THE KOREAN JOURNAL OF MYCOLOGY
Vol. 21, No 4, p293-300 December 1993

Printed in S. KOREA

Comparisons of Isozyme Patterns in *Pythium* Species and Application to *Pythium* Systematics

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Isozyme을 이용한 *Pythium* species의 비교 및 *Pythium* systematics에의 이용

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ABSTRACT: For the enzymes AAT, GmDH, ME, GPI, LDH and IDH, nine, seven, four, nine, seven, and four different phenotypes, respectively, were observed. All six isolates of an unidentified sterile *Pythium* sp. isolated from field soil showed the same band positions for all six enzymes compared. These phenotypes were not similar to any of the known *Pythium* species. Two isolates of unknown *Pythium* species (145 and 299) showed the same band positions for all six enzymes. The phenotypes for all three unknown *Pythium* spp. were different from the other species in the experiment. Five isolates of *P. heterothallicum* showed the same band positions for all enzymes compared except one enzyme, IDH. Two isolates of *P. torulosum* showed the same band positions for enzymes AAT, GmDH and ME, and three isolates of *P. totulosum* showed the same positions for enzymes GPI, LDH, and IDH. Single isolates of *P. spinosum* and *P. irregulare* showed the same band positions for enzymes AAT, GmDH and GPI. In conclusion, sterile types of *Pythium* species showed 100% similarities among themselves but did not show any similarity with all isolates of *P. heterothallicum* and *P. spinosum* isolate, and showed very low similarities with other isolates in general except with unknown *Pythium* isolate 306. Similarity levels between different species were low in general with few exceptions.

KEYWORDS: enzymes, phenotypes, *Pythium* species

Introduction

Taxonomy of the genus *Pythium* is based primarily on morphology of reproductive structure. Eighty seven species were recognized in the most recent monograph on *Pythium* (Van der Plaats-Niterink, 1981). Most plant pathogenic *Pythium* species have wide host ranges and geographical distributions that are broad and overlapping (Van der Platts-Niterink, 1981; Waterhouse, 1968). Species identification based on disease symptoms is inadequate, and distinguishing between species based on differences in morphological features is often difficult (Hendrix and Papa, 1974, Van der Platts-Niterink, 1981; Waterhouse, 1968). These factors have contributed to difficulties in species identification and disease diagnosis.

There is accumulating evidence of the taxonomic value of electrophoretic patterns of enzymes and other proteins from a variety of animals (Allendorf *et al.*, 1977; Paul and Fottrel, 1961; Selander *et al.*, 1971; Sibley, 1962; Smithies, 1959; Yang *et al.*, 1972), plants (Beckman *et al.*, 1964; Conkle, 1981, Graham, 1963), fungi (Chang *et al.*, 1962; Clare, 1963; Clare *et al.*, 1968; De Vallavieille and Erselius, 1984; Huettel *et al.*, 1983; Krywienczyk

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and Dorworth, 1980), and nematodes (Huettel *et al.*, 1983).

Electrophoretic patterns of enzymes from *Pythium* isolates may aid in the identification of species. Therefore, the objective of this experiment was to determine if isozyme comparisons with known *Pythium* spp. could be used to identify apparently sterile *Pythium* species isolated from sugarcane roots, and to compare zymorphic patterns of isozymes for twenty five *Pythium* species using starch gel electrophoresis.

Materials and Methods

Collection and maintenance of cultures

Isolates were obtained from American Type Culture Collection (ATTC), Arizona, North Carolina, England, and various locations in Louisiana (Table 1). Identification of all obtained isolates was confirmed based on morphological traits (Van der Plaats-Niterink, 1981). Twenty five isolates representing eight *Pythium* species were studied. *Pythium* isolates were maintained on plugs of V-8 agar (200 m*l* of V-8 juice, 2 g of $CaCO_3$, 17 g of agar, and 800 m*l* of water) in sterile water at room temperature.

Table 1. Isolates of *Pythium* species used for isozyme comparison.

Pythium species	Isolate	Source	Host	Location
P. spinosum	11-2	root	sugarcane	St. Gabriel, LA
P. irregulare	7-4	soil	sugarcane	St. Gabriel, LA
P. catenulatum	16-8	ATCC[a]	–	–
P. catenulatum	13-5	root	turf	Arizona
P. arrhenomanes	147	root	sugarcane	St. Gabriel, LA
P. torulosum	7-2	soil	sugarcane	St. Gabriel, LA
P. torulosum	7-7	soil	sugarcane	St. Gabriel, LA
P. torulosum	18-5	root	turf	North Carolina
P. torulosum	18-8	root	turf	North Carolina
Unknown	145	root	sugarcane	St. Gabriel, LA
Unknown	299	root	sugarcane	Pearce Farms, LA
Unknown	306	root	sugarcane	Pearce Farms, LA
P. heterothallicum	272	root	sugarcane	Pearce Farms, LA
P. heterothallicum	285	root	sugarcane	Goldmine, LA
P. heterothallicum	11-1	root	sugarcane	St. Gabriel, LA
P. heterothallicum	16-13	ATCC	–	–
P. heterothallicum	16-14	ATCC	–	–
Sterile type	B7P1	soil	sugarcane	St. Gabriel, LA
Sterile type	B7P5	soil	sugarcane	St. Gabriel, LA
Sterile type	B7L1	soil	sugarcane	St. Gabriel, LA
Sterile type	B7L5	soil	sugarcane	St. Gabriel, LA
Sterile type	B7L8	soil	sugarcane	St. Gabriel, LA
Sterile type	B7L12	soil	sugarcane	St. Gabriel, LA
P. sylvaticum	13-2	–	lettuce	England
P. sylvaticum	13-3	–	lettuce	England

[a]ATCC=American Type Culture Collection, Rockville, MD. USA

Preparation of protein extracts

Twenty-five *Pythium* isolates were transferred to water agar, and after 24 hr, 4-5 plugs were cut from the margin of an actively-growing colony of each isolate and transferred to a 250 m*l* flask containing growth browth. Growth broth was prepared as follows: 5 g sucrose, 0.54 g asparagine, 0.15 g KH_2PO_4, 0.15 g K_2HPO_4, 0.1 g $MgSO_4$ $7H_2O$, 0.08 g $CaCl_2$ $7H_2O$, 2 mg thiamin HCl, 10 mg ascorbic acid, 1 m*l* $ZnSO_4$ $7H_2O$ (44 mg/10 m*l*), 1 m*l* $FeSO_4$ $7H_2O$ and 1 m*l* $MnCl_2$ $4H_2O$ (7 mg/10 m*l*) were mixed in 1 L of distilled water and stirred for 30 min. After colony transfers, flasks were placed into a rotary shaker and shaken at 120 rpm for 5 days at room temperature.

Mycelium grown in each flask was collected and freeze-dried by the following procedures: Each mycelial mat was collected on Whatman No. 1 filter paper and washed twice with 500 m*l* sterile-distilled water. Mycelial mats were then placed into petri dishes and frozen immediately at −50℃ for 30 min and subsequently placed into vacuum freeze-dryer, then allowed to dry overnight. Freeze-dried mycelium was ground with a pre-cooled mortar and pestle into a fine powder. The powder was then transferred to a 15 m*l* centrifuge tube standing in ice, and grinding buffer (50 mM Tris, pH 7.1) was added at a rate of 0.8 m*l* per 100 mg mycelium. After adding grinding buffer, each tube was allowed to stand in ice for 30 min and vortex-mixed every 10 min. After a final mixing, each tube was centrifuged at 10,000 rpm (13, 800 g) for 10 min at below 4℃. After centrifugation, the supernatant was transferred to a 1.5 m*l* microtube and centrifuged at 14,000 rpm (16,000 g) for 30 min in a refrigerator. The supernatant for each isolate was then collected and stored in a 0.5 m*l* microtube at −20℃.

Preparation of starch gels

Starch gels were prepared in two different buffers as follows: Gel buffer A was prepared by mixing 6 mM Tris and 3 mM citric acid and adjusting to pH 6.7. Buffer B was prepared by mixing 74 mM Tris and 9 mM citric acid and adjusting to pH 8.4. To make the starch gels, 56 g of starch powder poured into each of two 1 L side-armed flasks. Gel buffer A and gel buffer B were added to separate flasks. The starch-buffer mixtures were then mixed on a hot plate with a magnetic stirring bar. As the temperature increased, the starch dissolved, the solution thickened, and the flasks were then shaken by hand. Each flask was heated further for 2 min while shaking by hand over a flame. Gas inside the starch paste was removed by an aspiration. The starch was poured into a gel mold, and allowed to solidify.

Preparation of sample

To prepare samples, 3×15 mm filter paper pieces were cut and placed into cells of polystyrene microtiter plates. To each cell containing filter paper, 13 μ*l* of an isolate protein extract was added. Two filter paper pieces were infiltrated with each isolate sample (one for each starch gel). Filter paper soaked with 13 μ*l* of bromophenol (0.2%) was prepared as control marker.

Electrophoresis

Different electrode buffers A and B were used for the starch gels mixed with two different buffers. To make buffer A, 68 mM Tris and 37 mM citric acid were mixed and adjusted to pH 6.3. To make buffer B, 1.37 M Tris and 0.314 M citric acid were mixed and adjusted to pH 8.1. Filter paper pieces containing individual samples were applied to wells of the two gels. Buffer A was poured into negative and positive charged sections of electrophoresis apparatus which contained gels prepared with buffer A, and current was applied for 4 hr at 50 mA. Buffer B was used for the starch gel prepared with gel buffer B, and an electrode buffer : water mix (1 : 3, v/v) was poured into negatively charged section of the electrophoresis apparatus, and a 1 : 4 electrode buffer/water mix was poured into the positively charged section of the apparatus. Current then was applied for 6 hr at 65 mA. The current was checked every 30 min and adjusted if necessary.

Staining and fixation of the gel

The gels were cut horizontally into 7-8 pieces.

Table 2. List of enzymes with detectable activity, enzyme pH, enzyme abbreviations and enzyme commission number used in the study.

Enzymes and their pH	Enzyme Abbreviation	Enzyme Commission Number[a]
Glutamate dehydrogenase (pH 6.7)	GmDH	1. 4. 1. 2
Malic enzyme (pH 6.7)	ME	1. 1. 1. 40
Aspartate aminotransferase (pH 6.7)	AAT	2. 6. 1. 1
Isocitrate dehydrogenase (pH 8.4)	IDH	1. 1. 1. 42
Glucose phosphate isomerase (pH 8.4)	GPI	5. 3. 1. 9
Lactate dehydrogenase (pH 8.4)	LDH	1. 1. 1. 27

[a]Source: Enzyme Nomenclature-Recommendations (1972) of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry. 1973. Elsevier Sci. Pub. Co., New York, NY 443 Pp.

Single gel slices were stained with one of six different enzyme substrates at 37℃ (Table 2). After treatment with an enzyme substrate, isolate lanes in each gel showed the presence of protein bands. Gels were fixed by addition of a water : methanol : glacial acetic acid mixture at a ratio of 5 : 5 : 1 (v/v) when the presence of protein bands was detected with naked eye.

Data analysis

Data matrix was made by scoring the presence or absence of the bands as 1 and 0, respectively. Similarity coefficients between two races were then calculated using the following modified estimator of DNA fragment homology (F), $F=2n_{xy}/(n_x+n_y)$, where n_x and n_y were the number of fragments in each strain, and n_{xy} was the number of fragments shared by the compared strains (Nei and Li, 1979). The matrix of similarity coefficients was then subjected to an unweighted pair-group method with arithmetic average (UPGMA) cluster analysis, which produced a dendrogram (Rohlf, 1990).

Results and Discussion

It was reported that protein electrophoresis could be used to differentiate species of *Pythium* (Clare, 1963). 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 to identification of fungal species. More recently, Adaskaveg *et al.* (1988) used isoelectric focusing of proteins to study six *Pythium* species, and they concluded that protein banding patterns reliably distinguished the six species.

Protein band positions for six enzymes for each of the *Pythium* isolates studied are presented in Fig 1. For the enzymes AAT, GmDH, ME, GPI, LDH and IDH, nine, seven, four, nine, seven, and four different phenotypes, respectively, were observed. All six isolates of an unidentified sterile *Pythium* sp. isolated from field soil showed the same band positions for all six enzymes compared. These phenotypes were not similar to any of the known *Pythium* species. Two isolates of unknown *Pythium* species (145 and 299) showed the same band positions for all six enzymes. The phenotypes for all three unknown *Pythium* spp. were different from the other species included in the experiment. Five isolates of *P. heterothallicum* showed the same band positions for all enzymes compared except one enzyme, IDH. Two isolates of *P. torulosum* showed the same band positions for enzymes AAT, GmDH, and ME, and three isolates of *P. torulosum* showed the same positions for enzymes GPI, LDH and IDH. Single isolates *P. spinosum* and *P. irregulare* showed the same band positions for enzymes AAT, GmDH and GPI.

Similarity matrix and UPGMA dendrogram showing the relationships among the twenty-five *Py-*

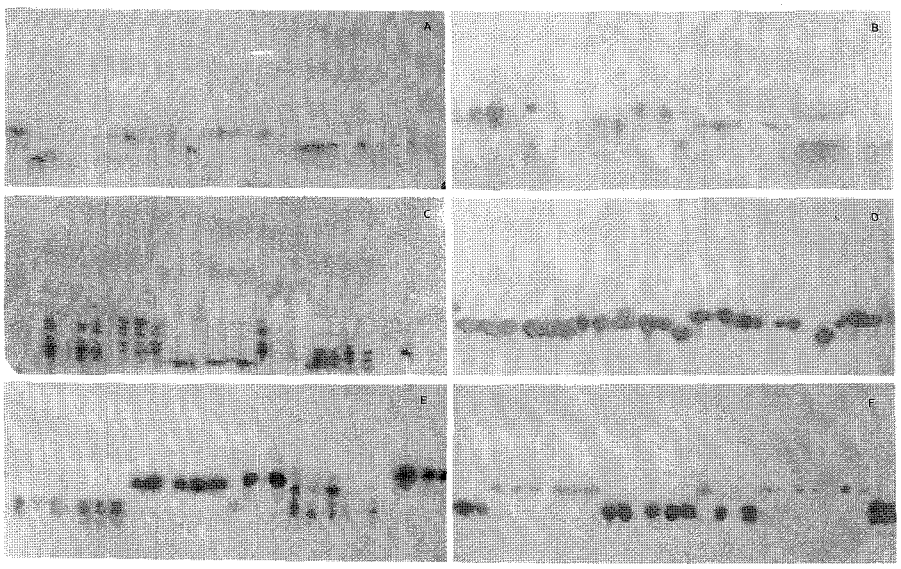

Fig. 1. Protein banding patterns of twenty five *Pythium* isolates after starch gel electrophoresis and staining with six different enzyme substrates (A: GmDH, B: ME, C: AAT, D: IDH, E: GPI and F: LDH) at 37℃.

thium species based on the number of shared bands by the compared *Pythium* isolates were presented in Table 3 and Fig. 2, respectively. *Pythium spinosum* isolate did not show any similarity with total eleven *Pythium* species including six sterile types. *Pythium irregulare* showed low levels of similarity for all other *Pythium* species and *P. catenulatum* isolates showed relatively low level of similarity to almost all other isolates, and did not show any similarity with four isolates including two *P. sylvaticum* isolates. *Pythium catenulatum* showed the lowest level of similarity among themselves compared to all the other isolates. *Pythium arrhenomanes* showed relatively high similarity with isolate 7-7 of *P. torulosum* and minimum similarities with all sterile *Pythium* isolates. These results partially explain the problems of identification among these three species encountered during identification based on morphology. *Pythium torrulosum* isolates showed low similarity with all other isolates, and they did not show any similarity with eight other isolates. Unknown *Pythium* species isolates 145 and 299 showed very low similarities with isolate 306. Isolates 145 and 299 showed very low similarities with all isolates of sterile type. Isolate 306 showed relatively high level of similarity with all sterile type isolates. Unknown species isolate 306 did now show any similarity with all isolates of *P. heterothallicum* and *P. sylvaticum*. *Pythium heterothallicum* isolates showed very high level of similarity among themselves and low levels of similarity with two *P. sylvaticum* isolates. All isolates of *P. heterothallicum* did not show any similarity with sterile type *Pythium* species. All sterile type isolates showed 100% similarity among themselves and all sterile type isolates and sterile types B7P1 and B7P5 showed very low similarities with 13-2 and 13-3, respectively. No similarities were observed among sterile type isolates B7L1, B7L5, B7L8 and B7L12, and *P. sylvaticum* isolate 13-3. In conclusion, sterile types of *Pythium* species showed 100% similarities among themselves, did not show any similarity with all isolates of *P. heterothallicum* and *P. spinosum* isolate, and showed very low similarities in general except with unknown *Pythium* isolate 306. Similarity levels between different species were low in general with few exceptions.

Results from this study only partially support the previous conclusions. Morphologically distinct

Table 3. Similarity matrix based on the number of shared bands by the compared *Pythium* isolates[a].

	7-4	16-8	13-5	147	7-2	7-7	18-5	18-8	145	299	306	272	285	11-1	16-13	16-14	B7P1	B7P5	B7L1	B7L5	B7L8	B7L12	13-2	13-3
11-2	0.67	0.50	0.00	0.20	0.00	0.00	0.13	0.00	0.33	0.33	0.00	0.17	0.17	0.31	0.17	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.44	0.50
7-4		0.42	0.18	0.39	0.13	0.12	0.22	0.14	0.43	0.43	0.11	0.43	0.43	0.40	0.43	0.43	0.11	0.11	0.11	0.11	0.11	0.11	0.36	0.36
16-8			0.36	0.29	0.27	0.24	0.22	0.27	0.43	0.43	0.11	0.43	0.43	0.43	0.43	0.43	0.22	0.22	0.22	0.22	0.22	0.22	0.00	0.00
13-5				0.18	0.33	0.27	0.27	0.36	0.00	0.00	0.13	0.36	0.36	0.36	0.36	0.36	0.13	0.13	0.13	0.13	0.13	0.13	0.00	0.00
147					0.13	0.72	0.11	0.14	0.29	0.14	0.56	0.14	0.14	0.14	0.14	0.14	0.56	0.56	0.56	0.56	0.56	0.56	0.00	0.00
7-2						0.78	0.74	0.67	0.00	0.00	0.42	0.27	0.27	0.25	0.25	0.27	0.42	0.42	0.42	0.42	0.42	0.42	0.00	0.00
7-7							0.95	0.82	0.12	0.12	0.29	0.47	0.47	0.44	0.47	0.47	0.29	0.29	0.29	0.29	0.29	0.29	0.00	0.00
18-5								0.78	0.11	0.11	0.09	0.44	0.44	0.42	0.44	0.44	0.27	0.27	0.27	0.27	0.27	0.27	0.13	0.14
18-8									0.14	0.14	0.11	0.43	0.43	0.40	0.43	0.43	0.22	0.22	0.22	0.22	0.22	0.22	0.00	0.00
145										1.00	0.11	0.43	0.43	0.40	0.43	0.43	0.11	0.11	0.11	0.11	0.11	0.11	0.36	0.40
299											0.11	0.43	0.43	0.40	0.43	0.43	0.11	0.11	0.11	0.11	0.11	0.11	0.36	0.40
306												0.00	0.00	0.00	0.00	0.00	0.72	0.72	0.72	0.72	0.72	0.72	0.00	0.00
272													1.00	0.93	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.18	0.20
285														0.93	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.18	0.20
11-1															0.93	0.93	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.37
16-13																1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.18	0.20
16-14																	0.00	0.00	0.00	0.00	0.00	0.00	0.18	0.20
B7P1																		1.00	1.00	1.00	1.00	1.00	0.13	0.14
B7P5																			1.00	1.00	1.00	1.00	0.13	0.14
B7L1																				1.00	1.00	1.00	0.13	0.00
B7L5																					1.00	1.00	0.13	0.00
B7L8																						1.00	0.13	0.00
B7L12																							0.13	0.00
13-2																								0.86

[a]Data matrix was made by scoring the presence or absence of the bands as 1 and 0, respectively. Similarity coefficients between two isolates were then calculated with modified formula of Nei and Li (1979) [$F=2n_{xy}/(n_x+n_y)$, where n_x and n_y were the number of bands in each strain and n_{xy} was the number of bands in each isolate and n_{xy} was the number of bands shared by the compared strains].

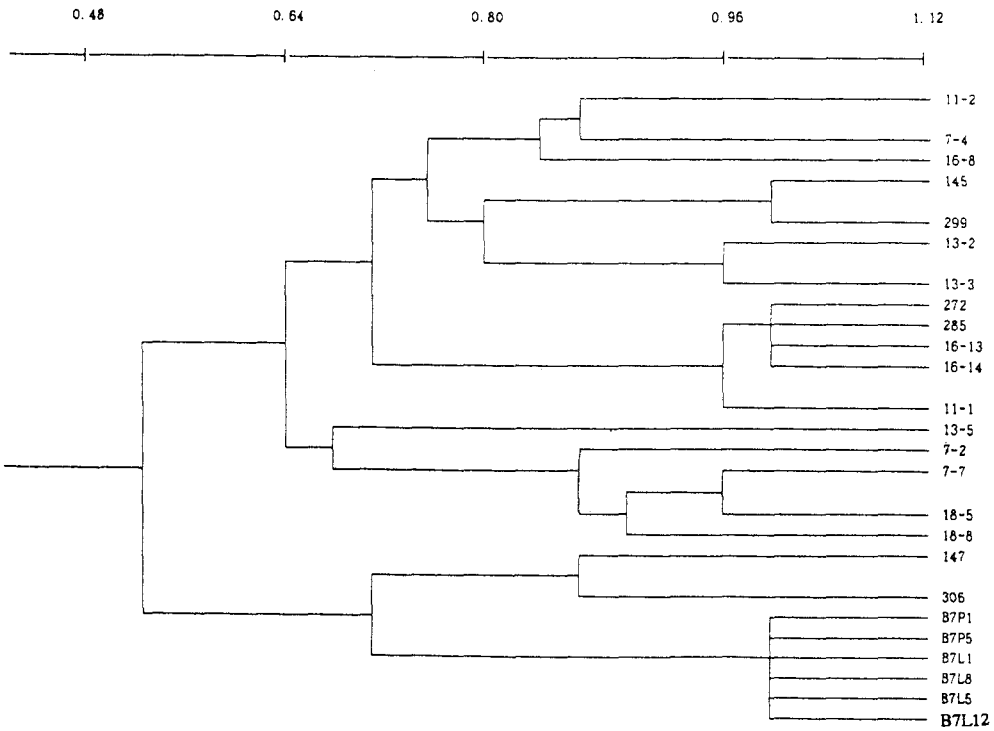

Fig. 2. UPGMA dendrogram showing the relationships among the twenty five *Pythium* isolates based on the bands formed in reaction to six differtent isozymes.

species were generally distinguishable based on visible observation and numerical analysis of soluble protein banding patterns. However, protein banding patterns could not be used to raliably differentiate species with a high degree of morphological similarity.

This study indicate that *Pythium* species differ in degrees of variability in biochemical traits. Therefore, *Pythium* speciation based on only minor differences in variable morphological characters may be unjustified. The number of species included in this study was insufficient to draw conclusions concerning broad species lineages of complexes. However, this study did demonstrate that *P. torulosum* and *P. arrhenomanes,* two species with similar sporangium types, have a low degree of biochemical similarity, so combination of species on a scale suggested by Hendrix & Papa (1974) is clearly not in order. Nucleic acid comparisons and additional isozyme analyses of other morphologically similar species, heterothallic species, and non phytopathogenic species are needed to provide more complete data base concerning *Pythium* systematics.

摘 要

Enzyme AAT, GmDH, ME, GPI, LDH 그리고 IDH에 대해 각각 아홉, 일곱, 넷, 아홉, 일곱 그리고 네개의 서로 다른 phenotype이 관찰되었다. Sugarcane field에서 분리한 종명이 밝혀지지 않은 여섯개의 sterile isolate 모두 여섯개의 enzyme에 대해 동일한 위치에 band를 형성하였다. 이 phenotype들은 이미 밝혀진 *Pythium* species와는 다른 위치에 band를 형성하였다. 종명이 밝혀지지 않은 *Pythium* isolate중 isolate 145와 299는 여섯개의 enzyme에 대해서 동일한 위치에서 band가 형성되었고, 다른 isoalte들은 다른 위치에서 band를 형성하였다. 다섯개의 *Pythium heterothallicum* isolate들은 IDH를 제외한 모든 enzyme에 대해서 동일한 위치에서 band를 형성하였다. 두개 그리고 세개의 *P. torulosum* isolate가 AAT, GmDH 그리고 ME와 GPI,

LDH 그리고 IDH에 대해 각각 동일한 위치에 band를 형성하였다. 한개의 *P. spinosum*과 *P. irregulare* isolate 각각이 enzyme AAT, GmDH 그리고 GPI에 대해 동일한 위치에 band를 형성하였다. 결론적으로, sterile *Pythium* isolate들은 intraspecies level에서는 similarity가 100%였지만, *P. heterothallicum* 그리고 *Pythium spinosum*과는 무관함이 밝혀졌고 isolate 306을 제외한 다른 *Pythium* species들과는 전반적으로 낮은 상관성을 나타내었다. 서로 다른 species간의 상관성은 몇몇 경우를 제외하고는 전반적으로 매우 낮았다.

Acknowledgment

The author would like to thank Dr. Hoy for his advice during the author's stay at Louisiana State University, Baton Rouge LA, USA.

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