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Classification of *Pythium* 'group F' based on mycelial protein and isozyme patterns

Hani M. A. Abdelzaher[1], Toshinobu Morikawa[1], Takio Ichitani[1] and Mohamed A. Elnaghy[2]

[1] College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 593, Japan
[2] Department of Botany, Minia University, Minia, Egypt

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Taxonomic characteristics were compared among 10 isolates of *Pythium* 'group F' in tems of the electrophoretic patterns of their mycelial proteins and isozymes. These isolates were obtained from water of three ponds in different seasons and have an identical morphology of zoosporangia. Attempts to cross the isolates with each other themselves and with other isolates from the same group failed. *Pythium* 'group F' is the most dominant of the pythia in the aquatic ecosystem and is difficult to identify because of the lack of sexual reproductive structures. Isozyme analysis proved useful in this respect. Comparisons of banding patterns of total soluble proteins and isozymes revealed five subgroups in *Pythium* 'group F'. Two isolates obtained from water of different ponds in different seasons showed the same protein and isozyme patterns. Our findings indicate that the use of total soluble protein and isozyme patterns for determining the variation within *Pythium* 'group F' could become a valuable adjunct to the morphological and physiological criteria.

Key Words——electrophoresis; isozymes; mycelial protein; *Pythium* 'group F'.

Introduction

The most recent monograph on the genus *Pythium* Pringsheim by Plaats-Niterink (1981) lists 87 species based on morphological criteria of both sexual and asexual structures. Identification of some pythia is sometimes impossible since many isolates produce no sexual reproductive structures. On the basis of sporangial characteristics the isolates that produce no sexual structures were divided into 5 groups: 'group F' (isolates with filamentous sporangia), 'group T' (isolates with lobulate sporangia), 'group G' (isolates with globose to elongate, non-proliferating sporangia), 'group P' (isolates with globose to elongate, proliferating sporangia, 20–30 μm in diam, without catenulate hyphal swellings), and 'group HS' (isolates with noncatenulate hyphal swellings only, not producing zoospores). Key characters to separate these isolates within each group are not provided (Plaats-Niterink, 1981). Although hyphal diameter, hyphal swelling diameter, temperature-growth response and phytopathogenicity are sometimes useful for separating these groups into sub-groups, these measurements could be altered by environmental conditions and the quality of the medium used. The variation in characters used as taxonomic criteria of the above groups prompted a search for characters that show less variation among isolates, and which could thus be used to further characterize a species and help to confirm an identification.

Both protein and isozyme electrophoretic analyses have been used to study taxonomic relationships of *Pythium* species. Clare (1963) found that the electrophoretic pattern of protein from fungal species including *Pythium* may aid in the identification of these fungi and in the delimitation of species. Clare et al. (1968) studied 11 *Pythium* species using general and specific protein stains, and observed little variation among isolates within species. They concluded that major proteins were characteristic of species, and protein banding patterns could be used in the identification of fungal species. Ma and Yu (1991) concluded that, for 27 isolates distributed in 23 *Pythium* species protein patterns of conspecific isolates were identical while those of isolates from different species were quite diverse. Species-specific isozyme banding patterns were also reported for species of *Pythium* (Clare et al., 1968; Nwaga and Citharel, 1987; Yu and Ma, 1989). On the other hand, Chen et al. (1991) in their study of the application of soluble protein and isozyme analyses in *Pythium* systematics concluded that their results only partially support using such techniques in differentiation of *Pythium* spp. They further pointed out that soluble protein and isozyme analyses have little value as techniques for routine identification of *Pythium* isolates in terms of species, and added that the species readily identified in their study could, in most cases, be distinguished with less trouble by microscopic examination of reproductive structures.

The objectives of our research were to evaluate the usefulness of SDS-polyacrylamide gel electrophoresis of total soluble proteins and starch gel electrophoresis of isozyme polymorphism from closely related morphological species of *Pythium* 'group F' and to determine if variation occurs among isolates within this group. A preliminary report of this work has been published (Abdelzaher et al., 1994b).

Materials and Methods

Fungi and isolates Isolates were obtained from water of three ponds in Sakai, Osaka, Japan, during an ecological study on aquatic pythia (Abdelzaher et al., unpublished data). These ponds are located in the northern part of the Senboku new town zone, namely, Nakatsu, Tatsumi and Komoda, and belong to the same water system. Ten isolates belonging to *Pythium* 'group F' were chosen for this study because they had been isolated from water of different ponds in different seasons. They were numbered 37, 88, 120, 169, 256, 279, 385, 391, 398 and 425, according to their isolation sequence.

Morphological studies All the isolates tested have filamentous zoosporangia which readily produce zoospores under aquatic conditions. To induce the ability to reproduce sexually, cultures were grown on V-8 juice agar, corn meal agar, potato carrot agar, lima-bean agar and potato dextrose agar, each supplemented with 500 μg/ml of wheat germ oil. Cross mating with each other and with other isolates within the same group was unsuccessful.

Evaluation of cultural characteristics Temperature-growth responses of isolates were determined on Bacto-corn meal agar (Bacto-CMA) at 5, 10, 15, 20, 25, 28, 30, 35, 37 and 40°C. Transfers were made from actively growing cultures on water agar, and fungi were allowed to grow overnight prior to beginning growth rate studies. Results are means of three measurements.

Cultural conditions and protein extraction Isolates were grown on 2.5% water agar for 48 h. Agar disks (7 mm diam) were taken from actively growing colonies and transferred to a modified Schmitthenner's synthetic, protein-free broth medium (Schmitthenner, 1979). This medium contained the following per 1000 ml: 5 g sucrose, 0.54 g asparagine, 0.15 g KH_2PO_4, 0.15 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 80 mg thiamine-HCl, 10 mg ascorbic acid, 4.4 mg $ZnSO_4 \cdot 7H_2O$, 1 mg $FeSO_4 \cdot 7H_2O$, and 0.7 mg $MnCl_2 \cdot 4H_2O$. Three agar disks were inoculated into 100 ml of medium in a 250-ml Erlenmeyer flask and incubated for 6 days on a rotary shaker (80 rpm) at 25°C.

During the protein extraction procedure, samples were always kept in an ice-bath unless otherewise indicated. Mycelial mats were collected by filtering through Whatman No. 1 filter paper, washed with chilled (4°C), sterile, distilled water, and frozen at −85°C for at least 2 h. The frozen mycelial mats were then ground to a fine powder with a chilled (−20°C) mortar and pestle using liquid nitrogen, and transferred to centrifuge tube (1 g mycelial powder/isolate). Extraction buffer [50 μl of 1 M Tris-HCl pH 8.0, 40 μl of 0.25 M EDTA, and 5 mg polyvinyl-pyrrolidone (PVP) per ml of distilled water] was then added at a rate of 1 ml per 100 mg of mycelium, and the suspension was mixed with a magnetic stirrer for 10 min. The extraction mixture was then centrifuged at 16,000 × *g* for 40 min at 4°C. A portion of the supernatant was used for SDS-polyacrylamide electrophoresis. The remainder was stored in small aliquots in micro centrifuge tubes at −20°C and used for starch gel electrophoresis. Each aliquot was used only once. All samples were used within one month after extraction (Chen et al., 1991, with modifications).

Polyacrylamide gel electrophoresis of soluble protein The protein samples were treated with sample buffer (250 mM Tris-HCl pH 8.0, 8% SDS, 40% glycerol and 0.04% bromophenol blue) in a reaction mixture containing 150 μl of sample, 50 μl of sample buffer and 17.4 μl of mercaptoethanol (8% of the total volume of reaction mixture). The protein-buffer-mercaptoethanol (PBM) mixture was boiled for 5 min in a boiling water bath. Polyacrylamide gels, consisting of 12.5, 10 and 7.5% separating gels and a 4% stacking gel, were 1 mm thick and 13.5 cm long. The SDS-treated protein samples were applied to the polyacrylamide gel at 10 μl (PBM) per sample and separated with an electric current of 150 V per gel for 2–3 h. At the end of the electrophoretic run gels were fixed and stained for 1 h in 0.25% Coomassie blue, 45% methanol and 10% acetic acid, and destained with 45% methanol and 10% acetic acid (Hames, 1990, with modifications).

Starch gel electrophoresis and enzyme staining Horizontal starch gel electrophoresis as described by Scandalios (1969), and Hayward and McAdam (1977) with some

Table 1. Cultural characteristics of *Pythium* 'group F' isolates[1].

Isolate no.	Pond	Date of isolation	Minimum temp. (°C) of growth	Optimum temp. (°C) of growth	Maximum temp. (°C) of growth	Daily growth rate mm^{-1}
37	Nakatsu	Oct., 1992	<5	30	37	13
88	Nakatsu	Nov., 1992	<5	28	37	16
120	Nakatsu	Dec., 1992	<5	28	35	7
169	Nakatsu	Feb., 1993	<5	28	35	17
256	Tatsumi	Mar., 1993	<5	30	37	16
279	Tatsumi	Mar., 1993	<5	28	37	17
385	Tatsumi	Jul., 1993	<5	28	37	16
391	Komada	Jul., 1993	<5	30	37	17
398	Komada	Jul., 1993	<5	28	37	16
425	Nakatsu	Aug., 1993	<5	28	37	18

[1] Temperature-growth responses were determined on Bacto-CMA.

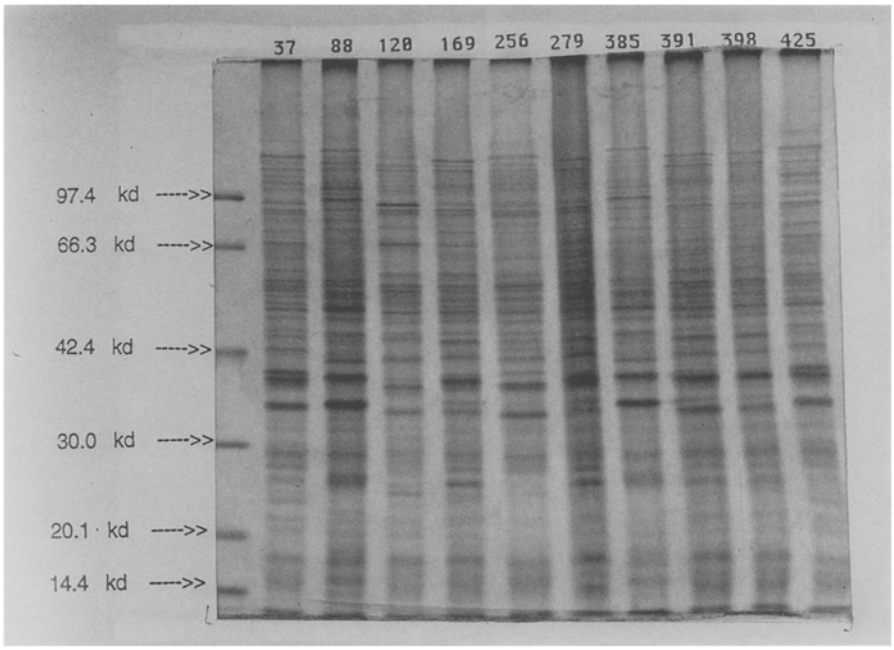

Fig. 1. Protein banding patterns of 10 isolates of *Pythium* 'group F' after discontinuous SDS-polyacrylamide gel (12.5%) electrophoresis, staining with Coomassie blue and drying. The lane at left contains the molecular weight markers.

modifications was used in this study. All starch gels (9% w/v in Histidine buffer and 12.8% w/v in O.M. buffer) were prepared with hydrolyzed potato starch (Sigma grade) in appropriate gel buffers. Proteins were extracted with Tris/HCl (pH 7.5) extraction buffer and absorbed into sample wicks (7 × 10 mm) made from Whatman No. 3 filter paper. Single paper wicks with protein extracts were placed vertically into sample slots 40 mm from the cathode of the gel. The gels were run at 200 V, 38 mA under 4°C for 4-5 h. After electrophoresis, gels were sliced horizontally into three pieces of 1.5 mm in thickness, each of which was used for an individual enzyme staining procedure. Each isolate was assayed for each enzyme at least twice.

A total of 15 enzymes was initially tested in two electrophoretic buffer systems for resolution and consistency. The banding patterns were detected using slight modifications of assays described by Shields et al. (1983). Nine enzymes that showed good resolution were selected for this study. The migration and presence or absence of a particular enzyme band were recorded, and the relative intensity of bands was ignored.

Results

Cultural characteristics of isolates Temperature-growth responses of the isolates are shown in Table 1. The minimum temperature was the same for all isolates studied, but some isolates had slightly higher optimum and maximum temperatures than others. Isolate no. 120 had the

Table 2. Detectable enzymes and buffer systems.

Enzyme	Abbreviation	Enzyme commission number	Buffer system[1)]
Esterase	Est	3.1.11	B
Glucose-6-phosphate dehydrogenase	G6PDH	1.1.1.49	B
Glutamate dehydrogenase	GDH	1.4.1.2	B
Glutamate oxaloacetate transaminase	GOT	3.6.1.1	A
Malate dehydrogenase	MDH	1.1.1.37	B
Malic enzyme	ME	1.1.1.40	B
Phosphoglucomutase	PGM	2.7.5.1	B
6-phosphogluconate dehydrogenase	6PGDH	1.1.1.44	B
Superoxide dismutase	SOD	1.15.1.1	A

[1)] A. Electrode buffer: 0.2 M brorate/lithium hydroxide, pH 8.3; Gel buffer: 0.2 M brorate/lithium hydroxide, pH 8.3, and 0.05 M Tris/citric acid, pH 8.3 (=1 : 9). B. Electrode buffer: 0.01 M Tris/citric acid, pH 7.0; Gel buffer: 0.01 M L-histidine monohydrochloride monohydrate/NaOH, pH 7.0.

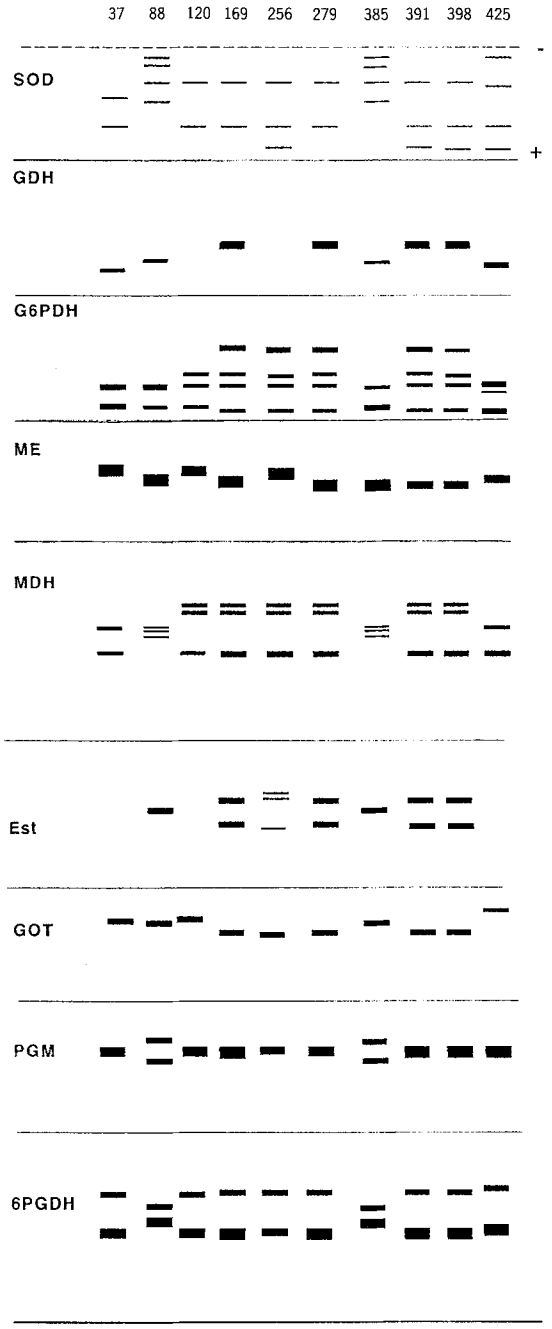

Fig. 2. Starch gel electrophoresis banding patterns of the nine variable isozymes of *Pythium* 'group F' listed in Table 2.

slowest growth rate (7 mm/24 h), isolate no. 37 was intermediate type (13 mm/24 h) while the rest had growth rates in the range of 16-18 mm/24 h.

Electrophoresis of soluble protein The Coomassie blue stain revealed sharp, clear protein bands. The isolates produced different protein patterns on SDS-gel electrophoresis. The 12.5% separation gel was found to give satisfactory results. Protein banding patterns for the isolates tested are presented in Fig. 1. The two major banding patterns between 42.4 kDa and 30.0 kDa were quite different among these isolates. According to

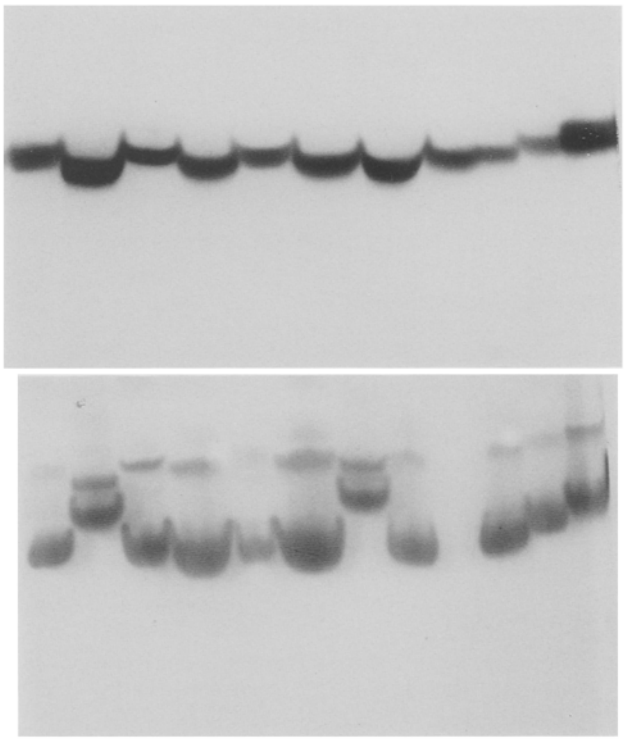

Fig. 3. Malic enzyme (ME) (above) and 6PGDH banding patterns (below) of 10 isolates of *Pythium* 'group F'.

the similarity of the protein banding patterns the isolates tested can be temporarily classified into 5 groups as follows: group 1 (37, 425), group 2 (88, 385), group 3 (256, 391, 398), group 4 (169, 279) and group 5 (120).

Starch gel electrophoresis and isozyme assays Initially, 15 enzymes were tested in two electrophoretic buffer systems. Poor resolution of electrophoretic bands and/or low levels of activity were obtained for acid phosphatase, alcohol dehydrogenase and leucine aminopeptidase. The 9 enzymes that produce well-resolved and consistent banding patterns are listed in Table 2 with their buffer systems. The temporary grouping according to SDS protein banding patterns was confirmed with the 9 isozymes, as follows: group 2 (88, 385), with all the same banding patterns; group 1 (37, 425), with minor differences in G6PDH and SOD; group 3 (256, 391, 398), with minor differences in Est; group 4 (169, 279), with all the same banding patterns; and group 5 (120), a unique isolate among those tested (Figs. 2, 3).

Discussion

Pythium 'group F' is the most dominant species group in the aquatic ecosystem (Plaats-Niterink, 1981; Pittis and Colhoun, 1984; Abdelzaher et al., unpublished data). This group of *Pythium* fails to reproduce sexually and a sexual stage is unknown so far. There are two possible ways for this group to survive under unfavorable conditions. The first is that it has heterothallic ability and produces sexual structures after mating with the oppo-

site strain. The second is that it survives as mycelia in plant or animal debris or even in the mud at the bottom or sides of a water body. A pond, lake or river water may provide conditions under which group F strains can repeat their life cycle asexually. This group of *Pythium*, has been isolated from a water basin mud (Abdelzaher et al., unpublished data). Abdelzaher et al. (1994a) in their study on zoospore production of some aquatic *Pythium* spp. pointed out that an isolate of *Pythium* 'group F' produced zoospores in the temperature range from 4-35°C and retained this ability to even after colonized grass blades had been kept in aquatic culture for 24 h at 40°C. During our ecological survey of *Pythium* species in three ponds in Sakai, Osaka, the temperature of the ponds ranged between 2 and 30°C (Abdelzaher et al., unpublished data). For this reason 'group F' can reproduce asexually all the year round.

Identification of this group of *Pythium* is incomplete since the asexual stage alone is not enough for identification. This study shows clear evidence that this group is heterogeneous and contains isolates differing in their genetic characteristics in terms of protein and isozyme variations.

Clare (1963) reported that protein electrophoresis could be used to differentiate the species of *Pythium*. Subsequently, Clare et al. (1968) studied oxidoreductase and other proteins from 27 isolates of 11 species of *Pythium* and species of *Fusarium, Phytophthora, Saccharomyces, Schizosaccharomyces* and *Rhizoctonia*. They concluded that banding patterns of major proteins could be used in identification of fungal species. Martin (1990) used mitochondrial DNA restriction patterns of 8 isolates of *Pythium ultimum* Trow and 11 isolates of *Pythium* 'group HS' (the same characters as *P. ultimum* but without a known sexual stage), and concluded that 7 of 11 HS isolates had banding patterns identical to the predominant *P. ultimum* pattern. He further pointed out that isolates of *Pythium* 'group HS' appear to be *P. ultimum* which have lost the ability to reproduce sexually.

On the contrary, Chen et al. (1991) concluded that soluble protein and isozymes analyses have little value as a technique for routine identification of *Pythium* species isolates. This might be due to the use of a very closed morphological species of *Pythium* such as *P. arrhenomanes* Drechsler and *P. graminicola* Subramaniam isolated from different countries.

The cultural characteristics of the isolates tested here are not sufficiently diverse to allow a conclusion to be drown about the variability among these isolates. However, one isolate (120) could be separated by its slow growth rate and its unique protein and isozyme patterns among isolates tested. Our results indicate that the isolates studied differ in their banding patterns and the data support their separation into 5 subgroups. The data also emphasize the heterogeneity of *Pythium* 'group F'. *Pythium* 'group F' might have a sexual stage but lost the ability to reproduce sexually for some reason. Further studies should be done using advanced molecular biological techniques such as total soluble protein and isozyme analyses, which proved to be useful in this respect. Comparisons between *Pythium* 'group F' isolates and the aquatic pythia with filamentous zoosporangia and a known sexual stage, such as *P. aquatile* Höhnk, *P. coloratum* Vaartaja, *P. diclinum* Tokunaga, *P. dissotocum* Drechsler, *P. monospermum* Pringsh. and others, should also be made on the basis of total soluble protein and isozyme analyses.

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