Comparison of Physical Maps of Ribosomal DNA Repeating Units in *Pythium*, *Phytophthora* and *Apodachlya*

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(Received 12 January 1987; revised 18 May 1987)

A satellite band in CsCl–bisbenzimidazole density gradients of *Pythium diclinum* DNA was found to consist of ribosomal DNA (rDNA) repeating units with a complexity of 10·3 kilobases (kb). Similar satellite bands were also obtained from a morphologically diverse selection of species from the Pythiaceae: *Pythium torulosum* (10·4 kb), *Pythium irregulare* (11·8 kb), *Pythium anandrum* (10·6 kb), *Pythium paddicum* (10·6 kb) and *Phytophthora cryptogea* (11·2 kb). Physical maps were constructed using seven endonuclease restriction enzymes and the maps were aligned on the basis of nine conserved restriction sites in a 6 kb region which was shown to be homologous to a DNA plasmid probe containing ribosomal genes from *Neurospora crassa*. A map of the rDNA region in *Apodachlya pyrifera* (10·1 kb), a species external to the Pythiaceae (Leptomitales), was also constructed to serve as a taxonomic reference. Percentage sequence divergence values indicated that *P. diclinum* and *P. torulosum* are closely related, but not identical. *P. anandrum* and *P. irregulare* also appeared closely related in spite of morphological differences. *P. paddicum* and *Phytophthora cryptogea* were the most outlying of the six species of Pythiaceae, but all six species formed a tight cluster when considered against *A. pyrifera*.

INTRODUCTION

Pythium and *Phytophthora* are two genera in the family Pythiaceae which contain economically important plant pathogens. About 100 species of *Pythium*, the largest genus of the family, are currently recognized, but they are difficult to identify because of considerable similarity in the morphology of their sexual reproductive structures (Dick *et al.*, 1984). The present study determines taxonomic affinities by comparing endonuclease restriction maps of ribosomal DNA (rDNA) sequences. This approach is feasible because *Pythium* and *Phytophthora* species have a prominent satellite band in CsCl–bisbenzimidazole gradients that consists of rDNA repeating units, as reported in this study.

The selection of a small group of *Pythium* species for comparison of rDNA restriction sites was based on sporangial morphology and sporangial renewal, traits which show the most striking differences within the genus. *Pythium diclinum* and *Pythium torulosum* both have a more or less filamentous sporangium, although that of *P. diclinum* is strictly filamentous though sometimes branched, while that of *P. torulosum* is contorted into distinctive toruloid swellings. *Pythium irregulare* seldom produces sporangia, but when they are formed they are small (about 1750 µm³), spherical, and terminal or intercalary. *Pythium anandrum* produces large (about 47 500 µm³) ellipsoid sporangia that sometimes show internal renewal, and has spiny oogonia. *Pythium paddicum* has sporangia of intermediate dimensions and has papillate oogonia. Thus, the five species can be regarded as spanning the range of sporangial diversity within the genus.

Abbreviation: rDNA, ribosomal DNA.

A well-known species of *Phytophthora*, *P. cryptogea*, was selected as an outgroup for the genus *Pythium* and *Apodachlya pyrifer* (Leptomitales) was selected as an outgroup for the Pythiaceae within the Pythiales.

METHODS

Species. *Pythium diclinum* (4110a), *Pythium anandrum* (4401d), *Pythium torulosum* (4212b), *Phytophthora cryptogea* (JM10) and *Apodachlya pyrifer* (502a) were provided by M. W. Dick from the Aquatic Phycomycete Culture Collection, University of Reading, UK; *Pythium irregulare* (174) was obtained from D. J. S. Barr (Biosystematics Research Institute, Ottawa, Canada); *Escherichia coli* C600SF8 carrying the plasmid pMF2 was a gift from Robert L. Metzenberg (Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin, USA). pMF2 was derived from pBR322 and contains a 6.5 kb insert from the *Neurospora crassa* rDNA repeat unit in the ampicillin resistance gene. The 17S, 5.8S and 26S rRNA coding regions are fully represented in pMF2 (Free *et al.*, 1979).

Growth of cultures. From stock axenic cultures (Clark & Dick, 1974; Dick, 1965) an agar plug was transferred to a Petri plate containing V8 broth (2.5%, v/v, V8 juice), and allowed to grow at room temperature into a thick mycelial mat. The mat was transferred to 500 ml V8 broth in a 1 litre flask, shaken vigorously and the contents dispensed into Petri plates. Cultures were allowed to grow into thick mats. Six mats were used to inoculate 2 l baffle flasks containing 500 ml PYG medium (Griffin *et al.*, 1974) which were shaken vigorously at room temperature for 3–5 d. Mycelium was harvested by vacuum filtration through Whatman no. 1 filter paper. The mycelial mat was rinsed thoroughly with distilled water and then with isolation medium (0.35 M-sucrose, 50 mM-EDTA, 10 mM-Tris/HCl buffer, pH 7.4). The mycelium was stored at -40°C when not used immediately.

Isolation of DNA. Frozen or fresh mycelial mats were broken into small pieces with a mortar and pestle and then blended in liquid nitrogen. Generally 20–40 g of frozen mycelium were ground for a typical isolation. DNA was prepared by phenol extraction and CsCl-bisbenzimidazole density centrifugation as described by Garber & Yoder (1983).

DNA digestion and electrophoresis. Restriction enzymes were obtained from Boehringer Mannheim or BRL and used according to manufacturers' instructions. Electrophoresis was done in TBE buffer (0.089 M-Tris, 0.089 M-boric acid, 2.5 mM-EDTA) using 0.8%, 1.0%, or 1.2% (w/v) agarose submarine gels. The BRL 1 kb ladder was used as a standard for determining fragment size. Gels were stained for 20 min in 0.5 µg ethidium bromide ml⁻¹ and viewed under UV light.

Hybridizations. pMF2 plasmid DNA was prepared from *E. coli* C600SF8 (Free *et al.*, 1979) using the preparative method of Birnboim & Doly (1979). Plasmid DNA was nick-translated according to the method of Rigby *et al.* (1977). Southern blots were prepared using Gene Screen Plus (New England Nuclear) according to the manufacturer's instructions. Prehybridization was done at 55 °C by agitating the membrane for 6 h in prehybridization fluid (1% SDS, 1 M-sodium chloride). The probe was denatured, added to the prehybridization fluid and incubated at 55 °C with constant agitation for 16–24 h. After hybridization the filters were washed twice in 2 × SSC (SSC: 0.15 M-sodium chloride, 0.015 M-sodium citrate, pH 7.0) at room temperature for 10 min each, then twice in 2 × SSC and 1% SDS at 55 °C with constant agitation for 30 min. Autoradiography was done for 24–48 h at -70°C .

Restriction mapping of the rDNA repeat unit. Mapping of DNA was done by analysis of single and double restriction enzyme digestions of DNA preparations containing the rDNA repeating unit. For *Phytophthora cryptogea*, which did not produce a well-resolved middle band on the CsCl density gradient, the chromosomal band, which was highly enriched in rDNA, was used as the source of rDNA. To map the *A. pyrifer* rDNA unit, bands were visualized by hybridization to the *N. crassa* rDNA probe pMF2, since restriction of chromosomal DNA did not yield clearly visible bands after ethidium bromide staining.

RESULTS AND DISCUSSION

rDNA satellite bands

When DNA from *Pythium diclinum* was isolated on a CsCl-bisbenzimidazole density gradient, three bands were visible under UV light (Fig. 1a). The top and bottom bands fluoresced blue while the middle band fluoresced green. This separation and colour pattern has been observed for all species of *Pythium* studied to date. The same band pattern was found with *Phytophthora cryptogea* DNA but with much less resolution of the middle and bottom bands. *Apodachlya pyrifer* DNA produced two sharp blue bands, well resolved from each other.

The width and intensity of each band on the gradient varied between different species and even between different preparations of the same species. In general the middle band was the broadest and most intense, but the relative amount of DNA recovered from each band could not

Fig. 1. Identification of rDNA in *P. diclinum*. (a) CsCl-bisbenzimidazole density gradient of *P. diclinum* DNA. (b) *Hind*III digestion of DNA from bands shown in (a). Lane 1, top band; lane 2, middle band; lane 3, bottom band. (c) Hybridization of pMF2 to the profiles in (b). Size estimations were based on the BRL 1 kb ladder.

be predicted from these appearances. The colour differences and lack of linearity between fluorescence and DNA concentration in bands may be due to spectral shifts occurring when bisbenzimidazole binds to DNA molecules of varying AT composition (Muller & Gautier, 1975).

DNA recovered from the three bands shown in Fig. 1(a) was digested with *Hind*III and the fragments separated by agarose gel electrophoresis (Fig. 1b). DNA from the top band produced a pattern typical of mitochondrial DNA (G. R. Klassen, unpublished results). DNA from the bottom band appeared to be of high complexity, producing no discernible restriction bands, and thus was presumed to be chromosomal DNA. Most of the DNA in the middle band was digested to form bands of 5.7 and 4.6 kb, indicating low sequence complexity.

Similar satellite bands have been described for other fungi and have in several cases been found to consist of rDNA repeating units (Garber & Yoder, 1983; Raeder & Broda, 1984; Specht *et al.*, 1984). To determine whether this was the case for *Pythium diclinum*, the gel

Fig. 2. Restriction maps of the rDNA repeating units from seven oomycetes. The twenty sites compared are listed in sequence on the top bar. Map regions hybridizing strongly to pMF2 are indicated by the solid parts of the bar; regions with weak or no hybridization are shown by the open part of the bar. The restriction sites are indicated by the following symbols: □, *EcoRV*; ○, *ClaI*; △, *EcoRI*; ◻, *PstI*; ●, *HindIII*; ▲, *BglII*; ▽, *BamHI*. The presence of these restriction sites and the relative lengths of the repeating units are shown below for each species. The 6 kb section analysed further in Figs 3 and 4 is that to the right of the diagram between *EcoRV*-1 and *PstI*-6, both inclusive.

(Fig. 1*b*) was blotted and the DNA probed with pMF2. The results (Fig. 1*c*) indicated that nearly all of the DNA of *P. diclinum* homologous to *N. crassa* rDNA was located in the 5.7 and 4.6 kb *HindIII* fragments derived from the middle CsCl band. Faint hybridization to the bottom band DNA at 5.7 and 4.6 kb indicated either that the bottom band was contaminated with middle band DNA or that a small fraction of the rDNA remained associated with chromosomal DNA. The middle band in *Pythium* species is far more prominent than corresponding bands from other fungi (Garber & Yoder, 1983).

Middle band DNA from *P. diclinum* was digested with six more restriction enzymes and in each case the size of the repeating unit of the DNA was found to be 10.3 kb. A restriction map of the sequence (Fig. 2) indicates that discrete termini do not exist, and thus that the molecule is either circular or consists of a number of tandem repeating segments. The DNA was partially digested with *BglII*, which cuts the sequence only once, and a ladder of approximately 10, 20 and 30 kb bands was discernible on a 0.4% agarose gel (data not shown). This indicates that at least three tandem repeats exist, either in a linear array or as a circular molecule.

Gels containing the DNA fragments from *Pythium diclinum* that had been used for the construction of the map of middle band DNA were blotted and probed with pMF2 in order to

		Number of sites shared by both species, with percentage divergence									
		<i>Pythium paddicum</i>	<i>Pythium irregulare</i>	<i>Pythium anandrum</i>	<i>Pythium diclinum</i>	<i>Pythium torulosum</i>	<i>Phytophthora cryptogea</i>	<i>Apodachlya pyrifera</i>			
Number of sites present for comparison in one or another of the species pair	<i>Pythium paddicum</i>		12 4.8	11 6.2	11 6.2	11 6.2	9 8.5	7	15.7		
	<i>Pythium irregulare</i>	16		13 1.2	11 6.2	11 6.2	10 5.6	8	12.6		
	<i>Pythium anandrum</i>	16	14		11 5.2	11 5.2	10 4.4	8	11.6		
	<i>Pythium diclinum</i>	16	16	15		13 0	10 4.4	7	14.8		
	<i>Pythium torulosum</i>	16	16	15	13		10 4.4	7	14.8		
	<i>Phytophthora cryptogea</i>	15	14	13	13	13		6	15.3		
	<i>Apodachlya pyrifera</i>	18	17	16	17	17	15				

Fig. 3. Matrix to show restriction enzyme sequence divergence between each pair of species for the 6 kb region of the rDNA repeating units depicted in Fig. 2. Percentage sequence divergence was calculated using the formula (Brown *et al.*, 1979)

$$-100 \ln S/N$$

where $\frac{\text{number of sites present in both species}}{\text{combined number of sites for both species}} = S$

and number of nucleotides recognized = $N = 6$

(*Bam*HI did not restrict within the 6 kb region, cf. Fig. 2.)

localize the ribosomal genes on the map. As shown in Fig. 2, a region of 6 kb showed strong homology to the probe. The homologous region was interrupted by a short (0.5–1.0 kb) region of heterology which divided the homologous region into long (3.0–3.5 kb) and short (2.5 kb) segments. Further studies are in progress to define the ribosomal genes within these regions.

rDNA physical maps

Physical maps of middle band DNA from *Pythium diclinum*, *P. torulosum*, *P. irregulare*, *P. anandrum*, *P. paddicum* and *Phytophthora cryptogea* were constructed and aligned on the basis of nine conserved restriction sites, all within the region shown to be homologous to *N. crassa* ribosomal genes (Fig. 2). The *A. pyrifera* map was constructed by restriction of chromosomal DNA and hybridization to the *N. crassa* probe. It was easily aligned with the other maps because six of the nine sites conserved in *Pythium* and *Phytophthora* were also conserved in *Apodachlya*. The regions of the repeating units not hybridizing to *N. crassa* ribosomal genes could not be aligned due to lack of a sufficient number of restriction sites and due to presumed insertions and deletions which are reflected in the differences in length of the repeating units.

The size of the rDNA repeating unit in the oomycetes investigated ranged from 10.1 kb (*A. pyrifera*) to 11.75 kb (*P. irregulare*), with an average of 10.7 kb. This is slightly larger than typical sizes reported for basidiomycetes (9.2–10 kb) (Raeder & Broda, 1984; Specht *et al.*, 1984) and for ascomycetes (7.7–11.1 kb) (Bell *et al.*, 1977; Lockington *et al.*, 1982; Russell *et al.*, 1984; Verbeet *et al.*, 1984).

Taxonomic analysis

Quantitative comparisons of the physical maps were based only on the 6 kb region which could be easily aligned (Fig. 2). The map positions of the twenty points scored for the presence or absence of restriction sites are given at the top of Fig. 2. In only one case is the interpretation dubious – the *Cla*I-3 site in *A. pyrifera* may in fact be the *Cla*I-4 site conserved in the other species. The effect of this alternative interpretation of the data is that the average percentage divergence of *A. pyrifera* is reduced from 14.1% to 11.9%. Since both values are substantially higher than values within the *Pythium*–*Phytophthora* group (Fig. 3) this alternate scoring of *Cla*I sites does not affect conclusions drawn from the data.

Fig. 4. Multidimensional diagram, based upon data in Fig. 3, to show relationships between percentage sequence divergences of the 6 kb region of the rDNA repeating units. Divergences within the Pythiaceae are drawn to scale. Symbols: dic, *Pythium diclinum*; tor, *P. torulosum*; ana, *P. anandrum*; irr, *P. irregulare*; pad, *P. paddicum*; cry, *Phytophthora cryptogea*; Apy, *Apodachlya pyrifer*. Divergences for *Apodachlya* are not to scale. Note the relative distances of *Phytophthora cryptogea* and *Pythium paddicum* with respect to the other *Pythium* species.

Morphological criteria (M. W. Dick, unpublished) suggest that there are perhaps five major centres of speciation within *Pythium*. *P. anandrum*, *P. torulosum*/*P. diclinum* and *P. irregulare* correspond to three of these groups. Molecular divergence data can be used to begin to test this suggestion. *P. diclinum* and *P. torulosum* showed no divergence in the region of rDNA being compared but differed in the remaining part of the repeating unit and differed slightly in the size of the repeating unit (Fig. 2). This suggests that the two species are distinct but quite closely related. If this conclusion is accepted, then the difference in appearance of sporangia caused by toruloid swelling is of slight taxonomic importance.

At a number of points more significant discrepancies appear between molecular and morphological criteria. The low percentage divergence between *P. anandrum* and *P. irregulare* is not consistent with major morphological differences in the size and shape of sporangia. Also, the fact that *P. anandrum* and *P. paddicum* both have ornamented oogonia was not reflected in a lower percentage divergence between the two species. The latter phenomenon suggests that this criterion may have relevance only to species delimitation within the major sections of the genus, as is the case with the *Achlya racemosa* group of the Saprolegniaceae (Dick, 1973) in another subclass of the Oomycetes. In addition, *P. paddicum* showed the highest percentage divergence from both *Phytophthora* and *Apodachlya* and was also an outlier species within the genus *Pythium* in spite of its intermediate spherical sporangia.

The relationship between *Phytophthora* and the *Pythium* group was striking. The percentage divergences (Fig. 3) of *Phytophthora cryptogea*, *Pythium paddicum*, *P. anandrum* and *P. irregulare* are all greater than those for *P. torulosum* and *P. diclinum*. This suggests that there are divergences in different directions as shown in Fig. 4. The degrees of divergence are comparable between the various species of *Pythium* and *Phytophthora* but not for the other outgroup, *Apodachlya*. Taxonomically, this could be taken as providing a *prima facie* case for examining

the generic boundaries within the Pythiaceae and for considering a subdivision of *Pythium*. Such a step must not be undertaken lightly because the type species of the genus, *P. monospermum*, belongs, on morphological evidence, to the *P. torulosum*–*P. diclinum* group. None of the common pathogens belongs to this group so nomenclatural changes would have severe repercussions.

A more comprehensive survey is required to determine whether the differences reported here are merely part of a continuum or whether there are identifiable discontinuities which correlate between biochemical and morphological data. The accessibility of oomycete rDNA repeating units due to the prominent satellite bands in CsCl–bisbenzimidazole gradients will make it more feasible to use molecular criteria to resolve taxonomic problems in the future.

This work was initiated as the result of research study leave granted to G. R. K. by the University of Manitoba and was supported by operating and travel grants provided by the Natural Sciences and Engineering Research Council of Canada. We are also indebted to Robert Metzberg for the gift of the plasmid pMF2. We thank Abbas Belkhir and Gillian Clark in Reading and David Boyd, Michael Bryan and Carla Chrusch in Winnipeg for laboratory assistance.

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