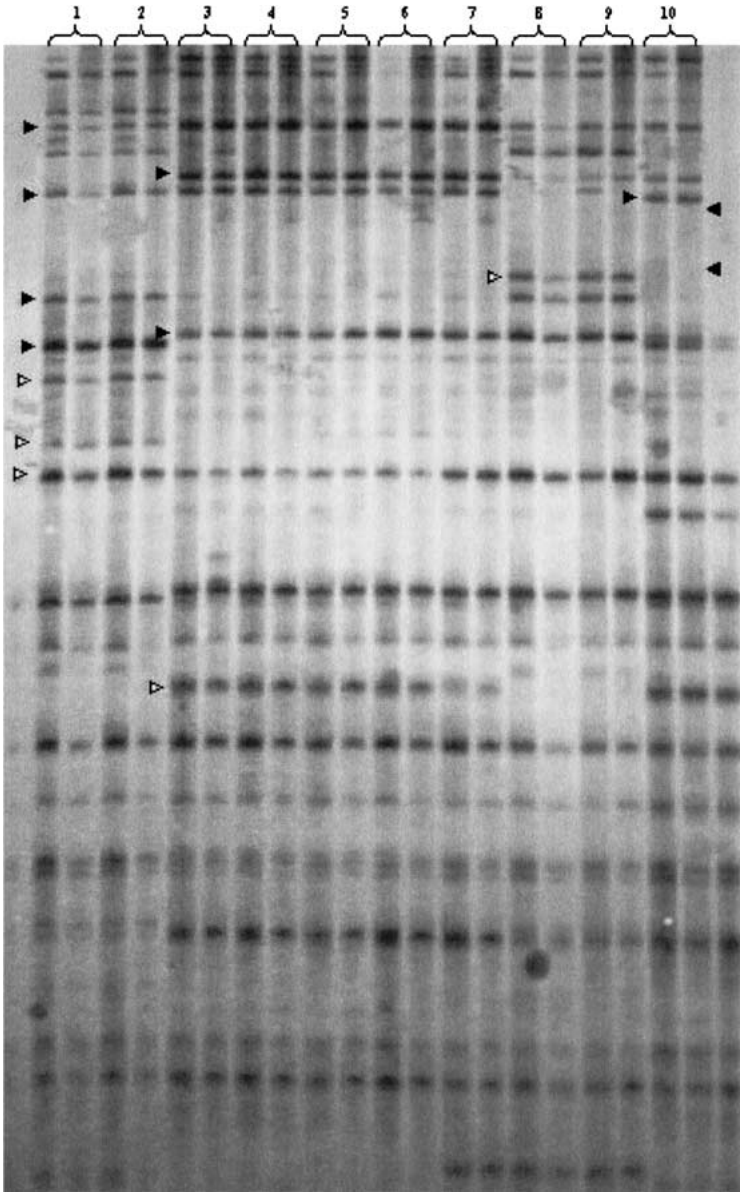

Fig. 2. Specific AFLP profiles generated from total genomic DNA of *Phaseolus vulgaris*. Lanes 1 and 2: individuals from population M7. Lanes 3 to 9: individuals from population M8. Lane 10: individual from population M3. Seven of nine PRLJ1 LRR markers assessed in this study are indicated with solid triangle. Two additional PRLJ1 LRR markers are not represented in these individuals. Their location is indicated by solid triangles on the right. Five polymorphic markers that have no homology with the PRLJ1 RGC family are indicated by open triangles.

Pathogen Diversity for RAPD and Phenotypic Resistance Markers

Pathogen strains isolated in the same wild bean populations, in 1994, have been previously characterized with RAPD markers and for virulence on a set of 12 differential cultivars, and pathogen populations were polymorphic for both markers (Sicard et al. 1997). Diversity was heterogeneously distributed and all the populations were differentiated with an overall differentiation reaching 0.43.

Uncorrelated levels of diversity

Polymorphism obtained for the LRR domain of the PRLJ1 RGC family was compared to both host and pathogen diversity. Considering intrapopulational levels of diversity, no significant correlation was detected between the PRLJ1 LRR markers and host diversity for resistance phenotypes or RAPD markers. Likewise, no significant correlation was detected between the PRLJ1 LRR markers and pathogen diversity given by RAPD or virulence phenotypes (Table 1, minimum $p = 0.08$). No significant correlation was detected between disease severity in each population and PRLJ1 LRR levels of diversity ($r = 0.07$, $p = 0.42$, $n = 10$; see Table 1).

Comparative Analysis of Host and Pathogen Population Structure

We compared overall differentiation levels and/or pairwise multilocus Φst matrices obtained separately for the PRLJ1 LRR markers and for the different

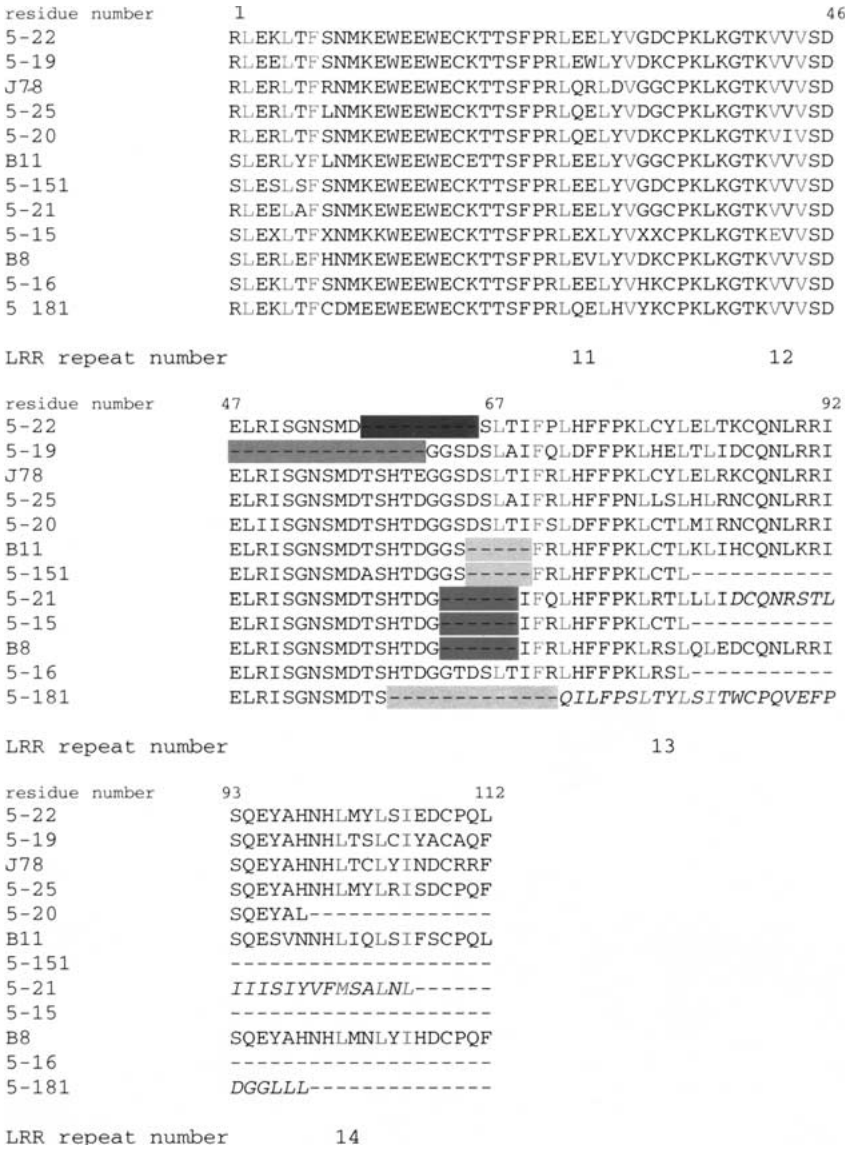

Fig. 3. Multiple alignment of amino acid sequences of the nine differently sized PRLJ1 LRR markers and three full-length PRLJ1 RGC (J78, B8, and B11) previously identified from cultivars. The different insertion/deletion events are indicated with different colors. LRR repeats were numbered according to the analysis of the full-length RGC and are indicated in red (Ferrier-Cana et al. 2003). Additional leucine-rich repeats have been identified whose consensus sequence has not been reported for the NBS-LRR class of resistance genes, but instead for the Cf class. These nonorthodox LRR repeats are indicated in green (not shown). Imperfect alignment zones are indicated in *italics* at the end of the sequences.

categories of markers used to characterize both host and pathogen diversity. No significant correlation was detected between host population structure for PRLJ1 LRR markers and pathogen population structure. Pairwise differentiation indices calculated for PRLJ1 LRR markers were not correlated with diversity in RAPD markers in *C. lindemuthianum* strains (Mantel test, $Z = 9.20$; $p = 0.6$; Table 7). Similarly, pairwise multilocus differentiation indices calculated for virulence were not correlated to differentiation indices calculated for PRLJ1 LRR markers (Mantel test, $Z = 8.23$; $p = 0.22$; Table 7).

Resistance phenotypes to the six pathogen strains and PRLJ1 LRR markers revealed correlated host population structures. Overall differentiation level for resistance phenotypes was not significantly different from differentiation for PRLJ1 RGC markers (Table 6). Analysis of pairwise multilocus differentiation detected a significant correlation between pairwise genetic distance calculated for resistance phenotypes and PRLJ1 LRR markers (Mantel test, $Z = 20.18$; $p = 0.0076$; Table 7).

Analysis of patterns of linkage disequilibria between resistance phenotypes and PRLJ1 LRR markers

In this section, we report the analysis of patterns of linkage disequilibria between PRLJ1 LRR markers and the ten resistance phenotypes whose underlying genetic determinism was mapped (Table 2). Nine resistance phenotypes were partly or fully determined by genes localized at the PRLJ1 locus. None of these phenotypes coincided with any of the presence/absence alleles of the PRLJ1 LRR markers, however statistically significant associations were observed. Five of nine PRLJ1 LRR markers were significantly linked to up to two of these PRLJ1 encoded phenotypes. Likewise, three of nine PRLJ1 encoded phenotypes were significantly linked with

Table 3. Presence/absence matrix obtained for PRLJ1 LRR markers on 68 individuals distributed in ten populations

	Population	M1	M2	M3	M4	M7
PRLJ1 LRR markers	5.25	**1**	**1**	**1**	**1**	**1**
	5.22	0	0	0	0	0
	5.21	0	0	**1**	0	0
	5.20	0	0	**1**	0	**1**
	5.19	0	0	0	**1**	0
	5.181	0	0	0	0	0
	5.16	0	0	0	**1**	**1**
	5.151	0	0	0	0	0
	5.15	0	0	**1**	0	**1**
Number of polymorphic markers		5	4	1	3	0
Number of haplotypes		4	4	2	2	1

	Population	M8	M6	M10	M12	M14
PRLJ1 LRR markers	5.25	**1**	**1**	0	**1**	0
	5.22	0	0	**1**	0	0
	5.21	**1**	**1**	0	0	0
	5.20	0	0	0	0	0
	5.19	0	0	0	0	0
	5.181	0	0	**1**	0	0
	5.16	**1**	0	**1**	0	0
	5.151	**1**	0	0	0	**1**
	5.15	0	0	**1**	0	0
Number of polymorphic markers		2	1	0	0	0
Number of haplotypes		2	2	1	1	1

The number of polymorphic markers obtained in each population is indicated as well as the number of haplotypes.

Table 4. Analysis of overall differentiation levels: differentiation index estimated with Weir and Cockerham θ for each locus over the ten bean populations

PRLJ1 LRR marker	θ	Significance ($p < 0.001$)
5.25	1	s
5.22	1	s
5.21	0.79	s
5.20	0.643	s
5.19	−0.005	ns
5.181	1	s
5.16	0.517	s
5.151	0.907	s
5.15	0.79	s

s: significant differentiation index.
ns: nonsignificant differentiation.

Table 5. Analysis of overall differentiation levels: over all Weir and Cockerman θ averaged over loci and associated confidence intervals estimated by bootstrapping over loci in the ten bean populations in the study

	θ over all loci	Confidence intervals
PRLJ1 LRR	0.77	[0.66;0.89]
RAPD	0.87	[0.76;0.9]

up to two PRLJ1 LRR markers (Table 2). In addition, the resistance phenotype to strain 3616, which is only partly influenced by a gene lying

Table 6. Analysis of overall differentiation levels: θ over all loci and associated confidence intervals calculated for PRLJ1 LRR markers, RAPD markers and resistance phenotypes over the seven populations that have been charecterized for resistance diversity at the phenotypic level

	θ over all loci	Confidence intervals
RRLJ1 LRR	0.78	[0.63;0.91]
RAPD	0.85	[0.71;0.95]
Resistance phenotypes	0.62	[0.49;0.74]

within the PRLJ1 RGC locus, was significantly linked to three PRLJ1 LRR markers (5-22, 5-181, and 5-16). However, four of nine PRLJ1 LRR markers showed no significant linkage disequilibrium with any of the PRLJ1 encoded resistance phenotypes. Likewise, five of nine PRLJ1 encoded resistance phenotypes, including the resistance phenotype to strain 80, which was assessed on a larger sample, showed no significant linkage disequilibrium with any of the PRLJ1 LRR markers.

The resistance phenotype to strain 100 is presumably not influenced by the PRLJ1 RGC locus, as its genetic determinism was mapped on a distinct linkage group (Geffroy et al. 1999). It shows significant linkage disequilibrium with three PRLJ1 LRR markers (5-20, 5-21, and 5-25).

Table 7. Results of Mantel test: genetic distances among pairs of populations, calculated for each category of markers in both host or pathogen populations, have been compared to genetic distance among pairs of populations obtained for the PRLJ1 LRR markers

		Pairwise population differentiation for PRLJ1 LRR markers		
		Number of populations compared	*Z*	*p*-value
Pathogen populations	Pairwise population differentiation for RAPD markers	10	9.20	0.6
	Pairwise population differentiation for virulence phenotypes	7	8.23	0.22
Host populations	Pairwise population differentiation for RAPD markers	7	54.5	0.10
	Pairwise population differentiation for resistance phenotypes	7	20.18	0.0076**

Z estimation and associated *p*-values are indicated.
**Significant correlation.

Table 8. Percentage of pairs of markers showing significant linkage disequilibrium among 68 plants; linkage disequilibrium was calculated among markers of the same class and tested by permutation

	PRLJ1		RAPD	
Number of pairs of polymorphic markers	36		66	
Number of pairs of markers significantly linked	13	36%	38	57%

Comparison of host population structure for PRLJ1 LRR and RAPD markers

In order to examine whether the host population structure observed for resistance at both molecular and phenotypic levels simply reflects genomewide evolutionary processes, the host population structure obtained for the PRLJ1 LRR markers was compared to host population structure obtained for RAPD markers. Overall θ for neutral markers (0.87) was not significantly different from that obtained for the PRLJ1 LRR markers (Table 5). However, the pairs of populations which were the most differentiated for PRLJ1 LRR markers were not the most differentiated for neutral markers, and vice versa (Mantel test, $Z = 54.5$; $p = 0.10$; Table 7). Similarly, the host population structure obtained for the resistance phenotypes was not correlated with that obtained for RAPD markers (data not shown). We tested the correlation obtained between ten pairs of subsamples of neutral markers in order to estimate the probability that pairwise differentiation levels among subsamples of neutral markers are correlated. All ten pairs of subsamples showed significantly correlated pairwise differentiation matrices (data not shown). The frequency distribution of the 9 PRLJ1 LRR and the 13 RAPD markers did not differ significantly (Kruskal-Wallis Rank Test, $H = 1.69$; $df = 1$; $p = 0.19$). By comparison with PRLJ1 LRR markers, an excess of marker pairs significantly linked was apparent among neutral markers (Chi2 = 4.29; ddl = 1; $p = 0.03$; Table 8).

Discussion

This study is, to our knowledge, the first attempt to compare locally occurring pathogen diversity with the spatial distribution of host molecular diversity at a complex R-locus. In addition, diversity was assessed at a putative primary target of selection by pathogens in the host (the LRR domain of the PRLJ1 RGC family in bean). The LRR domain indeed seems to be mainly involved in pathogen recognition (Dixon et al. 2000), although it is not the exclusive domain controlling this function and may also have other roles (Banerjee et al. 2001; Luck et al. 2000).

Results show that the markers used here to assess LRR diversity of the PRLJ1 RGC family in wild *Phaseolus vulgaris* populations may typify natural diversity existing at complex resistance loci. The comparison of population structures obtained for host populations and their co-occurring pathogen populations indicates that LRR diversity at the PRLJ1 RGC locus is not correlated to pathogen diversity. However, further comparison of host diversity for PRLJ1 LRR markers, RAPD markers and resistance phenotypes suggests that the antagonistic interaction between *P. vulgaris* and *C. lindemuthianum* has influenced the spatial distribution of LRR diversity.

The Specific AFLP Technique and the Assessment of a R-Gene Multigenic Family

To analyze specific polymorphism in R-gene LRR, we adapted the AFLP technique. This procedure requires that a primer can be defined that (i) allows amplifying a wide number of paralogs and (ii) is located near a highly variable domain that allows discrimination of each paralog. We designed such a

primer in the 5′ part of the LRR domain of four paralogs of the PRLJ1 RGC family. The majority of our polymorphic markers (9/14) were shown to belong to a subfamily of the PRLJ RGCs, as they aligned with three of four of the full-length PRLJ1 RGCs (J78, B8, B11).

It is possible that one single band corresponds to the amplification product of more than one paralog. However, because the *Mse*I restriction site is very frequent, and nucleotide polymorphism as well as insertion/deletion rates for the 5′ region of PRLJ1 LRRs are high, paralogs can be size discriminated effectively using our technique (see below). This assumption is supported by the fact that the four full-length RGCs would have yielded products that are distinct in length from the nine obtained from wild plants.

The patterns of diversity among LRR markers of the PRLJ1 RGC family of *P. vulgaris* are concordant with patterns of diversity identified at other complex R-loci harboring R-genes with known functions. Fragment length polymorphism is a result of restriction site polymorphism (i.e. point mutations) or insertion/deletion events. These events apparently affect repeats of the LRR differentially, with most deletion events having occurred within the same zone. Similar patterns of molecular diversity have been found for several other loci (reviewed in Bergelson et al. 2001; Young 2000). In addition, the LRR domains of the PRLJ1 family are variable both within and among populations in the field, as indicated by the presence/absence polymorphism of the nine differently sized PRLJ1 LRR markers. This furthermore corroborates results obtained for the RGC2 family in lettuce to a second complex R-locus (Sicard et al. 1999).

Divergence levels among PRLJ1 LRR markers were intermediate between those among RGC2 paralogs in lettuce and those among alleles at the L locus in flax (Bergelson et al. 2001; Meyers et al. 1998). Thus, it remains possible that some of the PRLJ1 LRR markers are alleles originating from the same paralog. Nonetheless, the analysis of intrapopulation polymorphism suggests that at least five of the nine markers correspond to polymorphism at distinct paralogs of the PRLJ1 RGC family. All individuals of population M3 were monomorphic for the presence of alleles at five markers (see Table 3). Given that this species reproduces through selfing, it is unlikely that all individuals in a population are heterozygous. As some of these putative paralogs showed no divergence at silent sites (data not shown), the analysis of divergence levels among markers did not allow us to draw more precise conclusions about the allelic relationships among markers. We thus assumed that each allele had originated from a distinct paralog of the PRLJ1 family. Allelic relationships are, however, likely to be extremely difficult to establish at complex R-loci because individuals varying in copy numbers have been shown to coexist within a single species (Noel et al. 1999; Van der Hoorn et al. 2001).

Impact of Currently Co-occurring Pathogen Populations

As a result of the dispersal of infected seeds, host and pathogen in the *P. vulgaris*/*C. lindemuthianum* system migrate together (Tu 1992). We did not observe the correlated patterns of diversity predicted by this shared life history. For diversity indices as well as for pairwise differentiation levels, no correlation was detected between PRLJ1 LRR diversity and either pathogen diversity for RAPD or virulence markers. Such an absence of correlation between the evolution of traits involved in a host/pathogen interaction has previously been observed for the interaction between flax and its rust, where host and pathogen migrate independently over fairly large distances (Burdon and Thrall 1999). This result was attributed to the impact of stochastic evolutionary processes. In the course of an epidemic, populations may decrease in size or become extinct, which as a consequence reduces the efficiency of natural selection as a whole and selection due to pathogens in particular (Frank 1992). Moreover, random migration in both host and pathogen can affect the diversity of the interacting populations following extinction (Burdon and Thrall 1999).

Nonetheless, the absence of correlation observed here between PRLJ1 LRR diversity in the host and diversity in the pathogen may be due to the fact that only one R-gene family has been studied and may not be representative of the whole pathogen recognition capacities of the host.

Comparison of Host Diversity for Resistance at the Molecular and Phenotypic Levels

Comparison of host diversity for resistance phenotypes to six strains of *C. lindemuthianum* and for PRLJ1 LRR markers reveals correlated population structures (Table 7). One reason for this could be that some of the resistance phenotypes are determined by the PRLJ1 locus. The correlation would then result from physical linkage. Although this hypothesis cannot be discarded, our results suggest that physical proximity among molecular or phenotypic markers located at the PRLJ1 locus has an unexpected limited influence. Presumably, PRLJ1 family members in wild bean plants map at the same unique chromosomal region than that identified on the progeny of two cultivars which originate from independent domestication events (Geffroy et al. 2000). Nonetheless,

the degree of linkage disequilibrium detected among PRLJ1 markers is lower than that detected among RAPD markers (Table 8). This result is not an artifact due to unequal frequencies among markers because RAPD and PRLJ1 LRR marker frequency distributions do not differ. The same was observed in another study involving South-Andean wild bean populations (de Meaux et al. 2003). This is in agreement with the limited amount of significant linkage disequilibrium that was detected between the PRLJ1 encoded resistance phenotypes and the PRLJ1 LRR markers (Table 2). The resistance phenotype to strain 3616, which is only partly a PRLJ1 encoded resistance phenotype, and resistance phenotype to strain 100, which maps elsewhere on the genome, appear to contribute equally to the correlation in population structure. Indeed, both resistance phenotypes are statistically linked to two distinct subsets of three PRLJ1 LRR markers. The resistance phenotypes to strains 80 and 3616 were also mapped at the PRLJ1 locus (Geffroy et al. 2000). But diversity for resistance phenotype 80 is low and consequently weakly responsible for host population differentiation patterns. The other three resistance phenotypes have not been mapped and there is no reason to predict that resistance genes located at the PRLJ1 RGC locus govern these three resistance phenotypes (Geffroy et al. 1999). Thus, the observed correlation between pairwise host population structures for resistance phenotypes and PRLJ1 LRR markers suggests that similar evolutionary processes have influenced the diversity of the LRR of PRLJ1 RGCs and the six resistance phenotypes assessed.

These evolutionary processes appear to affect specifically those loci that are related to the interaction. Although exactly the same individuals were assessed for RAPD and PRLJ1 LRR markers, a discrepancy was observed between host population structures. The RAPD markers seem to reliably mirror a neutral host population structure because subsamples of RAPD markers give correlated geographic structures. Indeed RAPD markers are frequently linked (Table 8). Thus, the discrepancy between population structures observed for PRLJ1 LRR and RAPD markers seems to result from differences in the evolutionary processes acting on these two categories of markers.

Conclusion

The fact that PRLJ1 LRR diversity is both correlated to phenotypic diversity for resistance in the host, and unrelated to host neutral diversity as revealed by RAPD markers, indicates that the interaction between *P. vulgaris* and *C. lindemuthianum* may have contributed to the observed spatial distribution of PRLJ1 LRR diversity. Nonetheless, our results do not detect an influence of contemporary pathogen populations.

We propose that the observed PRLJ1 LRR diversity could reflect historical rather than contemporary aspects of the host–pathogen interaction. PRLJ1 LRR diversity correlates with phenotypic diversity for resistance to strains which are not in contact with the wild populations studied here. A study of host resistance to strains from different origins has revealed that wild plants have a different genetic basis for resistance to wild versus cultivated, field-collected strains (Cattan-Toupance 1997; Geffroy et al. 1999). The phenotypic diversity for resistance assessed in this study could therefore mirror the ability of host plants to detect avirulence alleles that no longer exist in wild populations, although they have remained in cultivated fields. Thus, the discrepancy observed between PRLJ1 LRR and neutral markers could be a footprint of past selective episodes. Further characterization of host plant diversity for resistance to wild strains is necessary to confirm our hypothesis.

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