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Characterization of two morphological groups of isolates of *Pythium ultimum* var. *ultimum* in a vegetable field

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Comparisons were made between two morphological groups of *Pythium ultimum* var. *ultimum* strains isolated in a vegetable field in Japan. The groups were distinguished as having smaller or larger sexual organs by the sizes of their antheridia and oogonia. Morphological study indicated that the two groups comprised a single taxon, *P. ultimum* var. *ultimum*, by the current taxonomical keys. The smaller group grew faster in the lower temperature range of 4–15°C, whereas the larger group grew faster in the higher temperature range of 25–37°C. Random amplified polymorphic DNA (RAPD) and isozyme analyses revealed genetic dissimilarity between the two groups. Cluster analysis of the isozyme banding patterns with four other *Pythium* spp. demonstrated that the genetic dissimilarity between the two groups was equivalent to species level. In the field survey, the smaller group was frequently detected in February, May and September but not in July, while the larger group was detected mainly in July and September. The two groups were not distinguishable by their pathogenicity to spinach seedlings.

Key Words——intraspecific group; isozymes; morphology; *Pythium ultimum* var. *ultimum*; RAPD.

Pythium ultimum Trow var. *ultimum* is one of the commonest *Pythium* species and can cause severe damage to many plants in cool to moderately warm climates. It is of frequent occurrence in Japan (Watanabe et al., 1977; Kusakari and Tanaka, 1985; Ichitani et al., 1991, 1992), but intraspecific variations of *P. ultimum* var. *ultimum* are not well known. During a survey of *Pythium* flora in a vegetable field, a *P. ultimum*-like species referred to as *Pythium* sp. No. 6 in the previous report, was constantly observed as well as *P. ultimum* var. *ultimum* (Tojo et al., 1992). Nakazono et al. (1994) reported that the *P. ultimum*-like species was identified as *P. ultimum* var. *ultimum* by the current taxonomical keys (van der Plaats-Niterink, 1981; Dick, 1990), although it was distinguished from the *P. ultimum* var. *ultimum* isolates which coexisted in the same field by the larger sizes of its antheridia and oogonia. However, no comparative research was reported on these two intraspecific morphological groups.

In the present study, the characteristics of the two morphological groups of *P. ultimum* var. *ultimum* were compared. The groups were compared in morphology, growth temperature, random amplified polymorphic DNA (RAPD) analyses of total DNA, isozyme banding patterns, seasonal isolation frequency and pathogenicity. The groups with smaller and larger sexual organs are here referred to as S and L groups, respectively.

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Materials and Methods

Fungal isolates Isolates of *Pythium* spp. used in this study were listed in Table 1. Twelve isolates of *P. ultimum* var. *ultimum*, comprising OPU386, 387, 408, 413, 414 and 415 of S group, and 407, 416, 417, 418, 419 and 420 of L group, were examined for morphology, growth temperature and RAPD analysis. Ten isolates of *P. ultimum* var. *ultimum*, comprising OPU387, 388, 408, 413, 414 and 415 of S group, and 407, 416, 417, and 418 of L group, and 10 isolates of four *Pythium* spp., namely, *P. aphanidermatum* (Edson) Fitzp., *P. irregulare* Buisman, *P. spinosum* Sawada and *P. sylvaticum* Campbell & Hendrix, were subjected to isozyme analysis. Two isolates of *P. ultimum* var. *ultimum*, OPU408 of S group and 407 of L group, were examined for pathogenicity. All isolates of *P. ultimum* var. *ultimum* were obtained from a 15 m × 1 m plot in a vegetable field cropped with spinach or cucumber in rotation with onion at the University Farm, Osaka Prefecture University, Sakai. The isolates were maintained on corn meal agar (CMA), which was prepared with 20 g of corn meal (marketed as an assorted feed for chicken), 15 g of agar, and 1,000 ml of distilled water (Ichitani and Kang, 1988).

Morphology The morphological characters examined were: dimensions of oogonia, oospores, antheridia, hyphal swellings and oospore wall thickness. Percentages of terminal oogonia, monoclinous antheridia and intercalary hyphal swellings were also determined. Aplerotic and wall indexes devised by Dick (1990) were

Table 1. Isolates of *Pythium* spp. used in this study.

Species and isolate No.	Source	Isolation site and time
P. ultimum var. *ultimum*		
S group		
OPU386 (=IFO32424, MAFF02-35799)	spinach	Sakai, Osaka, Mar. 1991
OPU387 (=IFO32425, MAFF02-35800)	spinach	Sakai, Osaka, Mar. 1991
OPU388 (=IFO32426, MAFF02-35801)	spinach	Sakai, Osaka, Mar. 1991
OPU408	soil	Sakai, Osaka, Feb. 1992
OPU413	soil	Sakai, Osaka, Feb. 1992
OPU414	soil	Sakai, Osaka, Feb. 1992
OPU415	soil	Sakai, Osaka, Jul. 1992
OPU421	soil	Sakai, Osaka, Oct. 1992
P. ultimum var. *ultimum*		
L group		
OPU407	soil	Sakai, Osaka, Jul. 1992
OPU416	soil	Sakai, Osaka, Jul. 1992
OPU417	soil	Sakai, Osaka, Jul. 1992
OPU418	soil	Sakai, Osaka, Jul. 1992
OPU419	soil	Sakai, Osaka, Oct. 1992
OPU420	soil	Sakai, Osaka, Oct. 1992
P. aphanidermatum		
OPU286	spinach	Habikino, Osaka, Jul. 1978
OPU390	soil	Sakai, Osaka, Jul. 1991
P. irregulare		
OPU177	burdock	Oita, Oita, Nov. 1970
OPU423	soil	Sakai, Osaka, Feb. 1992
OPU424	soil	Sakai, Osaka, Feb. 1992
P. spinosum		
OPU385	soil	Sakai, Osaka, Feb. 1991
OPU391	soil	Sakai, Osaka, Oct. 1990
OPU422	soil	Sakai, Osaka, Nov. 1990
P. sylvaticum		
OPU319 (female isolate)	yum	Towada, Aomori, Jan. 1984
OPU402 (male isolate)	barley	Takamatsu, Kagawa, Feb. 1990

calculated. Isolates were pre-grown on CMA plates at 25°C, then 7-mm agar disks were taken from actively growing colony margins and inoculated onto CMA plates containing 500 μl/L wheat germ oil. Cultures were incubated for 7 to 30 d at 25°C for morphological observations. The isolates were also grown on grass blades for 3 to 7 d at 20°C to determine percentages of monoclinous antheridia (Martin, 1992). At least 30 organs were examined for each isolate for all the characters studied.

Growth temperature Growth response to temperature was investigated in the range of 1–40°C. Agar disks (7 mm in diam) of pre-grown culture on CMA were inoculated onto Bacto-CMA (Difco) plates and incubated in darkness. Colony diam was measured 1–2 d after inoculation, and the mean growth rate (mm/d) was calculated for each isolate. Two replicates were used for each isolate at each temperature. If no growth was observed, plates were subsequently incubated at 25°C to assess the viability of inocula.

DNA extraction Cultural conditions and DNA extraction followed the methods of Martin and Kistler (1990) with slight modifications. Seed cultures were grown on Bacto-CMA slant for 7 d at 25°C. A weft of mycelium was then scraped from slants and inoculated into the liquid medium (10 g glucose, 1.2 g asparagine, 0.5 g K_2SO_4, 0.6 g $MgCl_2 \cdot 6H_2O$, 0.2 g $CaCl_2$, 1 mg thiamine HCl, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, and 2.5 mg cholesterol per L of deionized water, pH 6.2) in 90 mm diam × 15 mm Petri plates. After 3 d of incubation at 25°C, hyphal mats were homogenized for 10 s at low speed in a blender, mixed with 500 ml of fresh liquid medium, and dispensed into Petri plates. They were incubated at 25°C for 3 d. Mycelia were harvested from plates, rinsed several times in deionized water, and collected on a filter paper (No. 1, Whatman) in a Buchner funnel. Excess water was removed from mycelia by pressing in a paper towel. A 1–2 g portion of mycelial mat was placed in a

prechilled mortar, frozen with liquid nitrogen, and ground into fine powder. Mycelial powder was suspended in 15 ml of extraction buffer (Martin and Kistler, 1990) in a polypropylene centrifuge tube and incubated at 65°C for 10 min, and 7.5 ml of 5 M potassium acetate was added per 15 ml of extraction buffer. The solution was incubated for 20 min in an ice bath and centrifuged at 3,000×*g* for 20 min at 4°C. The supernatant was poured through nylon fabric (161 μm opening, N-NO110S, NBC, Japan) into a fresh tube containing 15 ml of isopropanol. DNA was precipitated by overnight incubation at −20°C and pelleted by centrifugation at 3,000×*g* for 30 min at 4°C. Pellets were resuspended in 2 ml of buffer containing 20 mM Tris-HCl and 10 mM EDTA (pH 8.0), then treated with proteinase K (200 μg/ml, Sigma) and RNase (100 μg/ml, Sigma) for 60 min each. The suspension was treated once with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and twice with chloroform : isoamyl alcohol (24 : 1). DNA was precipitated by adding 0.7 vol of isopropanol, incubated at −20°C for at least 1 h, and centrifuged at 18,000×*g* for 15 min. DNA pellets were washed with 70% cold ethanol, dried in vacuo, and dissolved in 100 μl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

RAPD analysis RAPD assays of Williams et al. (1990) were modified using the GeneAmp kit (Perkin-Elmer Cetus). The reaction mixture was 25 μl, including 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.001% gelatin, 200 μM each of dNTP, 0.5 U of *Taq* polymerase, 0.4 μM primer, and approximately 25 ng of template DNA. The thermocycler was programmed for one cycle of 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 35°C, and 2 min at 72°C, and one cycle of 5 min at 72°C. The reaction product was electrophoresed on 0.7% agarose gel (Sigma) and stained with ethidium bromide (0.5 μg/ml). Sixty oligonucleotide decamer primers (Operon) were screened using two isolates of *P. ultimum* var. *ultimum*, OPU408 of S group and 407 of L group.

Isozyme analysis Cultural conditions, protein extraction, starch gel electrophoresis and enzyme staining were based on the procedure described by Abdelzaher et al. (1995). Each isolate was assayed for each enzyme at least twice. Banding patterns were detected with slight modifications of the assays described by Shields et al. (1983). A total of 13 enzymes were initially tested in two electrophoretic buffer systems for resolution and consistency. Acid phosphatase, alcohol dehydrogenase, glutamate oxaloacetate transaminase, and leucine aminopeptidase were excluded due to poor resolution of electrophoretic bands and/or low level of activity. Nine other enzymes with good resolution and consistent banding patterns are listed in Table 2 with their buffer systems. The presence or absence of a particular enzyme band was recorded, and the relative intensity of the bands was ignored. Simple matching coefficients (S) and dissimilarities (D) for each pair of isolates were calculated using the formula of Sneath and Sokal (1973), which was modified by unbiased estimates of Nei (1987) as follows: $S=m/(m+u)$, $D=-1n$ (S), where m=number of synonymous bands between two isolates, and u=total number of bands unique to each sample. Dissimilarity index was then subjected to a clustering algorithm. The unweighted pair group method with arithmetic average (UPGMA) was then used to construct a dendrogram by use of a computer program (SPSS, R4.0, SPSS Inc.).

Seasonal isolation frequency The fungus was isolated in the field from February to September 1992. At least 20 soil cores (3 cm diam×5 cm long) were taken at 1-m intervals from the plot, and a total of 2 kg of soil was combined as a composite sample. The soil was passed through a 2-mm mesh sieve, and 5 ml each of soil was immediately poured into 50 cells of plastic plug flats for rice seedbeds (Minoru-pot, Minorusangyo, Japan). One each of bait seed was buried in each cell. The soil was moistened to 35% (v/w) water content and incubated for 24–48 h at 10, 20, 28 and 15°C for February, April, July and October isolations, respectively. After incubation, seeds were washed in tap water for 1 h and incubated on the *Pythium* selective medium (Ali-Shtayeh et al., 1986) at the respective temperatures for up to 3 d. All samples with mycelia growing on the medium were transferred to the grass blade culture (Martin, 1992) and identified ac-

Table 2. Enzymes and buffer systems used for isozyme analysis.

Enzyme	Abbreviation	Enzyme commission number	Buffer system[a)]
Esterase	Est	3.1.11	A
Glutamate dehydrogenase	GDH	1.4.1.2	A
Glucose-6-phosphate dehydrogenase	G6PDH	1.1.1.49	A
Malate dehydrogenase	MDH	1.1.1.37	A
Malic enzyme	ME	1.1.1.40	A
6-phosphogluconate dehydrogenase	6PGDH	1.1.1.44	A
Phosphoglucoisomerase	PGI	5.3.1.9	A
Phosphoglucomutase	PGM	2.7.5.1	A
Superoxide dismutase	SOD	1.15.1.1	B

a) A. 0.01 M Tris/citric acid, pH 7.0; Gel buffer: 0.01 M L-histidine monohydrochloride monohydrate-NaOH, pH 7.1. B. Electrode buffer: 0.2 M borate-lithium hydroxide, pH 8.3; Gel buffer: 0.2 M borate-lithium hydroxide, pH 8.3; 0.05 M Tris-citric acid, pH 8.3.

cording to the keys of van der Plaats-Niterink (1981) and Dick (1990). The isolates of *P. ultimum* var. *ultimum* were further classified into S and L groups. Isolation frequency of *Pythium* species was shown as the number of colonies of a species grown on the medium from the 50 bait seeds. The experiments were conducted in duplicate.

Pathogenicity OPU407 and 408 were each cultured in V-8 juice broth for 3 wk at 25°C. Suspensions of propagules were prepared from the mycelial mats of the isolates by the method of Kusunoki and Ichitani (1982) and used as the inoculum. The suspension was added to the soil previously pasteurized by the method of Tojo and Ichitani (1992) and the final density was adjusted to 100 and 1,000 propagules per g dry soil. The soil was poured into 20 cells of plastic plug flats as described in the seasonal isolation frequency. Twenty germinated seeds of spinach (cv. King of Denmark) were sown into the cell with one seed per cell. The plug flats were placed in a plastic box in a growth chamber with continuous lighting (6,000 lx) at 15 and 25°C. Soil water contents were maintained at approximately 30% (v/w). The number of seedlings showing damping-off was scored at 10 to 14 d after sowing. Experiments were performed in duplicate.

Results

Morphology Morphology and dimensions of sexual organs and hyphal swellings in S and L groups of *P. ultimum* var. *ultimum*, and the related data of *P. ultimum* var. *ultimum* and *P. paroecandrum* Drechsler from van der Plaats-Niterink (1981) are presented in Table 3. S and L groups were distinguishable from each other on the basis of morphology of sexual structures (Figs. 1A, B, E, F). Antheridia of the S group tended to be shorter and thicker than those of the L group. Monoclinous antheridia were less frequently observed in the S group than in the L group. The number of antheridia per oogonium was usually one, sometimes two in all the isolates. Oogonia and oospores of the S group averaged 19.5±1.8 and 16.5±1.5 μm, respectively, whereas those of the L group were 23.3±2.0 and 20.4±1.6 μm, respectively. Oogonia were mostly terminal in all isolates. The thickness of oospore wall ranged 0.7–0.8 μm for S group and 0.7–1.3 μm for L group. The aplerotic index ranged 56.3–65.1% for the S group and 65.6–70.4% for the L group. The wall index ranged 24.0–27.7% for the S group and 19.4–34.1% for the L group. Hyphal swellings were mostly intercalary in all isolates and usually smaller in the S group than in the L group (Figs. 1C, D, G, H). The L group tended to produce spherical hyphal

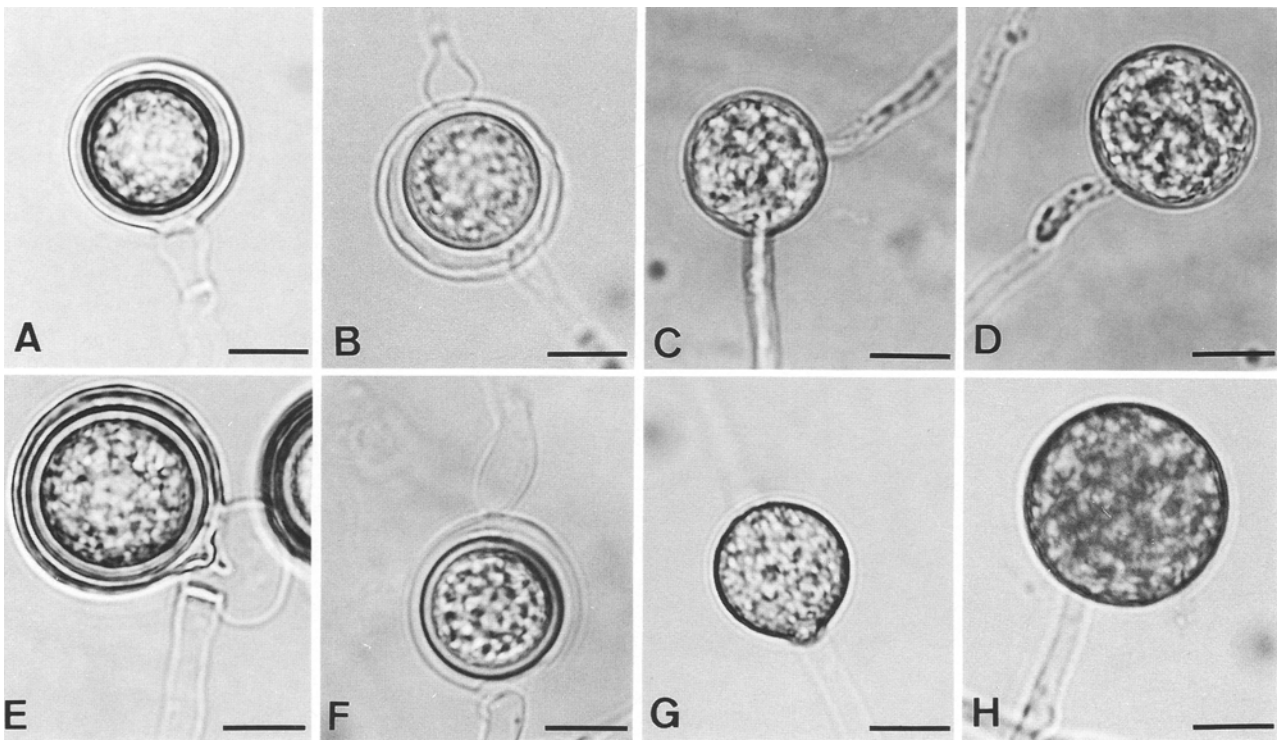

Fig. 1. Sexual organs and hyphal swellings of S and L groups of *Pythium ultimum* var. *ultimum*.
A. Oogonium and monoclinous antheridium of S group. B. Oogonium and diclinous antheridium of S group. C. Intercalary hyphal swelling of S group. D. Terminal hyphal swelling of S group. E. Oogonium and monoclinous antheridium of L group. F. Oogonium and diclinous antheridium of L group. G. Intercalary hyphal swelling of L group. H. Terminal hyphal swelling of L group. Scale=10 μm.

Table 3. Morphology and dimensions of sexal organs and hyphal swellings in S and L groups of *Pythium ultimum* var. *ultimum*.

Morphology	S group		L group		Reference data[a]	
	Overall mean ±SD[b]	Range of means	Overall mean ±SD[b]	Range of means	*P. ultimum* var. *ultimum*	*P. paroecandrum*
Antheridium length (μm)	10.6± 2.1	9.4–12.3	14.8± 2.7	13.9–15.7	sac-like	sac-like
Antheridium breadth (μm)	7.1± 1.4	6.3– 7.8	6.4± 1.0	6.1– 6.7		
Monoclinous antheridium (%)	59.0±11.4	38.7–70.0	82.2±10.1	66.7–93.5	monoclinous or diclinous	monoclinous, sometimes diclinous (occasionally intercalary)
Oogonium diam (μm)	19.5± 1.8	18.9–19.9	23.3± 2.1	22.9–24.0	(14–)20–24(–25)(av. 21.5)	(14–)20–24(–27)(av.19)
Terminal oogonium (%)	93.3± 6.7	83.3–100	97.2± 5.3	86.7–100	terminal, sometimes intercalary	intercalary, often in chains, rarely terminal
Oospore diam (μm)	16.5± 1.5	16.1–16.9	20.4± 1.6	19.9–21.2	(12–)17–20(–21)(av. 18)	(13–)15–21(–23)(av. 17)
Aplerotic index (%)	61.0± 2.7	56.3–65.1	68.3± 1.4	65.6–70.4	<60%*	<65%*
Oospore wall thickness (μm)	0.8± 0.1	0.7– 0.8	1.0± 0.4	0.7– 1.3	often 2 or more thick	1.0–1.5
Wall index (%)	25.9± 1.1	24.4–27.7	26.9± 5.4	19.4–34.1	>40%*	not given
Hyphal swelling length (μm)	12.1± 4.1	10.6–13.2	17.4± 5.6	15.0–20.1	globose, 20–25(–29)	(sub)globose or ellipsoidal, 12–33
Hyphal swelling breadth (μm)	9.1± 3.1	8.3–10.9	16.3± 5.8	14.5–18.9	not given	not given
Intercalary hyphal swelling (%)	81.3±13.2	62.5–93.5	64.1± 6.2	57.6–72.7	intercalary, sometimes terminal	intercalary or terminal

a) Data from van der Plaats-Niterink (1981) (not marked) and Dick (1990) (marked with *).
b) Six isolates of each group were examined with at least 30 organs.

swellings (1.07, length/breadth) as compared to the S group (1.33, length/breadth). All isolates were lacking in zoospore production at 5 and 20°C. These morphological characters of S and L groups were in accordance with the reference data of *P. ultimum* var. *ultimum* (van der Plaats-Niterink, 1981; Dick, 1990), except for oospore wall thickness and the wall index, and the aplerotic index. The characters of S and L groups were distinguishable from those of *P. paroecandrum* by their predominant terminal oogonia and the lack of diclinous antheridia.

Growth temperature The S group was distinguishable from the L group by its faster growth at 1–15°C and slower growth at 25–37°C (Fig. 2). The optimum and maximum temperature for growth of all isolates were 28 °C and 37°C, respectively. The minimum growth temperature was below 1°C for the S group, whereas it ranged 1–5°C for the L group. Growth of hypha was observed in all isolates when colonies were transferred to the optimum temperature following incubation at 40°C. Only slight variations in the data were found among isolates of the same group.

RAPD analysis Seven primers (Table 4) out of 60 produced clear and reproducible DNA bands for both S and L groups of *P. ultimum* var. *ultimum*. RAPD patterns of the 7 primers clearly demonstrated differences between the two groups (Fig. 3). Size of RAPD fragments ranged from approximately 0.5 to 3.0 kilobase pairs. The number of fragments of RAPD products varied from one to nine in all combinations of isolates and primers. Although some combinations lacked one or more fragments, the banding patterns were generally identical within each group.

Isozyme analysis Fifty-five discrete bands were resolved by the nine enzymes (Fig. 4). One enzyme system, 6PGDH, was monomorphic in all isolates examined. Eight of the enzyme systems were found to be poly-

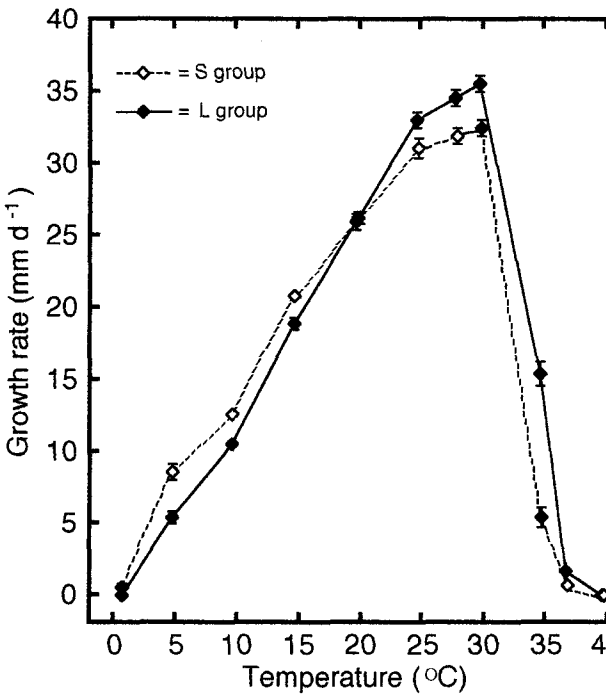

Fig. 2. Growth-temperature relations of S and L groups of *Pythium ultimum* var. *ultimum*.
Twelve isolates comprising OPU386, 387, 408, 413, 414 and 415 of S group, and 407, 416, 417, 418, 419 and 420 of L group, were used with duplicates. Bar represents standard deviation.

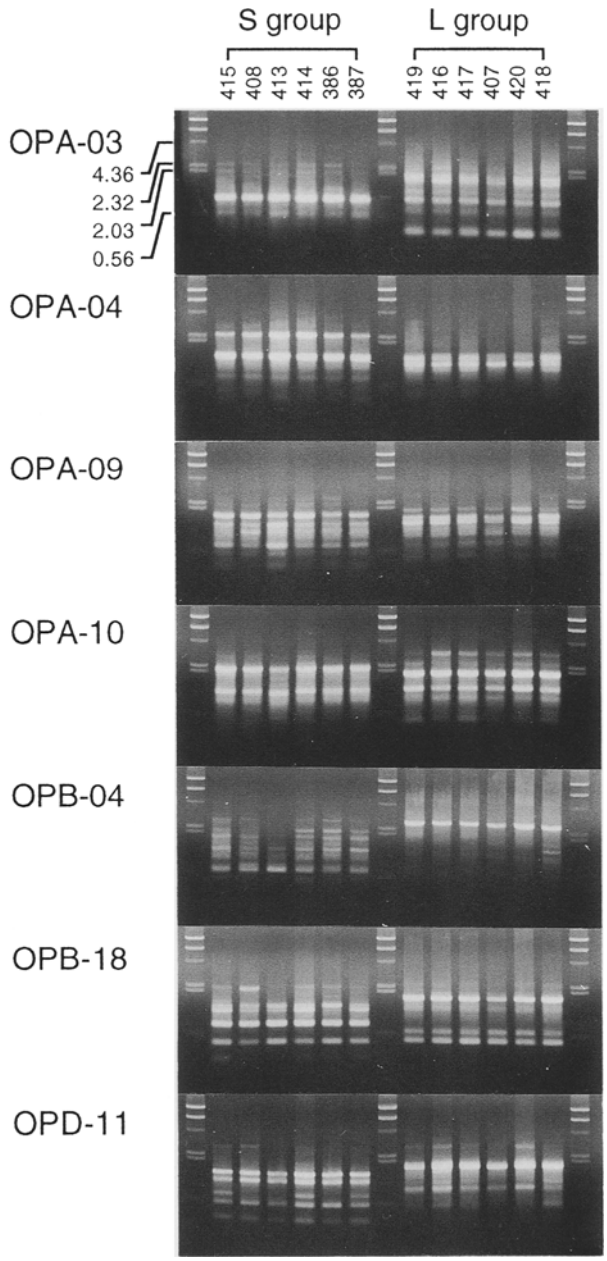

Fig. 3. RAPD banding patterns of S and L groups of *Pythium ultimum* var. *ultimum*.
Seven primers, OPA-03, OPA-04, OPA-09, OPA-10, OPB-04, OPB-18, and OPD-11, were used. Markers (λ-*Hind* III digest) appear on both sides and in the center (sizes in kbp).

morphic. Banding patterns of MDH were most distinctive in all isolates and clearly distinguished between S and L groups of *P. ultimum* var. *ultimum* (Fig. 5). UPGMA cluster analysis of the data for the eight isozymes indicated a dissimilarity value of 2.45 between S and L groups of *P. ultimum* var. *ultimum* (Fig. 6). This was higher than the value of 2.30 between the S group and *P. aphanidermatum*. The dissimilarity value between the L group and *P. sylvaticum* was 2.00, lower than that between S and L groups. There was a low level of heterogeneity within each group of *P. ultimum* var. *ultimum*.

Table 4. Primer sequences producing clear and reproducible bands for both S and L groups of *Pythium ultimum* var. *ultimum* in the RAPD analysis.

Primer code	Base sequence from 5′ to 3′
OPA-03	AGTCAGCCAC
OPA-04	AATCGGGCTG
OPA-09	GGGTAACGCC
OPA-10	GTGATCGCAG
OPB-04	GGACTGGAGT
OPB-18	CCACAGCAGT
OPD-11	AGCGCCATTG

Seasonal isolation frequency Isolation frequency of S and L groups of *P. ultimum* var. *ultimum* is shown in Fig. 7. The total of 127 isolates obtained were clearly divided into 74 of the S group and 53 of the L group. There were no isolates which had intermediate morphology between the S and L groups. The S group was frequently detected in February, May and September but not in July, while the L group was detected mainly in July and September.

Pathogenicity The pathgenicity of isolates of S and L groups of *P. ultimum* var. *ultimum* on spinach seedling was observed as shown in Table 5. Percentages of damping-off caused by the isolates were low with 100 propagules per g dry soil at 15°C and increased with 1,000 propagules per g dry soil at 25°C. The results of the pathogenicity test was similar for isolates of both S

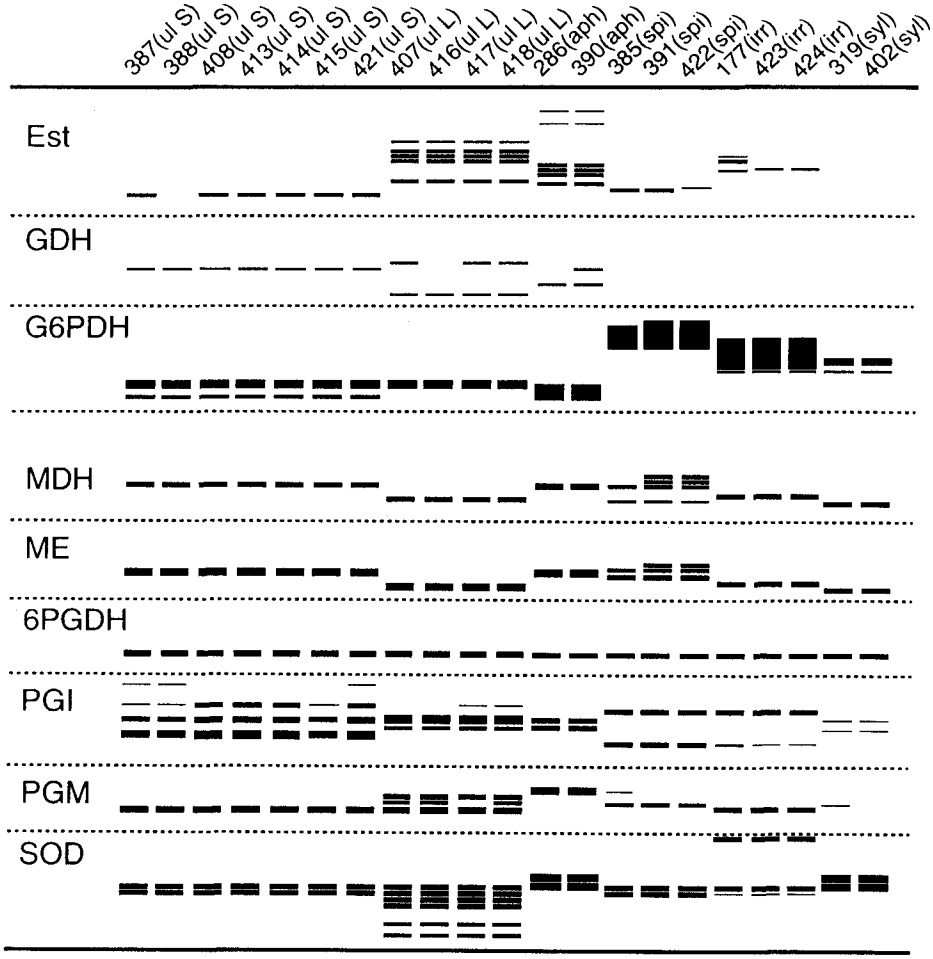

Fig. 4. Schematic summary of starch gel electrophoresis banding patterns of the nine variable isozymes of S and L groups of *Pythium ultimum* var. *ultimum* and four outgroup taxa.
ul S, *P. ultimum* var. *ultimum* S group; ul L, *P. ultimum* var. *ultimum* L group; aph, *P. aphanidermatum*; irr, *P. irregulare*; spi, *P. spinosum*; syl, *P. sylvaticum*.

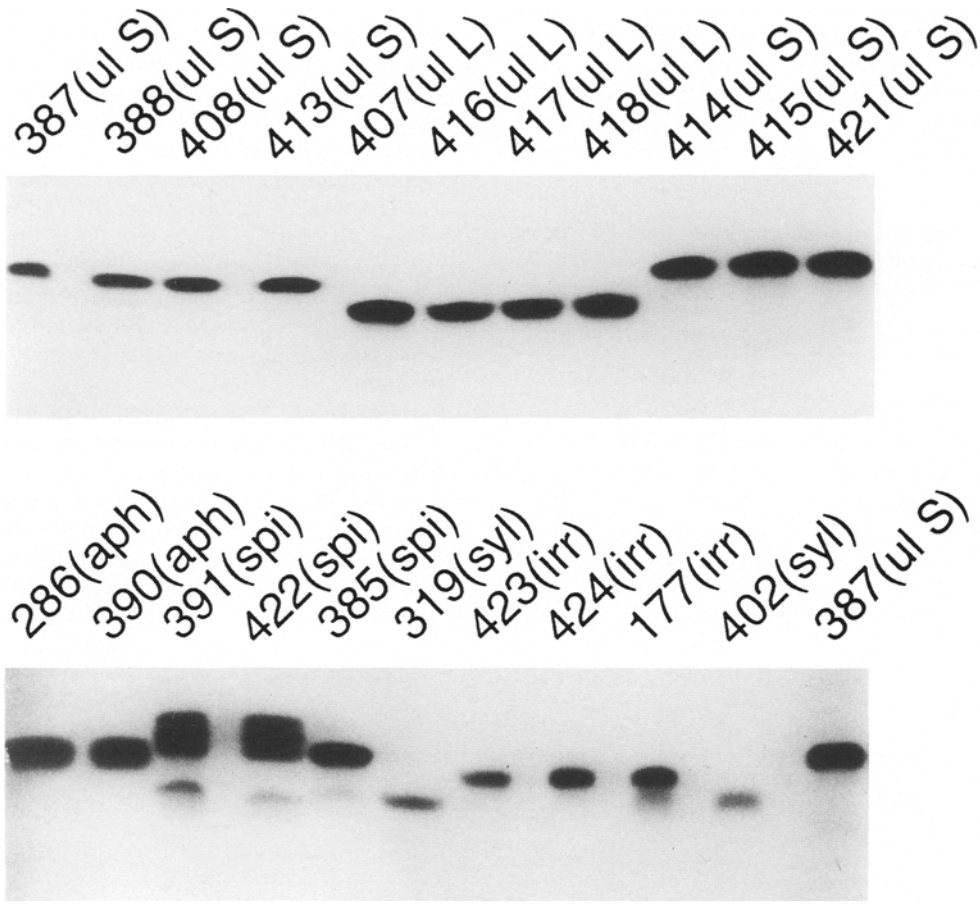

Fig. 5. Banding patterns of malate dehydrogease of S and L groups of *Pythium ultimum* var. *ultimum* and four outgroup taxa in starch gel electrophoresis.
ul S, *P. ultimum* var. *ultimum* S group; ul L, *P. ultimum* var. *ultimum* L group; aph, *P. aphanidermatum*; irr, *P. irregulare*; spi, *P. spinosum*; syl, *P. sylvaticum*.

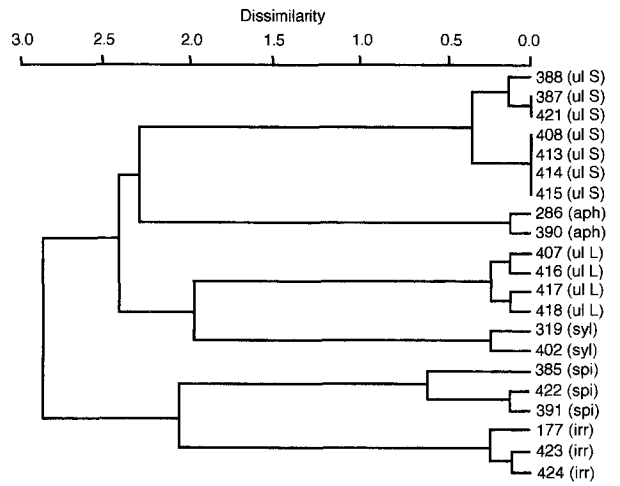

Fig. 6. UPGMA cluster analysis of S and L groups of *Pythium ultimum* var. *ultimum* and four outgroup taxa.
ul S, *P. ultimum* var. *ultimum* S group; ul L, *P. ultimum* var. *ultimum* L group; aph, *P. aphanidermatum*; irr, *P. irregulare*; spi, *P. spinosum*; syl, *P. sylvaticum*. Dissimilarity values were calculated by the formula of Sneath and Sokal (1973).

and L groups.

Discussion

Morphological comparisons of the reproductive structures, especially their dimensions, revealed clear differences between S and L groups of *P. ultimum* var. *ultimum*. The L group was readily distinguished from the S group by the combination of larger oogonia and elongated antheridia. The two groups were also distinguishable by growth temperature and banding patterns of RAPD and isozyme. Differences in the isozyme banding patterns demonstrated evident genetic dissimilarity at the species level between the two morphological groups. RAPD analysis indicated that no crossing occurred between S and L groups. This was supported by the seasonal isolation frequency, which indicated that all isolates of *P. ultimum* var. *ultimum* obtained were clearly divided into S and L groups, and no isolates with intermediate morphology were found. These results indicate that there are two separate groups in *P. ultimum* var. *ultimum* defined by the current taxonomical keys (van der

Table 5. Pathogenicty of S and L groups of *Pythium ultimum* var. *ultimum* on spinach seedling.

Isolate No. (groups)	Number of propagules per g dry soil	Percentages of seedlings damped-off[a]	
		15°C	25°C
OPU408 (S group)	100	5.0	22.5
	1,000	15.0	62.5
OPU407 (L group)	100	5.0	22.5
	1,000	57.5	62.5
Uninoculated control		0	0

a) From duplicates each with 20 plants.

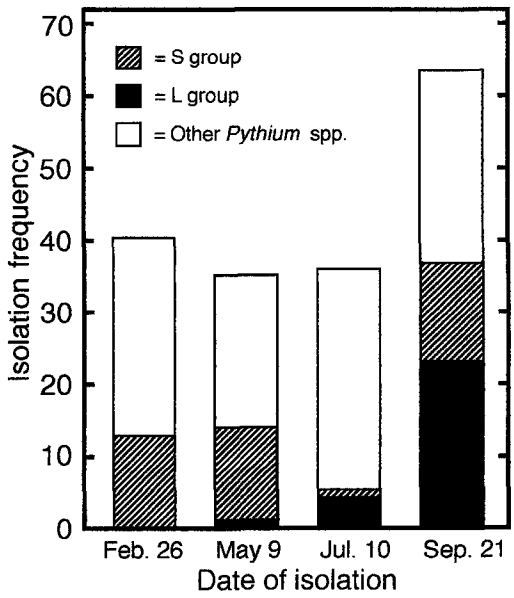

Fig. 7. Seasonal isolation frequency of S and L groups of *Pythium ultimum* var. *ultimum* and other *Pythium* spp. from a vegetable field of the University Farm, Osaka Prefecture University in 1992.
The frequency is shown as the number of colonies of a species grown on the medium from the 50 bait seeds. Averaged data of duplicated experiments are indicated.

Plaats-Niterink, 1981; Dick, 1990). This morphological variation accompanies physiological and genetic differences, although these characters were uniform within each group of *P. ultimum* var. *ultimum*. Sporangia of *P. ultimum* are thought to be a major propagules in soil (Stanghellini and Hancock, 1971; Stanghellini, 1974), and the homogeneity within each group implies the possibility of clonal propagation of only two morphologically distinctive strains of *P. ultimum* var. *ultimum* in the sampling site.

Several morphological characters of S and L groups differed from the reference data of *P. ultimum* var. *ultimum* (van der Plaats-Niterink, 1981; Dick, 1990). Oospore wall thickness and the wall index of S and L groups were thinner than those of the reference data. This morphological difference is difficult to explain, but both groups can be distinguished from other thin oospore wall species such as *P. irregulare* by other morphological features. The aplerotic index of the L group ranged 65.6–70.4%. Dick (1990) arbitrarily classified species with a mean aplerotic index over 60-65% as plerotic. Based on his criterion, oospores of the L group were plerotic. However, the oospores did not fill the oogonium and, therefore, they were aplerotic according to classical concepts. This concurs with the findings of Barr et al. (1996), who reported that the aplerotic index of *P. ultimum* oospores ranged 60.5–75.3% when the oospores did not fill the oogonium. Morphological features of the S group resemble those of *P. paroecandrum*, especially in oospore diam, but the former is distinguishable by predominant terminal oogonia and lack of intercalary antheridia.

The seasonal isolation frequency and pathogenicity results in the present work are difficult to interpret, because the geographical location and number of isolates examined were limited. However, the different phenology patterns between S and L groups is likely to correspond to their different growth temperature. Such phenological behavior should be reflected in the disease development under field conditions.

Although genetic variation exists in *P. ultimum* (Martin, 1990; Francis et al., 1994), morphological variation in the sexual organs has not been closely investigated in this fungus. Barr et al. (1996) indicated there was considerable uniformity in morphology among *P. ultimum* isolates collected worldwide, but they also detected an isolate which was distinct from the other isolates of *P. ultimum* in isozyme polymorphism, antheridial morphology and growth temperature. This suggest there remain unknown morphological and genetic variations within *P. ultimum*.

The present results corroborated the existence of two morphological groups in *P. ultimum* var. *ultimum*. However, taxonomic revision for these two groups should be made only after many isolates of *P. ultimum* var. *ultimum* of diverse origin have been fully examined morphologically, molecularly and ecologically.

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