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Molecular characterization of *Pythium* group F isolates by ribosomal- and intermicrosatellite-DNA regions analysis

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Abstract

Pythium group F is a ubiquitous, though minor, pathogen in several soilless and soil cultures; investigations were carried out to analyze different regions of the DNA and better understand the nature of this group. Fourty-two isolates were obtained from a variety of plants (cucumber, lettuce, tomato) grown in soil or soilless cultures collected in various countries (Canada, Denmark, France, Norway, Sweden and United Kingdom). All *Pythium* group F isolates displayed amplified ITS1-5,8S-ITS2 ribosomal DNA region (rDNA) of similar length, whereas polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) revealed that, among the seven enzymes used, polymorphism was only identified with *Hin*6I. After cloning of ITS1-5,8S-ITS2 rDNA region from *Pythium* group F isolates that displayed restriction polymorphism patterns with *Hin*6I, comparisons of sequence and restriction mapping data showed a slight variation consisting in a single base change. Inter Simple Sequence Repeat (ISSR)-PCR method was also used to obtain data related to the entire genome and not only to a single DNA region. It identified repeated motifs in the genome of *Pythium* group F isolates. Two primers $(CAC)_5$ and $(CCA)_5$ detected polymorphism, and isolates were classified among 11 molecular clusters. The genetic diversity of this group was not correlated with the geographical locations or the host plants from which the isolates originated. Polymorphism of *Pythium* group F isolates pointed out by ISSR is discussed.

Introduction

In recent years, beside well-studied attacks by recognized pathogenic *Pythium* species (e.g. *Pythium ultimum*, *P. aphanidermatum*, *P. irregulare*) known to cause severe damage on seedlings and on roots and stems of mature plants (Jenkins and Averre, 1983; Linde et al., 1994; Martin, 1995a), a growing interest has been devoted to other *Pythium* pathogens. A complex relationship between plants and these organisms has been generally assumed because of the frequent lack of visible symptoms (Salt, 1979; Stanghellini and Rasmussen, 1994; Rey et al., 1998b). Colonization by these organisms should not be ignored for two reasons: they may have a key impact on plant development, and they are quite common in soil and soilless greenhouses (Stanghellini and Kronland, 1986; Favrin et al., 1988; Rey et al., 1998a). Salt (1979) reported that eradication of root-invading *Pythium* spp., which are frequently isolated from apparently healthy roots, correlated with substantial increase in plant growth and yield. Several epidemiological studies have also shown that *Pythium* group F are the most common and ubiquitous *Pythium* isolated from tomato

hydroponic cultures (Rafin and Tirilly, 1995; Rey et al., 1998a), soils (Linde et al., 1994) and pot plants (Thinggaard and Middelboe, 1989). The frequency of recovery of this genus along with its deleterious effect on plant growth have led to investigations on *Pythium* group F pathogenesis. *Pythium* group F has been associated with frequent, though limited, root diseases responsible for yield losses (Rey et al., 1998a) and production of metabolites, which disturb plant growth (Rey et al., 2001). Moreover, severe necrosis and even root rotting occur when stressed plants are infected by *Pythium* group F (Chérif et al., 1997).

Despite the increasing interest devoted to *Pythium* group F, their taxonomic position is still debatable. Indeed, Plaats-Niterink (1981) used the term group F because fungi of this group produce only non-inflated filamentous sporangia on classical media, though sexual structures have never been observed. At least 17 *Pythium* species with similar sporangia as well as oospores are present in the literature (Plaats-Niterink, 1981). Therefore a high diversity among *Pythium* group F isolates is likely since it is probable that they are among *Pythium* species that have, for unknown reasons, lost their ability to produce sexual structures *in vitro*.

Over the last years, only a few experiments have been performed to highlight what this complex group really represents. To our knowledge, the only biochemical analysis of protein patterns was performed by Abdelzaher et al. (1995) who noticed great diversity among isolates. Only one study by Rafin et al. (1995) has focused on the molecular characterization of *Pythium* group F. Their investigation, carried out on isolates from roots of tomato plants cultivated within the same geographical area of France, produced similar banding patterns produced by polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism analysis (RFLP) of the ITS1-5,8S-ITS2 ribosomal DNA region. Thus, experiments on isolates collected from different geographical origins and host plants will allow a better characterization of *Pythium* group F. At the start of our study, we had no precise idea about the polymorphism level of *Pythium* group F isolates from worldwide distribution; we have therefore first focused our investigations on ITS DNA regions, since numerous studies have convincingly shown its usefulness to compare closely related *Pythium* species. In the literature, the use of restriction enzymes on the ITS rDNA region revealed evidence of intraspecific variations (Chen et al., 1992; Chen and Hoy, 1993; Wang and White, 1997). Similarly, Matsumoto et al. (1999) showed intraspecific variations in *P. graminicola*, *P. sulcatum*, *P. rostratum*, *P. vexans*, *P. violae* and *P. volutum* after comparison of ITS sequences. Schurko et al. (2003) using the ITS1-5, 8S-ITS2 rDNA region separated 23 isolates of *P. insidiosum* into three clades and showed a correlation between genetic clustering of the isolates and their geographical origins. Recently, Lévesque and de Cock (2004) compared the ITS rDNA sequences of 116 species of *Pythium*; the probability that a total homology in the ITS region equated to conspecificity was higher in *Pythium* than in ascomycetes because together the ITS-1 and ITS-2 spacers have approximately a length twice that in ascomycetes and as a consequence, the probability of polymporphism is elevated. These authors also revealed a wide variation within several strains of the same species.

Analysis of another segment of *Pythium* group F-DNA should provide complementary information. Moorman et al. (2002) have used ras-related gene sequencing to characterize *Pythium* isolated from greenhouse floral crops but they did not differentiate several species. In the present study, we have focused on the intermicrosatellite DNA region since Inter Simple Sequence Repeat (ISSR)-PCR has proven high discrimination capability in the successful analysis of mycorrhizal (Longato and Bonfante, 1997), pathogenic (Tenzer et al., 1999), and endophytic fungi (Groppe et al., 1995; Grünig et al., 2001). To obtain a better insight on this complex *Pythium* group, all these considerations led us to focus on the investigations reported here, first on the ITS, then on the intermicrosatellite regions. This report provides evidence of high homology in ITS1-5,8S-ITS2 rDNA of *Pythium* group F isolates and suggests that *Pythium* group F should not be considered as a group, but as a *Pythium* species. Furthermore, no correlation was found between ISSR-PCR polymorphism and the geographical origin of isolates.

Materials and methods

Pythium *culture*

Among the 42 isolates of *Pythium* group F investigated in this study (Table 1), some were collected

Table 1. Distribution of *Pythium* group F isolates according to restriction fragments sizes following digestion with *Hin*6I of amplified rDNA ITS region and ISSR banding patterns obtained with $(CCA)_5$ and $(CAC)_5$ primers

ITS group	*Hin*6I base pairs	ISSR clusters	Isolates	Origin	
				Host	Geographic
I	750	4	L15, L22	Tomato roots	Pays de la Loire, France
		8	Sw9	Unknown	Sweden
II	800	3	Ra7, Ra11, Ra14	Lettuce roots	Rhône-Alpes, France
		7	Gb17	Tomato or cucumber roots	United Kingdom
III	750 and 800	1	Br1, Br2, Br3	Tomato roots	Bretagne, France
		1	Gb3, Gb4	Tomato or cucumber roots	United Kingdom
		2	Br4	Tomato roots	Bretagne, France
		2	Gb2, Gb8	Tomato or cucumber roots	United Kingdom
		3	Br5	Tomato roots	Bretagne, France
		3	L4, L8	Tomato roots	Pays de la Loire, France
		3	Ra2, Ra3, Ra5, Ra6	Lettuce roots	Rhône-Alpes, France
		3	Gb9	Tomato or cucumber roots	United Kingdom
		4	L10, L26, L30	Tomato roots	Pays de la Loire, France
		4	Nor82	Cucumber roots	Norway
		4	Nor99	Tomato roots	Norway
		5	Can49	Muck soil	Canada
		5	Dk3, Dk5	Watering system	Denmark
		5	Nor308	Tomato roots	Norway
		5	Sw1, Sw2, Sw3, Sw7, Sw8	Unknown	Sweden
		6	L17	Tomato roots	Pays de la Loire, France
		9	L19, L28	Tomato roots	Pays de la Loire, France
		10	Dk2	Watering system	Denmark
		11	Dk4	Watering system	Denmark

by our team from three different French regions, Bretagne, Pays de la Loire and Rhône-Alpes, respectively denoted Br, L and Ra. Others came from the following countries: Canada denoted Can (from A. Lévesque, CRECO, Ontario), Denmark Dk (from K. Thingaard, Danish Institute of Agricultural Sciences, Aarslev), Norway Nor (from M.L. Herrero, Plant Protection Centre, Fellesbyget), Sweden Sw (from M. Wikström, Nestlé R&D Centre, Bjuv) and the United Kingdom Gb (from G. White, Horticulture Research International, Wellesbourne). Isolates were obtained from cucumber and tomato roots grown in soilless culture (Br, L, Nor and Gb), and others from lettuce roots in soil (Ra), the watering system for pot plants nurseries (Dk), muck soil (Can) or were of unknown origin (Sw). All the *Pythium* group F isolates studied were single-encysted zoospores obtained from water cultures produced as follows: 25 mm agar discs taken from the margins of 2-day-old cultures on V8 agar medium were placed in Petri dishes containing 20 ml of sterile water. After 24 h incubation at 25 °C, zoospores were released. One milliliter of the zoospore suspension was spread on Corn Meal Agar (CMA, Difco) medium. Single germinated encysted zoospores were located by microscopic observation, transferred to CMA plates and incubated at 25 °C.

Pythium *DNA extraction*

A plug of 2-day-old mycelium of *Pythium* group F isolates (about 1 cm^2) was taken directly from the V8 agar medium. The mycelium was placed in an Eppendorf tube, crushed with a microfuge pestle and mixed with 0.5 ml of the extraction buffer previously heated to 65 °C (1% CTAB (w/v), 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.7 M NaCl). The mixture was incubated for 50 min at 65 °C. The extract was emulsified by gentle inversion with an equal volume of chloroform/isoamyl alcohol (24/1, v/v). After centrifugation (13,000 × *g* for 15 min), the aqueous phase was removed and transferred to a fresh Eppendorf tube with 450 μl of cold isopropanol. The tube was gently inverted and left at 4 °C for 1–2 h. After centrifugation (13,000 × *g* for 15 min at 4 °C), the DNA pellet was air-dried for 10–20 min, then dissolved in 100 μl

TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). RNAse (1 mg ml^{-1}) was added to each sample to a final concentration of 0.1 mg ml^{-1} before incubation at 37 °C for 45 min. The DNA samples were re-precipitated with ethanol, dried and suspended in 100 μl TE buffer. DNA concentration was evaluated by spectrophotometry.

Amplification of ITS regions of Pythium *group F by polymerase chain reaction*

Amplifications of the ITS regions of *Pythium* group F were performed in a programmable thermal cycler (PTC-100^{TM}, MJ Research, Inc.). The primers used were PN3 (5′ CCGTTGGTGA ACCA GCGGAGGGATC 3′) and PN10 (5′TC CGCT TATTGTATGCTTAAG 3′) (Viaud et al., 2000). Both primers were employed to amplify the nuclear rDNA region of internal transcribed spacers (ITS-1 and -2) including the 5.8 S gene. PCR was performed in a 20 μl reaction mixture containing 0.3 μM of each primer, 0.5 unit of *Taq* DNA polymerase (Promega), 0.2 mM of each dNTP (Boehringer Mannheim), 2 μl of thermophilic buffer 10× (Promega), 1.5 mM $MgCl_2$ and 4 ng of purified DNA. Reactions were amplified for 31 cycles: DNA denaturation was at 95 °C for 1 min, primer annealing at 50 °C for 1.5 min and primer extension at 72 °C for 1 min.

The amplification products were analyzed by running 8 μl of each mixture through a 1% agarose gel (Eurogentec, France) in Tris–borate–EDTA (TBE) buffer 1× (Sambrook et al., 1989) stained with ethidium bromide and visualized under ultraviolet light. A molecular weight ladder (DNA molecular weight marker XIV, Boehringer Mannheim or 100-bp DNA ladder Gibco BRL) was used as the size standard. Amplification was repeated two or three times for each sample. The lengths of the DNA fragments were estimated by using the DNAStrider 1.2 software.

Restriction analysis of PCR products

Each amplified DNA sample (8 μl) was digested separately with seven restriction enzymes *Hae*III, *Hin*6I, *Hin*fI, *Msp*I, *Nde*II, *Rsa*I and *Taq*I according to manufacturer's instructions (Eurogentec, France). The DNA restriction fragments were separated by electrophoresis in 2% agarose gel prepared with TBE buffer 1×, stained with ethidium bromide and photographed under ultraviolet light.

ISSR amplification of Pythium *DNA*

Amplifications of the ISSR regions were performed using the material as described above. For the primers $(CAC)_5$, $(CCA)_5$ and $(GACC)_4$, the first issue was the determination of the annealing temperatures to be used: values of 60, 62, 64 and 68 °C were tested and 64 °C gave the best informative patterns; this was therefore chosen for further experiments. The reaction mixture (20 μl) contained respectively 2 μM of primer [5′DBDB$(CAC)_5$], [5′DBDB$(CCA)_5$] or [5′HBD B $(GACC)_4$]; the following designations are used for degenerate sites: H (A, T or C), B (G, T or C) and D (G, A or T), synthesized by Proligo France SAS, 0.2 mM of each dNTP, 2 mM of $MgCl_2$, 2 μl of thermophilic buffer 10×, 0.45 unit of *Taq* DNA polymerase and 20 ng of purified DNA. Reactions were amplified for 30 cycles: DNA denaturation was at 95 °C for 4 min for the first cycle only, and 30 s for subsequent cycles, primer annealing at 64 °C for 45 s and primer extension was at 72 °C for 2.5 min. The ISSR amplification products were also separated by electrophoresis in 2% agarose gel and detected as described above.

ISSR data analysis

For each isolate, banding patterns were scored. A matrix was computed for ISSR data using similarity index $S = 2N_{AB}/(N_A + N_B)$, where N_A and N_B are the number of fragments in individuals A and B, and N_{AB} is the number shared by both (Meyer et al., 1993; Longato and Bonfante, 1997). A dendrogram based on this matrix was constructed using the unweighted paired group arithmetic average method (UPGMA) of the software PHYLIP package (Felsenstein, 1989).

Sequencing of PCR products

The PCR products of two *Pythium* group F isolates, Ra7 and L15, referred to as clone 2 and clone 4, which displayed polymorphism after digestion by enzyme *Hin*6I, were purified by a GFX gel band kit (Pharmacia Amersham Biotech) as indicated by the manufacturer. DNA templates were sequenced on a 310 DNA apparatus (Applied

Biosystems) by using non-labelled primers and the Taq Dye Deoxy Terminator cycle sequencing kit according to manufacturer's instructions. Sequences were analyzed using the DNAStrider 1.2 software.

Results

Amplification and RFLP analysis of the ITS1-5,8S-ITS2 rDNA region of Pythium *group F*

The 42 *Pythium* group F isolates from different geographical origins and host plants displayed the same amplification product of about 860 bp (data not shown). PCR-amplified products from the ITS1-5,8S-ITS2 rDNA region of the different *Pythium* group F isolates digested by one of the enzymes *Hae*III, *Hin*fI, *Msp*I, *Nde*II, *Rsa*I and *Taq*I resulted in the same restriction patterns. According to the digestion patterns, the number of restriction enzyme sites as well as the size in base pairs (bp) of the restriction fragments were as follows: no site for *Msp*I, one site each for *Nde*II (500, 350) and *Hae*III (600, 250), two sites each for *Hin*fI (400, 300, 150) and *Taq*I (320, 250, 200) and four sites for *Rsa*I (220, 200, 180, 130, 100). Digestion with *Hin*6I resulted in different restriction patterns among *Pythium* group F isolates (Figure 1). Under our experimental conditions (2% agarose gel), three different banding patterns were observed: the first consisted of a 750 bp band, the second a 800 bp band, and the last was composed of two bands of 750 and 800 bp, respectively (Table 1).

Sequencing of the ITS rDNA region of Pythium *group F isolates with different banding patterns after* Hin6*I digestion*

Sequencing was performed to identify the polymorphism detected with the enzyme *Hin*6I. ITS rDNA regions of the isolates that displayed either one 800 bp (isolate Ra7) or 750 bp band (isolate L15) were cloned (clones 2 and 4, respectively), sequenced, and the sequences aligned and compared (Figure 2). Two restriction sites for the enzyme *Hin*6I were located at 728 and 794 bp for clone 4. Clone 2 contained only one *Hin*6I restriction site located at 794 bp; the change of one G into A at 728 bp caused the loss of the second *Hin*6I restriction site.

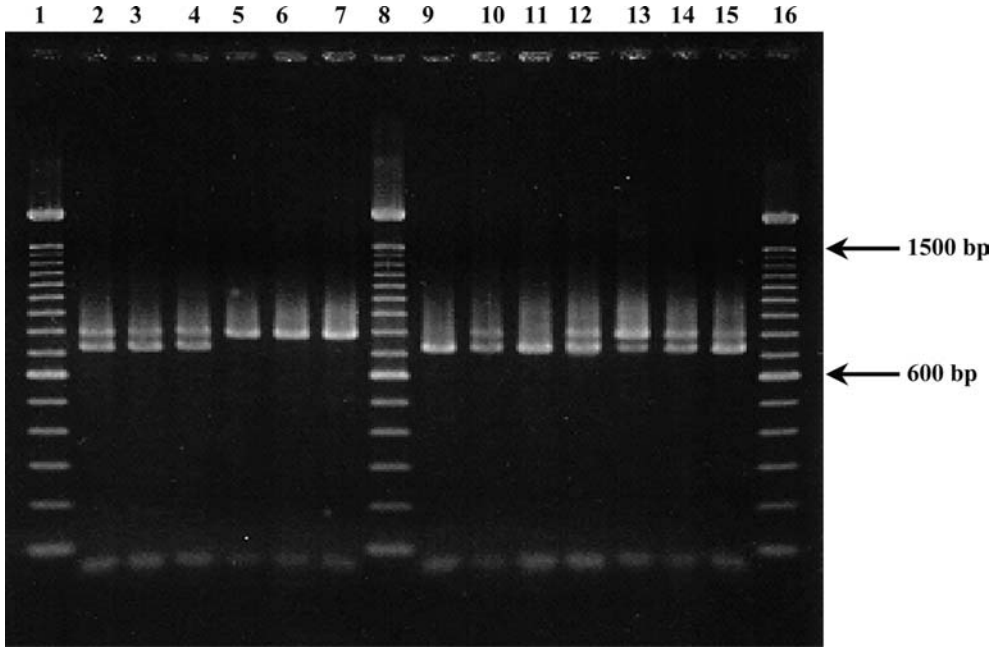

Figure 1. Agarose gel showing restriction banding patterns of the ITS rDNA from different isolates of *Pythium* group F digested with the endonuclease *Hin*6I. Lanes 1, 8, 16: ladder marker (100 bp DNA ladder, Gibco BRL); lane 2: Br1 ; lanes 3–7: Ra2, Ra3, Ra7, Ra11, Ra14; lanes 9–10: L22, L4; lanes 11–13: Sw9, Sw1, Sw2 ; lanes 14–15: Nor82, Nor99.

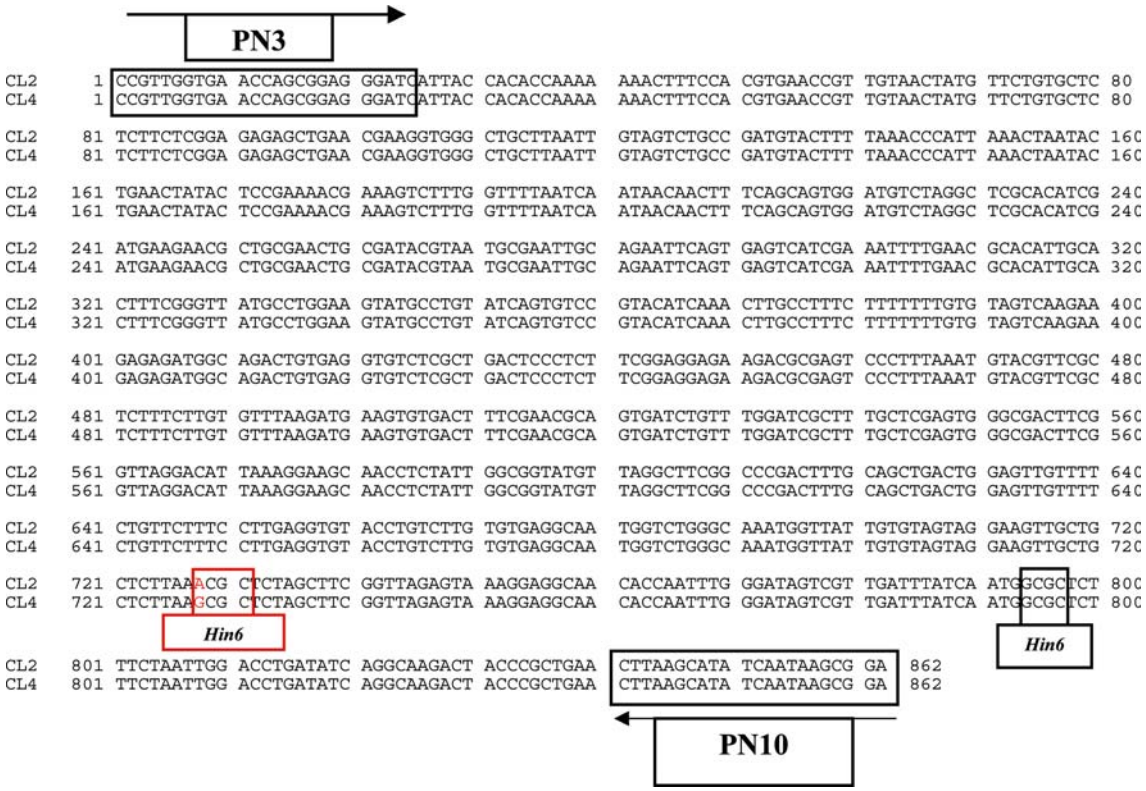

Figure 2. Nucleotide comparison of the ITS rDNA region of two *Pythium* F isolates amplified with primers pair PN3 and PN10. CL2: clone 2 (isolate Ra7), CL4: clone 4 (isolate L15).

ISSR amplification of Pythium *group F isolates*

Each of the primers $(CAC)_5$, $(CCA)_5$ and $(GACC)_4$ successfully amplified DNA fragments from all *Pythium* group F isolates. The ISSR banding patterns of each isolate were compared. Isolate-specific bands of length within 0.4 and 2.6 kb were observed. Each tested primer produced a pattern of 5–11 bands, on average, per isolate. With primer $(GACC)_4$, all *Pythium* group F isolates, except Gb17, exhibited the same banding patterns regardless of their geographical and host plant origin (data not shown). In contrast, the $(CAC)_5$ PCR fingerprints identified variation among individual isolates (Figure 3). Observation of the gel, $(CAC)_5$ allowed us to group into the same cluster the isolates that displayed the same amplification pattern. Finally, 11 banding patterns were distinguished. Table 1 shows that, for example, cluster no. 1 consists of isolates from France and the UK. Each of the cluster nos. 6, 7, 8, 10, 11 contains only one isolate. Except for isolates collected from roots of lettuce grown in the Rhône-Alpes region and grouped into cluster no. 3, no clear correlation was found between a given cluster and the geographical origin or host from which the isolates originated (Table 1). $(CCA)_5$ gave the same 11 banding patterns as those described above with $(CAC)_5$ (data not shown). With the primer $(CAC)_5$, the degree of likeness among isolates was expressed in terms of band-sharing coefficients. A similarity matrix was calculated and used to construct a dendogram according to the UPGMA method (Figure 4). Similarity indices ranged from 11 to 88%. A hierarchical clustering combined the clusters into six groups. The first contained cluster nos. 1, 2 and 3, whereas the second consisted of clusters nos. 4, 5 and 6. Clusters nos. 8 and 9 constituted the third group. Groups 4, 5 and 6 respectively corresponded to cluster nos. 7, 10 and 11.

Discussion

The phylogenetic assignment of *Pythium* group F isolates is uncertain since significant morphological

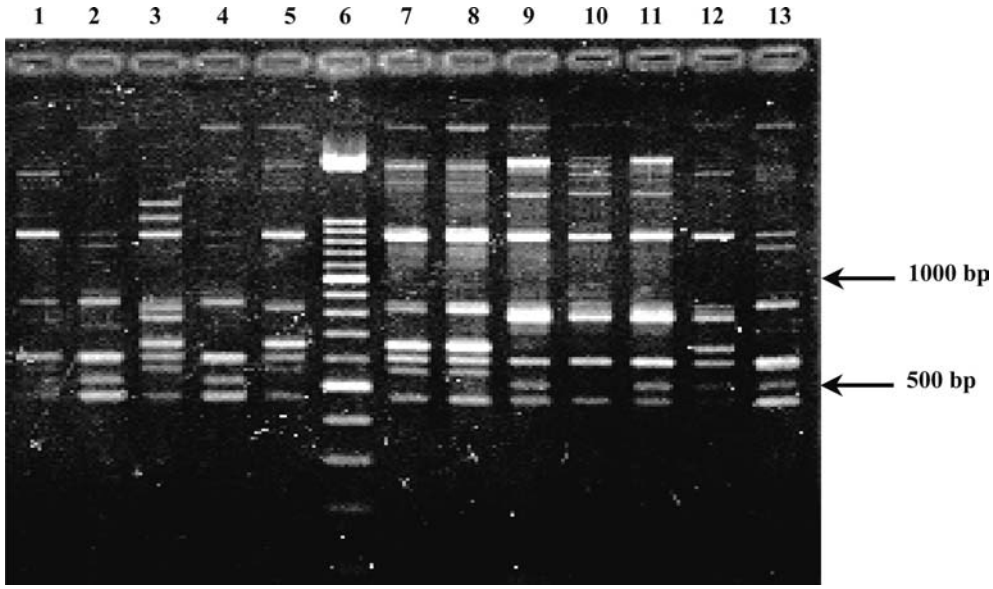

Figure 3. Amplified DNA of different *Pythium* group F isolates with the primer $(CAC)_5$. Lane 1: Br1 (cluster no. 1); lane 2: Can49 (cluster no. 5); lane 3: Dk2 (cluster no. 10); lane 4: DK3 (cluster no. 5); lane 5: Ra7 (cluster no. 3); lane 6: ladder marker (DNA molecular weight marker XIV, Boehringer Mannheim); lane 7: Ra11 (cluster no. 3); lane 8: Ra14 (cluster no. 3); lane 9: L15 (cluster no. 4); lane 10: L17 (cluster no. 6); lane 11: L22 (cluster no. 4); lane 12: Gb2 (cluster no. 2); lane 13: Sw3 (cluster no. 5).

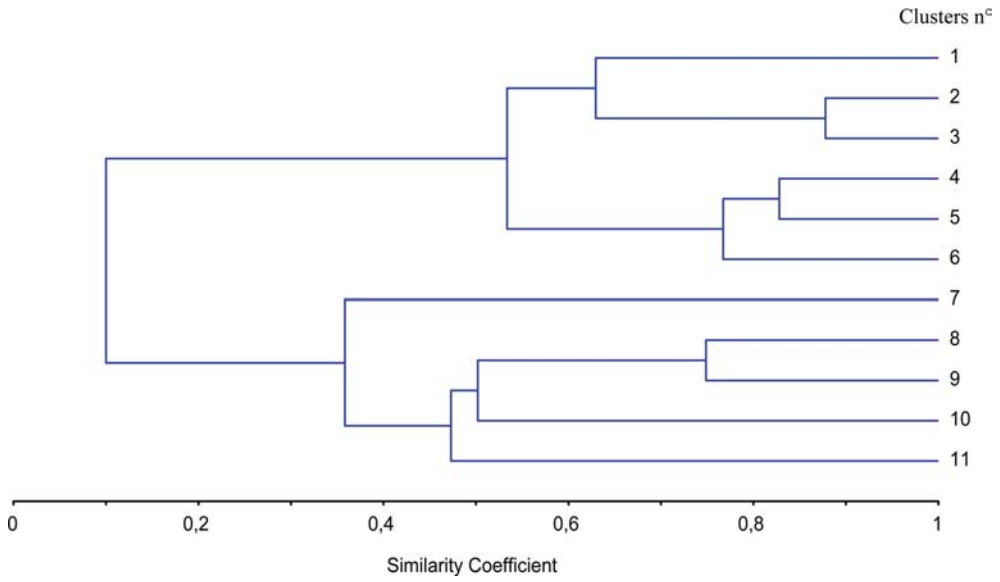

Figure 4. Dendrogram constructed for the *Pythium* group F isolates generated from genetic similarity coefficient obtained with the primer $(CAC)_5$ and based on the UPGMA method.

structures are limited to filamentous sporangia (Plaats-Niterink, 1981). In the present study, different DNA regions from *Pythium* group F isolates from various origins were investigated, for the first time, to determine whether conspecificity or high diversity could be found among them.

The analysis of ITS1-5,8S-ITS2 rDNA region indicates that *Pythium* group F isolates are closely related organisms. Indeed, comparison of PCR products and PCR-RFLP data from the *Pythium* group F isolates collected from different countries and from different host plants showed great similarities. In fact, little heterogeneity was observed only with *Hin*6I, one of the seven restriction enzymes used in this study. Polymorphism in the size of the restriction fragments was pointed out because several isolates showed a single band of either 750 or 800 bp. However, most isolates were distinguished from others because they displayed two bands of 750 and 800 bp. In this case, the sum of the fragment sizes was greater than the size of the original PCR product. This was not due to a partial digestion but may suggest the presence of multiple ITS variants within a single isolate. This assumption is supported by comparison of sequences and restriction mapping of the cloned ITS1-5,8S-ITS2 rDNA regions from these *Pythium* group F isolates. Martin (1990a) also noticed the presence of rDNA polymorphic forms in single isolates of *Pythium* species with globose (*P. iwayami*, *P. paroecandrum*, *P. pulchrum*, *P. ultimum*), inflated (*P. aphanidermatum*, *P. graminicola*) or proliferating sporangia (*P. violae*). *Pythium paroecandrum* and *P. spinosum*, which display two major forms of rDNA, differ in the non-transcribed spacer region for numbers and locations of restriction sites and for insertion-deletion of a fragment of about 60 bp (Martin, 1990a). Therefore, in the *Pythium* genus, the existence of polymorphic forms of the rDNA seems quite common.

ITS1-5,8S-ITS2 rDNA sequences showed only 1 bp change between the *Pythium* group F isolates studied; this observation agrees with that made by Schurko et al. (2003) on *P. insidiosum* isolates where variations among aligned ITS sequences were found to result from 1 to 3 bp insertion or deletion events. The high homogeneity in *Pythium* group F ITS region is remarkable and could suggest conspecificity among these isolates. This assumption is supported in a recent study by Lévesque and de Cock (2004); the probability that a 100% homology in the ITS region means conspecificity is higher in *Pythium* than in ascomycetes because together the ITS-1 and ITS-2 spacers have approximately a length twice that in ascomycetes. As a consequence, the probability of polymorphism is increased. Our suggestion that *Pythium* group F is a *Pythium* species therefore agrees with Lévesque and de Cock (2004). Additionally, it is also worth noting that ITS1-5,8S-ITS2 rDNA sequence of isolate L15 (clone 4) of *Pythium* group F and those of *P. dissotocum* and *P. coloratum* reported by Matsumoto et al. (1999) showed a high homology, 100 and 99.9% respectively. We also confirmed a close relationship of ITS1-5,8S-ITS2 PCR-RFLP patterns of these two *Pythium* species with those of *Pythium* group F isolates (data not shown). Lévesque and de Cock (2004) reported identical ITS sequences of *P. coloratum*, *P. dissotocum*, *P. lutarium* and *P. marinum*. Except for *P. dissotocum* currently found in several greenhouse crops of lettuce and tomato, other species seem to be far less common. In terms of pathogenicity, *Pythium* group F and *P. dissotocum* share numerous characteristics, for example, typical symptoms on plants and frequent isolation from soilless cultures. *Pythium* group F might therefore be considered as *P. dissotocum*-like isolates unable, for some unknown reason, to form sexual structures on classical media. This assumption is also supported by the findings that the *Pythium* group with hyphal swelling (HS) and *P. ultimum* are not genetically distinct and that HS isolates have probably lost their ability to sexually reproduce (Martin, 1990b; Huang et al., 1992; Francis et al., 1994; Kageyama et al., 1997, 1998). The pre-requisite for drawing a definitive conclusion on *Pythium* group F relatedness with *Pythium* species will be to firstly select *Pythium* species isolates from various plants and geographical origins; this will facilitate further studies. However, based on the ITS sequence alone, no definite conclusion about conspecificity can be drawn at this point (Lévesque and de Cock, 2004); further examinations of independent characters are needed to set species boundaries.

To obtain a better insight on this intriguing group, multi-DNA site analysis to demonstrate phylogenetic species could be used with very closely related species. Therefore, we have not restricted our analysis to a single DNA region, but used ISSR-PCR that will produce quite variable DNA fingerprints due to a large number of fragments throughout the genome. To our knowledge, this tool has not yet been used for *Pythium* spp., though it has been successfully applied for investigations to several other fungi (Groppe et al.,

1995; Field and Wills, 1996; Longanto and Bonfante, 1997; Tenzer et al., 1999; Burgess et al., 2001; Grünig et al., 2001). In the present study, amplification patterns showed the existence of repeated motifs in the genome of *Pythium* group F isolates. Among the three primers we used, the PCR fingerprints produced by $(GACC)_4$ failed to allow differentiation of the isolates apart from one. On the other hand, $(CAC)_5$ and $(CCA)_5$ clearly displayed differences among isolates of *Pythium* group F. From these ISSR data, the dendrogram established the degree of likeness among the 42 isolates, and allowed the constitution of 11 clusters of *Pythium* group F. Whatever the chosen level of partition, neither geographical origin nor host plant was associated with these clusters. Consequently, this method is useful to point out intraspecific variations among closely related isolates. For instance, even when *Pythium* group F isolates came from the same region (Bretagne) and host plant, we observed diversity in many cases; conversely, isolates from different countries and those from Bretagne displayed identical ISSR profiles. This shows that different genetic materials exist in different geographic areas. The high discriminatory power (Meyer et al., 1993; Burgess et al., 2001; Grünig et al., 2001) of this technique explains why, conversely to rDNA analysis, it revealed a great heterogeneity among isolates. It is important to stress that recombination or mutation may be responsible for genetically based variations which have dramatic consequences for the diversity of pathogenic populations (Mills and McCluskey, 1990; Burdon and Silk, 1997; Francis and St Clair, 1997). As the vegetative cells of *Pythium* have many co-existing nuclei within the same cytoplasm the potential for heterokaryosis could be great. Moreover, according to Martin (1995b) repetitive sequences may increase the potential for recombination and hence the extent of polymorphisms.

In conclusion, it is relevant to postulate that ITS and ISSR-region analyses provide different, but complementary, data about *Pythium* group F isolates. Study of the ITS1-5,8S-ITS2 rDNA region suggests conspecificity, whereas ISSR molecular analysis highlights differences at the intraspecific level. Further ISSR studies on closely related *Pythium* species from other regions of the world should help to determine relatedness and divergence with *Pythium* group F isolates.

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References

Abdelzaher HMA, Morikawa T, Ichitani T and Elnaghy MA (1995) Classification of Pythium group F based on mycelial protein and isozyme patterns. Mycoscience 36: 45–49

Burdon JJ and Silk J (1997) Sources and patterns of diversity in plant-pathogenic fungi. Phytopathology 87: 664–669

Burgess T, Wingfield MJ and Wingfield BW (2001) Simple sequence repeat markers distinguish among morphotypes of *Sphaeropsis sapinea*. Applied and Environmental Microbiology 67: 354–362

Chen W and Hoy JW (1993) Molecular and morphological comparison of *Pythium arrhenomanes* and *P. graminicola*. Mycological Research 97: 1371–1378

Chen W, Hoy JW and Schneider RW (1992) Species-specific polymorphisms in transcribed ribosomal DNA of five *Pythium* species. Experimental Mycology 16: 22–34

Chérif M, Tirilly Y and Bélanger RR (1997) Effect of oxygen concentration on plant growth lipidperoxidation, and receptivity of tomato roots to *Pythium* F under hydroponic conditions. European Journal of Plant Pathology, 103: 255–264

Favrin RJ, Rahe JE and Mauza B (1988) *Pythium* spp. associated with crown rot of cucumbers in British Columbia greenhouses. Plant Disease 72: 683–687

Felsenstein J (1989) PHYLIP-Phylogeny Inference Package (version 3.2). Cladistics 5: 164–166

Field D and Wills C (1996) Long, polymorphic microsatellites in simple organisms. Proceedings of the Royal Society of London Series B Biological Sciences, 263: 209–215

Francis DM and St Clair DA (1997) Population genetics of *Pythium ultimum*. Phytopathology 87: 454–461

Francis DM, Gehlen MF and St Clair DA (1994) Genetic variation in homothallic and hyphal swelling isolates of *Pythium ultimum* var. *ultimum* and *P. ultimum* var. *sporangiferum*. Molecular Plant Microbe Interactions 7(6): 766–775

Groppe K, Sanders I, Wiemken A and Boller T (1995) A microsatellite marker for studying the ecology and diversity

of fungal endophytes (*Epichloe* spp.) in grass. Applied and Environmental Microbiology 61: 3943–3949

Grünig CR, Sieber TN and Holdenrieder O (2001) Characterisation of dark septate endophytic fungi (DSE) using inter-simple-repeat-anchored polymerase chain reaction (ISSR-PCR) amplification. Mycological Research 105: 24–32

Huang HC, Morrison RJ, Muendel HH, Barr DJS, Klassen GR and Buchko J (1992) *Pythium* spp. group G a form of *Pythium ultimum* causing damping-off of safflower. Canadian Journal of Plant Pathology, 14: 229–232

Jenkins SF and Averre CW (1983) Root diseases of vegetables in hydroponic culture systems in North Carolina greenhouses. Plant Disease 67: 968–970

Kageyama K, Ohyama A and Hyakumachi M (1997) Detection of *Pythium ultimum* using polymerase chain reaction with species-specific primers. Plant Disease 81: 1155–1160

Kageyama K, Uchino H and Hyakumachi M (1998) Characterization of the hyphal swelling group of *Pythium*: DNA polymorphisms and cultural and morphological characteristics. Plant Disease 82: 218–222

Lévesque CA and de Cock AWAM (2004) Molecular phylogeny and taxonomy of the genus *Pythium*. Mycological Research 108 (12): 1363–1383

Linde C, Kemp GH and Wingfield MJ (1994) *Pythium irregulare* associated with *Pinus* seedling death on previously cultivated lands. Plant Disease 78: 1002–1005

Longato S and Bonfante P (1997) Molecular identification of mycorrhizal fungi by direct amplification of microsatellite regions. Mycological Research 101: 425–432

Martin FN (1990a) Variation in the ribosomal DNA repeat unit within single-oospore isolates of the genus *Pythium*. Genome 33: 585–591

Martin FN (1990b) Taxonomic classification of asexual isolates of *Pythium ultimum* based on cultural characteristics and mitochondrial DNA restriction patterns. Experimental Mycology 14: 47–56

Martin FN (1995a) *Pythium*. In: Eukaryotes, Vol. 2. (pp. 17–36) Pergamon Press, Oxford

Martin FN (1995b) Electrophoretic karyotype polymorphisms in the genus *Pythium*. Mycologia 87 (3): 333–353

Matsumoto C, Kageyama K, Suga H and Hyakumachi M (1999) Phylogenetic relationships of *Pythium* species based on ITS and 5,8 s sequences of the ribosomal DNA. Mycoscience, 40: 321–331

Meyer W, Mitchell TG, Freedman EZ and Vilgalys R (1993) Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish isolates of *Crytococcus neoformans*. Journal of Clinical Microbiology 31: 2274–2280

Mills D and McCluskey K (1990) Electrophoretic karyotypes of fungi: the new cytology. Molecular Plant Microbe Interactions 3(6): 351–357

Moorman GW, Kang S, Geiser DM and Kim SH (2002) Identification and characterization of *Pythium* species associated with greenhouse floral crops in Pennsylvania. Plant Disease 86: 1227–1231

Plaats-Niterink AJ, van der (1981) *Monograph of the genus Pythium*. Studies in Mycology. No. 21. Centraalbureau voor Schimmelcultures, Baarn, Netherlands

Rafin C and Tirilly Y (1995) Characteristics and pathogenicity of *Pythium* spp. associated with root rot of tomatoes in soilless culture in Brittany France. Plant Pathology, 44: 779–785

Rafin C, Brygoo Y and Tirilly Y (1995) Restriction analysis of amplified ribosomal DNA of *Pythium* spp. isolated from soilless culture systems. Mycological Research 99: 277–281

Rey P, Nodet P and Tirilly Y (1998a) *Pythium* F induce a minor but ubiquitous disease in tomato soilless cultures. Journal of Plant Pathology 79: 173–180

Rey P, Benhamou N and Tirilly Y (1998b) Ultrastructural and cytochemical investigation of asymptomatic infection by *Pythium* spp. Phytopathology 88: 234–244

Rey P, Leucart S, Désilets H, Bélanger RR, Larue JP and Tirilly Y (2001) Production of indole-3-acetic acid and tryptophol by *Pythium ultimum* and *Pythium* group F: possible role in pathogenesis. European Journal of Plant Pathology 107: 895–904

Salt GA (1979) The increasing interest in "minor pathogens". In: Soilborne Plant Pathogens (pp. 289–312) Academic Press, New York

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular Cloning: A Laboratory Manual. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Schurko AM, Mendoza L, Lévesque CA, Desaulniers NL, de Cock AWAM and Klassen GR (2003) A molecular phylogeny of *Pythium insidiosum*. Mycological Research 107: 537–544

Stanghellini ME and Kronland WC (1986) Yield loss in hydroponically grown lettuce attributed to subclinal infection of feeder rootlets by *Pythium dissotocum*. Plant Disease 70: 1053–1056

Stanghellini ME and Rasmussen SL (1994) Hydroponics a solution for zoosporic pathogens. Plant Disease, 78: 1129–1138

Tenzer I, Ivanissevich S, Morgante M and Gessler C (1999) Identification of microsatellite markers and their application to population genetics of *Venturia inaequalis*. Phytopathology 89: 748–753

Thinggaard K and Middelboe AL (1989) *Phytophthora* and *Pythium* in pot plant cultures on ebb and flow bench with recirculating nutrient solution. Journal of Phytopathology 125: 343–352

Viaud M, Pasquier A and Brygoo Y (2000) Diversity of soil fungi by PCR-RFLP of ITS. Mycological Research 104: 1027–1032

Wang PH and White JG (1997) Molecular characterization of *Pythium* species based on RFLP analysis of the internal transcribed spacer region of ribosomal DNA. Physiological and Molecular Plant Pathology 51: 129–143