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DNA fingerprinting of *Peronospora parasitica*, a biotrophic fungal pathogen of crucifers

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Abstract The fungus *Peronospora parasitica* (Pers. ex Fr.) Fr. is an obligate biotroph infecting a wide range of host species in the family Cruciferae. Isolates from different hosts are morphologically similar, and pathotypes are usually distinguished on the basis of host range. Random Amplified Polymorphic DNA (RAPD) fingerprints were generated from a range of *P. parasitica* isolates from different *Brassica* species. Reaction conditions, in particular DNA template, primer and Mg^{2+} concentrations, were optimized to ensure that amplifications were reproducible. Possible artefacts arising through host plant DNA were assessed by including such DNA in control reactions. Confirmation that diagnostic RAPD bands were generated from fungal DNA was also obtained by Southern hybridization of a RAPD band to genomic fungal DNA. By screening 20 decamer primers, 2 were found to detect sufficient genetic variation to allow complete differentiation between pathotypes. These results illustrate the potential value of RAPDs for detecting polymorphisms between isolates of a non-culturable plant pathogenic fungus.

Key words *Peronospora parasitica* · Non-culturable pathogenic fungus · DNA fingerprinting · RAPDs · Genetic variation

Introduction

The biotrophic fungus *Peronospora parasitica* (Pers. ex Fr.) Fr. causes downy mildew disease of crucifers. It occurs worldwide and has an extensive host range (Channon 1981), which includes the cultivated *Brassica* species, ornamentals and wild crucifers such as *Arabidopsis thaliana* (Koch and Slusarenko 1990). Isolates originating from different hosts are morphologically similar (Yerkes and Shaw 1959) but vary in host range (Chang et al. 1964; McMeekin 1969; Dickinson and Greenhalgh 1977; Kluczewski and Lucas 1983; Lucas and Sherriff 1988). Different pathotypes can be distinguished on the basis of this host specificity (Sherriff and Lucas 1990), but such tests are time consuming and may not reveal the full extent of variation present. Other phenotypic markers such as sexual compatibility type (Sherriff and Lucas 1989) or fungicide sensitivity (Crute et al. 1985; Crute 1987) can be assessed, but they provide limited information for epidemiological studies or genetic analysis. There is a need, therefore, for alternative molecular markers (Michelmore and Hulbert 1987) to further define variation in the pathogen.

Randomly amplified polymorphic DNAs (RAPD) have been proposed as genetic markers that overcome many of the technical limitations of restriction fragment length polymorphism (RFLP) analysis (Williams et al. 1990; Welsh and McClelland 1990). RAPDs can be used in the construction of linkage maps (Williams et al. 1990; Reiter et al. 1992), in the identification of strains and varieties by genomic fingerprinting (Welsh and McClelland 1990; Goodwin and Annis 1991; Hu and Quiros 1991; Schafer and Wostemeyer 1992; Kresovich et al. 1992; Klein-Lankhorst et al. 1991; Koller et al. 1993; Stiles et al. 1993) and, following the cloning of amplified fragments, may also serve as conventional RFLP probes. In the study reported here, the potential use of RAPDs as a source of genetic markers in *P. parasitica* was evaluated. Several isolates from different host species were compared to determine whether reproducible banding patterns correlated with host specificity. As *P. parasitica* can only be cultured on living plant tissues, particular attention was paid to possible artefacts arising through contamination of sample DNA from plant or other microbial sources.

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Materials and methods

Fungal isolates

RAPD analyses were carried out using genomic DNA from 16 isolates of *Peronospora parasitica* (Pers. ex Fr.) Fr. (Table 1). These included 7 field isolates from the *B. napus* pathotype and 4 field isolates from the *B. oleracea* pathotype (Sherriff and Lucas 1990) collected in different years from various sites in the UK. Within each pathotype, isolates were chosen on the basis of differential virulence to specific host cultivars; the oospore-derived progeny P003 and P033 segregate for virulence to the oilseed rape (*Brassica napus* var '*oleifera*') cv 'Cresor' (Lucas et al. 1988), while P005 and P006 differ in virulence to the cauliflower (*Brassica oleracea* var 'cauliflora') cv 'Palermo Green' (Moss et al. 1991). Other variable characters were mating type and differences in sensitivity to the acylalanine fungicide metalaxyl.

DNA isolation

From fungal isolates

Genomic DNA was extracted from conidia of 4- to 5-day-old *P. parasitica* cultures maintained on host seedlings (*B. napus* cv 'Mikado', *B. napus* cv 'Cresor' or *B. oleracea* cv 'Offenham Compacta') in a growth room at a temperature of 15° ± 2 °C under a 14-h photoperiod at a light intensity of 77 $\mu Em^{-2}s^{-1}$. Conidia were dislodged from sporulating cotyledons by washing with sterile distilled water. The conidial suspension was centrifuged, and the conidial pellet washed at least three times in sterile distilled water. Clean conidia were then vortexed with a mixture of 1- and 6-mm diameter Ballotini glass beads (R.W. Jennings & Co, Nottingham) in a lysis buffer containing 100 m*M* TRIS- HCl (pH 7.2), 100 m*M* EDTA, 10% (w/v) SDS and 2% (v/v) 2-mercaptoethanol. DNA was recovered from the suspension of broken conidia using the protocol described by Lee and Taylor (1990). The measurement of DNA concentrations was done using a TKO 100 Dedicated Mini Fluorometer (Hoefer Scientific Instruments).

From host plants

Genomic DNA was extracted from cotyledons of uninfected 9- to 10-day-old seedlings of maintenance hosts using the protocol described by Edwards et al. (1991).

Polymerase chain reaction (PCR) materials

The 10-mers used as random primers in the PCR were purchased from Operon Technologies (Alameda, Calif.). Taq DNA polymerase, together with 10 × concentrated buffer, was supplied by Boehringer Mannheim (FRG). Amplifications were carried out in a Model 60 Tempcycler (Coy Lab Products, Ann Arbor Mich.) and in a PHC-3 Dri-Block Thermal Cycler (Techne, Cambridge). Agarose (Ultrapure) was supplied by Gibco, BRL.

Amplification conditions

The amplification conditions were rigorously tested in optimization experiments described in the Results. A standard procedure was determined based on the protocol of Williams et al. (1990). The PCR volume was 25 µl and contained 0.2 µ*M* of primer, 100 µ*M* each of dATP, dCTP, dGTP and dTTP (Pharmacia LKB Biotech), 25 ng template DNA and 0.5 U of Taq polymerase (Boehringer Mannheim) in 1 × PCR buffer (10 m*M* TRIS-HCl pH 8.3, 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.1 mg/ml gelatine). During manipulations, the tubes were kept on ice. The reaction mixtures were overlaid with 25 µl mineral oil. Standard amplifications were performed in a Coy Model 60 Tempcycler programmed for 45 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C or in a Techne PHC-3 Dri-Block Thermal Cycler programmed for 45 cycles of 30 s at 93 °C with ramp time of 30 °C/min, 40 s at 37 °C with a ramp time of 30 °C/min and 1 min 20 s at 72 °C with a ramp time of 20 °C/min. When programmed as above, both machines produced similar temperature profiles and amplification products. After the last cycle, the samples were kept at 72 °C for an additional 10 min and then cooled to 4 °C. Samples of 15 µl were analysed by electrophoresis in a 1.5% (w/v) agarose gel containing 0.2 µg/ml ethidium bromide with 0.5 × TBE as buffer.

Hybridization conditions

Selected amplification products obtained with template genomic DNA from isolate P003 were recovered from the gel and purified using Gene-Clean II (Bio 101 Inc, La Jolla, Calif.). Random primed DNA labelling (Boehringer Mannheim) was carried out using $dCTP^{32}$. These fragments were used as probes in hybridization experiments with genomic DNA digested with *Eco*RI and *Hin*dIII. Genomic DNA (0.1 µg) was digested with 2 U of restriction enzyme, separated by electrophoresis and transferred to GeneScreen Plus membrane (Dupont), according to the manufacturer's instructions. The filter was prehybridized for 4 h at 42 °C in a buffer containing 50% (v/v) formamide, 5 × Denhardt's solution (Denhardt 1966), 3 × SSPE, 0.5% (w/v) SDS. Hybridization was carried out overnight under the same conditions. The filter was washed once in 3 × SSC with 0.1% (w/v) SDS, followed by another wash in 2 × SSC containing 0.1% (w/v) SDS, each for 20 min at 65 °C. A final rinse in 2 × SSC was carried out at room temperature, and the filter was exposed to X-ray film (Fuji Medical).

Table 1 Isolates used for RAPD analysis

Isolate code	Source host	Year of isolation	Geographical origin	Mating type[a]	Response to metalaxyl[b]
P001	*B. napus*	1982	Leicestershire	Homothallic	S
P1072	*B. napus*	1992	Hertfordshire	Homothallic	nt
P1118	*B. napus*	1993	Leicestershire	Homothallic	nt
P1119	*B. napus*	1993	Leicestershire	Homothallic	nt
P1120	*B. napus*	1993	Nottinghamshire	Homothallic	nt
P1121	*B. napus*	1993	Essex	Homothallic	nt
P1122	*B. napus*	1993	Hertfordshire	Homothallic	nt
P003[c]	*B. napus*	1983	–	P1	S
P004[c]	*B. napus*	1983	–	P1	S
P033[c]	*B. napus*	1983	–	P2	S
P1100[c]	*B. napus*	1992	–	P2	nt
P1105[c]	*B. napus*	1992	–	P1	nt
P005	*B. oleracea*	1977	Tyne and Wear	P1	S
P006	*B. oleracea*	1983	Lincolnshire	P2	R
P1091	*B. oleracea*	1991	Lincolnshire	nt	nt
P1092	*B. oleracea*	1991	Lincolnshire	nt	nt

[a] Mating defined as in Sherriff and Lucas (1989)
[b] S, Sensitive; R, resistant; nt, not tested
[c] Single spore oospore progeny from selfs of isolate P001; P003 and P033 segregated for virulence to the oilseed rape (*B. napus* var *oleifera*) cv 'Cresor'

Results

Influence of different parameters on amplification

DNA concentration

To determine the optimal template concentration for amplification, genomic DNA ranging from 0.1 to 200 ng was added to the standard reaction mixture; the concentration range from 0.1 to 50 ng yielded reproducible patterns (Fig. 1). This consistency was particularly evident for strongly amplified bands. DNA concentrations greater than 100 ng produced non-specific amplification, which was visible as high-molecular-weight smears.

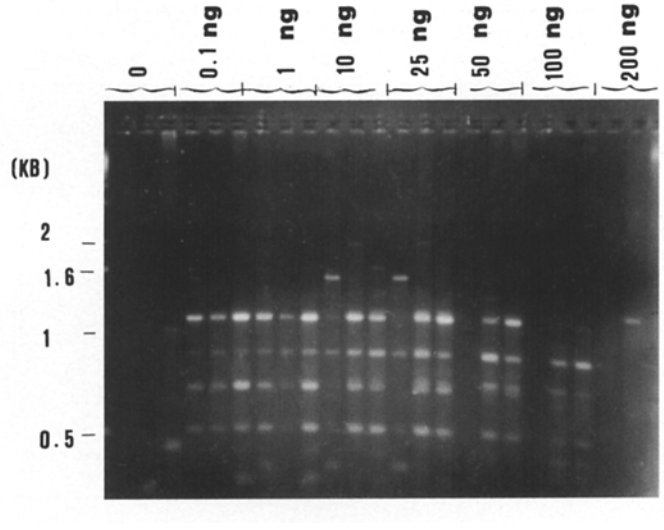
Mg^{2+} concentration

Mg^{2+} has been reported to influence the efficiency and fidelity of RAPDs (Innis and Gelfand 1990; Saiki 1990; Hosta and Flick 1992; Devos and Gale 1992). To examine the effects on RAPDs generated from *P. parasitica*, $MgCl_2$ was added to the reaction buffer to give final concentrations of 1.5 m*M*, 2.0 m*M* and 3.0 m*M*. Specific and reproducible results were obtained in the presence of both 1.5 m*M* and 2.0 m*M* $MgCl_2$ using primer A12 (Fig. 2). However, for some other primers, e.g. A2, which gave few bands, increasing the Mg^{2+} concentration resulted in the generation of a more complex banding pattern (data not shown).

Primer performance

All primers yielded amplification patterns using the standard reaction conditions; however the amounts of amplification product varied depending on primer concentration. For most primers, reproducible RAPDs were generated at a concentration of 0.2 μ*M* per 25 μl reaction (Fig. 3). Primer sequence altered the efficiency of amplifications; some primers gave poorly amplified bands, while others produced bands that were not consistently reproducible. Initial screening of a set of primers rapidly identified those that give reproducible RAPD patterns.

Fig. 1 Effect of template DNA concentration on generation of amplification products from genomic DNA of isolate P001 using primer A7. Reactions carried out in triplicate on separate occasions with different DNA samples of the same isolate



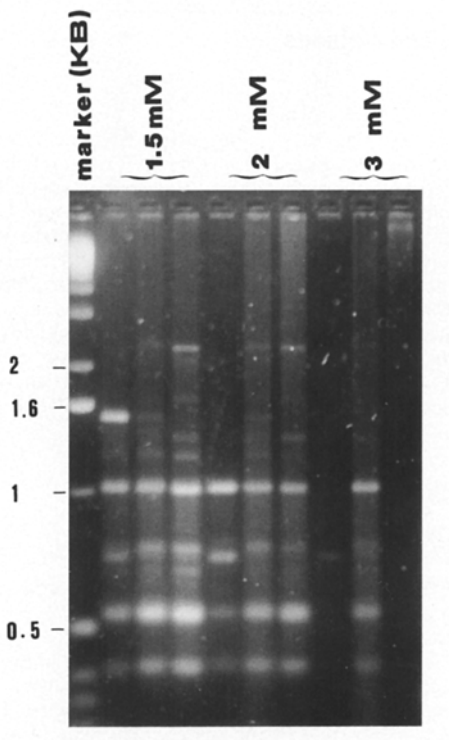


Fig. 2 Effect of Mg^{2+} concentration on generation of amplification products from genomic DNA of isolate P001 using primer A12. Reactions carried out in triplicate on separate occasions with different DNA samples of the same isolate

Origin of RAPD bands

To verify that the RAPD bands were generated from fungal DNA, genomic DNA from the maintenance host plant was isolated and amplified. Amplifications were also carried out with fungal DNA samples deliberately contaminated with host DNA. The results (Fig. 4) showed that RAPDs generated from host and fungal DNA were distinct, with the exception of one band that was common to all reactions. A novel band not present in either host or fungal RAPD fingerprints was observed in 'spiked' reactions, but all other bands were consistent with single sample patterns.

To further confirm that fungal DNA was amplified, DNA from a RAPD band, derived from isolate P003 using primer A19, was hybridized to a blot of genomic DNA from isolates P001, P003 and P033.

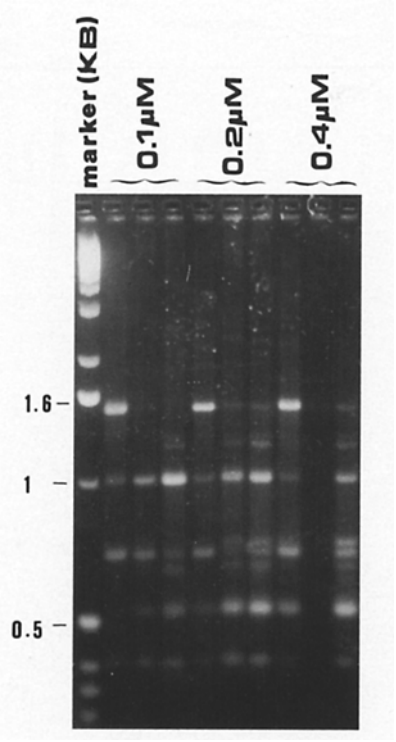


Fig. 3 Effect of primer concentration on generation of amplification products from genomic DNA of isolate P001 using primer A12. Reactions carried out in triplicate on separate occasions with different DNA samples of the same isolate

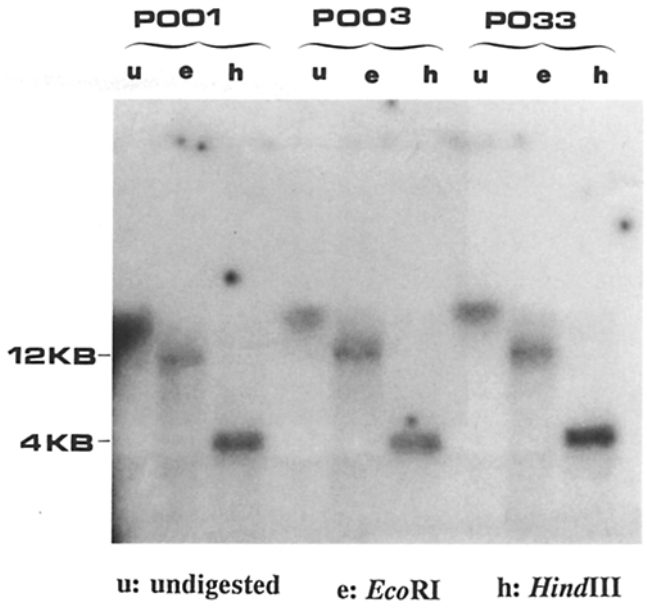
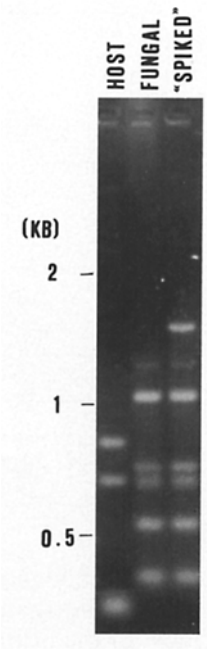


Fig. 5 Hybridization of a RAPD band ($B19^{(3)800}$) labelled with [^{32}P] to a blot of genomic DNA from isolates P001, P003 and P033 digested with restriction enzymes *Eco*RI and *Hin*dIII

Fig. 4 Comparison of RAPDs generated from host plant DNA, fungal DNA and fungal DNA contaminated with plant DNA. Primer A12 was used



The RAPD band hybridized to a single-copy sequence in all 3 fungal genomes (Fig. 5); no RFLPs were detected when the genomic DNAs were digested with *Eco*RI or *Hin*dIII.

Preliminary screening for RAPD markers

In an initial screening for polymorphisms between the *B. napus* and *B. oleracea* pathotypes 20 primers were tested. Amplifications were performed on three separate occasions with different DNA samples extracted from the same isolates. Of these primers 10% showed reproducible polymorphisms between the pathotypes and were capable of distinguishing between different isolates within a pathotype.

Amplification patterns were observed for all isolates using all primers, although variation was observed between sequential RAPD runs. Most bands were consistently amplified, while a few varied in their appearance. Only bands that appeared consistently in all three replicates were used to identify a set for each isolate in data analyses.

When the fingerprints of the 16 isolates analysed were compared, some bands were seen to be common to all isolates, while others were pathotype or isolate specific. Figures 6 and 7 show the DNA profiles of the different isolates amplified by 2 primers that differentiate between the *B. napus* and *B. oleracea* pathotypes. When the bands that appeared in all isolates were disregarded 3–4 RAPD markers remained from each of the 2 primers that can be used to clearly distinguish between *B. napus* and *B. oleracea* isolates. These primers also detected polymorphisms between different field isolates of the *B. oleracea* pathotype. This information is schematically represented in Figs. 8 and 9. Scoring for presence or absence of these markers results in a unique binary code for each isolate (Tables 2 and 3).

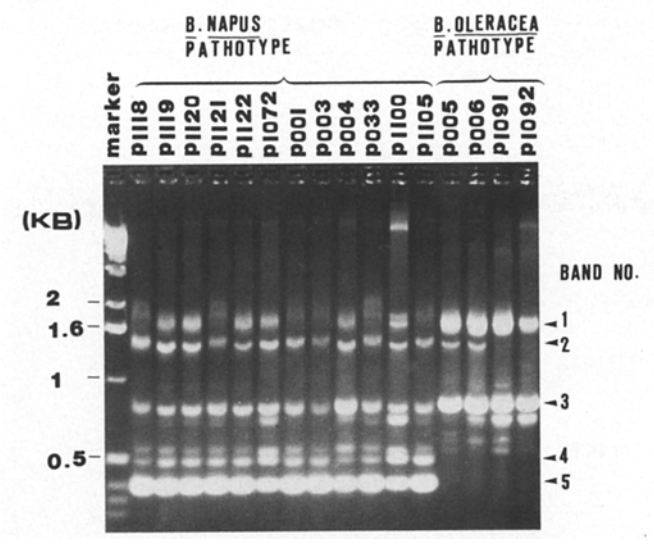


Fig. 6 RAPDs from 16 isolates of *Peronospora parasitica* using primer B12

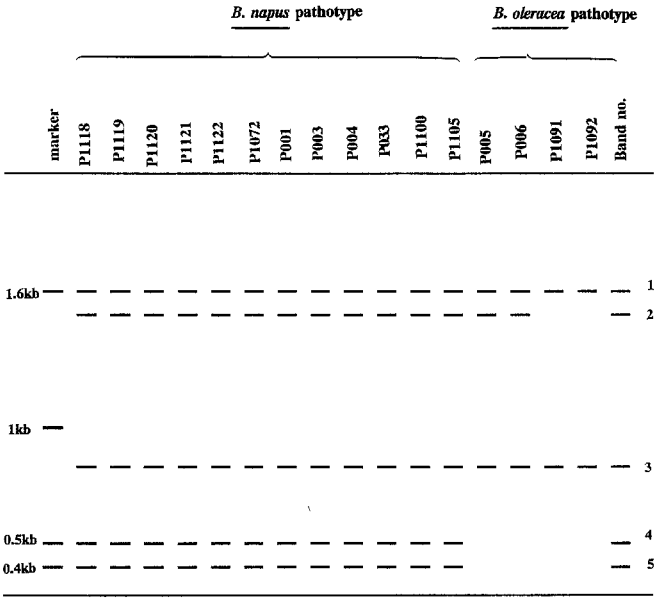


Fig. 8 Banding pattern with primer B12 created by selecting consistent bands from Fig. 6

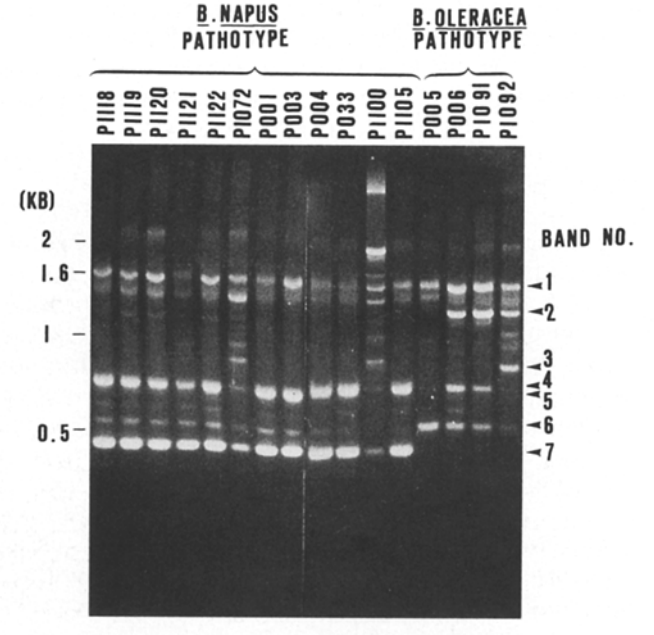


Fig. 7 RAPDs from 16 isolates of *Peronospora parasitica* using primer B17

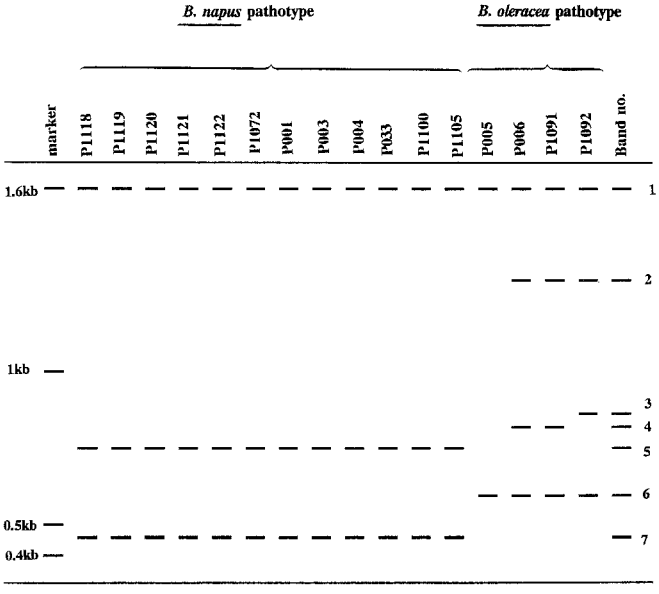


Fig. 9 Banding pattern with primer B17 created by selecting consistent bands from Fig. 7

Discussion

RAPDs generated from *P. parasitica* isolates were shown to be robust over a range of DNA template concentrations (0.1–50 ng); reproducible banding patterns could be obtained even with very low concentrations of DNA. This feature is particularly useful for genomic fingerprinting a biotrophic fungus such as *P. parasitica* where DNA is isolated from conidia and yields are relatively low compared to the quantities obtained from mycelial samples of culturable fungi. The smears of high-molecular-weight fragments obtained using more than 100 ng DNA may suggest that once most of the primers have been converted to amplification products, further priming probably involves the annealing of these products to genomic DNA or to themselves. Extension and random termination of these molecules may be the cause of the observed smear (Bell and DeMarini 1991).

The poorly amplified bands obtained with certain primers, as well as those which were not reproducible,

Table 2 System for differentiation of 16 *P. parasitica* isolates based on the presence (1) or absence (0) of chosen RAPD bands amplified using primer B12 (Fig. 8)

	Band number				
	1	2	3	4	5
P001	1	1	1	1	1
P1072	1	1	1	1	1
P1118	1	1	1	1	1
P1119	1	1	1	1	1
P1120	1	1	1	1	1
P1121	1	1	1	1	1
P1122	1	1	1	1	1
P003	1	1	1	1	1
P004	1	1	1	1	1
P033	1	1	1	1	1
P1100	1	1	1	1	1
P1105	1	1	1	1	1
P005[a]	1	1	1	0	0
P006[a]	1	1	1	0	0
P1091[a]	1	0	1	0	0
P1092[a]	1	0	1	0	0

[a] *B. oleracea* pathotype. Others are *B. napus* pathotype

Table 3 System for differentiation of 16 *P. parasitica* isolates based on the presence (1) or absence (0) of chosen RAPD bands amplified using primer B17 (Fig. 9)

	Band number						
	1	2	3	4	5	6	7
P001	1	0	0	0	1	0	1
P1072	1	0	0	0	1	0	1
P1118	1	0	0	0	1	0	1
P1119	1	0	0	0	1	0	1
P1120	1	0	0	0	1	0	1
P1121	1	0	0	0	1	0	1
P1122	1	0	0	0	1	0	1
P003	1	0	0	0	1	0	1
P004	1	0	0	0	1	0	1
P033	1	0	0	0	1	0	1
P1100	1	0	0	0	1	0	1
P1105	1	0	0	0	1	0	1
P005[a]	1	0	0	0	0	1	0
P006[a]	1	1	0	1	0	1	0
P1091[a]	1	1	0	1	0	1	0
P1092[a]	1	1	1	0	0	1	0

[a] *B. oleracea* pathotype. Others are *B. napus* pathotype

are probably due to a lack of suitable priming sites in the genomic DNA. It has been suggested (He et al. 1992) that these unstable bands may also result from the formation of heteroduplexes between amplified fragments or from non-specific amplification where primer/template homology is not perfect. The ability of 'poor performing' primers to generate more complex banding patterns when Mg^{2+} concentration was increased suggests that varying Mg^{2+} concentration stabilizes primer/template annealing in such instances.

The RAPD technique has been reported to be very sensitive to experimental variables (Devos and Gale 1992), and RAPD assay conditions described for one species may not necessarily be successful with another (Klein-Lankhorst et al. 1991). However, current results suggest that once optimum conditions for generating RAPDs are established for a species, specific fingerprints can be generated with different batches of DNA (Hunt and Page 1992; Koller et al. 1993; Caetano-Anolles et al. 1992; Schafer and Wostemeyer 1992). Reproducibility is most critical in the ultimate inclusion of bands for analyses. Amplification reactions with host plant DNA as a control confirmed that diagnostic bands were generated from the *P. parasitica* template. This was further verified by Southern hybridization of a labelled RAPD band to genomic fungal DNA. The probe hybridized to a single-copy sequence in the fungal genome. If RAPDs are to be used as genetic anchors for physical mapping or as starting points for chromosome walking, it is important to determine the genomic sequence complexity of the amplification site. Further polymorphic bands will be used in hybridization experiments with genomic DNA to determine the proportion of RAPD bands which may be useful as RFLP probes.

RAPDs have already been used to discriminate between isolates of culturable plant pathogenic fungi, such as *Leptosphaeria maculans* (Schafer and Wostemeyer 1992). In the current study, by screening only 20 decamer primers, 2 were found that detected sufficient genetic variation to permit complete differentiation between 2 *Brassica* pathotypes of *P. parasitica*. These results illustrate the potential of RAPDs for detecting polymorphisms between isolates of a non-culturable plant pathogenic fungus. The technique should prove of value to identify isolates from different crucifer hosts, including not only brassica crops but wild species such as *Arabidopsis thaliana* (Koch and Slusarenko 1990). This will facilitate analysis of the extent of variation within the pathogen population and also clarify relationships between pathotypes.

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