Repetitive DNA Polymorphism Analysis as a Tool for Identifying *Phytophthora* Species

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ABSTRACT

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A method has been developed for the rapid extraction of the total DNA of *Phytophthora*. Up to 100 μg of DNA, with a size greater than 23 kb, could be recovered from 0.25 g of frozen mycelium. The DNA was digested with a restriction enzyme, then subjected to electrophoresis in 1% agarose gels. After staining with ethidium bromide, the gels were examined under UV light. Repetitive DNA fragments appeared as numerous discrete bands over a faint smear. The method was applied to 39 isolates belonging to 12 species of *Phytophthora*. Isolates belonging to a single species exhibited the same digestion pattern, whereas different species had clearly different patterns. This held true when different restriction enzymes were used, except that minor intraspecific variations were occasionally detected with methylation-sensitive enzymes. The analysis of repetitive DNA profiles discriminated between the very similar species *P. cryptogea* and *P. drechsleri*. It also supported the heterogeneous status of *P. megasperma* by differentiating the soybean isolates. In contrast, complete homogeneity was observed among profiles from 12 isolates of *P. parasitica*, including eight isolates from tobacco. It is suggested that, because of its simplicity, the method might be useful, not only in investigating taxonomical problems, but also for identification purposes.

Additional keywords: restriction pattern, taxonomy.

A group of pathogenic fungi of prime importance, the genus *Phytophthora* has always been difficult to study. It is complex both for the taxonomist, who tries to delineate the species, and for the plant pathologist, who must identify isolates (8). The classification of the genus is based mainly on morphological features. However, some physiological characters, such as cardinal temperatures for growth in culture of host-specific pathogenicity, have been added (21). Moreover, in a few instances, the number and size of chromosomes have also been used for diagnosis. For identification purposes, several of these characteristics are difficult or time consuming to determine. Obtaining sexual or asexual forms may require cultivating the fungus under different conditions, and other criteria often involve statistical analysis of quantitative features, which demands tedious measurements of sometimes highly variable parameters. Recently, chemical procedures that used the patterns of total soluble proteins or of particular isozymes separated by gel electrophoresis or electrofocusing have been proposed for inter- or intraspecific discrimination (5,8). A shortcoming of these methods is that they rely on gene expression and, as such, can be affected by cultural conditions. The analysis of restriction fragment length polymorphism (RFLP), which is based on DNA structure, obviates this difficulty. It has been successfully applied to the taxonomy of many groups of eukaryotes, but has not yet been frequently used in the case of phytopathogenic fungi (15). As a rule, this technique involves digesting a total DNA preparation with a restriction enzyme and sorting the fragments according to their size by gel electrophoresis. After transfer onto a membrane, it is possible to localize a unique or newly repeated sequence by hybridization with an appropriate radioisotope-labeled probe. Applying this method, we have observed that, after digestion and electrophoresis, the total DNA of *Phytophthora* exhibits a high number of discrete bands (due to repetitive DNA) giving a distinctive pattern. We present here a comparative study of 12 species of *Phytophthora* that indicates that these patterns are characteristic at the species level. This could provide a relatively simple method for the identification of *Phytophthora* species. In addition, our results yield new information on some of the yet unresolved nomenclature problems evoked by Erwin (6).

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MATERIALS AND METHODS

Isolates. Twelve among the most important species were examined: *P. cactorum* (Leb. & Cohn) Schröeter (Waterhouse's group I, [20]), *P. palmivora* (Butler) Butler (Group II), *P. megakarya* Brasier & Griffin (Group II), *P. parasitica* Dastur, including *P. parasitica* var. *nicotianae* sensu Tucker (19) (Group II), *P. capsici* Leonian (Group II), *P. citrophthora* (Sm. & Sm.) Leonian (Group II), *P. infestans* (Mont.) de Bary (Group IV), *P. megasperma* Drechsler (Group V), *P. cambivora* (Petri) Buisman (Group VI), *P. cinnamomi* Rands (Group VI), *P. cryptogea* Pethybr. & Laff. (Group VI), and *P. drechsleri* Tucker (Group VI). Within each species, two or more isolates, selected for differences in host and/or geographical origin, were compared. Table 1 lists the 38 isolates used in this study.

Culture conditions. The mycelium was grown in a defined liquid glucose asparagine medium (GYP medium [9] without yeast extract and peptone), 100 ml per 250-ml flasks. Each flask was inoculated with five 4-mm-diameter plugs cut from the margin of a culture on 1% malt agar. These cultures were incubated in the dark at 24 C on a New Brunswick orbitary shaker (100 rpm) for 4–14 days, depending on the growth rates of the different isolates. Cultures were harvested by filtration on paper filter under vacuum, rinsed with 250 ml of distilled water, and stored frozen. Some isolates (nos. 195, 295, 293, P32, and P33) were cultivated on green pea agar plates covered with a cellophane film, which was peeled off to collect the mycelium.

DNA preparation. The frozen mycelium (250 mg) was ground in liquid nitrogen. The resulting powder was suspended in 0.5 ml of NIB buffer (100 mM NaCl, 30 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 10 mM β-mercaptoethanol), 0.5% (v/v) NP-40, and centrifuged at 12,000 *g* for 1 min. The pellet was treated once as above, then resuspended in 0.8 ml of homogenization buffer (0.1 M NaCl, 0.2 M sucrose, and 10 mM EDTA) before adding 0.2 ml of lysis buffer (0.25 M EDTA, 0.5 M Tris, pH 9.2, 2.5% sodium dodeyl sulfate). The homogenate was incubated for 30 min at 55 C, extracted twice with one volume of phenol-chloroform-isoamyl alcohol (50:48:2), and then twice with one

volume of ether. One volume of ethanol was added, and the DNA was immediately collected by centrifuging for 1 min in a microcentrifuge, at room temperature. Under these conditions, mainly high molecular weight DNA was recovered in the pellet, while small molecules remained in the supernatant. The pellet was washed with 70% ethanol, centrifuged again, and resuspended in 50 μl of TE (10 mM Tris, pH 8.0, 1 mM EDTA). The A_{260nm}/A_{280nm} ratio of these DNA preparations was between 1.7 and 2.0 and their A_{260nm}/A_{230nm} ratio between 1.6 and 2.0, suggesting that they were essentially free of protein and carbohydrate. All DNA preparations were kept at −20 C.

DNA digestion and electrophoresis. Approximately 5 μg of total DNA was digested overnight with 20 units of restriction enzyme (Boehringer, Mannheim), according to the manufacturer's recommendations. DNA fragments were separated on 1% agarose gels at 5 V/cm in 90 mM Tris borate buffer, pH 8.3. Ethidium bromide-stained gels were photographed under UV light. In some experiments, the DNA was digested for 2 hr under the same conditions without any difference in the electrophoretic pattern.

RESULTS

Preparations of *Phytophthora* DNA. The method presented here offers many advantages: it is quick and simple and yields relatively high amounts of DNA. Starting with 0.25 g (fresh weight) of frozen mycelium, it was possible, within 2 hr, to purify up to 100 μg of total DNA, with a size greater than 23 kb.

Species specificity of the electrophoretic patterns of repeated DNA. The total DNA of 38 isolates belonging to 12 species was digested with various restriction enzymes having hexanucleotide-recognition sites. In each case, the electrophoretic pattern contained discrete, brightly staining bands that were due to repetitive DNA. A typical experiment, employing enzyme *Sal*I, is shown in Figure 1. Different species always exhibited distinct patterns, although some bands might be common to several species (Fig. 1). In contrast, within any single species, all isolates showed identical profiles (Fig. 2), except for *P. megasperma* (see below). Repeating this experiment with various other restriction enzymes (*Pst*I, *Hin*dII, *Eco*R1, *Bam*H1) gave different patterns (not shown), which in each case were species specific.

Distinction between *P. cryptogea* and *P. drechsleri*. The value of repetitive DNA analysis as a criterion for discriminating between morphologically very similar species was tested by comparing *P. cryptogea* and *P. drechsleri*. Four isolates were used: two belonging to *P. drechsleri* (characterized by their normal growth at 35 C) and three ascribed to *P. cryptogea* (having the ability to rapidly colonize *Gerbera* leaf disks). As shown in Figure 3, the two species exhibited distinct patterns. It seems that *P. drechsleri* and *P. cryptogea* have more bands of repeated DNA in common than the other species that were compared. We doubt, however, that this method would be suitable for examining interspecific relatedness.

Diversity within *P. megasperma*. Our experiment included four cultures of *P. megasperma*: two soybean isolates from the United

TABLE 1. Origin of the isolates of *Phytophthora* examined in this study

Species	No.	Host	Country	Source and date of isolation
P. cactorum	177	*Malus communis*	France (Gironde)	*ex* Simone, 1975
	195	*Fragaria* sp.	France (Alpes Maritimes)	*ex* S. Mercier, 1978
P. palmivora	178	*Ficus carica*	Venezuela	*ex* R. Garnier
	292	*Theobroma cacao*	Togo (Kloto)	I.R.C.C.
P. megakarya	293	*Theobroma cacao*	Togo (Litimé)	I.R.C.C
	295	*Theobroma cacao*	Cameroon	*ex* M. Partiot
P. parasitica	26	*Dianthus caryophyllus*	France (Alpes Maritimes)	*ex* S. Mercier, 1971
	44	*Citrus* sp.	France (Corsica)	*ex* G. Augé, 1972
	51	*Dianthus caryophyllus*	France (Alpes Maritimes)	*ex* S. Mercier, 1972
	116	*Citrus*	Ivory Coast	*ex* E. Laville
	181	*Nicotiana tabacum*	U.S.A. (Kentucky)	*ex* C. C. Litton
	183	*Nicotiana tabacum*	U.S.A. (Kentucky)	*ex* C. C. Litton
	300	*Nicotiana tabacum*	U.S.A.	*ex* J. L. Apple
	301	*Nicotiana tabacum*	U.S.A.	*ex* J. L. Apple
	308	*Nicotiana tabacum*	Cuba	*ex* N. Peñalver
	309	*Nicotiana tabacum*	Cuba	ex N. Peñalver
	310	*Nicotiana tabacum*	Australia (North Queensland)	*ex* G. Johnston
	311	*Nicotiana tabacum*	Australia (North Queensland)	*ex* G. Johnston
P. capsici	65	*Cucumis melo*	France (Alpes Maritimes)	*ex* S. Mercier, 1973
	140	*Lycopersicon esculentum*	France (Vaucluse)	*ex* P. M. Molot, 1974
	147	*Capsicum annuum*	France (Vaucluse)	*ex* M. Clerjeau
P. citrophthora	218	*Arbutus unedo*	France (Var)	*ex* S. Mercier, 1981
	233	*Citrus clementina*	France (Corsica)	*ex* C. de Vallavieille, 1979
P. infestans	P32	*Solanum tuberosum*	The Netherlands	*ex* L. C. Davidse
	P33	*Solanum tuberosum*	The Netherlands	*ex* L. C. Davidse
P. megasperma	151	*Forsythia intermedia*	France (Eure)	*ex* I. Vegh, 1975
	203	*Daucus carota*	France (Manche)	*ex* Marzin, 1981
	210	*Glycine max*	U.S.A.	courtesy of N. T. Keen
	212	*Glycine max*	U.S.A.	courtesy of N. T. Keen
P. cambivora	143	*Castanea sativa*	France (Gironde)	*ex* Simone, 1975
	253	*Acer pseudoplatanus*	France (Jura)	*ex* C. Delatour, 1982
P. cinnamomi	122	*Chamaecyparis lawsoniana*	France (Loiret)	*ex* I. Vegh
	127	*Azalea* sp.	France (Hauts de Seine)	*ex* I. Vegh
	189	*Persea americana*	France (Corsica)	G.E.R.D.A.T
P. cryptogea	52	*Gerbera jamesonii*	France (Alpes Maritimes)	*ex* S. Mercier, 1972
	159	*Chamaecyparis lawsoniana*	France (Hauts de Seine)	*ex* I. Vegh, 1975
P. drechsleri	109	*Amygdalis communis*	Greece	*ex* H. Kouyeas, 1976
	165	*Chrysanthemum* sp.	Argentine	*ex* Van der Weyen

States (*P. megasperma* f. sp. *glycinea* sensu Kuan & Erwin) (13) and two French isolates from other hosts. After growth on pea broth and transfer to 0.1% KNO_3, each of these cultures produced numerous sex organs. Measurements of the size of 30 oogonia in each culture indicated that the soybean isolates nos. 210 and 212 (32 ± 4 μm and 33 ± 3 μm in diameter, respectively) belonged to *P. megasperma* var. *sojae* (20). Isolates nos. 151 and 203 (45 ± 4 μm and 41 ± 3 μm in diameter, respectively) corresponded to *P. megasperma* var. *megasperma*. Figure 3 shows that the two groups have markedly different repetitive DNA patterns.

Homogeneity within *P. parasitica*. Emphasis was given to use of repetitive DNA analysis for discriminating black shank isolates (*P. parasitica* var. *nicotianae* sensu Tucker [19]; eight cultures from several parts of the world) from other less specialized isolates of *P. parasitica* (four cultures from two different hosts). Figure 2 shows the result of one among many experiments performed with 6-base or 4-base pair recognizing restriction enzymes. It was not possible to differentiate among the patterns produced by the various isolates, irrespective of their host and geographical origin. Only the carnation isolate (no. 51) exhibited a slight difference from the typical profile of the species when its DNA was digested with *Msp*I. The same isolate showed an additional modification when *Msp*I was replaced by *Hpa*II, which has the same restriction site but is sensitive to the methylation of its target sequence (Fig. 4). Because this behavior was not found with the other cultures, including a second carnation isolate (not shown), it cannot be related to host specificity.

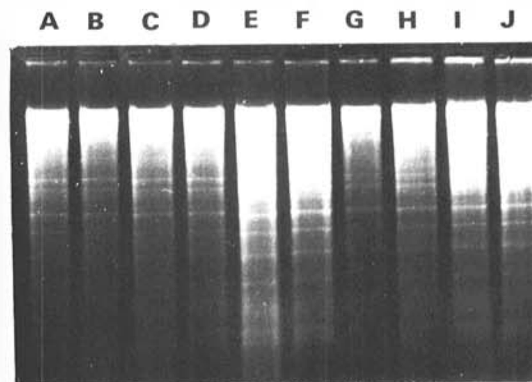

Fig. 2. Restriction patterns of DNA from 10 isolates of *Phytophthora parasitica*, digested with *Sal*I. **A to H,** tobacco isolates no. 309, 308, 301, 300, 183, 181, 311, 310. **I,** citrus isolate no. 44. **J,** carnation isolate no. 26.

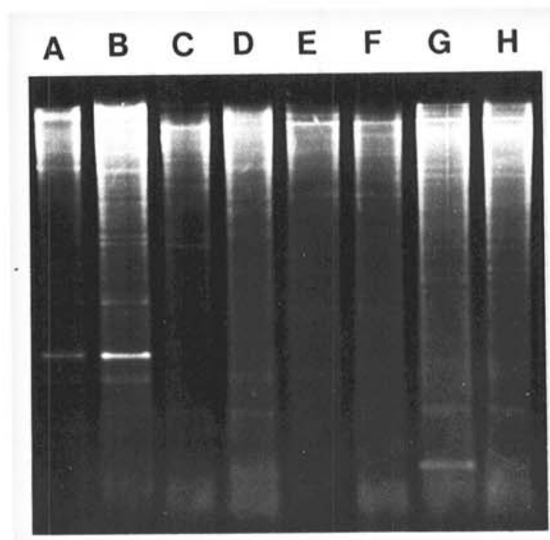

Fig. 3. Restriction patterns of DNA from three species of *Phytophthora*, digested with *Sal*I. **A and B,** *P. cryptogea* isolates no. 52 and 159. **C and D,** *P. drechsleri* isolates no. 109 and 165. **E and F,** *P. megasperma* f. sp. *glycinea* isolates no. 212 and 210; **G and H,** *P. megasperma* f. sp. *megasperma* isolates no. 151 and 203.

DISCUSSION

Most of the difficulties encountered in the taxonomy of *Phytophthora* come from the extreme variability within and among species, resulting in the lack of "good" taxonomic characters; it has been suggested that biochemical features would provide more reliable criteria (8). Location of a specific restriction site in repetitive DNA can be expected to be a stable character, as it will not be altered by random mutations in one or a few copies. The results we have obtained indicate that it is indeed a reliable criterion for classification and identification of *Phytophthora* species.

Recently, RFLP methodology has been applied to pathogenic fungi, in particular to study the taxonomy of fusaria (4,11,14). However, except for Coddington et al (4) who classified races of *Fusarium oxysporum* f. sp. *pisi* from ethidium bromide-stained electrophoretic patterns of total DNA, the authors had to develop radioisotope-labeled probes to detect RFLPs by hybridization.

The method we propose here has many practical advantages. Only a small amount of vegetative mycelium has to be produced, and there is no need for spore formation. As the method addresses

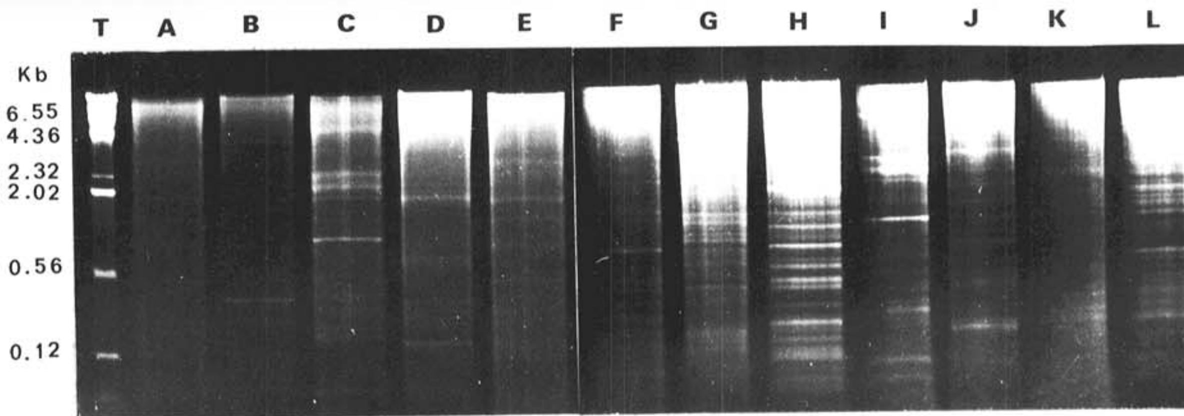

Fig. 1. Restriction digestion patterns of DNA from 11 species of *Phytophthora*. DNA was digested with *Sal*I as described in Materials and Methods. **T,** size markers (λ/*Hin*dIII). **A,** *Phytophthora parasitica* no. 26. **B,** *P. cryptogea* no. 52. **C,** *P. cinnamomi* no. 127. **D,** *P. capsici* no. 147. **E,** *P. parasitica* no. 183. **F,** *P. cactorum* no. 177. **G,** *P. cambivora* no. 253. **H,** *P. infestans* no. P32. **I,** *P. cinnamomi* no. 122. **J,** *P. palmivora* no. 292. **K,** *P. citrophthora* no. 218. **L,** *P. megakarya* no. 293.

DNA structure, uniform cultural conditions are not required; instead, each species can be grown in its own most suitable conditions. DNA extraction and analysis are technically simple, rapid, and reproducible; it is possible to prepare DNA, digest it with a restriction enzyme, and obtain its electrophoretic pattern in a single day. Contrary to the case of fusaria (14), *Phytophthora* total DNA digests give many visible discrete bands over a very faint smear, providing a significant means of determining difference or identity between patterns (Fig. 1).

These discrete bands were assumed to represent repetitive DNA sequences. In the case of some of the species here investigated, we can ascertain that most of this repetitive DNA is of nuclear origin. Indeed, Förster et al (7) have established restriction maps of the mitochondrial DNA from *P. parasitica* and from two forma specialis of *P. megasperma*; a previous work of Klimczak and Prell (12) allows to establish a similar restriction map for *P. infestans*. These different maps exhibit only one or two *Sal*I sites. Therefore the *Sal*I fragments of mitochondrial DNA cannot account for the complex DNA banding patterns of these species.

A major result of our study is that grouping of the 38 isolates according to identity in repetitive DNA pattern led to the same delineations as classical taxonomic procedures. Although we had selected the isolates to maximize potential variation due to host or geographical origin, isolates ascribed to the same species exhibited identical patterns, except for *P. megasperma*, which was split into two groups. This result indicates that the patterns of *Phytophthora* repetitive DNA are characteristic at the species level. It also largely supports the current taxonomic organization of the genus.

However, the characters currently used to separate some species appear rather elusive. For example, Tucker (19) differentiated *P. drechsleri* from *P. cryptogea* because of its more elongated sporangia and growth at 35 C. It has often proved difficult to ascribe isolates to one or the other species on this basis and, after Bumbieris (3), several authors suggested that they should be merged (6). However, Athanassiou et al (1) noted that *P. cryptogea* isolates were distinct from those of other species, including *P. drechsleri*, in their ability to rapidly colonize wound-inoculated surviving leaf disks of *Gerbera*, irrespective of their original host-plant. Our observations (Fig. 3) support the existence of two separate entities in the *P. cryptogea-P. drechsleri* group.

Analysis of repetitive DNA should also help to clarify nomenclature problems in *P. megasperma*. The great diversity of this species is recognized, but authors disagree on whether this diversity justifies the formation of subgroups (2). Waterhouse (20) distinguished *P. megasperma* var. *sojae* from *P. megasperma* var. *megasperma* by its smaller oogonia (<40 μm in diameter). This separation was later supported by the finding that representatives of the two varieties have different chromosome numbers (17). Kuan and Erwin (13) discarded this notion on the grounds that the range of oogonium size appears continuous when many isolates are compared. These authors introduced the forma specialis concept for isolates specifically pathogenic to soybean and alfalfa. On the basis of their repetitive DNA pattern, the four isolates of *P. megasperma* examined here can be split into two groups: the two isolates of the small oogonial type from soybean form one group and those of the large oogonial type from carrot and *Forsythia* form another (Fig. 4). Examination of a larger set of isolates would be needed to determine whether the difference in the electrophoretic pattern is related to host specialization, oogonium size, or geographical origin; nevertheless, our data certainly supports the view, expressed by Hansen et al (10), that *P. megasperma* includes more than one taxon.

P. parasitica (syn. *P. nicotianae*) is another species in which the separation into varieties, in the botanical sense, has created controversy and some ambiguity (6). Several authors have objected to this separation (5,18), and the black shank pathogen was found to be serologically identical to *P. parasitica* (16). We have carefully examined the repetitive DNA patterns of 12 isolates of *P. parasitica* from Europe, America, and Australia after digestion with several enzymes without detecting a difference (Fig. 2), except with some enzymes sensitive to the level of DNA methylation. In particular, the eight tobacco isolates could not be distinguished from the others. Also identical was isolate no. 44, in which isoelectric focusing of total proteins had revealed an additional "citrus" band (5). Repetitive DNA, as we have seen, is able to differentiate very similar taxa; therefore, we believe that pattern uniformity within *P. parasitica* provides a strong evidence that this species is homogeneous. In this case, the need for maintaining two varieties can be questioned and, should the black shank isolates be singled out, the terminology *P. parasitica* f. sp. *nicotianae* suggested by Erwin (6) would probably be the most appropriate.

New contributions to the taxonomy of *Phytophthora* can be expected when repetitive DNA analysis will be expanded to the other species. This method, however, has two limitations: it does not provide information at the subspecific level, contrary to what occurs in *Fusarium* (4), and it is not suitable for determining the degree of similarity between different species. The simplicity of the technique nevertheless allows the examination of the large number of isolates necessary to ascertain species homogeneity. For the same reason, the method can also be useful for those faced with complex pathological situations where several *Phytophthora* species are involved, as occurs on cocoa. In this respect, it is interesting to note that *P. palmivora, P. megakarya, P. citrophthora,* and *P. capsici* give clearly distinct patterns (Fig. 1). Besides being a taxonomic tool, repetitive DNA analysis might also serve in practice to identify new isolates by comparison with reference strains.

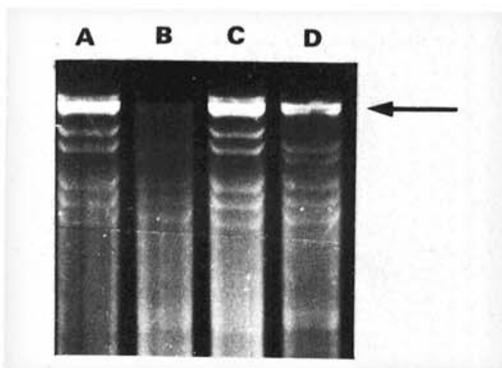

Fig. 4. Restriction patterns of DNA from two isolates of *P. parasitica*: no. 300 **(A,C)** and 51 **(B,D)**, digested with *Msp*I **(A,B)** and *Hpa*II **(C,D)**, respectively, insensitive and sensitive to the methylation of their target sequence. The arrow indicates the band present only after digestion with the methylation sensitive enzyme.

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