FULL PAPER

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Structure and organization of the rDNA intergenic spacer region in *Pythium ultimum*

Abstract Nucleotide sequences of the rDNA intergenic spacer (IGS) region in *Pythium ultimum* were determined in 16 clones obtained from three isolates differing in production of sexual organs. Several sequences with different lengths were detected in each isolate, showing heterogeneity in the IGS region. In addition, several tandem repeat regions were detected in all the clones. The sequences, length, and number of each copy largely varied among repeat regions. Length heterogeneity arose from the complex combination of the number of copy within the repeat regions. Furthermore, the nucleotide sequence of each copy and the number of repetition varied not only between isolates but also between clones from an isolate. Based on the sequence similarity and the number of copies in repeat regions, specific patterns different between homothallic *P. ultimum* and the *Pythium* group HS (hyphal swellings) were recognized in a few regions. These results suggest that these two groups have slight genetic differences in the IGS region, although the differences in most of the repeat regions were not enough to identify each group.

Key words Heterogeneity · Intergenic spacer (IGS) · *Pythium ultimum* · Tandem repeat

Introduction

The genus *Pythium* belongs to the order Pythiales, and appropriately 150 species have been reported up to the present (Kirk et al. 2008). Identification of *Pythium* is mainly based on morphological features such as size and shape of sporangia or oogonia (van der Plaats-Niterink 1981). However, members of the *Pythium* are considered as a very difficult group for identification of species, because these characteristics are similar among species and are sometimes not produced when they are cultured on media. Molecular techniques have greatly enhanced species identification and description (Chen et al. 1992; Wang and White 1997; Matsumoto et al. 1999; Martin 2000; Lévesque and de Cock 2004; Kageyama et al. 2005).

Pythium ultimum Trow is an important plant pathogen causing severe diseases of various plants in the world, especially crop plants. Although *P. ultimum* has few morphological characteristics suitable for population studies, variation by formation of zoospores is known. The most common isolates of *P. ultimum* are homothallic and fail to produce zoospores (van der Plaats-Niterink 1981). In contrast, *P. ultimum* var. *sporangiiferum* Drechsler produces abundant zoospores at room temperature (van der Plaats-Niterink 1981). Some isolates of *Pythium* only form sporangia or hyphal swellings but do not form any reproductive organs in single or dual cultures. van der Plaats-Niterink (1981) defined five groups for these isolates based on morphological characteristics of the asexual stage. In this definition, isolates with hyphal swellings were grouped as group HS. Some reports suggest that several isolates classified as group HS belong to *P. ultimum* based on sporangial morphology, growth rate, restriction fragment length polymorphism (RFLP) of the rDNA internal transcribed spacer (ITS) region, and mitochondrial DNA and genomic DNA polymorphic analyses (Martin 1990; Kageyama et al. 1998). Saunders and Hancock (1994) also demonstrated that some isolates of group HS functioned as a male form and produced antheridia when paired with a homothallic *P. ultimum* isolate. On the other hand, differences between group HS and *P. ultimum* were shown in pathogenicity, colony type, and growth rate (O'Sullivan and Kavanagh 1992). Therefore, it is suggested that *P. ultimum* includes three types, although not all group HS isolates are *P. ultimum*. The genetic relationships among these three types have been reported. The phylogenetic difference between *P. ultimum* var. *ultimum* Trow and *P. ultimum* var. *sporangiiferum* was detected in the mitochondrially encoded cytochrome *c*

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oxidase subunit II gene (Martin 2000). In contrast, genetic relationships between *P. ultimum* var. *ultimum* and some *Pythium* group HS strains remain unclear. These two types were not genetically differentiated from each other based on the mitochondrial polymorphisms and sequencing, RFLP, and protein banding patterns (Adaskaveg et al. 1988; Martin 1990, 2000; Chen et al. 1992; Francis and St. Clair 1993; Francis et al. 1994), whereas a few HS strains differed from the *P. ultimum* var. *ultimum* isolates by differences of the mitochondrial DNA restriction patterns or sequences (Martin 1990, 2000).

Various regions of the nuclear rDNA have been extensively used for phylogenetic studies and taxonomy in many organisms. The rDNA gene is divided into coding regions such as large subunit (LSU), small subunit (SSU), 5.8S, two internal transcribed spacers (ITS1 and ITS2), and an intergenic spacer (IGS) region. The 5S gene is also located in the IGS region in some *Pythium* species with filamentous sporangia (Belkhiri et al. 1992). The IGS, composed of the nontranscribed spacer (NTS) and extratranscribed spacer (ETS), plays an important role in cellular processes including initiation and termination of transcription. The three coding regions evolved relatively slowly and are generally used to study distantly related organisms from kingdom to genera or species. The noncoding regions consisting of ITS and IGS evolved more rapidly and can be used for comparisons at the species or population level. The sequences of the regions are highly different between most *Pythium* species, although intraspecific variations have been recently reported in several species such as *P. arrhenomanes* Drechsler, *P. graminicola* Subraman., *P. helicoides* Drechsler, *P. irregulare* Buisman, *P. mercuriale* Belbahri, Paul & Lefort, *P. myriotylum* Drechsler, *P. sylvaticum* W. A. Campb. & F. F. Hendrix, and *P. ultimum* (Chen and Hoy 1993; Martin 1995; Matsumoto et al. 2000; Kageyama et al. 2005, 2007; Perneel et al. 2006; Belbahri et al. 2008). Sequences of the ITS were also different between *P. ultimum* var. *ultimum* and *P. ultimum* var. *sporangiiferum* (Lévesque and de Cock 2004), but the differences between *P. ultimum* var. *ultimum* and the *Pythium* group HS were not recognized (Matsumoto et al. 1999).

Although few studies have focused on the IGS of *Pythium*, two variations of this region have been reported in *P. pachycaule* Ali-Shtayeh (Belkhiri and Klassen 1996). Also, the heterogeneity of the region has been reported in *P. ultimum*, in which two different regions comparable with heterogeneity were detected (Buchko and Klassen 1990; Klassen and Buchko 1990). One region was located about 1 kilobase (kb) downstream of the 3′-end of the LSU and comprised a segment with multiple versions differing in size by as much as 0.9 kb. The other region was located near the center of the IGS and consisted of a family of fragments differing in length by increments of 385 bp. As shown in PCR amplification of the IGS region, this heterogeneity was also observed after digestion with *Hin*dIII, *Pst*I, or *Hinc*II because their restriction sites were located near each other between the two regions of heterogeneity. However, nucleotide sequences and structures of the IGS region responsible for the heterogeneity are not yet understood.

The objective of this study was to determine the complete nucleotide sequences of the IGS region of *P. ultimum* and identify the regions responsible for heterogeneity. In addition, we assessed whether this region is useful for characterization of differences in the production of sexual organs in pure cultures of *P. ultimum* by comparing the structures of IGS between and within isolates.

Materials and methods

Isolates

One hundred and nine isolates of *Pythium ultimum* were used for PCR-RFLP analysis. The isolates were obtained from soil samples in various regions of Japan by a baiting technique (Watanabe 1981) (Table 1). Among them, 71 isolates produced the sexual organs and were identified as *P. ultimum* var. *ultimum* according to the description (van der Plaats-Niterink 1981). Thirty-eight isolates failed to produce the sexual stage, so these isolates were considered as the *Pythium* group HS (van der Plaats-Niterink 1981).

DNA extraction

DNA of isolates was extracted from mycelium grown on potato dextrose agar (PDA). Mycelia were suspended in 10 µl lysis buffer [50 mM Tris-HCl, pH 8.0; 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0; 0.5% Tween 20] and homogenized by a pipette tip; then, 10 µl more of the lysis buffer and 0.01% Proteinase K were added. The mixture was vortexed and incubated at 37°C for 3 h. After the incubation, 30 µl sterile distilled water (SDW) was added, and the mixture was incubated at 95°C for 10 min, then stored at –20°C until used for analysis.

Table 1. Location of *Pythium ultimum* and group HS (hyphal swellings) isolates used in this study

| Species	Location	No. of isolates
P. ultimum	Hokkaido	1
	Aomori	3
	Fukushima	2
	Gunma	4
	Nagano	
	Ueda	6
	Karuizawa	16
	Minamimaki	11
	Fujimi[a]	8
	Asahi	5
	Shiojiri	8
	Minamiminowa	1
	Ina	5
	Toyama	1
Group HS	Hokkaido	5
	Aomori	3
	Nagano	
	Minamimaki[b]	2
	Minamiminowa	28
Total		109

[a]The strains UZ085 and UZ087 are included
[b]The strain UZ065 is included

Table 2. Primers used for polymerase chain reaction (PCR) and DNA sequencing of the rDNA intergenic spacers (IGS)

Primer	Sequence (5′→3′)	Reference
Q	ACGCCTCTAAGTCAGAATC	Belkhiri et al. (1992)
P	GGCTCCCTCTCCGGAATC	Belkhiri et al. (1992)
IGS1	CACAGTCGCTTTCGTACTCTC	This study
IGS2	CTGGAACATGTGCATATTCGCG	This study
IGS3	GAAGCACGACAAGTACCTCTC	This study
IGS4	TACATTAGTACCGACTCGTTC	This study
IGS5	CCTGTCATATCTGTTGACAG	This study
IGS6	GAGATATGTCCTTTGTGCTG	This study
T7	TAATACGACTCACTATAGGG	T7 Promoter Primer (Promega)
SP6	TATTTAGGTGACACTATAG	SP6 Promoter Primer (Promega)
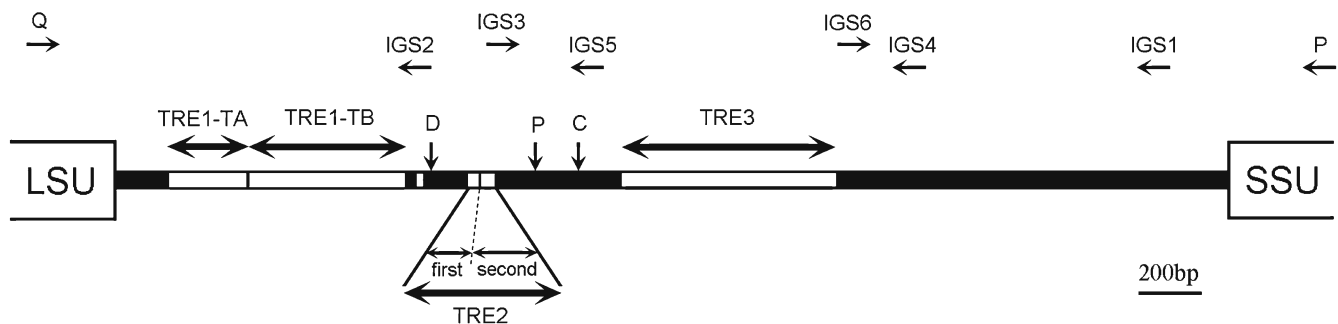
Fig. 1. Schematic diagram of the rDNA intergenic spacers (IGS) of *Pythium ultimum*. The locations of the primers used for amplifications and sequencing of the region are indicated on the map. Positions of all tandem repeat elements (TRE) are indicated as *white bars*; nonrepeat regions are indicated as *black bars*. The symbols for restriction sites are as follows: D, *Hin*dIII; P, *Pst*I; C, *Hin*cII. *LSU*, large subunit RNA; *SSU*, small subunit RNA. *Scale bar* shows approximate length because the total length of the IGS and the number and length of repeats within each repeat element are different between clones

PCR-RFLP analysis of the IGS

The IGS of *P. ultimum* was amplified with primers Q and P (Table 2, Fig. 1). Fifty microliters of the PCR reaction mixture contained 5.0 μl 10× Ex Taq buffer (20 mM Tris-HCl, pH 8.0; 100 mM KCl), 4.0 μl 2.5 mM dNTP mixture, 0.5 μM each primer, 1.25 units *Taq* DNA polymerase (Takara Bio, Shiga, Japan), and 3.0 μl template DNA. The PCR reactions were carried out in a PCR System 9700 (Applied Biosystems, Tokyo, Japan) according to the following amplification program: an initial denaturation at 95°C for 5 min followed by 30 cycles including denaturation at 95°C for 30 s, annealing at 64°C for 30 s and extension at 72°C for 2.5 min, and the final extension step at 72°C for 10 min. Visual quantification was made by comparison to a 500-bp DNA ladder (Takara Bio) following electrophoresis on a 0.8% agarose gel stained with ethidium bromide.

The amplified DNA of the IGS was used for RFLP analysis. Digestions with *Hin*dIII or *Pst*I (Takara Bio) were conducted according to the manufacturer's specifications, and the reaction fragments were electrophoresed in a 1.0% agarose gel followed by staining with ethidium bromide and visualization with UV light.

Cloning and sequencing of the IGS

For sequencing the IGS amplicons from the PCR products, the PCR products were electrophoresed on 0.8% agarose gel, separated from the agarose gel using an UltraClean 15 DNA Purification Kit (MO BIO Laboratories, Solana Beach, CA, USA), and cloned into the pGEM-T Easy Vector Systems (Promega, Madison, WI, USA). Positive colonies were placed in a separate test tube with Luria–Bertani (LB) broth and incubated overnight at 37°C while shaking. After approximately 12 h, plasmids were purified by QIAprep Spin Miniprep Kit (Qiagen, Tokyo, Japan), digested with *Pst*I, and quantified by visual comparison on a 0.8% agarose gel stained with ethidium bromide.

The terminal sequence of the IGS amplicon was determined using the T7 and SP6 vector primers (Promega; Table 2) from the cloning vector. Internal primers were subsequently designed from the obtained sequence data until the entire sequence of all clones was determined. All sequence reactions were performed with BigDye Terminator V3.1 Cycle Sequencing Reaction Kit (Applied Biosystems). The final volume of 20 μl was composed of 3.0 μl 5× sequencing buffer, 2.0 μl BigDye Terminator sequencing mix, 2.0 μl 1.6 μM primer, 0.7 μl DNA template, and 12.3 μl

Table 3. GenBank accession numbers of IGS sequences on clones of Pythium ultimum

Isolate	Clone number	Direction in the vector	GenBank accession no.
UZ065	5	Reverse	AB370091
	10	Reverse	AB370092
	12	Forward	AB370093
	22	Reverse	AB370094
	23	Forward	AB370095
	24	Forward	AB370096
UZ085	4	Forward	AB370097
	5	Reverse	AB370098
	9	Reverse	AB370099
	10	Forward	AB370100
	11	Forward	AB370101
	12	Forward	AB370102
UZ087	2	Forward	AB370105, AB370106
	5	Forward	AB370103
	7	Forward	AB370107, AB370108
	12	Reverse	AB370104

SDW. Reactions were performed in a PCR System 9700 (Applied Biosystems) with an initial denaturation at 96°C for 2 min, followed by 25 cycles of 96°C for 10 s, 55°C for 10 s, and 60°C for 4 min. The sequencing reaction mixtures were purified by ethanol precipitation and resuspended in 15 μl Hi-Di Formamide (Applied Biosystems), and the mixtures were run on an ABI 3130 Genetic Analyzer (Applied Biosystems). The IGS sequence data of all clones are deposited in GenBank (Table 3).

The IGS nucleotide sequences of all clones were aligned using the ClustalX program (Thompson et al. 1997). Based on the alignment, IGS regions were classified and then further analyzed separately.

Results

PCR-RFLP analysis of IGS

Amplification of the IGS region from 109 isolates of *P. ultimum* resulted in an incremental set of fragments ranging from approximately 3.5 to 7.0 kb (data not shown). Digestions with *Hin*dIII and *Pst*I of the IGS showed banding patterns similar to each other, although the length of the bands varied slightly by the difference of the locations of two restriction sites. *Hin*dIII digestions included an incremental set of fragments ranging from approximately 2.1 to 4.0 kb and some short fragments located between approximately 1.1 and 1.4 kb (Fig. 2). Incremental size was very uniform within each isolate, and similar patterns were shown among isolates. The length of dominant bands varied among isolates, but the differences did not correlate with morphological differences in formation of sexual organs.

Sequencing and designation of primers

Based on the differences in the RFLP patterns and the formation of sexual organs, three isolates, UZ065, UZ085, and UZ087, were selected and used for cloning and sequencing analysis to identify the regions responsible for heterogene-

Fig. 2. *Hin*dIII fragments produced by digestion of the rDNA IGS of three isolates (UZ065, UZ085, UZ087). *Lane M* indicates molecular size maker
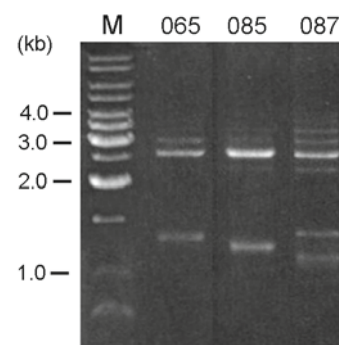
ity in the IGS (Fig. 2). These isolates were deposited in Microbiological Benebank, National Institute of Agrobiological Sciences (MAFF) as accession numbers MAFF241062, MAFF241063, and MAFF241064, respectively. The UZ065 isolate was from *Pythium* group HS, because the isolate abundantly formed hyphal swellings but could not produce sexual organs in a single culture. The others were from homothallic isolates of *P. ultimum*. The numbers of positive clones obtained from the IGS region were 11, 6, and 4 from UZ065, UZ085, and UZ087, respectively. Based on the length of *Hin*dIII-digested fragments, 16 clones that consisted of 6 each of UZ065 and UZ085 and 4 of UZ087 were selected and sequenced (Table 3). First, the nucleotide sequences near the 5′- or 3′-ends of the IGS were determined with the T7 or SP6 vector primers. Following alignment of the 3′-IGS sequences, the IGS1 reverse primer was designed, and subsequently an IGS4 reverse primer was also designed from the sequences determined with the IGS1 primer. On the basis of sequences determined with the IGS4 primer, an IGS6 forward primer was designed (see Fig. 1). On the other hand, tandem repeat regions with a large number of copies were recognized in the 5′-end of the IGS sequence. To determine the 5′-end of the IGS sequence, some clones were digested with *Pst*I, and self-ligated *Pst*I fragments were removed from the vector. The sequences of the internal regions of the IGS were determined in the products, and IGS2 reverse and IGS3 forward primers were designed (see Fig. 1). Furthermore, an IGS5 reverse primer
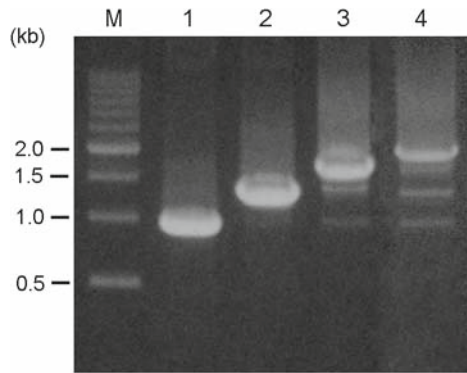
Fig. 3. Polymerase chain reaction (PCR) products produced by IGS3 and IGS4 primers. Each lane indicates a repetitive number (*1*, *2*, *3*, *4*, respectively), in TRE3. Two clones, UZ087–2 and -7, show the pattern of lane 4. *Lane M*, molecular size maker

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a1  GCAAGTCATTTTGCTATTATAGCTGGCTCGGCTTTCG-GTTCCTTTTCAGTTTGCTGAAAA-GCG  63
a2  ·····································T·C···························  63
a3  ·······················································A  63
b1  ······-G····T·G···--··----··----··················A··A  55
c1  C·········C··G·····T··············C·---·······T···  63
c2  C·········G·····T··············C·---·······T···  63
c3  C·········C·····T··············C·---·······T···  63
c4  C·········C·····A··············C·---·······T···  63
c5  C·········C·····A··············C·---·······A··A  63
d1  CA········C·····A·······T·C·········G·CA-A--····A··A  64
d2  C·········C·····A·······T·C·········G·CA-A--····A··A  64
d3  C·········C·····A···············G·CA-A--····A··A  64
d4  C·········GC····T···········T·C·········G·CA-A--····A··A  64
    ****  *** * *   **   ***   ***** * ** ****  *   * ***** **
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Fig. 4. Sequence alignment of 13 sequence types in TRE1-TA. Four distinct sequence types (a to d) and their variations (a1 to a3, c1 to c5, and d1 to d4) based on sequence homology are shown in the left margin. Lengths of each sequence type (bp) are shown on the right side
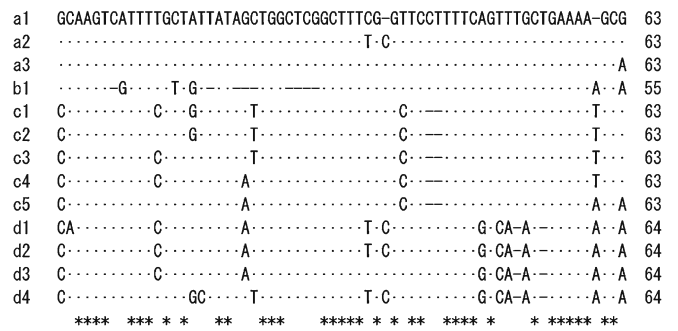
was designed from the sequences obtained by the IGS3 primer (Fig. 1).

Nucleotide sequence and structure of the IGS

Although the complete nucleotide sequences of the IGS were determined in most clones with the constructed primers, the sequences of the tandem repeat elements TRE3 (Fig. 1) were not clear in two clones, UZ087–2 and UZ087–7. Because further development of primers within the element was not successful, the numbers of the copy were estimated by PCR using the IGS3 and IGS4 primers. As a result, the two clones appeared to include four repetitions (Fig. 3). The total length of the IGS determined in all clones except for the above two clones ranged from 3019 bp (UZ065–22) to 3889 bp (UZ065–24).

The IGS region of *P. ultimum* contained three tandem repeat elements: TRE1, TRE2, and TRE3 (Fig. 1). In all clones, the first element, TRE1, was located 173 bp downstream from the 3′-end of the rDNA LSU. This element was further divided into two tandem repeat subelements, namely, TRE1-TA and TRE1-TB. The second element, TRE2, consisting of two shorter tandem repeat subelements, was located between the *Hin*dIII and *Pst*I restriction sites in all clones. The third element, TRE3, was located near the center of the IGS region in all clones (Fig. 1). Additionally, two repeated sequences of 28 bp located upstream of the *Hin*dIII site were recognized in only two clones of UZ085–5 and UZ085–11.

TRE1-TA consisted of three or four tandemly repeated copies of a 55- to 64-bp sequence in all clones. By estimating the homology among sequences of each copy, 4 distinct sequence types named from "a" to "d" were identified. Each sequence type included an additional sequence variation ranging from one to five nucleotide substitutions, so 13 sequence types in total were detected (Fig. 4). According to combinations of the sequence types, 9 combination types were recognized, and they were further grouped into four subelement groups (Fig. 5). Two groups were composed of the distinct sequence types "a," "d," and "c," and the other two groups were composed of "b," "d," and "c" (Fig. 5).

Fig. 5. Nine combination types within TRE1-TA based on the 13 sequence types shown in Fig. 4. Clone names are shown on the *left side* of each group. Two basic groups and one variation by combination of four subelement groups are shown on the *right side*

All the clones from UZ065 identified as the *Pythium* group HS belonged to the former two subelement groups, whereas all clones from UZ085 and UZ087 identified as homothallic *P. ultimum* to the latter two groups.

TRE1-TA was followed by TRE1-TB with tandemly repeated copies of a 36- to 39-bp sequence. The minimum number of copies was 9, in UZ087–5, and the maximum was 17 in three clones, UZ065–5, -12, and -24. In addition to the difference of the number of copies among clones, the sequence of each copy varied among or within clones, but no specific relationships were recognized between *Pythium* group HS and homothallic *P. ultimum*.

In TRE2, all clones included two tandem repeat subelements. The first subelement consisted of five or seven tandemly repeated copies of 4 or 5 bp. The copies were composed of four different short sequence types, each type named "α1" to "α4." The second subelement was adjacent to the first subelement and consisted of three to six tandemly repeated copies of 7 bp. The copies were composed of five different short sequence types, named "β1" to "β5." According to combinations of the four short sequence types in the first subelement and the five short sequence types in the second subelement, nine combination types were recog-

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Clone	Combination
UZ065 5, 12, 22	α1 α2 α2 α4 α2 ‖ β1 β2 β4 β2 β2
10, 23	α1 α2 α1 α2 α2 α4 α2 ‖ β1 β3 β2 β2 β2 β2
24	α1 α2 α2 α4 α2 ‖ β1 β5 β2 β2 β2
UZ085 4	α1 α2 α2 α4 α2 ‖ β1 β3 β2 β2
5, 9, 10, 11	α1 α2 α2 α4 α2 ‖ β1 β2 β2 β2
12	α1 α2 α2 α4 α2 ‖ β1 β2 β2
UZ087 2, 5	α1 α2 α2 α4 α2 α4 α2 ‖ β1 β2 β2
7	α1 α2 α3 α4 α2 α4 α2 ‖ β1 β2 β2
12	α1 α2 α2 α4 α2 α4 α2 ‖ β1 β4 β2 β2

α1: TACAA
α2: TACAG
α3: CACAG
α4: TACA
β1: CGCAAAT
β2: GCCAAAT
β3: GCCAAAC
β4: GCAAAAT
β5: GCCGAAT

Fig. 6. Nine combination types within TRE2 based on the four short sequence types (α1 to α4) and five short sequence types (β1 to β5). Each sequence of short sequence types is shown on the *right side* of the combination types. Clone names are shown on the *left side*

nized (Fig. 6). Specific relationships between *Pythium* group HS and homothallic *P. ultimum* were not recognized.

TRE3 was located near the center of the IGS region and appeared to have the elements previously reported (Buchko and Klassen 1990; Klassen and Buchko 1990). The lengths of the element varied among clones. Three distinct sequence types composed of long tandemly repeated copies were recognized and named "I" to "III." These three distinct sequence types were different in length: 366 bp, 270 bp, and 264 bp, respectively. In addition, types "I" and "III" included five ("Ia" to "Ie") and seven ("IIIa" to "IIIg") variations by nucleotide substitutions (Fig. 7). Type "II" was recognized in only one clone, UZ065–10. The 5′-sequences from 1 to 121 bp were nearly identical among the three distinct sequence types. Moreover, the nucleotide sequences to 236 bp were almost identical between types "I" and "II". On the other hand, the remaining sequence of type "II" was identical to type "III." The 96-bp sequence at the 3′-end of type "I" was present only in type "I" (Fig. 7). Five combination types were recognized based on comparison of three distinct sequence types (Fig. 8). The number of copies ranged from one to four in each clone. The last copy was type "III" or "II" in all clones, and only type "I" was repeated. Specific relationships between *Pythium* group HS and homothallic *P. ultimum* were not recognized in TRE3.

The nucleotide sequences of the remaining region ranging from the 3′-end of TRE3 to the 5′-end of the IGS were also aligned across clones. A few nucleotide substitutions or indels were present among clones from the same isolate as well as from different isolates. However, the nucleotide sequences showed high similarity both within and between isolates (data not shown).

Discussion

The present study demonstrates that the IGS region of *Pythium ultimum* includes three tandem repeat elements, TRE1, TRE2, and TRE3, and variations in the copy number of these elements are responsible for IGS size heterogeneity

as detected by PCR and RFLP analyses. Two previous studies showed that the IGS in *P. ultimum* is heterogeneous because of the presence of variant arrays of tandem repeats (Buchko and Klassen 1990; Klassen and Buchko 1990). However, sequences responsible for the tandem repeats were not clear, because the evidence presented was deduced from variable PCR and digested product size. Our study is the first report to determine the complete nucleotide sequence of the rDNA IGS region and demonstrate the structures causing intra-isolate variations of this region in *P. ultimum*. Furthermore, based on detailed examinations of some repeat elements detected in the IGS, relationships between *P. ultimum* and group HS strains are discussed.

PCR amplification of the IGS region produced a family of fragments in all isolates of *P. ultimum* and revealed that this region includes size heterogeneity. The sizes of these PCR products were up to approximately 7.0 kb, with the dominant fragments between 3.5 to 4.0 kb in all isolates (data not shown). The IGS sizes determined by sequencing were up to approximately 4.1 kb, so the dominant fragments could be inserted into the vector efficiently and analyzed in this study. In PCR-RFLP using *Hin*dIII, several dominant fragments were also detected. However, the fragment sizes estimated by the nucleotide sequence were overlapped among the tested clones from the same isolate, and no characters supporting the RFLP results were recognized. These facts suggested that nucleotide sequences of fragments except for dominant fragments were also analyzed in this study. Similarly, many more fragments than analyzed in this study are included in the IGS.

The full nucleotide sequences of the rDNA IGS region of three *P. ultimum* isolates revealed three tandem repeat elements: namely, TRE1, TRE2, and TRE3. Similarly, repeat elements in IGS have been identified from different organisms such as the swimming crab (Ryu et al. 1999) and *Lessingia* (Markos and Baldwin 2002), as well as in fungi such as *Verticillium dahliae* (Pramateftaki et al. 2000), *Trichophyton rubrum* (Jackson et al. 2000), *Trichophyton tonsurans* (Gaedigk et al. 2003), and *Lentinula edodes* (Saito et al. 2002). Three tandem repeat elements in *P. ultimum* varied from 55 to 64 bp (TRE1-TA) and 36 to 39 bp (TRE1-TB), from 4 to 7 bp (TRE2), and from 264 to 366 bp (TRE3).

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I a   AATGGCAATGCGAGTCCCGAGATTTGAGTGGTATAGGGGGTTATTCATCACGGGGAACACGCTCGTGCACTCGTATCAAGTCTCCTGTGAACGCATCATGG 100
I b   ·····················································································A·············
I c   ·································································································C··
I d   ····················································································································
I e   ···················G·················································································
Ⅱ     ····················································································································
Ⅲa    ····················································································································
Ⅲb    ····················································································································
Ⅲc    ····················································································································
Ⅲd    ····················································································································
Ⅲe    ····················································································································
Ⅲf    ·············································C·····················································
Ⅲg    ····················································································································
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I a   TCAAACGCGCGTTTTCGTGCTCCAAAAGTGGTACCATGGTATCAAATGTTGGAGGTTTTTGCGAGCTGGCGGGCTAGGGGTG-ACACGTAGACCACCAGA 199
I b   ····················································································································
I c   ····················································································································
I d   ····················································································C·············
I e   ····················································································································
Ⅱ     ···················································································································· 199
Ⅲa    ······A············——···A··—······AT·TG·GTT···—·············A·TTT·A··ACA·CT··AC·C····—··C········· 193
Ⅲb    ······A············——···A··—······AT·TG·GTT···—·············A·TTT·A··ACA·CT··AC·C····—····C·······
Ⅲc    ······A············——···A··—·····AT·TG·GTT·T—·············A·TTT·A··ACA·CT··AC·C······C·······
Ⅲd    ······A············——···A··—·····AT·TG·GTT·T—·············A·TTT·A··ACA·CT··AC·C······C·······
Ⅲe    ······A············——···A··—·····AT·TG·GTT···—·············A·TTT·A··ACA·CT··AC·C······C·······
Ⅲf    ······A············——···A··—·····AT·TG·GTT···—·············A·TTT·A··ACA·CT··AC·C······C·······
Ⅲg    ······A············——···A··—·····AT·TG·GTT···—·············A·TTT·A··ACA·CT··AC·C·····—·AC··G·····
      ***** ************** *** *** **** * *   * * *********** *   * **   * **  * **** **  ** *****

I a   TGTTTTTGCAATCGTATTGTCCTACTTGCGTACATATATTTAGAGGCCAGCAGCGTGTCGAAATGTTTTCTGGATCAAAAGTTAGGACCATTTGCGTCAA 299
I b   ····················································································································
I c   ····················································································································
I d   ····················································································································
I e   ····················································································································
Ⅱ     ···················································G·····CC···············T·C········ 270
Ⅲa    ···················································G·····CC···············T·C········ 264
Ⅲb    ···········································G····G·····CC···············T·C········
Ⅲc    ····································C······G····G·····CC···············T·C········
Ⅲd    ···········································G····G·····CC···············T·C········
Ⅲe    ···········································G····G·····CC···············T·C········
Ⅲf    ···········································G····G·····CC···············T·C········
Ⅲg    ···········································G····G·····CC···············T·C········
      ****************************** **** ****** ************ * ********

I a   AAGTTGGAGTGAAATGGTGAGCAGGTCGTGGTCGCTCGGACGTTGGCGCGGCCCACCGATTGTGCTA 366
I b   ··········································································
I c   ··········································································
I d   ··········································································
I e   ··········································································
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Fig. 7. Sequence alignment of 13 sequence types in the TRE3. Three distinct sequence types (I to III) and their variations (Ia to Ie and IIIa to IIIg) based on sequence homology are shown on the *left side*. Lengths of each sequence type (bp) are shown on the *right side*

Different sizes of repeat elements were previously reported in organisms other than *P. ultimum* (Ryu et al. 1999; Gaedigk et al. 2003). Based on the sequence similarities among the repeat elements, Ryu et al. (1999) demonstrated that each repeat element of the swimming crab (*Charybdis japonica*) derived from one common prototype sequence. However, the low sequence similarities among the three tandem repeat elements suggest that these elements occurred independently in *P. ultimum*.

Nucleotide sequences of the rDNA ITS are currently used to species-level identification in the genus *Pythium*, because they are highly variable among species. ITS and *cox*II gene sequences were different between the two varieties of *Pythium*, var. *ultimum* and var. *sporangiiferum*
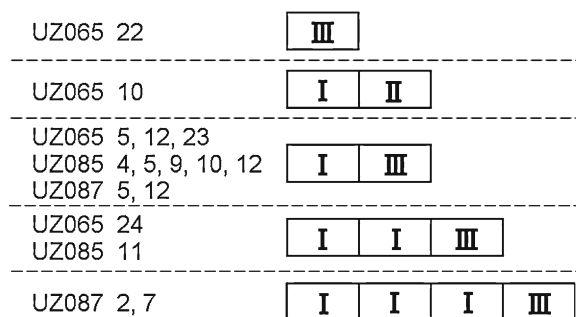
Fig. 8. Grouping of each copy within TRE3 based on the three distinct sequence types shown in Fig. 7. Clone names are shown on the *left side* of each group

(Martin 2000; Lévesque and de Cock 2004). On the other hand, the ITS sequence of *Pythium* group HS, defined as a hyphal swelling producer, was identical to that of *P. ultimum* var. *ultimum* (Matsumoto et al. 1999), although only one isolate of each group was investigated. Additionally, even though intraspecific variations in the ITS have been found in *P. ultimum* var. *ultimum* isolates from different hosts and geographic origins (Kageyama et al. 2007), no difference was detected between *P. ultimum* var. *ultimum* and group HS isolates. Similarly, no sequence variation was identified within the ITS region of the three isolates used in this study (data not shown). Martin (2000) indicated that *P. ultimum* var. *ultimum* and group HS isolates were placed in the same clade, except for two of four HS isolates tested, based on the *cox*II gene analysis. However, the differences observed in the two HS isolates were much lower than the interspecific differences between different species. Therefore, some isolates defined as *Pythium* group HS may be variations of *P. ultimum* having only an asexual stage in their life cycle.

In other organisms, the IGS region is frequently used not only for species identification but also for strain differentiation, because the region usually evolved faster than the ITS (ex. Jackson et al. 2000). In this study, therefore, the IGS sequence was investigated to determine if it would be effective to distinguish *P. ultimum* var. *ultimum* and *Pythium* group HS, having identical ITS sequences. IGS of these isolates showed heterogeneity with high length variation, and structures of the region largely varied among clones not only from different isolates but also from the same isolate. These results revealed that the IGS region could not be a useful tool to easily distinguish between *P. ultimum* var. *ultimum* and *Pythium* group HS. However, one tandem repeat subelement, TRE1-TA, distinguished the *Pythium* group HS (UZ065) from *P. ultimum* var. *ultimum* (UZ085 and UZ087). Although further examinations based on more isolates of both groups are needed, it is suggested that these two groups have slight genetic differences.

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