

Molecular markers in Australian isolates of *Rhizoctonia solani*

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Isolates of *Rhizoctonia solani* from different locations within Australia and Japan were analysed for restriction fragment length polymorphisms (RFLPs). These isolates belong to different anastomosis and pectic zymogram groups. Southern blots of restriction enzyme digested DNA were probed with a cloned 18S ribosomal RNA (rRNA) gene, or random cloned fragments of *R. solani* DNA. The patterns obtained with the rRNA probe revealed significant variation within some of the groups whereas other groups were more uniform. Group specific patterns could not be identified for all groups. There was less variation in the RFLP patterns when random cloned fragments of DNA were used as probes and group specific patterns could be identified for all groups.

Isolates of many species of plant pathogenic fungi show tremendous diversity in characteristics such as morphology and pathogenicity. A useful concept has been to assign these isolates to groups based on pathogenicity (pathotypes), or anastomosis behaviour (anastomosis groups).

In recent years the application of molecular biological techniques such as restriction fragment length polymorphism (RFLP) have made great advances in clarifying the taxonomic relationships of numerous species of fungi (Manicom *et al.*, 1987; Braithwaite, Irwin & Manners, 1990; Levy *et al.*, 1991). These studies have revealed a hitherto unsuspected level of variation in a number of species of phytopathogenic fungi, and have shed light on the mechanisms of variation in those species (Hulbert & Michelmore, 1987).

Rhizoctonia solani Kuhn is an important pathogen of cereal and legume crops in Australia. The incidence of disease due to this pathogen has been increasing in recent years, and there are no effective measures to control the spread of the pathogen (Anderson, 1982; McNish, 1986). Isolates show tremendous variation in morphological, and pathogenic characteristics. These isolates are divided into anastomosis groups (Ogoshi, 1987; Sneh, Burpee & Ogoshi, 1991). To some extent these groups correlate with pathogenicity, but there is still considerable variation within groups, and new groups are regularly identified (Sneh *et al.*, 1991). Due to the lack of stable markers in *R. solani* there is no information of the mechanisms by which this variation is generated. Lack of markers has also hindered studies on the population dynamics of the pathogen in the soil, and on the epidemiology of the pathogen. In this study we have explored the possibility of using DNA polymorphisms as genetic markers for *R. solani*. The results show that markers can be detected using either ribosomal RNA genes, or random cloned fragments to probe Southern blots of restriction digested *R. solani* DNA.

MATERIALS AND METHODS

Fungal strains

The names and characteristics of the isolates used in this study are outlined in Table 1. All of the isolates are multinucleate, and have as their teleomorph *Thanatephorus cucumeris* (Frank) Donk. The isolates are divided into pectic zymogram groups (ZG) on the basis of the pectic enzymes produced when grown on pectin (Sweetingham, Cruickshank & Wong, 1986; Neate, Cruickshank & Rovira, 1988; Cruickshank, 1990). These pectic zymogram groups correspond to anastomosis groups. The pathogenicity of the isolates is: ZG1, cereals and legumes; ZG2, cereals and legumes; ZG3, legumes; ZG4, legumes; ZG5, crucifers; ZG6, legumes; ZG7, potato; ZG8, soil saprotroph; AG1, cereals and legumes; AG5, soil; AG6 and 7, saprotroph (Sneh *et al.*, 1991).

Plasmids

To generate probes for RFLP analysis, DNA from the ZG3 isolate RI6 was digested with *Hind* III and the digest cloned into the bacterial plasmid pUC18. Plasmids were chosen at random from this library and used as probes. The ribosomal RNA clone pTA250.10 was supplied by R. Appels, CSIRO Division of Plant Industry, Canberra. This plasmid contains a 1 kb sequence from the 18S rRNA gene of wheat cloned into pBR322 (Appels & Dvorak, 1982). Plasmid DNA was prepared from cultures of *E. coli* strain JM83 by the alkaline-SDS method (Sambrook, Fritsch & Maniatis, 1990), and stored in TE buffer at -20°C .

Growth and isolation of DNA

For analysis of RFLPs mycelium was grown in Petri dishes

Table 1. Isolates of *Rhizoctonia solani* used in this study

	ZG	AG	Geographical origin*	Source
R163	1.1	8	WA	1
R146	1.1	8	WA	1
JW92	1.1	8	SA	3
R1232	1.1	8	WA	1
1475	1.1	8	SA	2
H155	1.1	8	SA	2
H140	1.2	8	SA	2
1453	1.3	8	SA	2
1440	1.3	8	SA	2
1188	1.3	8	SA	2
R829	2	8	WA	1
R132	2	8	WA	1
R881	2	8	WA	1
R138	2	8	WA	1
1517	2	8	SA	2
R880	2	8	WA	1
R16	3	ND	WA	1
R103	3	ND	WA	1
R1011	3	ND	WA	1
R1012	3	ND	WA	1
R1013	3	ND	WA	1
R917	3	ND	WA	1
R1026	3	ND	WA	1
R56	4	2.2	WA	1
R57	4	2.2	WA	1
R586	4	2.2	WA	1
R990	4	2.2	WA	1
R817	4	2.2	WA	1
R812	4	2.2	WA	1
BC108	4	2.2	SA	3
C96	4	2.2IIIB	Jap	3
R1-64	4	2-2IV	Jap	3
R75	5	2.1	WA	1
R120	5	2.1	WA	1
PS4	5	2.1	Jap	3
T9-1	5	2.1	SA	3
I321	5	2.1	SA	2
1519	5	2.1	SA	2
R98	5	2.1	WA	1
TasD	6	ND	Tas	2
NZT7	6	ND	NZ	2
P40	7	3	SA	2
TasA	7	3	Tas	2
TasC	7	3	Tas	2
STG	7	3	SA	3
1323	8	4	SA	3
SCR117	8	4	SA	3
AH-1	—	4HG-I	Jap	3
RH-165	8	4HG-II	Jap	3

* WA, Western Australia; SA, South Australia; Tas, Tasmania; Jap, Japan; NZ, New Zealand.

1, Isolates obtained from M. Sweetingham, Dept. of Agriculture, Perth; 2, isolates obtained from R. Cruickshank, University of Tasmania, Hobart, Tasmania; 3, isolates obtained from K. Sivasithamparam, University of Western Australia, Perth; ZG, pectic zymogram groups (Sweetingham *et al.*, 1986; Cruickshank, 1990); AG, anastomosis groups (Ogoshi, 1987); ND, not determined.

containing 20 ml of V8 medium for 6 d at 25° in the dark without shaking. The mycelium was then harvested, washed with ice cold TE buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA), lyophilized, and DNA isolated as described by Raeder & Broad (1985).

Construction of an *R. solani* DNA library

R16 mycelium was inoculated into 500 ml of GPY medium (1% glucose, 1% peptone, and 1% yeast extract) in a 2 l Ehrlenmeyer flask. The culture was incubated for 6–7 d at 25° and 120 rpm. The mycelium was harvested, and extracted for DNA as described above. The DNA was further purified by equilibrium centrifugation in a CsCl gradient (Sambrook *et al.*, 1990). The DNA was digested for 15 h with *Hind* III (5 units of activity μg^{-1} DNA). *Hind* III digested pUC18 was treated with calf intestinal phosphatase (1 unit of activity μg^{-1} DNA) at 37° for 30 min. Both DNA preparations were then extracted with phenol-chloroform, with chloroform, and ethanol precipitated. The pellet was washed with 70% ethanol, dried and dissolved in TE buffer. The *Hind* III digested *R. solani* DNA was ligated to pUC18 overnight at 15°, and the products of the reaction transformed into *E. coli* JM83 by the method of Hanahan (1983). Transformants were selected on LB agar containing 50 $\mu\text{g ml}^{-1}$ ampicillin, and X-gal (Sambrook *et al.*, 1990).

Southern blotting

R. solani DNA was digested with restriction enzyme (5 units of activity μg^{-1} DNA) for 15 h under conditions specified by the supplier. The digest was fractionated by electrophoresis through a 0.8% agarose gel in TAE buffer at 1 V cm^{-1} . The DNA was then denatured and blotted onto Zeta Probe (Bio-Rad) for 16 h using 1 M ammonium acetate 20 mM NaOH as the transfer buffer (Rigaud *et al.*, 1987). The membrane was baked at 80° for 2 h, and prehybridized for 1 h at 65°. Rapid hybridization buffer (Amersham) was used at 0.1 ml cm^{-2} for both prehybridization and hybridization. After prehybridization, heat denatured probe was added to the bag and hybridization carried out overnight at 65°. The membrane was rinsed briefly in 2 × SSC, 0.1% SDS at room temperature and at 65° for 30 min in the same solution. This was followed by two washes in 0.1 × SSC, 0.1% SDS for 30 min at 65°. The membrane was then wrapped in cellophane film and exposed to Kodak X-Omat AR X-ray film at –80°.

Labelled hybridization probes were prepared by nick translating 100 ng plasmid DNA with ^{32}P -dCTP. The nick translation reagents were obtained from Amersham, and used in accordance with their instructions. The reaction was terminated by adding EDTA to 20 mM. The probe was used directly after heat denaturation.

RESULTS

RFLPs in ribosomal RNA genes

A Southern blot prepared with *Eco*R I digested *R. solani* DNA was probed with a wheat rRNA gene. A representative sample of the data from this experiment is shown in Fig. 1, and the results obtained with a larger number of isolates is given in Table 2. A prominent 4.8 kb band was common to all of the groups, and was a consistent feature of the isolates within these groups. The exceptions to this were ZG3, and ZG5. All of the ZG3 isolates contain a 2.2 kb band which is unique to this group. However, only one isolate, R16, contains the 4.8 kb band in addition to the 2.2 kb band. Of the six ZG5

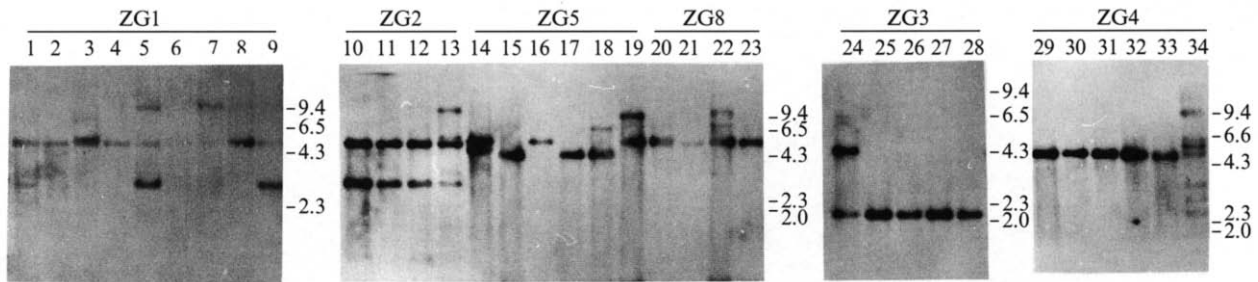


Fig. 1. RFLPs in the ribosomal RNA genes of *R. solani*. *EcoR* I digested DNA was electrophoresed on a 0.8% agarose gel, and blotted onto a nylon membrane. The membrane was probed with nick translated pTA250.10. Lanes: (1) R163; (2) R146; (3) R1232; (4) JW92; (5) 1475; (6) H155; (7) H140; (8) 1453; (9) 1440; (10) R829; (11) R132; (12) R138; (13) R880; (14) R75; (15) R120; (16) PS4; (17) T9-1; (18) 1321; (19) 1519; (20) 1323; (21) SCR117; (22) AH-1; (23) RH1-65; (24) R16; (25) R103; (26) R1011; (27) R1013; (28) R1012; (29) R56; (30) R586; (31) R813; (32) BC108; (33) C96; (34) R1-64.

Table 2. Size of *EcoR* I fragments homologous to pTA250.10

AG	8				8		ND		2.1		ND		3		4					
ZG	1				2		3		4		5		6		7		8			
Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	14
	—	—	9.7	9.7	—	9.7	—	—	—	9.7	—	—	—	—	9.7	—	—	9.7	—	9.7
	—	—	—	—	—	—	—	—	—	—	—	—	—	8	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—	6.3	—	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—	5.8	—	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—	—	5.6	—	—	—	5.6	—	—	—	—	—
	—	—	—	—	—	5.1	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—	5	—	—	—	—	—	—	—	—	—	—
	4.8	4.8	4.8	4.8	4.8	4.8	4.8	—	4.8	—	—	—	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8
	—	—	—	—	—	—	—	—	—	—	4.5	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—	—	—	4.4	—	4.4	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—	3.7	—	—	—	—	—	—	—	—	—	3.6
	—	—	—	—	—	—	—	—	—	2.9	—	—	—	—	—	—	—	—	—	—
	2.7	—	—	2.7	2.7	2.7	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—	—	2.6	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	2.2	2.2	—	—	—	—	—	—	—	—	—	—	—	—
	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
No. of isolates	2	4	2	1	4	1	1	5	9	1	1	2	1	1	1	2	1	2	3	1

Isolates: lane (1) R163, 1440; (2) R146, JW92, H155, 1232; (3) H140, 1453; (4) 1475; (5) R829, R132, R881*, R138; (6) R880; (7) R16; (8) R103, 41011, R1012, R1013, R1026*; (9) R56, R813, R57*, R586, R990*, R817*, R812*, BC108, C96; (10) R1-64; (11) R75; (12) R120, T9-1; (13) PS4; (14) 1321; (15) 1519; (16) TasD*, NZT7*; (17) P40*; (18) TasA*, TasC*; (19) 1323, SCR117, RH-165; (20) AH-1.

Data for these strains are not shown in Fig. 1. Fragment sizes are in kbp.

isolates tested, only two contain the 4.8 kb band (Fig. 1). Other bands observed in common among isolates from different groups included a 9.7, and a 0.6 kb band. All isolates tested contained the 0.6 kb band (Table 2). In contrast the 9.7 kb band was not a consistent feature within any one group. It was observed in some of the isolates from ZG1, 2, 4 and 5 (fig. 1).

Bands in addition to the 4.8 kb band were observed in a number of groups. These are unique to the groups they were observed in, but unlike the 4.8 kb band were not a consistent feature of the isolates from those groups. The ZG1 and ZG2 isolates contain a 2.8 kb band. All of the ZG2 isolates tested contain the 2.8 kb band, but its presence in the ZG1 isolates is variable. The ZG5 (AG2-1) isolates were highly polymorphic. Five different patterns were obtained for six isolates. On the basis of anastomosis behaviour this group is known to be highly polymorphic (Sneh *et al.*, 1991).

The variability in banding pattern corresponded more

closely with the classification of isolates into AG than with the ZG concept. Of the nine ZG4 isolates analysed (Table 2), eight gave an identical pattern with a single 4.8 kb band. The remaining isolate R1-64 gave a very different pattern from the other ZG4 isolates. This may be a reflection of the fact that R1-64 belongs to a different anastomosis subgroup than C96. From the RFLP patterns the Australian isolates belong to the same subgroup as C96. Similarly, the ZG8 isolate AH-1 which can be differentiated from the other ZG8 isolates by its RFLP pattern (Fig. 1, lane 22; Table 2), belongs to a different AG4 subgroup than the other AG4 isolates (Table 1). ZG1 and 2 isolates all belong to AG8, although these two ZG cannot be clearly distinguished with the rRNA probe since the characteristic 2.8, and 9.7 kb bands appear in both groups.

The results obtained with the rRNA probe show that this probe would be very useful for detecting variation within the group, and in clarifying relationships between clones from the same group, but less useful for the identification of groups.

Table 3. Differentiation of groups of *R. solani* by RFLP analysis with random cloned DNA fragments as probes

Probe	pRGL2-1					pRGL2-10					pRGL2-12					pRGL2-A1							
	7	8	2.2	2.1		8	8	2.2	2.1		8	8	2.2	2.1		8	8	2.2	2.1	3	4		
AG	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	7	8	
ZG	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	7	8	
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	> 20	—	—	—	
	—	—	—	—	—	—	—	—	13	—	—	—	—	—	—	11	11	—	—	—	—	—	
	10	—	—	—	—	—	—	—	11	—	—	—	—	—	—	—	—	—	—	—	—	—	
	8.2	8.2	—	—	8	—	—	—	—	—	—	—	—	—	—	—	8.1	—	—	—	—	8.2	
	—	—	—	—	—	—	—	—	7.5	—	—	—	—	—	—	—	—	—	—	—	—	—	
	—	—	—	7.1	—	—	—	—	7	—	—	—	—	—	—	—	—	—	—	—	—	—	
	—	—	—	—	—	—	—	—	—	—	—	—	—	6.5	—	—	—	—	—	—	—	—	
	—	—	6.1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	6	—	—	
	—	—	—	—	—	—	—	5.2	—	—	—	—	5.8	—	—	—	—	—	—	—	—	—	
	—	—	—	—	—	—	—	—	—	—	—	—	5.2	—	—	—	—	—	—	—	—	—	
	—	—	4.2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4.4	—	—	3.8	—	
	—	—	—	—	—	—	—	—	—	—	3.6	3.6	3.4	—	—	—	—	—	—	—	—	—	
	—	—	—	3.2	—	—	—	—	—	—	—	3	—	—	—	—	—	—	—	—	—	—	
	—	—	—	—	—	—	—	—	2.9	—	—	—	—	—	—	—	—	—	—	—	—	—	
	—	—	2.7	2.8	—	2.8	—	—	—	—	2.8	2.8	—	—	—	—	—	—	—	—	—	—	
	—	—	—	—	—	—	—	—	2.6	—	—	—	2.6	2.6	—	—	—	—	—	—	—	—	
	2.4	2.4	—	—	—	2.3	2.3	—	—	2.3	—	—	—	—	—	—	2.5	—	—	—	—	—	
	—	—	1.9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	—	—	1.8	1.7	—	—	—	—	1.7	—	—	—	—	—	—	—	—	1.8	—	—	1.8	—	
	—	—	1.5	—	—	—	—	—	—	—	1.4	—	1.5	—	—	—	—	—	—	—	—	—	
	—	—	1.2	—	—	1.1	1.1	—	—	—	—	—	1.2	—	—	—	—	—	—	—	—	—	
	—	—	—	—	—	—	—	0.9	—	—	—	—	—	—	—	—	—	0.8	—	—	—	—	
	—	—	—	—	—	—	—	0.7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	

Southern blots of *EcoR* I digested DNA were probed with nick translated random cloned fragments from isolate R16. Isolates: ZG1, R163; ZG2, R829; ZG3, R16; ZG4, R56; ZG5, R75; ZG7, STG; ZG8, SCR117. Fragment sizes are in kbp.

Table 4. Size of *EcoR* I fragments homologous to pRGL2-10

AG	8	8	2.2	2.1	3	4				
ZG	1	2	3	4	5	7	8			
Lane	1	2	3	4	5	6	7	8	9	10
	—	—	—	—	—	13	—	—	—	—
	—	—	—	—	—	11	—	—	11	—
	—	—	—	—	—	7	—	—	—	—
	—	—	—	5.2	5.2	—	—	—	—	—
	2.8	—	—	—	—	2.9	—	—	—	—
	—	—	—	—	—	—	—	2.7	—	—
	—	—	—	—	—	2.6	—	—	2.6	—
	—	—	—	—	—	—	—	—	—	2.5
	2.3	2.3	2.3	—	—	—	2.3	—	2.3	—
	—	—	—	—	—	—	—	—	—	2
	—	—	—	—	—	1.7	—	—	—	—
	—	—	—	—	—	—	—	—	—	1.5
	1.1	1.1	1.1	—	—	—	—	—	—	—
	—	—	—	0.9	—	—	—	—	—	—
	—	—	—	0.7	0.7	—	—	—	—	—
No. of isolates	1	5	6	1	5	4	3	1	1	1

Isolates: lane (1) R163; (2) R146, R1232, 1475*, H155*, H140*; (3) 1514*, 1517*, R829, R132, R881, R880; (4) R16; (5) R103, R1011, R1012, R101; (6) R56, R57, R586, R990; (7) R75*, R120*, R98*; (8) T9-1; (9) STG*; (10) SCR117.

* Data for these strains are not included in Fig. 2. Fragment sizes are in kbp.

RFLPs with random cloned fragments

To generate additional probes for RFLP analysis, *R. solani* DNA was digested to completion with *Hind* III, and the digest cloned into the plasmid pUC18 to generate a library. Clones

were selected from this library at random, and screened to determine the insert size. Clones with different sized inserts were assumed to be different and were used as probes in hybridization reactions.

Initially a number of restriction enzymes were tested to determine which one would be most useful in generating polymorphisms. Of the enzymes tested (*EcoR* I, *Hind* III, *Bam*HI, *Pst* I, and *EcoR* V), *EcoR* I generated the most polymorphisms (data not shown). *Hind* III and *EcoR* V were also very useful whilst *Pst* I, and *Bam*HI generated very few bands and thus were likely to be less useful in differentiating groups.

A number of randomly cloned fragments were tested for their ability to detect RFLPs in isolates from different groups. Southern blots of *EcoR* I digested DNA were probed with nick translated random cloned fragments of DNA from ZG3 isolate R16. Although the probes were derived from a ZG3 isolate, they hybridized to DNA from isolates in other groups (Table 3) except for the following two probes (pRGL2-6, and pRGL2-8) that were specific for ZG3 isolates. All of the probes were able to differentiate ZG3, ZG4, ZG5, and pRGL2-A1 was able to differentiate ZG8 from the other ZG (not tested for pRGL2-10). In each case the fragment pattern produced for the ZG3 isolate R16 was more complex than the pattern produced for the isolates from the other groups. This may be a reflection of the fact that the probes originated from R16. Only one out of the four probes (pRGL2-1) was able to differentiate the ZG1 and ZG2 isolates (Table 3). However, the ZG1 isolate used for this experiment was subsequently found to be different from the other ZG1 isolates (Table 3 & 4).

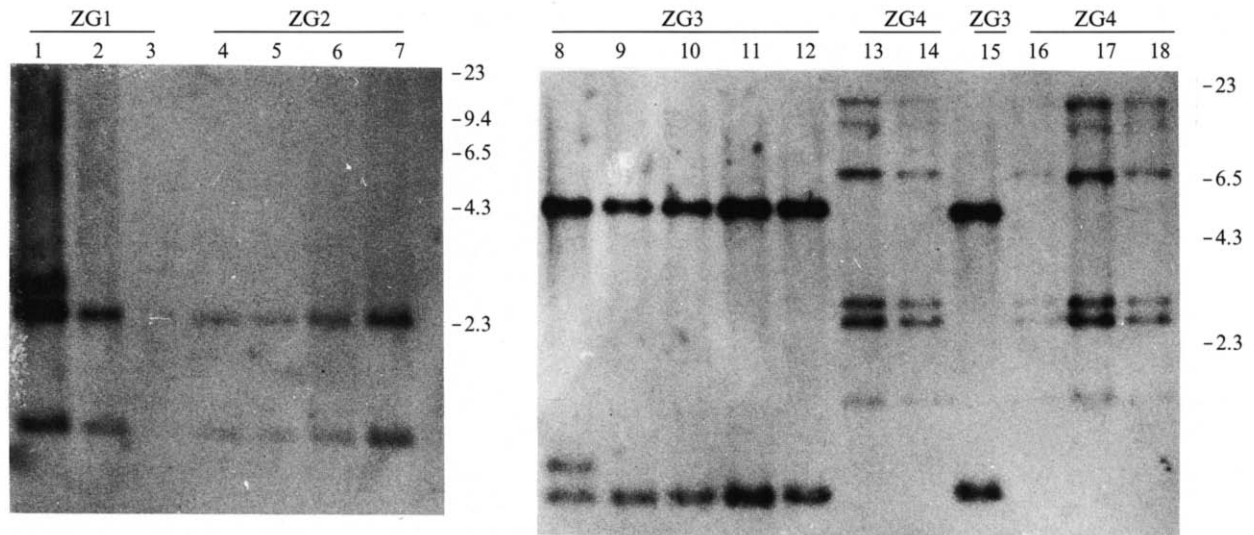


Fig. 2. Southern blot of *EcoRI* digested *R. solani* DNA probed with nick translated pRGL2-10. Lanes: (1) R163, (2) R146, (3) 1232; (4) R829, (5) R132, (6) R881, (7) R880; (8) R16, (9) R103, (10) R1011, (11) R1012, (12) R1013, (13) R56, (14) R57, (15) R917, (16) R817, (17) R817, (18) R812. Size of λ *Hind* III fragments are given in kbp.

Table 5. Size of *EcoRI* fragments homologous to pRGL2-A1

	AG	8	8	ND	2.2	2.1	3	4				
	ZG	1	2	3	4	5	7	8				
	Lane	1	2	3	4	5	6	7	8	9	10	11
		—	—	—	—	—	—	20	—	—	—	—
		11	11	11	—	—	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	8.2
		—	—	—	8.1	—	—	—	—	—	—	—
		—	—	—	—	—	—	—	6.6	—	—	—
		—	—	—	—	—	—	—	6	—	—	—
		—	—	—	—	5.8	5.8	—	—	—	—	—
		—	—	—	4.4	—	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	3.8	—
		—	—	—	—	3.2	3.2	—	—	—	—	—
		—	—	—	—	2.9	2.9	—	—	—	—	—
		2.5	—	2.5	—	—	—	—	—	—	—	—
		—	—	—	—	2.2	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	1.8	—
		—	—	—	1.8	1.8	—	—	—	—	—	—
		—	—	—	0.8	0.8	—	—	—	—	—	—
	No. of isolates	1	2	4	1	2	3	5	1	1	1	1

Isolates: lane (1) R16; (2) R146, R1232; (3) R829, R132, R881, R880; (4) R16; (5) R103, R1011; (6) R1013, R1012, R917; (7) R56, R57, R586, R817, R812; (8) R75; (9) R120; (10) STG; (11) SCR117. Fragment sizes are in kbp.

To assess the level of variation between isolates of the same group, a number of isolates from each group were analysed with the probes pRGL2-10 and pRGL2-A1. The results of these experiments are given in Tables 3, and 4, and a representative sample of the data is given in Fig. 2. For both of the probes the level of intragroup variation was less than observed with the rRNA probe.

Of twelve ZG1 + 2 (AG8) isolates tested with pRGL2-10, all were identical except for R163 which gave a different pattern from the other isolates with both probes (and also with the rRNA probe). Identical patterns were also obtained for these isolates with the pRGL2-A1 probe. No variation was observed between the ZG4 isolates with either probe. The ZG3 isolates could be distinguished by the presence of a 0.7

and a 5.2 kb bands in the pRGL2-10 patterns. The exception to this was the isolate R16 which in addition to these two bands, contained a 0.9 kb band. Probe pRGL2-A1 gave a more complex pattern with the ZG3 isolates than with isolates from other ZG. On the basis of the patterns obtained with this probe, the ZG3 isolates can be divided into three subgroups. One of these consists of R16. This probe also appeared to differentiate between groups 4, 5, 7 and 8 although the sample size for groups 5, 7 and 8 was very small.

There was also less variability in the ZG5 isolates. Identical patterns were obtained for three of the four isolates tested with pRGL2-10, Table 4). The number of ZG5 isolates tested with pRGL2-A1 was too small to draw any conclusions regarding intragroup variation, as was the number of ZG7 and

ZG8 isolates tested. Nevertheless, these isolates could be differentiated from each other with both of the probes (Tables 4 & 5). This differentiation was particularly clear with pRGL2-A1.

DISCUSSION

Ribosomal RNA genes are present in hundreds of copies per genome in fungi, and they contain sequences that are highly variable (Olsen *et al.*, 1986). Consequently they are very useful for analysing relationships between groups of organisms, and levels of variation within these groups. The RFLP patterns obtained for *R. solani* with a rRNA gene probe revealed the presence of group specific bands in some of the pectic zymogram groups. However, except for the presence of the 2.2 kb band in all of the ZG3 isolates, these bands were not a consistent feature of the groups, and therefore cannot be used to identify the groups. ZG5 showed a higher level of intragroup variability than any of the other groups. Jabaji-Hare *et al.* (1990), and Vilgalys & Gonzalez (1990) analysed RFLPs in North American isolates of *R. solani* and reported a high level of variation within AG2. Neither of these studies were able to differentiate between AG2-1 (ZG5) and AG2-2 (ZG4). In this study the ZG4 (AG2-2) isolates comprise a very homogeneous group except for isolate R1-64 which belongs to a different Ag subgroup (Table 1).

When random cloned fragments were used as probes the level of intragroup variation was less than that observed with the rRNA gene probe. With probe pRGL2-10, only two patterns were obtained for the five ZG5 isolates tested, compared with four patterns when the same isolates were analysed with the rRNA probe. Similar results were obtained with the ZG1 isolates, which showed a greater degree of complexity when the rRNA probe was used, compared with the random cloned fragments. This may be a consequence of the fact that the rRNA genes can undergo significant variation without affecting biological function (Olsen *et al.*, 1986). The rRNA genes are organized as clusters which may contain hundreds of copies of a basic repeat unit. Each unit contains the 23S, 5S and 18S rRNA genes with spacer regions between each gene. Also, between each unit there are nontranscribed spacer sequences. It is the spacer sequences both within, and between the units, as well as sequences within the genes which are highly variable. In contrast, other genes are more constrained with respect to variation in sequence. Only those changes which do not impair the biological function of the gene can be accepted. The nature of the sequences to which the pRGL probes hybridize is not known. These probes are very useful in that they reduce the level of variability in the pattern, thus allowing us to generate patterns that can be used to identify groups, that cannot be identified using the rRNA probe.

Group specific probes can be easily isolated from libraries of *R. solani* DNA. Two out of six clones chosen at random from our library were specific for ZG3 (data not shown). One of these pRGL2-8 gave a complex pattern indicating a repetitive sequence. This should be very useful in differentiating clones within the ZG3 population. Jabaji-Hare *et al.* (1990) reported that when they adopted a similar approach, all

of the cloned fragments were specific for a single group, and would not cross hybridize with isolates from other groups. Group specific probes can be of great benefit in identification of isolates and would be very useful in studies on population dynamics, and epidemiology of the pathogen.

Although the non rRNA probes gave RFLP patterns that were less variable than the rRNA probe, they also showed that there was variation within each of the groups. In some cases this variation is very marked, e.g. the ZG1 isolate R163, and the ZG3 isolate R16 can clearly be differentiated from the other members of the group. This differentiation is evident with all of the probes used. In other cases, the differentiation from the group is less marked and can only be detected with some tests, and not with others. R1232 can only be differentiated from the other ZG1 isolates with the rRNA probe. With pRGL2-A1 and pRGL2-10 as probes R1232 is the same as the other ZG1 isolates. Similarly, the patterns obtained with the probe pRGL2-A1 shows that R917 and R1012 are different from the rest of the ZG3 isolates (Fig. 3). These differences are not evident when pTA250.10 or pRGL2-10 are used as probes. This pattern of variability may reflect the degrees of divergence from the group. Isolates such as R1232, R917 and R1012 may be in the process of diverging from ZG1 and ZG3 respectively, whereas isolates such as R163 and R16, may be much further along the path.

The results of the RFLP analysis support the classification of isolates on the basis of anastomosis behaviour, but failed to distinguish all of the pectic zymogram groups; for example, isolates from ZG1 and 2 (all of which belong to AG8) could not be differentiated by RFLP patterns.

All of the ZG4 isolates show the same pattern with the three probes (Tables 2–4), except for the isolate R1-64. Ogoshi (1987) has subdivided a number of AG into smaller groups termed intraspecific groups (ISG) on the basis of anastomosis behaviour. R1-64 belongs to a different ISG than the other Japanese isolate C96 (Table 1). The data indicate that the WA and SA isolates belong to the same ISG as C96.

ZG4 and ZG5, appear to be quite different groups despite the fact that they belong to the same anastomosis group, AG2. However, they represent different subdivisions within AG2. ZG4 isolates belong to AG2.2, whilst ZG5 isolates belong to AG2.1 (Table 1). Unlike ZG4 isolates which are very homogenous in their RFLP patterns, ZG5 isolates are very heterogeneous except perhaps with probe pRGL2-10. Jabaji-Hare *et al.* (1990), and Vilgalys & Gonzalez (1990) who studied RFLPs in the rRNA genes of *R. solani*, also reported a high level of variation within AG2. However, neither of these studies were able to differentiate between AG2.2 (ZG4), and AG2.1 (ZG5). The difference between their results and the results reported here may stem from the fact that both of those studies used a more complex rRNA probe which contained the 18S, 5S and 23SrRNA genes. In addition Vilgalys & Gonzalez (1990) used two restriction enzymes simultaneously to digest the DNA. Both of these factors would be expected to increase the level of variation observed.

The results of this study demonstrate that random cloned fragments of *R. solani* DNA can be used to generate RFLP patterns that can be used to identify different anastomosis groups. The analysis also revealed significant variation in the

RFLP patterns between isolates of the same group. The variable bands within each of the groups may potentially be very useful in studying the genetic heterogeneity of populations of *R. solani*, and in studying factors which affect the survival and spread of the pathogen.

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