Organization and evolution of ribosomal RNA gene families in the genus *Pythium*

by

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ORGANIZATION AND EVOLUTION OF RIBOSOMAL RNA GENE FAMILIES IN THE GENUS *Pythium*

BY

ABBES BELKHIRI

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

A method was devised for locating 5S rRNA genes with respect to other rRNA genes in *Pythium* species using the polymerase chain reaction. The 5S rRNA gene was found in the nontranscribed spacer (NTS) of the rDNA repeat of *Pythium* species with filamentous zoosporangia, but with only several exceptions it was absent from the rDNA repeat of those species which have globose or unknown zoosporangia. When present in the NTS, the gene was located about 1 kb downstream of the large-subunit rRNA gene and on the strand opposite to that on which the other rRNA genes were located. When not in the NTS, the 5S rRNA genes were found organized in tandem arrays, unlinked to the rDNA. Oomycetes related to *Pythium* were also found to have the 5S gene in the NTS, although sometimes in the opposite orientation. This indicates that the presence of the gene in the NTS may be the ancestral arrangement for the Oomycetes.

The intergenic regions (IGRs) between adjacent 5S genes in tandem arrays were fully characterized in three *Pythium* species with globose and unknown zoosporangia. In *P. ultimum* and *P. spinosum*, a large proportion of the IGR consisted of simple sequences such as CA dinucleotide repeats near the 5′ end and a GT-rich region near the 3′ end. In *P. irregulare*, however, the 5S IGR does not have CA or GT rich regions, nor does it have a significant amount of simple sequence.

A novel 5S rRNA gene arrangement was detected and characterized in the rDNA repeat of *Pythium pachycaule*. Here the NTS exists in two versions, one being 200 bp longer than the other one. Both versions contain a conserved 5S rRNA gene in the inverted orientation at the predicted location and an adjacent 5S pseudogene positioned as a tandem repeat with a spacer of about 180 bp between them. The relationship between the functional 5S genes and the pseudogenes suggest that the 5S sequence family of *P. pachycaule* originated by duplication of an ancestral gene, followed by divergence. After length heterogeneity was introduced, divergence of both pseudogenes continued, resulting in the current organization.

In order to characterize the 5S tandem arrays in *Pythium irregulare*, a lambda EMBL3 genomic library was constructed and an intact array of 5S genes was characterized. This array comprises 9 possible functional 5S rRNA genes separated by conserved 541 bp spacers, and flanked at one end by a possible 5S pseudogene. Sequencing of numerous subclones of 5S genes derived by amplification from the lambda clone indicate that the genes in the array have identical sequences and suggests that the repeats in the array are highly homogeneous.

For phylogenetic studies, 24 isolates representing 18 *Pythium* species, and one isolate of *Phytophthora cryptogea*, were analyzed by RFLP analysis of a variable region of the

large subunit ribosomal RNA gene (LSrRNA). The results indicate that *Pythium* species with filamentous zoosporangia form a relatively tight monophyletic cluster distant from other species.

To go beyond the RFLP data to a more definitive approach, the 5S rRNA gene was sequenced in 31 isolates representing 27 *Pythium* species, and one isolate of *Phytophthora cryptogea*. The 5S rRNA sequence in all isolates studied comprises 118 bp with only two variable positions-39 and 107. Generally, these two "signature" positions appear to confirm the phylogenetic split between *Pythium* species with filamentous zoosporangia and those species with globose and unknown zoosporangia. *P. periplocum*, which has filamentous zoosporangia, however, seems to group with those species with globose and unknown zoosporangia, suggesting that convergent evolution has occurred.

Partial external transcribed spacer (ETS) sequences from 26 isolates representing 15 *Pythium* species were used for phylogenetic analysis, and this allowed partial resolution of the relationships within the filamentous zoosporangial group that was included. In addition, *P. tardicrescens*, a species that is difficult to classify because it does not produce zoospores, is placed within a monophyletic group which also includes *P. arrhenomanes*, *P. aristosporum* and *P. volutum*. One of the species with globose zoosporangia, *P. hypogynum*, was placed with the filamentous

zoosporangial group on the basis of ETS sequence, and thus appears to be a case of convergent evolution with respect to zoosporangial form. And although *P. vanterpoolii* is morphologically similar to *P. torulosum*, it appears to be phylogenetically the most distant from all other *Pythium* species with filamentous zoosporangia.

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LIST OF ABBREVIATIONS

A	adenine
bp	base pairs
C	cytosine
CTAB	hexadecyltrimethyl ammonium bromide
cm	centimetre
DNA	deoxyribonucleic acid
dATP	2'-deoxyadenosine 5'-triphosphate
DMSO	dimethyl sulfoxide
dNTP	2'-deoxyribonucleoside 5'-triphosphate
EDTA	ethylenediamine-tetra-acetic acid
ETS	external transcribed spacer
Fig(s)	figure(s)
G	guanine
g	gram(s)
h	hour(s)
ICR	internal control region
IGR	intergenic region
INS	insertion
IPTG	isopropylthiogalactoside
ITS	internal transcribed spacer
kb	kilobase pairs
L	litre(s)
LSrRNA	large subunit ribosomal RNA
mg	milligram(s)

min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
nm	nanometre
NTS	nontranscribed spacer
PCR	polymerase chain reaction
pfu	plaque forming unit(s)
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
rDNA	ribosomal DNA
RNA	ribonucleic acid
rRNA	ribosomal RNA
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SSC	sodium saline citrate
SSrRNA	small subunit ribosomal RNA
T	thymine
U	uracil
μg	microgram(s)
μl	microlitre(s)
μm	micrometre(s)
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

INTRODUCTION

The rRNA genes are universal and their products are functionally and evolutionarily homologous in all organisms (Bruns *et al.* 1991). In the nuclear genome of eukaryotes, the rDNA generally consists of tandemly repeated units comprising the 18S, 5.8S, and 28S rRNA genes which are separated by transcribed and nontranscribed spacers. The 5S gene, however, is transcribed separately and may or may not be a component of the rDNA repeat unit. As in plants and animals the 5S genes may be organized in tandem arrays unlinked to the rDNA repeat (Brown and Sugimoto 1973; Gerlach and Dyer 1980), dispersed throughout the genome in a complex pattern as in several ascomycetous fungi (Selker *et al.* 1981; Bartnik *et al.* 1986), or linked to the rDNA repeat unit as in some other fungi (reviewed in Gerbi 1985). Except for its detection in the rDNA of *Achlya ambisexualis* (Rozek and Timberlake 1979), 5S gene family organization has not been characterized in the class Oomycetes.

Based on nutrition and mycelial form, Oomycetes are considered to be fungi. However, sequences of the 18S rRNA gene have shown that these organisms are unrelated to the true fungi (Gunderson *et al.* 1987; Förster *et al.* 1990). It has also been reported that the structure of the NTS in *Pythium ultimum* is as in plants and not as in fungi (Klassen

and Buchko 1990). Extending our knowledge of 5S rRNA gene organization in Oomycetes may shed some light on the evolution of this gene and on that of the rRNA gene family in a relatively unexplored part of the phylogenetic world.

The rDNA has been used extensively in phylogeny becaused it constitutes a significant component of the nuclear genome, and high yields can be recovered from all organisms. In addition, some portions of the rDNA sequence are fairly variable, while others are highly conserved across the primary kingdoms. While conserved regions are useful for mapping and designing primers for PCR (Bruns *et al.* 1991), it has also been suggested that they are useful for assessing affinities above the genus level, while the more variable regions of the rDNA such as the transcribed and the nontranscribed spacers could be adequate to resolve inter- and intraspecific relationships (Hillis and Dixon 1991).

Pythium, which is the largest genus in the class Oomycetes, comprises more than 120 described species (Dick 1990) that are economically important due to their consistent association with root diseases of various plants such as cereals and sugar cane (Plaats-Niterink 1981). However, the phylogeny of the genus has not yet been resolved, although there have been several preliminary studies (Chen and Hoy 1993; Chen *et al.* 1992; Martin and Kistler 1990). Thus the aim of this study was to investigate

whether the charateristics the ribosomal gene family could be used to resolve the phylogenetic relationships amongst the various *Pythium* species.

LITERATURE REVIEW

Early history and discovery of 5S rRNA

In the late 1950s the ribosome was being studied intensively, mainly by ultracentrifugation. The small subunit and the large subunit of the ribosome had been found to comprise the 16-18S and the 23-28S rRNA molecules, respectively, and soluble rRNA (transfer RNA) had also been discovered. Hamilton and Petterman (1959) noticed for the first time a low molecular weight particle in ultracentrifuge gradients of rat liver microsomes, and based on its size they called it "5S". A similar particle was dissociated from the *E. coli* 50S subunit, and although it had none of the properties of tRNA, the hypothesis was that it was a species of ribosome-bound tRNA (Elson 1961, 1964). The 5S rRNA was identified in the eukaryotes *Blastocladiella emersonii*, KB cells, and HeLa cells, and again identified as a species of tRNA or a tRNA precursor (Comb and Katz 1964; Galibert *et al.* 1965). Careful characterization and attempted hybridization of tRNA to 5S demonstrated that 5S and tRNA were unrelated (Rosset *et al.* 1964; Zehani-Willner and Comb 1966). Brown and Littna (1966) fully characterized the 5SrRNA, short of sequence determination, in *Xenopus laevis*, and found it was expressed coordinately with the other rRNA species. The nucleotide sequence of 5S RNA was determined in *E. coli* and KB cells (Brownlee *et al.* 1967; Forget *et al.* 1967), HeLa cells (Hatlan *et al.* 1969), and *Saccharomyces carlsbergensis* (Hindley and Page 1972). The

genes encoding for 5S rRNA were detected and quantified first in *X. laevis* by hybridization to 5S rRNA (Brown and Dawid 1968). Different classes of 5S genes in oocytes and somatic cells of *X. laevis* were detected, and a high level of inferred sequence heterogeneity was observed, mainly in the oocyte genes (Mairy and Denis 1971; Wegnez and Monier 1972) and between kidney and ovary cells (Ford and Southern 1973). Hybridization and electron microscopy studies showed that the 5S gene family in *Xenopus* species consisted of about 24,000 genes in a number of arrays and on different chromosomes within which the gene and the spacer (1 to 5 size ratio) alternated regularly with uniform lengths but with some sequence heterogeneity in the spacer (Brown *et al.* 1971; Pardue *et al.* 1973). A similar arrangement was found in HeLa cells (Aloni *et al.* 1971), but in *Drosophila melanogaster*, while the genes were in tandem, they were found in a single array (Wimber and Steffensen 1970). Brown and Sugimoto (1973) compared two closely related species of *Xenopus* (*X. laevis* and *X. mulleri*) and found great differences in the 5S gene family sizes (24,000, 9000), and in the repeat unit size (840, 1700 bp); they also observed no homology between spacers. Pardue *et al.* (1973) located the 5S genes near the telomere of most *X. laevis* chromosomes, suggesting that all of the genes would be clustered during crossing over to facilitate recombination between 5S arrays on different chromosomes. Sequence studies

of the rDNA repeat in *X. laevis* revealed that the 710 bp repeat consisted of a gene (121 bp), a short linker, a pseudogene (the first 101 bp of the gene with about 9 substitutions), and a 400 bp A-T rich spacer composed of 15 bp imperfect tandem repeats (consensus: AAAACTCAAACTTTG) (Brownlee *et al.* 1974; Jacq *et al.* 1977). Using restriction enzyme analysis, individual organisms were shown to have length heterogeneity due to variation in the number of these short repeats in the spacer (Carroll and Brown 1976a, 1976b). Similar analysis of *Drosophila melanogaster* revealed a shorter repeat unit (380 bp), smaller family size (165 copies), and less dispersion, and much less heterogeneity (Hershey *et al.* 1977). It was also shown that in *Saccharomyces cerevisiae*, there are about 150 copies of the rDNA repeat and that each repeat includes a linked 5S gene (Rubin and Sulston 1973). With the arrival of Maxam and Gilbert sequencing in 1977, the 5S gene and its flanking regions in *S. cerevisiae* became the first regions to be sequenced by this method (Valenzuela *et al.* 1977a, 1977b; Maxam *et al.* 1977).

The structure and expression of 5S rRNA gene

Weinmann and Roeder (1974) found that transcription of the 5S gene (118-121 bp) was performed, not by RNA polymerase I like the other rRNA genes, but by RNA polymerase III. In order to function at maximum efficiency,

the cell must coordinate the synthesis of ribosomal proteins, rRNA , and 5S rRNA needed for biogenesis of the ribosome. The 5S RNA is found as part of the rRNA precursor in prokaryotes, but in eukaryotes the 5S rRNA genes may be physically unlinked from the rDNA. One mechanism to ensure molar equality of the ribosome components, is to have a similar number of repeated copies of 5S rRNA genes and rRNA genes; *D. melanogaster* has about 160 copies of 5S rRNA genes and a comparable number of rDNA repeat units per haploid genome (Ritossa and Spiegelman 1965; Vermeulen and Atwood 1965; Tartof and Perry 1970; Quincey 1971), while *Drosophila hydei* has 320 5S rRNA genes per haploid genome (Renkawitz-Pohl 1978) and 250 rDNA repeat units (Henning and Meer 1971; Schafer and Kunz 1976). In other cases, however, the number of gene copies of 5S rRNA differs drastically from that for rDNA repeats. Humans have about 2000 copies of 5S rRNA genes per haploid genome (Hatlen and Attardi 1971), but less than 200 copies of rDNA (Schmickel 1973; Gaubatz and Cutler 1975; Henderson *et al.* 1976; young *et al.* 1976). This excessive 5S rRNA gene copy number relative to rDNA may indicate that either all the 5S rRNA genes are not transcribed or the excess amount of 5S rRNA that is made may simply be degraded to ensure equimolar amounts of 5S rRNA and rRNA (Gerbi 1985). The fact that in *X. laevis* embryos, 5S rRNA synthesis occurs at the blastula stage independently of rRNA synthesis, the latter does not begin until gastrulation,

demonstrates that 5S rRNA transcription can be independent of rDNA control (Miller 1973, 1974).

In *X. laevis* there are two types of multigene families that encode 5S rRNA; 400 somatic-type 5S rRNA genes (Peterson *et al.* 1980) are located on one chromosome within a cluster of oocyte-type 5S rRNA genes (Harper *et al.* 1983) which are also found on the telomeres of all chromosomes; this adds up to 20,000 gene copies (Pardue *et al.* 1973; Brown and Sugimoto 1973; Peterson *et al.* 1980). Both types of 5S rRNA are synthesized in oocytes, but the oocyte-type 5S rRNA genes are not expressed in somatic cells (Gerbi 1985). After fertilization and development of the embryo, the oocyte-type 5S rRNA genes are repressed, whereas the somatic-type genes remain active (Wolffe and Brown 1988). Based on deletion analysis in *X. laevis*, it was shown that a control region in the center of the 5S gene (residues 50-55 to 80-83) was needed to direct specific initiation of transcription (Bogenhagen *et al.* 1980; Sakonju *et al.* 1980, 1981). Oligonucleotide-directed mutagenesis within the 5S gene internal control region (ICR) has shown that the promoter extends discontinuously from residues 50 to 97 of the 5S gene, and based on *in vitro* transcription studies, three separate promoter elements were identified. Box A element (residues 50-64) has a relatively low affinity for the transcription factor IIIA (TFIIIA) and is involved in the binding of TFIIIC; the intermediate element (residues

67-72) and box C (residues 80-97) are the main determinants of affinity for TFIIIA (Pieler et al. 1985a, 1985b; Pieler *et al*. 1987). Initiation of transcription is facilitated by binding TFIIIA to the internal control region (Sakonju and Brown 1982). Due to base differences at positions 53 and 55 between the two 5S rRNA species, it was demonstrated that somatic 5S rRNA genes bind four times tighter to TFIIIA than to oocyte 5S rRNA genes (Wormington *et al*. 1981). It has been suggested that a stable transcriptional complex which involves transcription factors TFIIIA, B, and C, prevents repression of the 5S rRNA gene by chromatin structure (histone H1) (Brown 1984; Schlissel and Brown 1984). When oocyte 5S rRNA genes become unoccupied by transcription factors, a repressive chromatin structure forms and subsequently the binding of the factors is excluded (Wolffe and Brown 1988).

Based on 5′ and 3′ deletion analysis it was shown that the *S. cerevisiae* 5S gene ICR does not extend outside residues 57 to 99 (Taylor and Segall 1985). Unlike *X. laevis*, the transcription initiation site of *Neurospora crassa* 5S genes is determined primarly by a TATA box located at residue 29 (Tyler 1987). It has also been demonstrated that the transcription rate of 5S genes is reduced to 2 to 30-fold when the normal TATA box is deleted. The ICR of *N. crassa* 5S rRNA genes is subdivided into three distinct sub-regions. The A (residues 44-57) and C (residues 73-103)

regions, correspond to ICR (A and C boxes) of *X. laevis* 5S genes (Tyler 1987). Although the structure of *Xenopus* and *Neurospora* A elements is similar, they have a fundamental functional difference. In *X. laevis*, the A box plays a primary role in fixing the start point of transcription of 5S rRNA gene (Hall *et al.* 1982; Ciliberto *et al.* 1983). In *N. crassa* 5S genes, however, the TATA box has this function (Tyler 1987). The D region, the third element of the *N. crassa* 5S gene ICR, has no *Xenopus* corresponding region. Its function, however, seems to be related to those of A and C elements. A possible function for the D region is that it may be essential for the interaction of the transcription factors bound to the TATA box and other elements (Tyler 1987).

Fox and Woese (1975) proposed the general secondary structure of prokaryotic 5S rRNA which contains four helices, referred to as the molecular stalk, the prokaryotic loop, the tuned helix, and the common arm base. Based on comparative analyses of 5S rRNAs (Studnicka *et al.* 1981; Mackay *et al.* 1982; Delihas and Andersen 1982; Kuntzel *et al.* 1983) another model which includes a fifth helix for eubacteria was proposed (Wolters and Erdmann 1988). The common secondary structure of eukaryotic 5S rRNA which is composed of five helices, A to E, connected by loops a to e, was proposed by Nishikawa and Takemura (1974). The presence of helix D in eubacteria has been confirmed biochemically by

MacDonell and Colwell (1985), and Wolters and Erdmann (1988). The length and base-pairing structure of this region remain the major difference between eubacteria and eukaryotes as well as between different groups of archaebacteria (Wolters and Erdmann 1988).

Certain regions within the 5S rRNA exhibit remarkable conservation in sequence and secondary structure among diverse species. Some functional constraints may play a role in preserving certain sequences whose alteration would impair the 5S rRNA function. Compensating substitutions may also impose structural constraints upon other regions to maintain base-paired stems as part of the 5S rRNA secondary structure (Gerbi 1985). Comparative analysis of three species of *N. crassa* 5S rRNA sequences (α, β, γ) showed that the differences occur in two regions (residues 12-20 and 59-70). The overall secondary structure for the three 5S rRNA species, however, is conserved; all but one of the sequence differences are compensated (Selker *et al.* 1981). Extensive secondary structure analyses have been carried out on 5S rRNA sequences for prokaryotes (Wolters and Erdmann 1988) and eukaryotes (Qi *et al.* 1988; Hendriks *et al.* 1986; Wolters and Erdmann 1988).

5S rRNA gene location and organization

1- Prokaryotes

Based on hybridization studies, seven rDNA operons

were detected in *E. coli*, a representative of the eubacteria (Kiss *et al.* 1977). Each of these operons contains a 5S rRNA gene downstream of the 23S rRNA gene, and it is regulated by two promoters found upstream of the 16S rRNA gene generating a 30S RNA precursor. In other words, the 5S gene is part of one transcription unit comprising the other rRNA genes (Morgan 1982).

A similar gene arrangement was also found in *Halobacterium halobium*, a member of archaebacteria, but only a single operon was detected in the genome (Hofman *et al.* 1979). However, a common rRNA precursor has not been found, and the possibility remains that each of the rDNA genes may have its own promoter (Hofman *et al.* 1979). Four rDNA operons and a single unlinked 5S rRNA gene were found in the archaebacterium *Methanococcus vannielii* (Jarsch *et al.* 1983). It would seem that the 16S, 23S, and 5S rRNA genes were once part of a single transcription unit, as in *E. coli*, but later became independent and, possibly, each obtained its own promoter (Gerbi 1985).

2- Mitochondria

The 5S rRNA gene has been lost from the mitochondria of most species of fungi and animals (Borst and Grivell 1971, O'Brien and Matthews 1976; Buetow and Wood 1978). The loss of 5S gene in the mitochondrial genome raises the question of its function in the ribosome. Thurlow *et al.* (1984)

speculated that the 5S rRNA may be substituted for by an insertion in domain V of the large rRNA of yeast mitochondria. This insertion was not found in the same location in human mitochondrial rRNA, but it has been suggested that a 23 -nucleotide remnant of the 5S gene may function as the 5S rRNA gene (Nierlich 1982).

The mitochondria of flowering plants, however, have the 5S rRNA gene (Leaver and Harmey 1976; Cunningham and Gray 1977). The 5S gene was located close to the 18S rRNA coding region, in the mitochondrial genome of both maize and wheat (Bonen and Gray 1980; Bonen *et al.* 1980; Stern *et al.* 1982).

3- Chloroplasts

The organization of rRNA genes in chloroplasts has been characterized in several flowering plants (Palmer and Thompson 1982; Kossel *et al.* 1983). The rRNA genes are found in the order 5'-16S-23S-5S-3', and are usually organized in an inverted repeat of about 22-25 kb. Based on restriction map alignments it was concluded that one of the repeat units was lost in the chloroplast of pea (Kolodner and Tewari 1979; Palmer and Thompson 1981) and broad bean (Koller and Delius 1980). In the chloroplast genome of *Euglena gracilis*, however, three tandem direct repeats of the rDNA operon and an additional copy of the 16S rRNA gene were detected (Gray and Hallick 1978; Jenni and Stutz 1978; Rawson *et al.* 1978). Unlike the case in *E. coli*, the 5S rRNA has not been found

as part of the chloroplast rRNA transcript (precursor), indicating that it has its own promoter (Hartley 1979; Kossel *et al.* 1982). The chloroplast 5S rRNA gene has been investigated in spinach (Romby *et al.* 1988; Romby *et al.* 1991), a red algae; *Porphyra umbilicalis* (Van den Eynde *et al.* 1988), and a brown algae; *Pylaiella littoralis* (Somerville *et al.* 1992).

4- Eukaryotes

The large rRNA genes in the nuclear genome of eukaryotes are organized in repeated tandem units. Generally the repeating unit consists of the three main genes in a fixed order; 5'-18S-5.8S-28S-3'. The three genes are preceded by the external transcribed spacer (ETS) which comprises a common promoter involved in transcribing a 45S rRNA precursor (Long and Dawid 1980b). In Eukaryotes the 5S rRNA genes may be either linked to the other rRNA genes, dispersed in the genome as single copies, or dispersed as tandem arrays outside the rDNA repeats.

4.1- 5S rRNA gene linked with respect to rRNA genes

In some fungi and protozoa the 5S rRNA gene is within the rDNA repeat unit, and located in either orientation downstream of the 28S rRNA gene (Gerbi 1985). The direction of transcription of 5S gene may differ within the same genus. The basidiomycetous fungus *Coprinus cinereus* has the

5S gene transcribed in the same direction as the other rRNA genes, whereas in *Coprinus comatus* it is in the opposite orientation (Pukkila and Cassidy 1987). A survey of the 5S gene-linked arrangement is summarized in Table 1.

The fact that the 5S rRNA gene in eukaryotes had gained its own promoter and RNA polymerase, made it independent of the other rRNA genes and able to move to either of the DNA strands or leave the rDNA cluster altogether (Gerbi 1985).

4.2- 5S rRNA genes dispersed as single copies

In most Ascomycota (true fungi) the 5S rRNA genes are not part of the rDNA cluster, but are dispersed as single copies throughout the entire genome (Gerbi 1985). Free *et al.* (1979) characterized the rDNA repeat unit of *N. crassa* and found that the 5S rRNA gene was unlinked. Intensive restriction and hybridization analyses of several 5S gene library clones indicated that *N. crassa* has heterogeneous and homogeneous dispersed 5S genes. Five different 5S gene types (α, β, β', γ and δ) were detected, and analysis of 5S rRNA showed that it consists mainly of one form, the α-type (Selker et al. 1981). Based on the secondary structure analysis of α, β, and γ 5S rRNAs it was concluded that the overall structure and function is maintained because about 80% of the substitutions are compensatory (Selker *et al.* 1981). It is likely that the relatively large number of compensating substitutions in *N. crassa* 5S rRNA exist because of the dispersed arrangement of 5S genes. In other

Table 1. Organisms where the 5S rRNA genes are linked to the rDNA

Organism	Orientation*	Reference
Basidiomycetes		
Coprinus cinereus	+	Pukkila & Cassidy (1987)
Coprinus atramentarius	+	Pukkila & Cassidy (1987)
Coprinus micaceus	+	Pukkila & Cassidy (1987)
Coprinus comatus	-	Pukkila & Cassidy (1987)
Agaricus bisporus	+	Pukkila & Cassidy (1987)
Flammulina velutipes	+	Pukkila & Cassidy (1987)
Armillaria spp.	+	Duchesne & Anderson (1990)
Puccinia graminis	+	Kim *et al.* (1992)
Tilletia caries	+	Zerucha *et al.* (1992)
Tilletia controversa	+	Zerucha *et al.* (1992)
Ascomycetes		
Saccharomyces cerevisiae	-	Bell *et al.* (1977)
Saccharomyces carlsbergensis	?	Bell *et al.* (1977)
Saccharomyces rosei	?	Verbeet *et al.* (1983)
Torulopsis utilis	-	Tabata (1980)
Kluyvermyces lactis	?	Verbeet *et al.* (1984a)

Hansenula wingei	?	Verbeet *et al.* (1984a)
Candida albicans	+	Magee *et al.* (1987)
Candida stellatoidea	+	Magee *et al.* (1987)
Candida guilliermondii	+	Magee *et al.* (1987)
Candida tropicalis	+	Magee *et al.* (1987)
Candida glabrata	+	Magee *et al.* (1987)
Pyrenophra graminea	?	Amici & Rollo (1991)
Zygomycete		
Mucor racemosus	?	Cihlar & Sypherd (1980)
Oomycetes		
Achlya ambisexualis	?	Rozek & Timberlake (1979)
Saprolegnia ferax	+	Howlett *et al.* (1992)
Phytophthora vignae	+	Howlett *et al.* (1992)
Phytophthora cinnamomi	+	Howlett *et al.* (1992)
Phytophthora megasperma	+	Howlett *et al.* (1992)
Phytophthora glycinea	+	Howlett *et al.* (1992)
Slime mold		
Dictyostelium discoideum	?	Maizels (1976)
Protozoa		
Euglena gracilis	?	Curtis & Rawson (1981)
Copepods (zooplankters)		

Calanus finmarchicus	-	Drouin *et al.* (1987)
Calanus glacialis	-	Drouin *et al.* (1987)
Calanus helgolandicus	-	Drouin *et al.* (1987)

*The orientation of 5S rRNA gene transcription with respect to the other rRNA genes is indicated by the symbols: + = same orientation; - = opposite orientation; ? = undetermined orientation.

words, dispersed repeated genes cannot be deleted by unequal crossing-over as tandemly arranged genes, and as a result, they may accumulate a large number of mutations. To restore proper function, a defective dispersed 5S gene would have to undergo a second compensating mutation (Selker *et al.* 1981). It has been speculated that the dispersed 5S gene arrangement in *N. crassa* could have arisen from a presumed ancestor which had tandemly arranged 5S genes, as a result of several transpositions. Based on the assumption that natural selection acts to maintain an optimal number of 5S genes and the transposition ability of 5S genes, it was suggested that tandem arrays of 5S genes would have been eliminated (Selker *et al.* 1981). It has also been speculated that duplicative or conservative transposition and gene conversion would be expected to have a homogenizing effect on a family of dispersed repeated 5S genes (Selker *et al.* 1981; Metzenberg *et al.* 1985; Morzycka-Wroblewska *et al.* 1985).

The dispersed arrangement of 5S genes was also detected in *Aspergillus nidulans* (Bartnik *et al.* 1981, 1984, 1986; Bartoszewski *et al.* 1987) and *Schizosaccharomyces pombe* (Mao *et al.* 1982). Several dispersed 5S pseudogenes have been sequenced and characterized. The nucleotide sequence of *N. crassa* 5S pseudogenes contain a number of substitutions and insertions, in agreement with the usual features of pseudogenes (Selker *et al.* 1981). In *A. nidulans*, however,

the 5S pseudogenes comprise a highly conserved 5′ half and about 200 bp insertion in the 3′ end (Bartnik *et al.* 1986; Borsuk *et al.* 1988).

4.3- 5S rRNA genes dispersed as tandem arrays away from rDNA

In plants and animals the 5S rRNA genes are generally organized in tandem arrays unlinked to the rDNA repeat unit (Brown and Sugimoto 1973; Hemleben and Grierson 1978; Gerlach and Dyer 1980; Benes and Cave 1985). In plants 5S rRNA genes are located at at least 5 loci comprising several thousands of tandemly arranged 5S gene repeat units. Each unit consists of a 120 bp gene and a 100-700 bp-long spacer (Appels and Baum 1992). In mammals, however, in addition to tandemly arranged functional 5S rRNA genes with a fairly large (1.6 -2.3 kbp) repeat unit size (Sorensen and Frederiksen 1991; Suzuki *et al.* 1994), variant 5S genes or pseudogenes are dispersed in the genome as single copies (Stambrook 1976; Hart and Folk 1982; Little and Braaten 1989; Leah *et al.* 1990). A survey of tandemly arranged 5S rRNA genes unlinked to rDNA is summarized in table 2.

The oocyte-type 5S repeat unit of *X. laevis* was found to comprise a pseudogene, with 85% homology with respect to the DNA sequence of the functional 5S gene (Jacq *et al.* 1977). This pseudogene may be involved in maintaining sequence homogeneity within the tandem repeats (Fedoroff and Brown 1978), or it may act as a template for transcription

(Miller and Melton 1981). The newt, *Notophthalmus viridescens* contains two types of 5S gene repeat units (231 bp, 269 bp), and a 36 bp pseudogene which corresponds to the 3′ part of the 5S rRNA gene, arranged in an interspersed pattern within the 269 bp repeat units (Kay and Gall 1981).

In mammals, functional 5S rRNA genes are highly conserved and arranged tandemly, and their homogeneity must be maintained by concerted evolution within each reproductive population (Brown *et al.* 1972; Arnheim 1983; Leah *et al.* 1990). Suzuki *et al.* (1994) investigated the 5S gene tandem arrays in closely related mice and indicated that the 5S spacer region exhibits substantial sequence and length heterogeneity due to nucleotide substitutions, insertions and deletions. It was also noticed that most of the small size variations were due to fluctuations in the number of simple tracts of microsatellites generated by the slippage phenomenon during DNA replication (Tautz *et al.* 1986; Levinson and Gutman 1987).

Brown *et al.* (1972) studied the rDNA tandem repeats of *X. laevis* and *X. borealis*, two closely related species, and noted that the sequence of the tandem repeats was the same within one species, but the NTS sequences differed in the two species although the coding genes were fairly conserved. This phenomenon has been termed concerted evolution and it does somewhat apply to tandemly arranged 5S rRNA genes (Arnheim 1983). When mutations arise in a tandemly arranged

gene family, homogenization of the repeated copies (genes and spacers) in one or several loci takes place by means of unequal crossing-over, gene conversion, and transposition (Dover 1982; Dover and Flavell 1984). Due to functional constraints, however, selective pressures would affect the evolution rates of different components of the repeat unit (Kimura 1983; Gerbi 1985).

Table 2. Organisms in which the 5S rRNA genes are tandemly arranged and unlinked to rDNA

Organism	Unit size (kbp)*	Reference
Mammals		
Humans	1.600 2.300	Sorensen & Frederiksen (1991)
Mus musculus domesticus (mouse)	1.600	Suzuki *et al.* (1994)
Syrian hamster	2.200	Hart & Folk (1982)
Amphibians		
Xenopus laevis	0.70°	Carroll & Brown (1976a)
	0.88[s]	Peterson *et al.* (1980)
Xenopus tropicalis	0.264[o&s]	Nietfeld *et al.* (1988)
Plants		
Pinus radiata (conifer)	0.524	Gorman *et al.* (1992)
Mathiola incana (Brassicaceae)	0.510	Hemleben & Werts (1988)
Vigna radiata (mung bean)	0.215	Hemleben & Wertz (1988)
Glycine max (soybean)	0.330	Gottlob-McHugh *et al.* (1990)
Acer rubrum (red maple)	0.338	Gottlob-McHugh *et al.* (1990)
Albizia lebbeck (siris tree)	0.477	Gottlob-McHugh *et al.* (1990)
Cucumis sativus (cucumber)	0.341	Gottlob-McHugh *et al.* (1990)

Cynara cardunculus (artichoke)	0.320 0.330 0.480	Gottlob-McHugh *et al.* (1990)
Gymnocladus dioicus (Kentucky coffee tree)	0.215	Gottlob-McHugh *et al.* (1990)
Nicotiana tabacum (tobacco)	0.416 0.617	Gottlob-McHugh *et al.* (1990)
Phaseolus vulgaris (broad bean)	0.389	Gottlob-McHugh *et al.* (1990)
Spinacia vulgaris (spinach)	0.336	Gottlob-McHugh *et al.* (1990)
Allium cepa (onion)	0.336	Gottlob-McHugh *et al.* (1990)
Hordeum vulgare (barley)	0.304 0.442	Gottlob-McHugh *et al.* (1990)
Zea mays (corn)	0.323	Gottlob-McHugh *et al.* (1990)
Cycas revoluta (sago palm)	1.572	Gottlob-McHugh *et al.* (1990)
Eruca sativa (Brassicaceae)	0.500 1.000	Singh *et al.* (1994)
Lycopersicon esculentum (tomato)	0.400	Lapitan *et al.* (1991)
Triticum aestivum (wheat)	0.410 0.500	Gerlach & Dyer (1980)
Arabidopsis thaliana	0.497	Cambell *et al.* (1992)
Insects		
Drosophila melanogaster	0.375	Hershey *et al.* (1977)
Calliphora erythrocephala	0.480	Rubacha *et al.* (1984)
Crustacean		

Artemia	8.500[h] 9.000[h]	Cruces *et al.* (1989)
Newt		
Notophthalmus viridescens	0.231 0.269	Kay & Gall (1981)
Protozoa		
Eimeria tenella	0.728	Stucki *et al.* (1993)
Euplotes eurystomus	0.930	Roberson *et al.* (1989)
Oxytricha nova	0.600 0.680	Roberson *et al.* (1989)
Stylonychia lemnae	0.600	Roberson *et al.* (1989)
Onychodromus quadricornutus	0.640	Roberson *et al.* (1989)
Trypanosoma cruzi	0.481	Hernandez-Rivas *et al.* (1992)
Trypanosoma rangeli	0.913[l]	Aksoy *et al.* (1992)
Tetrahymena thermophila	-	Pederson *et al.* (1984)

*Organisms may have more than one array of tandemly arranged 5S rRNA genes. 5S arrays are found in oocytes[o] and somatic cells[s]. [h]Histone genes are associated with 5S rRNA gene repeat units in *Artemia*. [l]Spliced leader RNA (SLRNA) genes are organized within the 5S rRNA tandem repeats in *Trypanosoma rangeli*.

Classification of the genus *Pythium*

The Oomycetes are considered to be different from most other fungi because they produce biflagellate zoospores with one tinsel flagellum directed forward and one whiplash flagellum directed backward. While their cell walls consist mainly of glucans, they also contain cellulose, and most species lack chitin. They are diploid and their sexual reproduction is oogamous and meiosis gametangial (Alexopolous and Mims 1979). Their rRNA gene sequences have also shown them to be unrelated to the true fungi (Gunderson *et al.* 1987; Förster *et al.* 1990). And there are also adequate biochemical and cytological data suggesting that Oomycetes are related to the heterokont algae (Barr 1992). Cavalier-Smith (1989) classified Oomycetes along with Hyphochytridiomycetes under the subphylum Pseudomycotina, phylum Heterokonta, and the kingdom Chromista. The true fungi, however, were placed in a separate kingdom, Eumycota (Cavalier-Smith 1989). The Oomycetes are mostly aquatic fungi which might be parasitic on algae, water molds, small animals, and other forms of aquatic organisms. The most complex of the Oomycetes are terrestrial parasites of plants passing their entire life cycle in the host, relying on the wind to disperse their spores or spore-like sporangia. Nevertheless, the production of zoospores continues to be generally common, an indication of their aquatic ancestral life (Alexopolous and Mims 1979).

Sparrow (1976) subdivided the class Oomycetes into six orders: Eurychasmales, Saprolegniales, Lagenidiales, Thraustochytriales, Labyrinthulales, and Peronosporales. Of the six orders in this class, only the Peronosporales have great economic value because they include some of the most destructive plant parasites. The classification of the Peronosporales is based mainly on the shape of the sporangia and the sporangiophores. Alexopolous and Mims (1979) divided the order Peronosporales into four families: Albuginaceae, Peronosporaceae, Peronophythoraceae, and Pythiaceae. While the species of Pythiaceae generally produce their sporangia directly on the somatic hyphae, the most complex species do however, produce sporangiophores, but of indeterminate growth. Waterhouse (1973) published a key to the Pythiaceae that included eight genera, the most common of these being *Phytophthora* and *Pythium*. The former genus includes many important plant pathogens, such as *Phytophthora infestans*, the cause of late blight of potatoes. The genus *Pythium* includes more than 120 described species with wide distribution and host ranges (Dick 1990; Plaats-Niterink 1981). They live in the soil saprobically on dead organic matter or parasitically on the young seedlings of a great number of susceptible species of economic seed plants. They also affect algae, other marine plants, and fungi (Hendrix and Campbell 1973).

The genus *Pythium* Prings., nom. cons., was introduced

by Pringsheim in 1858, but was antedated by *Pythium* Nees in 1823. The genus was placed in the family Saprolegniaceae. *P. monospermum* Pringsh. was chosen as the type species. Schröter (1897), however, on the basis of the description of many new species and the clarification of taxonomic details, included the genus in a new family, Pythiaceae. The taxonomy of the genus *Pythium* is mainly based on the morphological characters of the reproductive structures, such as zoosporangium presence, shape, and size; zoospore production; oogonium location, ornamentation, size, and shape; oospore size and wall thickness; and the number, shape, and origin of antheridia (Plaats-Niterink 1981). A number of species are differentiated by quantitative biometric measurements, e.g. the oogonial and oospore diameters (Dick 1969; Ho 1975; Reischer 1949a, 1949b). Hendrix and Campbell (1974), however, questioned the value of biometric data because the variability it measured could be due to the influence of environmental conditions. Identification of some *Pythium* species may be very difficult because they do not readily produce certain reproductive structures. Heterothallic species are among them; they need the opposite mating types to be identified. *Pythium* isolates that do not reproduce sexually are the most difficult to identify. Plaats-Niterink (1981) grouped these isolates into five groups according to the types of sporangia or hyphal swellings they produced: F, sporangia are filamentous, non-

inflated; T, sporangia are filamentous, inflated; G, sporangia are globose to elongate and non-proliferating; P, sporangia are smaller (20-30 um diam); and HS, hyphal swellings are not catenulate. Molecular characters may be very useful in identifying morphologically closely related species and delineating different taxonomic groupings of species in the genus.

Molecular systematics

For hundreds of years, biologists have attempted to identify, describe, and elucidate diversity in the biological world; this quest is known as systematics. The hierarchical system of nomenclature established by Linnaeus (1758) created a framework for describing and categorizing biological diversity. This hierarchical system was initially independent of evolutionary theory, but later workers (Darwin 1859; Haeckel 1866) developed the notion that classification should be based on phylogenetic relationships. Until the 1960s, classification of organisms was based largely on analysis of morphological, physiological, anatomical, and ecological features. The characters which appeared to be useful either in describing an organism or suggesting a common descent or relatedness with other taxa were defined, and these became taxonomic characters. However, with the elucidation of the molecular basis of inheritance, biological macromolecules (DNA, RNA,

proteins) were used increasingly in evolutionary studies. These macromolecules are found in all organisms and contain regions that are conserved in structure and function (Hillis and Moritz 1990).

Very little is known about evolutionary relationships within fungi due to their simple and frequently convergent morphology, and lack of a useful fossil record (Bruns *et al.* 1991). A variety of methodologies have been developed to characterize DNA differences between fungal strains.

1- DNA base composition (G+C content)

Nuclear DNA base composition values can be determined using either thermal denaturation profiles of nuclear DNA (Kurtzman 1985a), or by cesium chloride buoyant density gradient ultracentrifugation methods (de Hoog and Gueho 1984; Martini and Kurtzman 1988). In general, G+C content appears to have very little value for taxonomic purposes because of both the wide percentage range (30-70%) in G+C content reported for individual fungal species and the overlapping values obtained from unrelated fungi (Storck 1965, 1967; Kurtzman 1985a). Moreover, in cases where closely related species are examined, G+C content can be misleading; e.g. *Saccharomyces dairensis* and *Saccharomyces servazzii* have identical G+C values, but cross-hybridization between these two species shows only 13% DNA relatedness (Martini and Kurtzman 1988). Belkhiri and Dick (1988)

investigated the DNA base composition in 14 *Pythium* species and found that the G+C content range for mitochondrial DNA (18.7-23.6%) was smaller than for chromosomal DNA (48.5-58.1%). These results indicated that DNA base composition had very limited resolving power at the species level. However, the G+C content mean difference between mitochondrial and chromosomal DNA separated the Oomycetes from the true fungi.

2- DNA-DNA hybridization

The fact that fungal genomes are small in size and contain a smaller proportion of repetitive sequences with respect to plants and animals, would seem to make them ideal for hybridization studies. However, the technique has very limited usefulness due to the way in which fungal genomes evolve. In general, DNA complementarity values for fungi have been obtained by determining the percentage of cross-hybridization between total DNA extracts (Kurtzman 1985b; Jahnke 1987). Several variations of both isotopic and spectrophotometric assays have been used and are reported to yield similar results if the percentage of cross-hybridization is greater than 90%. At lower percentages the results of different methods vary dramatically (Jahnke and Bahnweg 1986; Kurtzman *et al.* 1980). DNA-DNA hybridization studies have been useful in some systematic studies of ascomycetous and basidiomycetous yeasts (Lachance *et al.*

1986; Kurtzman and Phaff 1987; Martini and Kurtzman 1988; Kurtzman 1990). They were also used to a limited extent for filamentous fungi (Kurtzman 1985b; Vilgalys 1988). The main conclusion from these studies is that the percentage of DNA that cross-hybridizes between closely related species is very low, typically less than 20%, while the percentage cross-hybridization between members within a species is generally greater than 90% (Bruns *et al.* 1991).

3- Restriction enzyme analysis

Restriction enzyme analysis is the most common technique used to characterize various regions within the fungal genome. Restriction patterns are generated by cleavage of DNA with type II restriction endonucleases, and size separation of the DNA restriction products is effected using agarose gel electrophoresis. In most cases the DNA fragments are transferred onto a filter, and then hybridized to a labeled DNA probe which permits the autoradiographic visualization of specific DNA bands. Restriction fragment length polymorphisms (RFLPs) are the result of differences in the DNA sequence that affect either the location or the nucleotide sequence of the restriction endonuclease target sites. The specificity of cleavage by restriction endonucleases means that complete digestion of a particular sequence of DNA will yield a reproducible array of fragments. Changes in the number and size of fragments can

be caused by sequence rearrangement, transposition events and the inversion or translocation of DNA fragments, the addition or deletion of DNA, and base substitutions within cleavage sites (Upholt 1977). The assumption made in RFLP studies is that while closely related species will show similar hybridization patterns when their restriction fragments are hybridized to an appropriate probe, distantly related species will exhibit a significantly different hybridization pattern (Olsen *et al.* 1986).

The RFLP patterns that result from a specific restriction endonuclease-probe of different strains are compared, and the phylogenetic relatedness is determined by the number of restriction fragments in common (Koszlowski and Stapien 1982; Egger *et al.* 1991; Gardes *et al.* 1991). Garber and Yoder (1984) viewed the entire RFLP pattern of one restriction enzyme as one discrete character. Thus, RFLP patterns for a large number of restriction endonucleases should be generated to obtain sufficient discrete characters for phylogenetic reconstruction (Dowling *et al.* 1990).

RFLP enzyme analyses can be relatively fast, permitting simultaneous examination of a fairly large number of samples, and they are useful for developing molecular markers in population genetics (Förster *et al.* 1989; Jacobson and Gordon 1990; Correll *et al.* 1992) and to infer evolutionary relationships (Natvig *et al.* 1987; Smith and Anderson 1989). They are also useful in DNA fingerprinting

of economically important fungal strains (Bruns *et al.* 1991). Bruns (1991) noted that RFLP analysis had a limited application in phylogenetic reconstruction because of length mutations due to insertions and deletions; these appear to occur frequently in fungal genomes. Thus restriction fragments should not be taken as independent characters. Therefore RFLP data analysis should be limited to phenetic methods, since cladistic approaches are based on independent characters.

The most common target regions for restriction analysis are the nuclear ribosomal DNA (rDNA) repeat unit and the mitochondrial genomes. In addition to its small size (17-176 kb) (Bruns *et al.* 1991), high copy number, and relative ease of purification (Garber and Yoder 1983), the mitochondrial genome seems to be functionally conserved in all eukaryotic organisms (Gray 1982; Wallace 1982). Mitochondrial genomes are rich in RFLPs at the intraspecific level (Bruns *et al.* 1988; Smith and Anderson 1989; Taylor *et al.* 1986), and length mutations are the major source of RFLP variation (Bruns *et al.* 1988; Sanders *et al.* 1977; Taylor *et al.* 1986). Although length mutations can cause some analytical problems, their major advantage is that unlike site changes which are unique to a specific enzyme, they can be detected by virtually any restriction enzyme (Bruns *et al.* 1991). Length mutations seem to be very common in fungal mtDNA (Taylor 1986; Taylor and Natvig 1989), and effective

restriction fragment analysis requires mapping of mtDNA. This permits the detection of nucleotide substitutions, length mutations, DNA rearangements, and the evaluation of their respective contribution to genetic variability (Taylor 1986; Bruns *et al.* 1991). In general, RFLP analysis of mtDNA suggests that while restriction patterns are very similar within biological species, they are quite different between species (Förster *et al.* 1988; Kohn *et al.* 1988; Taylor and Natvig 1989).

RFLP analysis of mtDNA has been used to assess phylogenetic relationships between different species from several groups of fungi; the Ascomycotina (Suzuki *et al.* 1988; Taylor and Natvig 1989), the Basidiomycotina (Jahnke *et al.* 1987; Weber *et al.* 1986), and the Oomycetes (Förster *et al.* 1988; Förster *et al.* 1990b; Martin 1989). RFLP analysis of mtDNA from 29 *Pythium* species representing several morphological groupings, indicated a high degree of interspecific variation and a low level of intraspecific variation (Martin and Kistler 1990).

In fungi, the nuclear rDNA exists as a tandemly repeated array of rDNA units consisting of 16-18S, 5.8S, and 23-28S rRNA genes which are separated by transcribed and nontranscribed spacers. The 5S gene may or may not be part of the rDNA repeat unit (Gerbi 1985). The multiple copies of the repeat unit appear to homogenize quickly via concerted evolution and, as a result, they generally behave like a

single copy gene (Bruns *et al.* 1991). Oomycetes, however, may be an exception because some of them contain different copies of repeats which vary in size within the tandem array due to different copy numbers of small subrepeats within the nontranscribed spacer (NTS) (Martin 1991; Klassen and Buchko 1990; Buchko and Klassen 1990). In addition to its abundance in the nuclear genome and its ease of isolation (Garber and Yoder 1983; Kim *et al.* 1990), rDNA has highly conserved genes and variable spacers; these make it very useful in phylogenetic studies. The small ribosomal subunit gene (SSrRNA) and the large subunit gene (LSrRNA), can provide adequate sequence information to allow statistically relevent analysis. In general, the conserved regions of the rDNA provide the means of assessing affinities above the genus level, whereas the more variable sections of the rDNA such as the NTS and internal transcribed spacer (ITS), might be useful for resolving inter- and intraspecific relationships (Hillis and Dixon 1991). Restriction mapping of rDNA was used to delineate some species of *Pythium* which lack definitive morphological characters and thus were not amenable to traditional taxonomic techniques (Klassen *et al.* 1987). Mapping rDNA was also used to study evolution in five yeast genera (Verbeet *et al.* 1984b). To resolve phylogenetic relationships at the intraspecific level, RFLP analysis of the rDNA has been utilized with *Phytophthora* spp.(Förster *et al.* 1990a), *Neurospora* spp. (Russell *et al.* 1984), and

Fusarium oxysporum (Kistler *et al.*1987).

The polymerase chain reaction (PCR) which was first invented by Mullis and Faloona (1987) and then further developed by Saiki *et al.* (1988), permits *in vitro* amplification of specific target DNA sequences using a thermostable DNA polymerase and oligonucleotide primers which anneal at both ends of the region of interest. Since a number of ribosomal genes have already been sequenced (Neefs *et al.* 1990; Hillis and Dixon 1991), oligonucleotide primers can be synthesized based on the conserved regions of rDNA genes. Universally conserved sequences within the ribosomal genes make ideal priming targets for PCR amplification, and thus to generate DNA fragments for RFLP analysis. Little genomic DNA (nanograms) is required as template for a PCR reaction (Saiki *et al.* 1988). It has been demonstrated that sufficient genomic DNA for PCR amplification can be obtained from a single fungal spore (White *et al.* 1990). This is of great importance in the study of both obligate by parasitic fungi and those that grow poorly in culture. Genomic DNA suitable for PCR analysis could also be extracted from herbarium specimens, opening the possibility for molecular analysis of species that are no longer available as living cultures (Bruns *et al.* 1990).

RFLP analysis of PCR amplified rDNA was used to study various species of the genus *Lentinus* (Hibbet and Vilgalys 1991). The restriction analysis of PCR products from several

Cryptococcus species produced complex restriction patterns that were used to deduce the interspecific genetic relationships (Vilgalys and Hester 1990). RFLP analysis of PCR amplified ribosomal genes and the internal transcribed spacer (ITS) was used to study the genetic variability in twenty-five isolates representing five *Pythium* species. Restriction patterns revealed three polymorphic groups indicating inter- and intraspecific genetic relationships (Chen *et al.* 1992). Restriction analysis of the ITS indicated that the morphologically similar species *Pythium arrhenomanes* and *Pythium graminicola* were genetically distinct, and intraspecific variation was detected only in *P. arrhenomanes* (Chen and Hoy 1993). It has been demonstrated that restriction mapping of highly variable regions within the rDNA permits cladistic analysis of molecular data to resolve phylogenetic relationships at the intraspecific level (Liu *et al.* 1992; Chen 1992). It is also more practical and easier to map PCR products than it is to obtain the entire rDNA repeat unit map via extensive probing and Southern hybridizations.

Random amplified polymorphic DNA (RAPD), another PCR technique, will be very useful in addressing taxonomic problems at the intraspecific level, and it has the potential to find markers that discriminate between closely related species when suitable morphological features are lacking (Welsh and McClelland 1990; Williams *et al.* 1990;

Williams *et al.* 1991). RAPD, however, provides very limited resolution when applied to distantly related organisms due to extreme length polymorphism.

4- Ribosomal DNA sequence analysis

The use of ribosomal DNA/RNA sequences for evolutionary studies can solve many of the problems associated with RFLP analysis. Compared sequences contain a large number of characters which provide a better resolution. Phylogenetic analysis can take into account the mode of sequence variation; whether a change is a transition or transversion, selected or silent, and the degree of nucleotide bias (Bruns *et al.* 1991). It also allows results from different laboratories to be compared and integrated more easily, and sequences can be deposited in electronic databases (GENBANK, EMBL) to facilitate access to information.

Direct sequencing of amplified DNA fragments and sequencing ribosomal RNA are the most popular methods for obtaining rDNA sequences for phylogenetic analysis (White *et al.* 1990). These two methods are substantially less laborious and time consuming than the recombinant DNA techniques used to generate DNA libraries necessary to eventually obtain clones of rDNA genes. Therefore it is feasible to study a large number of species in a reasonable time. The 5S rRNA gene was the first rRNA to be sequenced extensively (Blanz and Gottschalk 1986; Hori and Osawa 1987;

Walker and Doolittle 1982). However, due to its small size and conservative secondary structure which is maintained by compensatory substitutions, the 5S gene is not viewed as being very informative for resolving most fungal phylogenetic relationships (Bruns *et al.* 1991; Haylanych 1991). In addition, some filamentous Ascomycota, including *Neurospora* (Selker *et al.* 1985) and *Aspergillus nidulans* (Bartoszewski *et al.* 1987), contain multiple heterogeneous 5S gene species which present further analytical problems. Nevertheless, the conservative nature of the 5S rRNAs have been used to determine the broad evolutionary relationships in the Ascomycotina (Chen *et al.* 1984; Walker 1985) and in the Basidiomycotina (Walker and Doolittle 1982; Huysmans *et al.* 1983; Gottschalk and Blanz 1984).

It appears that sequencing one strand of rRNA is more prone to a relatively high frequency of errors (1-5%) than direct sequencing of rDNA PCR products, because ambiguous sequences can not be resolved by comparison with the opposite strand (Bruns *et al.* 1991). Direct sequencing of amplified DNA, however, might be problematic if errors were introduced during synthesis by the DNA polymerase. The observed error frequency for the Taq DNA polymerase was as high as one substitution per four hundred base pairs after 30 cycles of amplification (Scharf *et al.* 1986). But this error frequency can be substantially reduced to less than one substitution in 15000 bp by optimizing PCR conditions

(Gelfand and White 1990).

Sequences of the small subunit ribosomal gene and those of the large subunit ribosomal gene seem to contain adequate sequence variability to be useful in fungal phylogenetic studies (Lane *et al.* 1985; Sogin *et al.* 1986; Qu *et al.* 1988). Gunderson *et al.* (1987) and Förster *et al.* (1990a) sequenced the SSrRNA gene, and demonstrated that the fungal-like protoctists such as the Oomycetes and the acellular slime molds should not be included within the kingdom Fungi. The Chytridiomycetes, however, were included with the true fungi (Dore and Stahl 1991; Bowman *et al.* 1992; Li and Heath 1992). The phylogenetic analysis of nucleotide sequences has been generally based on two main categories of methods for inferring trees. The distance matrix methods create trees by reducing all information on genetic characters to pairwise estimates of similarity or disimilarity (Felsenstein 1988). The parsimony methods, however, are character based and attempt to reconstruct the course of events that led an assumed ancestral sequence to evolve into its various presumed descendent lineages. The general assumption in parsimony analysis is that evolution takes the shortest route, so that the correct tree should have the minimum number of character state changes (Felsenstein 1981; Swofford and Olsen 1990). Recently, great interest has been shown in the degree of confidence in phylogenetic trees. Bootstrapping is one of the statistical methods which

addresses the question of confidence by resampling the data and assessing the strength of internal branches of parsimony trees (Felsenstein 1985).

MATERIALS AND METHODS

Culture methods

From the margin of a vigourous culture growing on malt extract agar, an agar plug (approx. 5 mm a diameter) was transferred to a 2 l shaker flask containing 200 ml - yeast extract - glucose medium (PYG) containing 3 g glucose, 1 g peptone, and 1 g Difco yeast extract per litre, and allowed to grow in shake culture for 3-5 days at room temperature. Mycelium was then harvested by vacuum filtration onto Whatman No. 1 filter paper (Whatman Laboratory Products, Clinton, N.J.), thoroughly washed with distilled water, and then freeze dried. Mycelium harvested from two shaker flasks (300 mg dry weight) was generally sufficient for DNA extraction. Isolates employed in this investigation, and their sources, are listed in Table 1 under their designated catalogue names and accession codes.

Table. 1. A list of the *Pythium* species isolates and related species studied.

Species	Accession code	Host/ Source	Culture origin
P. acanthophoron Sideris	APCC4000a BR276	Soil	USA
P. anandrum Drechsler	APCC4401d	Soil	UK
P. aphanidermatum (Edson) Fitzpatrick	BR206	Red pine	Ontario
P. aristosporum Vanterpool (Type)	BR136	Wheat	USA
P. arrhenomanes Drechsler	APCC4201b	Sugar cane	Australia
P. arrhenomanes Drechsler	BR140 CBS294.32	-	Netherl.
P. arrhenomanes Drechsler	APCC4201c	Sugar cane	Australia
P. arrhenomanes Drechsler	BR607	Maize	Manitoba
P. australe Shahzad (2)	APCC4041a IMI332 970	-	Australia
P. australe Shahzad (1)	IMI331 762	-	Australia
P. coloratum Vaartaja	BR483	Cucumber	B.C.
P. coloratum Vaartaja	BR323	Carrot	New Zealand
P. diclinum Tokunaga	APCC4110a	-	-
P. dissimile Vaartaja	BR160	Wheat	Ontario
P. dissimile Vaartaja	APCC4204b IMI308 135	Soil	UK
P. erinaceous Robertson	SS78621	-	-
P. erinaceous Robertson	SS78622	-	-

P. graminicola Subramaniam	APCC4205a IMI034 768	Sugar cane	Australia
P. graminicola Subramaniam	APCC4205b IMI091 329	Sugar cane	Australia
P. graminicola Subramaniam	APCC4205d	Sugar cane	Australia
P. graminicola Subramaniam	APCC4205g	Sugar cane	Australia
P. graminicola/ aristosporum Subramaniam	BR608	Maize	P.Ed.I
P. graminicola/ aristosporum Subramaniam	BR166	Wheat	USA
P. hydnosporum Schröter	APCC4006a IMI147 441	-	-
P. hypogynum Middleton	BR635 IMI242 092	-	-
P. hypogynum Middleton	BR389	Alfalfa	Alberta
P. irregulare Buisman	BR486 CBS250.28	-	-
P. irregulare Buisman	BR174	Spinach	Ontario
P. irregulare Buisman	BR706	-	-
P. iwayamae S. Ito	APCC4405g IMI308 160	Soil	UK
P. mamillatum Meurs	APCC4311a IMI120 409	-	USA
P. mamillatum Meurs	APCC4311b IMI308 166	-	UK
P. myriotylum Drechsler	APCC4216a IMI308 182 CBS254.70	-	-
P. nagae S. Ito & Tokunaga	APCC4321a IMI308 183	Soil	UK
P. nagae S. Ito & Tokunaga	APCC4321b IMI308 184	Soil	UK

P. oligandrum Drechsler	APCC4410b IMI308 324	Soil	UK
P. pachycaule Ali-Shtayeh (Isotype)	APCC4117b IMI308 331	Soil	UK
P. paddicum Hirane	480a	-	-
P. paroecandrum Drechsler	BR574	Black spruce	Ontario
P. paroecandrum Drechsler	BR163	-	-
P. paroecandrum Drechsler	BR568	raspberry	B.C.
P. paroecandrum Drechsler	BR601	-	Australia
P. paroecandrum Drechsler	BR419	Snapdragon	Alberta
P. paroecandrum Drechsler	BR479	Juniper	B.C.
P. parvum Ali-Shtayeh (Type)	APCC4009a IMI310 520	Soil	UK
P. parvum Ali-Shtayeh	APCC4009b	Soil	UK
P. periplocum Drechsler	APCC4461a	Soil	UK
P. periplocum Drechsler	APCC4461b	Soil	UK
P. rostratum Butler	APCC4329j	Soil	UK
P. rostratum Butler	APCC4329c	Soil	UK
P. Salpingophorum/ conidiophorum Drechsler	APCC4331a IMI308 282 CBS471.50	-	-
Pythium sp.	BR147[a]	Wheat	Ontario
Pythium sp.	BR671[a]	Oats	Manitoba
Pythium sp.	BR625[a]	Grass	Regina
Pythium sp.	BR620[a]	Maize	Quebec
Pythium sp.	BR667[a]	Barley	Manitoba

P. spinosum Sawada	APCC4012a IMI134 459 CBS377.72	-	Japan
P. spinosum Sawada	APCC4012d IMI308 286	Soil	UK
P. spinosum Sawada	APCC4012e IMI308 287	Soil	UK
P. sulcatum Pratt & Mitchell	BR653	-	-
P. sulcatum Pratt & Mitchell	BR157	-	-
P. sulcatum Pratt & Mitchell	BR146	-	-
P. tardicrescens Vanterpool	BR569	Barley	Ontario
P. torulosum Coker & Patterson	APCC4212d	Soil	UK
P. torulosum Coker & Patterson	APCC4212e	Soil	UK
P. torulosum Coker & Patterson	BR158	Wheat	Ontario
P. torulosum Coker & Patterson	BR489 CBS316.33	-	Netherl.
P. ultimum Trow	BR406	-	-
P. ultimum Trow	BR418	Alfalfa	Ontario
P. ultimum Trow	BR471	-	-
P. ultimum Trow	BR600	Bean	B.C.
P. ultimum Trow var. *ultimum* Trow	APCC4016a IMI82 514	-	Australia
P. ultimum Trow var. *ultimum* Trow	APCC4016c IMI308 273	-	UK
P. ultimum Trow var. *sporangiiferum* Drechsler	APCC4333b IMI308 276	Soil	UK

P. ultimum Trow var. *sporangiiferum* Drechsler	APCC4333c	Soil	UK
P. ultimum Trow var. *sporangiiferum* Drechsler	APCC4333d	Soil	UK
P. vanterpoolii V. Kouyeas & H. Kouyeas (Type)	BR488 CBS295.37 APCC4213a IMI308 281	-	UK
P. vanterpoolii V. Kouyeas & H. Kouyeas	APCC4213a	-	-
P. violae Chesters & Hickman	MA2024	-	UK
P. violae Chesters & Hickman	MA2160		
P. volutum Vanterpool & Truscott	APCC4214a IMI280 171	Snow rot	Japan
Phytophthora cryptogea	JM10	-	-
Phytophthora cryptogea	BR521	Tomato	New Zealand
Laginidium giganteum[b]	-	-	-
Pachymetra chaunorhiza	-	-	-
Verrucalvus flavofaciens	-	-	-
Achlya klebsiana	157a	-	-

APCC: Aquatic Phycomycete Culture Collection, University of Reading, U.K.
CBS: Central Bureau voor Schimmelcultures, Baarn, the Netherlands.
IMI: International Mycological Institute, Kew garden, London, U.K.
BR: Biosystematics Research Centre, Ottawa, Ontario, Canada.
[a]Not assigned to species because of uncertainties.
[b]DNA was a gift from Michael E. S. Hudspeth, University of North Illinois.
All other strains were obtained from the aquatic Phycomycete Culture Collection, University of Reading, U.K.

DNA extraction and purification

Initially, large scale DNA extraction was employed whereby 4-5 shaker flasks were harvested and, immediately after washing, the mycelium was extracted by grinding in a precooled mortar with pestle for 20 min in the presence of liquid nitrogen. The DNA was then purified by phenol extraction and CsCl-bisbenzimide density centrifugation (Garber and Yoder 1983). However, as this procedure was cumbersome and time consuming, a more rapid one was developed which also required less mycelium for extraction of suitable amounts of DNA.

A DNA "mini" preparation procedure based on the methods of Murray and Thompson (1980) and Kim *et al.* (1990) was used to extract "polymerase chain reaction grade" DNA (Saiki *et al.* 1988). Frozen mycelium (100-200 mg) was added to sterile Falcon polystyrene conical tubes (Becton Dickinson Labware, Lincoln Park, N. J.), each of which contained 4 ml of ice cold lysis buffer (150 mM NaCl, 50 mM EDTA, 10 mM Tris, pH 7.4), 20 μg/ml proteinase K (Sigma, St. Louis, Mo.), and 9 g of acid-washed and baked-dry 0.5 mm glass beads (Braun Melsungen). The mixtures were then vortexed for 2 to 3 min, and an additional 3 ml of lysis buffer was added to each tube. Sodium lauryl sulfate (SDS; Fisher Scientific, Nepean, Ont.) was added to a final concentration of 1%, and the tubes then incubated at 55°C for at least 1 h. NaCl and hexadecyltrimethyl ammonium bromide (CTAB; Sigma) were added

to the tubes to a final concentration of 1M and 1% respectively, and the tubes were then incubated for an additional 30 min at 55°C. Next the glass beads were pelleted by centrifugation at 2000 rpm for 2 min, and the supernatant transferred aseptically to sterile 15 ml glass Corex tubes (Canlab, Winnipeg, Man.), and the CTAB-protein complex and SLS were removed by two chloroform/isoamyl alcohol (24:1, V:V) (Fisher Scientific) extractions. Approximately 100 μg of DNA was recovered from each strain by precipitation with 2.25 volumes of 95% ethanol (Fisher Scientific). This miniprep method was self contained within separate sterile tubes for each strain, thus cross contamination by DNA from different samples was avoided. The DNA was redisolved in 150 to 500 μl of TE buffer (10 mM-Tris/HCl; 1 mM-EDTA; pH 7.6) and stored frozen at -20°C. Although the quality (size range: 20-40 kb) and yield of the DNA was somewhat variable, one miniprep procedure yielded sufficient DNA from each sample to carry out genomic RFLP and PCR analysis.

DNA digestion and electrophoresis

Endonuclease digestions were performed using enzymes obtained from Pharmacia (Canada) Ltd. Dorval, Que. and BRL (Bethesda Research Laboratories Inc., Gaithersburg, MD), according to the manufacturer's recommendations. Electrophoresis was carried out in TBE buffer (89 mM Tris,

89 mM boric acid, 2.5 mM EDTA, pH 7.6) on 15 X 20 X 0.4 cm horizontal 0.8 or 1.2% agarose (Boehringer Mannheim Corporation, Indianapolis, Ind.) submarine gels at 2 V/cm for 14 to 20 h. Electrophoresis was also carried out on 20 X 22.3 X 0.4 cm vertical 4% polyacrylamide: 50 ml TBE buffer, 9 ml 30% polyacrylamide- 4 M acrylamide (Bio-Rad Laboratories, Hercules, CA), 64 mM bisacrylamide (Sigma Chemical Co., St. Louis, MO)- 3.5 mM ammonium persulfate, 3.2 mM Temed (Bethesda Research Laboratories Inc., Gaithersburg, MD), using a Protean II electrophoresis unit (Bio-Rad Laboratories, Hercules, CA). Polyacrylamide gels at 4% were required for resolving restriction patterns obtained from treating Polymerase Chain Reaction DNA with endonucleases that restrict at sites consisting of 4 nucleotides. The BRL 1-kb (Bethesda Research Laboratories) ladder was the molecular weight standard used to estimate fragment size. Gels were stained for 15 min with ethidium bromide (0.5 μg/ml in TBE buffer) (Sigma) and illuminated with UV (310 nm) transilluminator (Fotodyne Incorporated, Mississauga, Ont.), and photographed using polaroid 667 film.

Southern hybridizations

Nick translation probe

pMF2 plasmid DNA, which contains the portion of the rDNA repeat unit of *Neurospora crassa* with the 18S, 5.8S,

and 25S ribosomal RNA cistrons but little of the nontranscribed spacer (NTS), was prepared from *Esherichia coli* C600SF8 (Free *et al.* 1979) according to Birnboin and Doly (1979) and labeled with [α-^{32}P]dATP (Dupont, New Research Products, Boston, Ma.) (Rigby *et al.* 1977; also see Maniatis *et al.* 1982). Blots were prepared using Hybond-N nylon membrane (Amersham International, Oakville. Ont.) according to the manufacturer's instructions. Prehybridization of the blots was at 55°C for 2 h in 1 M NaCl (Fisher Scientific) and 1% SDS (Fisher Scientific) with constant agitation. The probe was denatured by boiling for 10 min, then added to the hybridization fluid and incubated at 55°C with constant agitation for 12-14 h. Following hybridization, the membrane was washed twice in 2X sodium saline citrate (SSC; 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at room temperature for 5 min each, then three times in 2X SSC and 1% SDS at 55°C for 30 min each with constant agitation.

5'-end labeled oligonucleotide probes

Oligonucleotide primers were 5'-end labeled using T4 polynucleotide kinase (Sambrook *et al.* 1989). Synthetic oligonucleotides were synthesized without a phosphate group at their 5' termini and then labeled by transfer of the γ-^{32}P from [γ-^{32}P]dATP. The reactions were carried out at 37°C for 30 min and stopped with 0.5 M EDTA, pH 8.0 (Sigma

Chemical Co., St. Louis, MO). The oligoprobes were then precipitated with 2.25 volumes of 95% ethanol and each redissolved in 50 μl of TE buffer. Prehybridization of DNA blots was at 42°C for 2 h in 1 M NaCl and 1% SDS with slow agitation. The probes were then added separately (boiling was not needed) to the hybridization fluid and incubated at 42°C with slow and continuous agitation for 12-14 h. Following hybridization, the membranes were washed with a series of decreasing concentrations of SSC (5X, 3X, 1X, and 0.1X).

Random primers DNA labeled probes

This labeling DNA system was suitable for 0.5-1.0 kb PCR products. The labeling reaction was performed using a kit obtained from Gibco BRL (Life Technologies, Inc., Burlington, Ont.), according to the manufacturer's recommendations. DNA blots were prehybridized, hybridized and washed as described above for the nick translation probe. Autoradiography employed Kodak X-Omat RP film with a Dupont Hi-Plus intensifying screen at -70°C for 48-96 h.

PCR primers

All oligonucleotide primers used for the polymerase chain reaction (PCR) amplifications and DNA sequencing are characterized in Table 2. Primers were obtained from the Department of Microbiology, University of Manitoba, where

oligonucleotides were synthesized with the PCR-MATE (391 DNA synthesizer, Applied Biosystems, Foster city, Ca.).

Table. 2. Primers used to amplify and / or sequence rDNA.

Primer	Location of the oligonucleotide	Sequence (5' to 3')
C	79-97[b]	GCCTTAGTAACGGCGAGTG
D	1479-1496[b]	GGAACCTTTCCCCACTTC
G	1866-1887[a]	CCAAGAATTTCACCTCTGAC
H	2863-2880[b]	CTTCGATGTCGGCTCTTC
N	34-52[d]	ATCCCGTTCGCTCTGCGA
N_c	5' end[d]	AATTCGTAGACGGCCATCTTAG
N_2	2-21[d]	TAGACGGCCATCTTAGGC
N_3	5S IGR from *Pythium spinosum*	TCTTGTTGTGTATACG
N_4	5S pseudogene from *Pythium pachycaule*	TTAGACAGGCAATGCATA
P	701-720[a]	GGCTCCCTCTCCGGAATC
P_2	80-98[a]	ATACTTAGACATGCATGGC
Q	3110-3128[b]	ACGCCTCTAAGTCAGAATC
T_3	Bluescript pM13	ATTAACCCTCACTAAAG
T_7	Bluescript pM13	AATACGACTCACTATAG
Y	34-52[d]	TCGCAGAGCGAACGGGAT
Y_c	3' end[d]	AATTCGAGCACTCGGACTTCCC
Y_2	5S 3'flanking region from *Pythium torulosum*	GTTCCACTTGAAGTTTG
Y_3	98-118[d]	GTAGACAGCACCCGGACTTC
Y_4	5S IGR from *Pythium irregulare*	ATCTGCGTTGACAGCAC
Y_4C	5S IGR from *Pythium*	GTGCTGTCAACGCAGAT

	irregulare	
Y_6	Downstream of 5S pseudogene in IGR of *Pythium pachycaule*	ATGGTCACTGTGGCGTTG

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[a]Based on the SSrRNA sequences of *S. cerevisiae* (Rubstov *et al.* 1980)
[b]Based on the LSrRNA sequences of *S. cerevisiae* (Gutell and Fox 1988)
[c]Based on the 5.8S rRNA sequences of *S. cerevisiae* (Gutell and Fox 1988)
[d]Based on 5S rRNA sequences of *Pythium hydnosporum* (Wolters and Erdman 1988)

Fragment amplification and preparation of sequencing templates

DNA fragments representing specific target sequences were amplified using the polymerase chain reaction (PCR) (Saiki *et al.* 1988) in a reaction mixture of 100 µl total volume containing the following components: 10 µl 10X Taq DNA polymerase reaction buffer (Promega Corp., Madison, Wis.); 8 µl of deoxyribonucleotide triphosphates (dNTP, Pharmacia) mixture (stock concentration 2.5 mM with final concentration of each dNTP, 200 µM); 1 µl (32 pmol) of each primer; 1 µl template DNA (50-100 ng of DNA); 78.5 µl ultrapure water (HPLC grade, Fisher Scientific); and 0.5 µl (2.5 units) Taq polymerase (Promega). The reaction mixtures were overlaid with mineral oil (Paraffin oil, Fisher Scientific) and subjected to 25-30 cycles in a Perkin Elmer-Cetus DNA thermal cycler (Norwalk, Conn.) under the following general temperature conditions: 1 min at 93°C, 1 min at 55°C, and 2 min at 72°C.

PCR products were purified by electrophoresis in 0.6-1% agarose followed by freeze-squeeze extraction of bands by a method similar to that of Tautz and Renz (1983), modified by Hausner *et al.*(1992) as follows. After staining with ethidium bromide, bands were cut out of the gel and frozen at -20°C. The gel plug was placed between two layers of parafilm (American National Can, Greenwich, Conn.) and gently thawed by steady finger pressure. The expressed

liquid was collected and made up to 1 M NaCl and 1% CTAB. After incubation at 55°C for 10 min, two chloroform/isoamyl alcohol (25:1 V/V) extractions were done, followed by precipitation of DNA by the addition of 2.25 volumes of 95% ethanol.

Sequencing of double stranded PCR products

Double-stranded templates were sequenced by a method similar to a rapid denaturation-annealing-sequencing (RDAS) technique suggested by L.E. Pelcher (personal communication), modified by Hausner *et al.* (1992). Approximately 1 μg of lyophilized template DNA was dissolved in 3 μl of water and mixed with 12 μl of tricine buffer (0.6 M tricine (Sigma), 2% NP-40 (Sigma), 100 mM $MgCl_2$ (Fisher Scientific), 4 μl 0.6 N NaOH (Fisher Scientific), and 5 μl of primer solution. The standard amount of primer was 5 pmol, but this amount was adjusted to optimize sequencing for each of the primers used. The mixture was boiled for 3 min and then transferred immediately to an ethanol bath at -70°C. The mixture was thawed on ice, and 4 units of Sequenase (0.5 μl) (United States Biochemical Co., Cleveland, O.) or T7 polymerase (Pharmacia) in 4 μl of Sequenase dilution buffer and 1 μl of 100 mM dithiothreitol (Sigma), and 2 μl of [α-^{32}P]dATP (1 mCi in 100 μl; Dupont) were added. Of this mixture, 7.1 μl were added to each of the four sequencing termination mixes prepared as prescribed

by the manufacturer for Sequenase (also see Sambrook *et al.* 1989). After incubation for 7 min at 37°C, the contents of each tube was diluted with 9 μl of water and precipitation by the addition of 51 μl of ethanol (95% ethanol made to 0.12 M sodium acetate). The DNA was pelleted by centrifugation for 30 min in a tabletop centrifuge, the supernatant decanted, and the ethanol evaporated by heating the tube in a waterbath. The pellet was resuspended in TE buffer containing the sequencing stop solution (Pharmacia) and loaded on the sequencing gels.

Sequencing reaction products were separated by electrophoresis using 6% polyacrylamide (polyacrylamide stock solution: 97.5% acrylamide (Bio-Rad Laboratories, Richmond, Cal.) and 2.5% N,N'-methylene-bis-acrylamide (Sigma), and 48% urea (BRL) denaturing gels (Maniatis *et al.* 1982). The preparation of gels, including the preparation of the acrylamide solution and the cleaning and taping of the sequencing gel plates, were as described by Sambrook *et al.* (1989). Two loadings were spaced approximately 2 to 2.5 h apart; this allowed for determination of 250 to 280 nucleotide stretches. Gels were vacuum dried at 80°C and exposed for 1-4 days to Kodak X-OMAT film at room temperature.

Construction of the *Pythium irregulare* genomic library

A Lambda EMBL3 genomic library was constructed using a

cloning kit (Stratagene, La Jolla.CA) according to the manufacturer's recommendations and Sambrook *et al.* (1989) as follows. A high molecular weight genomic DNA was prepared using a large scale method as described above (Garber and Yoder 1983). Conditions for partial digestion with *Mbo*I (Bethesda Research Laboratories Inc., Gaithersburg, MD) were established using 5 μg of total genomic DNA, 0.05 units of endonuclease enzyme, and a series of incubation times of 1 to 30 min at 37°C. A large scale preparation of DNA, partially digested with *Mbo*I to a size range of 20 kb, was prepared by digesting 300 μg of DNA with 8.5 units of enzyme for 10 min. The reaction was then stopped by placing on ice and adding 20 μl of 0.5 M EDTA, and the DNA was precipitated with 2.5 M ammonium acetate (Fisher Scientific) and 2.25 volumes of 95% ethanol. The DNA sample was redissolved in 250 μl of TE buffer, pH 8.0 and stored at 4°C.

The isolation of 20 kb fragments was achieved by sucrose gradient centrifugation as follows: A 12 ml, 10-40% sucrose (Fisher Scientific) density gradient was prepared in a 12.5 ml polyallomer tube (Beckman Instruments, Inc., Palo Alto, CA) using STE buffer (1 M NaCl, 20 mM Tris-HCl, pH 8.0, 5mM EDTA). Then 225 μl of *Mbo*I partially digested DNA were heated at 65°C for 10 min, cooled to room temperature and loaded on the sucrose gradient. Centrifugation was performed at 26000 rpm for 24 h at 20°C, using the SW41 Ti swinging bucket rotor (Beckman).

Using the Fraction Recovery System (Beckman), approximately 200 μl fractions were collected in a sample tray (Corning). A sample of 10 μl from each third fraction was mixed with 3 μl of Stop solution: 6.6% sucrose (Fisher Scientific), 0.04% bromophenol blue, 20.8 mM EDTA) and run on 0.4% agarose gel to determine the inclusive fractions that contained the 20 kb genomic DNA fragment. The chosen fractions were diluted with TE buffer, pH 8.0, so that the concentration of sucrose was reduced to 10%. The DNA was then precipitated with 0.15 M sodium acetate and 2.25 volumes of 95% ethanol, and washed with 70% ice cold ethanol. Next, the DNA was dissolved in 10 μl of TE buffer, pH 8.0, from which 1 μl was analyzed by agarose gel electrophoresis for a quality check.

The ligation of 20 kb genomic fragments to EMBL3 arms was performed along with a control test insert (pME), obtained from Stratagene, La Jolla, CA, in a total volume of 5 μl. The test reaction included: 1.0 μl of Lambda EMBL3 vector predigested with *BamHI*/*EcoRI* (Stratagene), 2.5 μl of 20 kb genomic fragments (insert), 0.5 μl of 10X ligation buffer (0.5 M Tris-HCl, pH 7.6, 100 mM $MgCl_2$, 100 mM dithiothreitol), 0.5 μl of 10 mM of dATP, pH 7.5, 0.5 μl (4 units) of T4 DNA ligase (Pharmacia, LKB Biotechnology AB, Uppsala, Sweden). The ligation reaction was incubated at 4°C for 20 h, and stopped by heating at 65°C for 15 min, and stored at -20°C before packaging. To check the DNA ligation,

1 μl ligated Lambda arms (EMBL3 + 20 kb insert) was run on 0.4% agarose gel, along with 1 μl of predigested Lambda arms, and 1 μl of 20 kb genomic fragments (insert).

In vitro packaging of Lambda DNA (genomic DNA-EMBL3 arms) was performed using Gigapack II Gold packaging Extract (Stratagene, La Jolla, CA), according to the manufacturer's recommendations as follows. One set of packaging extract from -70°C freezer was removed and placed on ice. At the same time a sonic extract was being thawed. The packaging extract was then thawed quickly and 2 μl of ligated Lambda DNA were added and the tube was placed on ice. To the tube, 15 μl of sonic extract were added and the contents were mixed well and spun down quickly. The tube was then incubated at room temperature for 2 h. 500 μl of phage dilution buffer (0.1 M NaCl, 0.02 M Tris-HCl, pH 7.4, 0.01 M $MgSO_4$) and 20 μl of chloroform were added and mixed gently. The contents were then spun briefly to sediment debris, and the supernatant was stored at 4°C before titration.

A culture of *E. coli* host bacterium P2392 (Stratagene, La Jolla, CA) was grown the night prior to packaging in LB medium; 0.17 M NaCl, 0.5% yeast extract (DIFCO Laboratotories, Detroit, MI), 1% tryptone (DIFCO Laboratories, Detroit, MI), supplemented with 10 mM $MgSO_4$ (Fisher Scientific) and 0.2% maltose (BDH; The British Drug Houses Ltd, Poole, England). The following day, 0.2 ml was subcultured into 10 ml fresh medium and allowed to grow by

shaking for 2.5 h at 37°C. The cells were spun down in a sterile screw capped centrifuge tube at 2000 rpm for 10 min. The supernatant was then decanted and the cells were resuspended in 5 ml of sterile 10 mM $MgSO_4$. A series of EMBL3 genomic library serial dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) were done in 0.5 ml of phage dilution buffer (SM buffer). Four sterile polypropylene tubes (Fisher Scientific) containing 0.2 ml plating bacteria (P2392) were set up and 0.1 ml of each EMBL3 genomic dilution was added separately. After mixing by shaking, the tubes were incubated at 37°C for 20 min to allow the bacteriophage particles to adsorb. To the first tube, 3 ml of melted 0.7% NZY top agar (85.5 mM NaCl, 8.1 mM $MgSO_4$.7H2O, 1% casein hydrolysate, 0.5% yeast extract, pH 7.5, 1.5 % agar; DIFCO Laboratories) was quickly added and the content was immediately poured onto a labeled LB plate, prewarmed at 37°C. The plate was swirled gently to ensure an even distribution of the bacteria and the top agar. The plates were left to stand at room temperature for 5 min to allow the top agar to harden. They were then inverted and incubated at 37°C for 12-16 h to allow plaques to appear and be counted. Based on the number of plaques in the four plates, the titre of the library, in terms of plaque forming units per ml (pfu/ml), was determined.

Amplification of the EMBL3 genomic library

A sterile tube containing 10 ml of LB broth,

supplemented with 0.2% maltose and 10 mM $MgSO_4$, was inoculated with a single colony of *E. coli* (P2392) and let grow overnight with shaking at 30°C. A tube containing fresh 10 ml LB broth containing tube, supplemented with maltose and $MgSO_4$, was inoculated with 1 ml of bacteria grown overnight, and incubated for 3 h by shaking at 37°C. The bacterial cells were then spun down at 2000 rpm for 10 min, and resuspended in 5 ml of sterile 10 mM $MgSO_4$. Four sterile tubes containing 0.2 ml of plating bacteria were set up and 100 ul of the genomic library preparation were added to each tube. The tubes were incubated at 37°C for 20 min, and 3 ml of melted NZY top agar were added to the first tube. The content was then poured onto a LB plate and let stand to solidify for 5 min. The same procedure was carried out with the other three tubes. The plates were incubated at 37°C and plaques began to appear after 8 h and matured after 12 h. To each plate, 3 ml of SM buffer were added and incubated overnight at 4°C. The supernatant from the four plates was collected in a sterile tube and chloroform was added to a final volume of 5%. The phage suspension was shaken for 15 min and the debris spun down. The supernatant was kept, and two drops of chloroform were added. This amplified library was titred as described previously, and 1 ml aliquots were stored at 4°C. For long term storage at -70°C, dimethyl sulfoxide (DMSO) (Fisher Scientific, Fair Lawn, NJ) was added to a final volume of 7%.

Plating the EMBL3 genomic library

To screen the *P. irregulare* genomic library for the presence of 5S genes, approximately 10^4 plaques were plated on a 150 mm diameter petri plate as follows. P2392 bacterial cells suitable for plating were prepared as described previously for titration. To a sterile tube containing 0.4 ml P2392 cells, 250 μl of 1/10 genomic library dilution were added. The tube was then incubated at 37°C for 20 min, and 8 ml of melted NZY top agar were added, and the tube contents were immediately poured onto a 150 mm diameter agar plate (LB). The plate was incubated at 37°C for 12h and then stored at 4°C before plaque blotting.

EMBL3 genomic library plaque blotting

Two 150 mm diameter Hybond-N membranes were carefully labeled with identification marking and date. The first membrane was placed on the agar surface and, using a sterile needle, the edges of the membrane were marked by piercing through the membrane into the agar. This ensured correct orientation of plaques. The membrane was removed after 1 min and placed, colony side up, on 3MM paper (Whatman International Ltd, Maidstone, England) soaked in denaturing solution (0.4 M NaOH, 0.6 M NaCl). The membrane was left for 7 min, then placed on 3MM paper soaked in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, 0.001 M Na2EDTA, pH 7.2). The membrane was left for 3 min, then transferred to

fresh 3MM paper soaked in neutralizing solution and left for another 3 min. The same procedure was performed on the second duplicate membrane, and the two membranes were washed by submerging for 1 min in 2X SSC buffer. They were then air dried, colony side up, on 3MM paper. The membranes were covered with Saran Wrap and exposed to UV light (320 nm), for 2 min, colony side next to a UV transilluminator. They were then stored at room temperature until ready to use.

Selection of positive clones

After hybridization to a 5S gene specific oligonucleotide probe as described previously, the blots were exposed to Kodak X-OMAT film for 16 h. Positive clones were located by plaques that hybridized to the probe. Autoradiograms with blots and mark orientation positions were aligned with the orientation marks on the plates, and the corresponding plaques were marked. For a positive plaque, a sterile Eppendorf tube containing 0.5 ml SM buffer and 3 drops of chloroform was set up. The plaque was picked by using a Pasteur pipette equipped with a rubber bulb. Mild suction was applied so that the plaque, together with the underlying agar, was drawn into the pipette. The agar fragment was added to SM buffer and let stand at room temperature for an hour to allow the phage particles to diffuse out of the agar. An average plaque yielded 10^5 to 10^6 phage particles that could be stored indefinitely at 4°C

without loss of viability. If the plaques were not well separated it was necessary to repeat the screening process to ensure that virions were derived from a single clone.

Screening clones with PCR

To an Eppendorf tube, 20 μl of phage suspension and 20 μl of a stock solution containing 2% CTAB and 2 M NaCl were added. The tube was then incubated at 55°C for 10 min, and the contents were extracted twice with equal volumes of chloroform. The resulting supernatant contained DNA template that could be used directly in PCR amplification as described previously.

Large scale isolation of phage DNA

Lambda DNA from phage lysates was purified based on the rapid biochemical method of Kaslow (1986) as follows. With a single colony of P2392, a tube containing 10 ml of LB broth supplemented with 0.2% maltose and 10 mM $MgSO_4$ was inoculated and let grow overnight by shaking at 30°C. The next day, 1 ml of bacterial cells was mixed with 1 ml of eluted phage (10^5 to 10^8 pfu/ml) and 1 ml of 10 mM $MgCl_2$, and incubated at 37°C for 20 min. The mixture was then transferred to 500 ml LB broth, supplemented with 0.2% maltose and 10 mM $MgSO_4$, and incubated at 37°C by shaking for 8 h. Chloroform was added to 2%, DNase I and RNase A (Sigma) were added to 1 μg/ml, and solid NaCl was added to 1

M final concentration. After incubation at 37°C for 30 min, the aqueous phase was clarified by centrifugation at 5000 rpm at 4°C for 10 min. Solid polyethylene glycol (PEG 800) (Sigma) was added to 10% w/v and the cloudy mixture stored at 4°C for at least one hour. The intact phage were recovered by centrifugation at 5000 rpm at 4°C for 20 min and resuspended in 3 ml of SM buffer. DNase I and RNase A were added to 5 μg/ml and 100 μg/ml, respectively. After a 30 minute incubation at 37°C, the phage were lysed by adding 10% SDS to 0.5%, 0.5 M EDTA (pH 8.0) to 20 mM, and proteinase K (GIBCO BRL) to 100 μg/ml, and heating to 68°C for 30 min. The phage DNA was extracted with equal volumes of buffer-saturated phenol (GIBCO BRL), phenol/chloroform, and then chloroform (Fisher Scientific), and precipitated by adding 0.5 volume of 5 M ammonium acetate and 2.25 volumes of 95% ethanol. After storing on ice for 15 min, the precipitate was recovered by centrifugation at 10000 rpm at 4°C for 15 min. To the dry pellet, 1.6 ml of HPLC grade H2O, 0.4 ml of 4 M NaCl and 2 ml of 13% PEG were added. The resulting precipitate was collected after an hours incubation on ice. This was followed by a 15 minute centrifugation at 10000 rpm, rinsed with 70% ethanol, dried and resuspended in TE buffer to a final concentration of 1 mg/ml.

Subcloning DNA fragments from phage clone into the Bluescript plasmid (pM13 KS+)

A partial characterization of a phage clone (C13A) from the *P. irregulare* library, revealed that it contained an array of tandem repeats of 5S genes, with a single *Pst*I endonuclease site per repeat. To further characterize the array and gain access to the insert for mapping and sequencing, *Pst*I/*Sal*I fragments with various sizes were subcloned into the Bluescript (pM13 KS+) (Stratagene, San Diego, CA) according to the manufacturer's recommendations and Maniatis *et al*. (1989) as follows. Approximately 1 μg of pM13 plasmid DNA was digested with 5 units of *Sal*I and *Pst*I endonucleases in a total volume of 10 μl, by incubating at 37°C for 3 h. The reaction was stopped by heating at 68°C for 15 min. Approximately 1 μg of C13A phage DNA was completely digested with 5 units of *Sal*I endonuclease, then partially digested with 0.5 units of *Pst*I endonuclease in a total volume of 10 μl, by incubating at 37°C for 20 min. The reaction was stopped by heating at 68°C for 15 min, and the DNA fragments were then precipitated with 0.5 volume of 5 M ammonium acetate and 2.25 volumes of 95% ethanol. The DNA was spun down and the pellet dried by vacuum. To ligate *Sal*I/*Pst*I fragments and pM13 plasmid vector, the dried DNA pellet was resuspended with 5 μl of predigested pM13/*Sal*I/*Pst*I. To the DNA mixture, 1 μl of 10X ligase buffer, 1 μl of 10 mM ATP (Sigma), 2 μl of HPLC grade H2O,

and T4 ligase (Pharmacia), were added to ligate the DNA fragments and the plasmid. After incubating overnight at 15°C, the reaction was stopped by heating at 65°C for 15 min. To prepare competent NM522 cells (Stratagene), 0.2 ml of an overnight culture of *E. coli* was added to 10 ml LB medium supplemented with 10 mM $MgCl_2$. After shaking at 37°C for 2.5 h, the cells were put on ice for 20 min. They were then centrifuged at 3000 rpm for 5 min at 4°C, and the pellet gently resuspended in 3 ml of 50 mM $CaCl_2$ and put on ice for another 20 min. The cells were again centrifuged at 3000 rpm for 5 min and resuspended in 0.5 ml of 50 mM $CaCl_2$ and put on ice ready for transformation. In a sterile Eppendorf tube, 10 μl of ligation mixture were mixed with 200 μl of competent cells (NM522), and put on ice for 15 min. The tube was then transferred to a 42°C water bath and incubated for 1 min. After 10 min at room temperature, 1 ml of prewarmed LB/Mg medium was added, and incubated at 37°C for 1 h. The cells were collected by centrifugation and resuspended in 200 μl of LB medium. LB-Ampicillin (Sigma) plates containing 40 μg/ml X-gal (Sigma) and 24 ng/ml IPTG (Sigma) were used to select for transformants and recombinants as described in Sambrook *et al.* (1989).

Cloning of 5S genes

Twenty-two clones containing a large portion of the 5S gene amplified from library clone C13A were sequenced and

found to be identical. The genes were amplified with Nc and Yc (Table 1), primers designed to have partial *EcoRI* sites at their ends. The PCR products were treated with T4 DNA polymerase in the presence of 10 mM GTP, to allow the endogenous nuclease to create the *EcoRI* site at the end of each product. The products were then phosphorylated with T4 polynucleotide kinase and cloned into the *EcoRI* site of the Bluescript vector by standard methods.

Purification of plasmid DNA

Small scale purification of plasmid DNA was carried out using a Magic Minipreps DNA Purification System (Promega Co., Madison, WI) according to the manufacturer's recommendations as follows. A 10 ml overnight culture of *E. coli* was pelleted by centrifuging for 5 min at 3000 rpm and resuspended in 200 μl of Cell Resuspension Solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 μg/ml RNase A). After transferring the resuspended cells to a microcentrifuge tube, 200 μl of Cell Lysis Solution (0.2 M NaOH, 1% SDS) were added, and the contents were mixed by inverting the tube several times. To the clear, lysed cell suspension, 200 μl of Neutralizing Solution (2.55 M Potassium acetate, pH 4.8) were added and mixed by inverting the tube. After centrifugation at top speed in a microcentrifuge for 5 min, the cleared supernatant was decanted to a new microcentrifuge tube. To the tube, 1 ml of the Magic

Minipreps DNA Purification Resin, was added and mixed thoroughly. Using a 3 ml disposible syringe, the DNA solution was run through a Magic Minicolumn and purified with 2 ml of Column Wash Solution (200 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, Diluted 1:1 with 95% ethanol). The Minicolumn was transferred to a 1.5 ml microcentrifuge tube and spun down at top speed for 20 seconds to dry the resin. The Minicolumn was again transferred to a new microcentrifuge tube and 50 μl of TE buffer were added. After 1 min, the DNA was eluted by spinning the Minicolumn at top speed in a microcentrifuge tube for 20 seconds. The plasmid DNA was then stored at 4°C or -20°C.

CHAPTER 1

The 5S ribosomal RNA gene in *Pythium* species: two different genomic locations

INTRODUCTION

In eukaryotes the nuclear 5S ribosomal RNA (rRNA) genes are relatively independent of the other rRNA genes. They are transcribed separately and may or may not be part of the ribosomal DNA (rDNA) repeat unit (reviewed by Gerbi 1985). In plants and animals the 5S rRNA genes are generally organized into tandem arrays unlinked to the rDNA repeat unit (Brown and Sugimoto 1973; Benes and Cave 1985; Hemleben and Grierson 1978; Gerlach and Dyer 1980). In *N. crassa* and in several other ascomycetous fungi, the 5S genes are dispersed throughout the genome in a complex manner (Selker *et al.* 1981; Bartnik *et al.* 1986; Mao *et al.* 1982). In other fungi, a 5S gene is embedded in the nontranscribed spacer (NTS) of the rDNA repeat unit. This is true for a diverse collection of fungi including Ascomycotina (Kramer *et al.* 1978; Tabata 1980; Amici and Rollo 1991), Basidiomycotina (Cassidy and Pukkila 1987, Duchesne and Anderson 1990), a Zygomycete (Cihlar and Sypherd 1980), and an Oomycete (Rosek and Timberlake 1979). In *Dictyostelium* the 5S gene is also linked to the other rRNA genes (Maizels 1976).

Obviously, the 5S rRNA gene family has been rearranged during eukaryote evolution. Which of the three arrangements outlined above is most similar to the original one? More specifically, have 5S genes become unlinked from the rDNA repeat in the course of gene dispersion (Clark and Gerbi

1982), or have 5S genes become linked to the rDNA repeat in some fungi and protozoa as an adaptation for tighter control of ribosome synthesis (Clark 1987)? These questions might be answered if we had a system in which the change from one arrangement to the other was in process. Previous studies in this laboratory have discovered that both linked and unlinked 5S genes exist in the oomycete genus *Pythium*, and it is expected that this system will be useful in resolving questions regarding 5S gene family change. Herein the 5S gene family arrangement will be described as it occurs in the two types of *Pythium*, and the results of a survey of the genus and other related genera regarding 5S gene presence and organization will be presented.

Although Oomycetes have been considered to be fungi on the basis of nutrition and mycelial form, based on rRNA gene sequences they are not true fungi (Gunderson *et al.* 1987; Förster *et al.* 1990). It has also been shown that the structure of the NTS in *P. ultimum*, with its arrays of subrepeats, resembles plants and not fungi (Klassen and Buchko 1990). Thus, this report extends knowledge of 5S rRNA gene organization into a relatively unexplored part of the phylogenetic world.

RESULTS

Location and orientation of the 5S rRNA genes in *Pythium torulosum*

A physical map of the rDNA repeat unit in *P. torulosum* was reported previously (Klassen *et al.* 1987), but as presented here it shows more restriction sites, gene placement, and primer sites for the present study (Fig. 1; McNabb 1989). A 5S gene was located in the NTS region by PCR amplification with oligonucleotides designed to recognize conserved regions within 5S genes. Amplification with primer pairs QN, YP, and QP resulted in products of 1.25, 3.35, and 4.6 kb respectively (Fig. 2). Use of primer pairs NP and QY did not yield amplification products. These results mean that a 5S gene is located 1.25 kb downstream of the Q primer site and that it is coded on the DNA strand opposite to the one on which the other rRNA genes are found; i.e. it is in the "inverted" orientation. The region between the large subunit rRNA (LSrRNA) gene and the 5S gene is designated as NTS1 and the region between the 5S gene and the small subunit rRNA (SSrRNA) gene as NTS2 (Planta and Raué 1988). Although this locates a 5S gene in the rDNA repeat of *P. torulosum*, other 5S genes may occur elswhere in the genome and it may be that not all of the rDNA repeats include the gene.

Sequence of the 5S rRNA gene and flanking regions

Using PCR products QN, QN_2, YP, and Y_2P as templates and primers N, N_2, Y, and Y_2 as sequencing primers, the sequence of the 5S gene and its flanking regions was determined (Fig. 3). Except for the N_2 primer region, the gene has been sequenced from both strands and each flanking region twice from the same strand. The ends of the 5S rRNA product of the putative coding region were not sequenced, but when the sequence was compared with the published 5S rRNA sequence of *Pythium hydnosporum* (Walker and Doolittle 1982) only two base substitutions were observed (T→C at position 39 and A→G at position 107). Thus, the extent of the 5S rRNA gene coding region is based on this assumed homology.

Detection of 5S rRNA genes in other *Pythium* species and related organisms

Amplification with QN and QY primer pairs was attempted with template DNA from 45 isolates of *Pythium* that represent at least 27 different species, and six representatives of other oomycete groups (Table 1). Three different types of results were obtained. When successful amplification with QN occured, this was taken to mean that the 5S gene was likely to be present in the rDNA repeat and that it was in the inverted orientation. And successful amplification with QY was taken to mean that the gene was also likely to be

present, but in the other orientation. In no case was amplification observed with both primer pairs. Failure to obtain amplification with either of the primer pairs was presumed either to some problem with the assay or to the absence of the gene from the rDNA repeat. When more than one isolate of the same species was investigated, the same results were obtained except for minor differences in the sizes of fragments produced. In many of the amplifications, multiple bands were produced ranging in size from 0.95 to 2.4 kb. All of the amplification products reported in Table 1 were hybridized to a 5S rRNA-specific probe to show that they contain 5S rRNA sequences. QN products were hybridized to Y_3 primer and QY products to N_2 primer (data not shown except for that in Figure 6).

In general, *Pythium* species with filamentous sporangia had the 5S gene in the rDNA repeat, and species with globose or absent sporangia did not (see Table 1). Exceptions were *P. periplocum* (filamentous but no 5S in rDNA), *P. hypogynum* and *P. salpingophorum* (globose but with 5S in rDNA), and *P. violae* (unknown sporangial type but with 5S in rDNA). All *Pythium* species that had the gene in the rDNA repeat, had it in the inverted orientation.

The two isolates of *Phytophthora cryptogea* studied, as well as *Achlya klebsiana*, were found to have the 5S gene in the non-inverted orientation, but *Lagenidium giganteum*, *Verrucalvus flavofaciens*, and *Pachymetra chaunorhiza* also

had the inverted orientation.

5S rRNA sequences located outside of the rDNA repeat in *Pythium ultimum*

5S rRNA sequences were not located in any *P. ultimum* isolates by the method described above. In order to locate the 5S genes of *P. ultimum* outside of the rDNA repeat, genomic DNA from two isolates (BR418, isolated from alfalfa in Ontario; BR600, isolated from bean in British Columbia) was digested simultaneously with *Hinc*II and *Eco*RV. The blotted profiles were hybridized to pMF2, a plasmid containing the ribosomal RNA genes of *N. crassa* (Free *et al.* 1979) and to 5S RNA-specific oligonucleotides Y and N_2 (Fig. 4A, lanes 1 & 4). These pMF2-probed profiles have been presented earlier (Klassen and Buchko 1990) and are included here for comparison. The profiles generated by the Y probe (lanes 2 & 5) and the N_2 probe (lanes 3 & 6) consisted of prominant bands at 2.2 kb and numerous minor bands larger and smaller than 2.2 kb. The 2.2 kb band did not correspond to any one of the bands from the rDNA repeat and thus is not part of the rDNA repeat since the entire repeat is accounted for in the bands hybridizing to pMF2 (Klassen and Buchko 1990). The two different 5S rRNA-specific probes produced identical profiles, indicating that all of the bands actually contain 5S rRNA or 5S rRNA-like sequences because it is highly unlikely that two different probes would

produce the same artifacts or non-specific hybridization patterns. It was also observed that when genomic DNA from *P. ultimum* BR471 was digested separately with *Hinc*II, *Eco*RI, *Cla*I, and *Kpn*I, prominant 2.2 kb bands were produced in each case (Fig. 4B). This was interpreted to mean that most of the 5S RNA genes of *P. ultimum* are in an array having a periodicity of 2.2 kb with single sites for *Eco*RI, *Cla*I, *Kpn*I and *Hinc*II (The 2.2 kb band generated by the *Hinc*II/*Eco*RV digestion is due to *Hinc*II digestion alone [Fig. 4B]). More than one 5S rRNA gene may be present in each repeat unit.

Although the major 2.2 kb band was conserved in different *P. ultimum* isolates, most of the minor bands were not. This could mean that 5S rRNA or 5S rRNA-like sequences found outside of the major array are being rearranged rapidly during subspecific evolution.

Absence of the 5S rRNA gene from the rDNA of other tested *Pythium* species

The above evidence which suggests strongly that the 5S rRNA gene is not located in the rDNA of *P. ultimum* could be obtained from the detailed physical map of the rDNA repeat unit in this species. For the other species which apparently lack the 5S gene in their rDNA, such evidence must await detailed knowledge of the physical maps of their rDNA repeats. For 8 of the 13 species in this category, however,

other evidence is available which supports the belief that the 5S gene is absent from their rDNA. The entire NTS was amplified using primers located at the 3′ end of the LsrRNA gene and at the 5′ end of the SSrRNA gene; this yielded products ranging in size from 4 to 5.5 kb (Fig. 5A). These products were hybridized to the ribosomal probe pMF2 which confirmed that they contain the expected ribosomal sequences (Fig. 5B). Finally, the products were hybridized to the 5S-specific probe Y (Fig. 5C), but no hybridization was observed to either the PCR products from the 8 species tested, nor to such products derived from *P. ultimum*. The two products from *P. torulosum* and *P. graminicola/aristosporum* (Fig. 5C, lanes 12 & 13), however, showed strong hybridization; this is consistent with the presence of the 5S gene in the target DNA. The NTS region from the 5 species that remain untested would not amplify and thus it is not certain that the 5S genes are absent in these cases.

Heterogeneous amplification products from NTS1

When multiple bands were generated by QN or QY amplification (Table 1) this was taken as preliminary evidence of template heterogeneity. Almost half of the successful amplifications shown in Table 1 produced multiple bands, indicating that heterogeneity of the NTS1 region could be common in the Oomycetes. Although it was shown that

all of the multiple fragments reported contained 5S rRNA sequences (see above), the production of multiple amplification products does not prove template heterogeneity. To do so would require detailed physical maps for each isolate, so *P. vanterpoolii* 4213a was chosen as a test case (Fig. 6). Amplification with the QN_2 primer pair produced a set of three fragments as reported in Table 1. To show that the three bands were not likely to be artifacts, amplification with the HN_2 primer pair was also done, and this produced a set of fragments each 250 bp larger than the corresponding fragment produced by QN_2 amplification, as predicted by the map (Fig. 1). All of these bands were recognized by the 5S rRNA-specific probe Y. To show that the genomic template had the same pattern of heterogeneity, DNA from *P. vanterpoolii* was digested with *Ava*I+*Cla*I and hybridized to the oligonucleotide Y_3. Assuming that the physical map of the rDNA repeat in *P. vanterpoolii* was similar to that in *P. torulosum* (Fig. 1), this digestion should produce a fragment including all of NTS1. Three bands in the profile were recognized by the probe. The three bands have the same ratio of sizes as the PCR products from this region and even the same relative intensities. The fragments are a little larger than expected from the map, indicating that *P. vanterpoolii* and *P. torulosum* differ with respect to the location of a *Cla*I site at the 3′ end of the LSrRNA gene.

Fig. 1. Physical map of *P. torulosum* 4212e rDNA repeat unit. Symbols above the map indicate oligonucleotide primer sites with arrows giving 5′-3′ orientation. Restriction sites are indicated below the map (V: *EcoR*V, S: *Sst*I, H: *Hind*III, C: *Cla*I, P: *Pst*I, N: *Hinc*II, B: *Bgl*II, X: *Xba*I, Z: *Sst*II, A: *Ava*I, E: *EcoR*I). LSrRNA: large subunit ribosomal RNA gene, SSrRNA: small subunit ribosomal RNA gene, NTS: nontranscribed spacer.

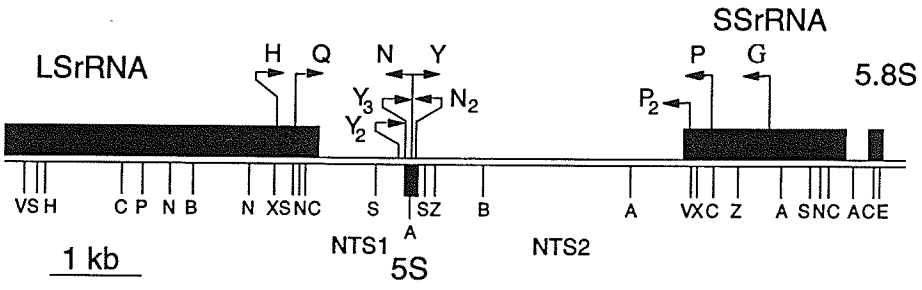

LSrRNA
H
Q
N
Y
SSrRNA
P
G
5.8S
Y_3
N_2
Y_2
P_2
VS H
C P N B
N XS NC
S
SZ
B
A
VXC Z
A SNC ACE
1 kb
NTS1
A
5S
NTS2

Fig. 2. PCR amplification products showing the position and orientation of the 5S rRNA sequence in *P. torulosum* 4212e. Lane L: BRL 1 kb ladder (Band sizes in kilobase pairs, from marked band at bottom: 0.5 [marked], 1.0, 1.6 [marked], 2.0, 3.1, 4.1, 5.1, 6.1, 7.1, 8.1, 9.1, 10.2, 11.2, 12.2). Lane 1: PCR product from amplification with NQ primer pair, Lane 2: PCR product from YP amplification, Lane 3: PCR product from PQ amplification.

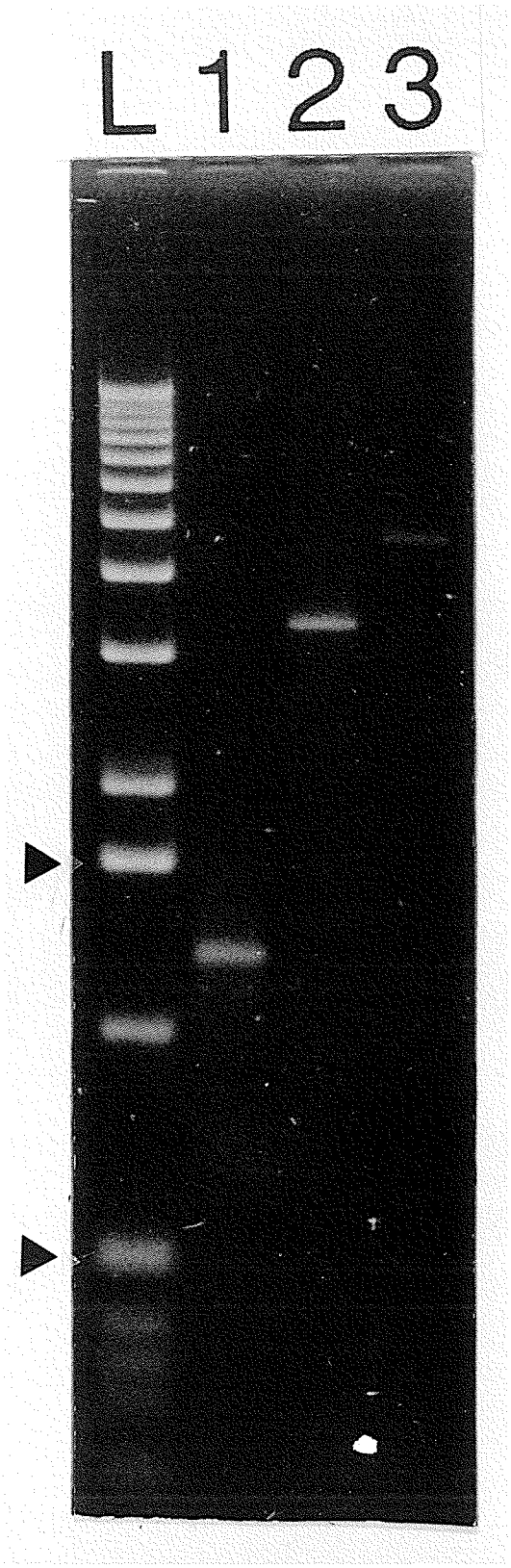

L 1 2 3

Fig. 3. The DNA sequence of the 5S rRNA gene and flanking regions of *P. torulosum* 4212e. Coding region of the gene is in bold face.

-120 -100 -80

ACCAGTTTCC TGTAACATGT GGTAGTGCCG GGCCGAGGAG TCCGGAGCTC

-60 -40

GAAATCGTCG TTTTTGGTGA AAGAGACGGT CCATGTTGGG AAGGCAGCTG

-20 1 20

TAGGGGTGGG TGGGAGTAGA GTGTC**GTAGA CGGCCATCTT AGGCTGAGAA**

40 60

CACCGTATCC CGTCCGCTCT GCGAAGTTAA GCAGCCTCAA GCTCGGGTAG

80 100 120

TACTCGGGTG GGTGACCACC GGGGAAGTCC GGGTGCTGTC TACTTTTTGC

140 160

TTTTTGCTTT TTGCTTTTGG TGGGCGGTGG ATATACAAAC TTCAAGTGGA

180

ACTGGG

Table 1. Presence or absence of 5S rRNA sequences in the rDNA repeat of *Pythium* isolates and those of related Oomycetes.

Isolate[a]	QN PCR	QY PCR	Band Size(kb)	Sporangial Form
P. coloratum BR483	+	-	1.75	Filamentous
P. coloratum BR323	+	-	1.40	Filamentous
P. diclinum 4110a	+	-	1.40	Filamentous
P. sulcatum BR157	+	-	1.65 1.50 1.30	Filamentous
P. pachycaule 4117b	+	-	1.50 1.30 1.00	Filamentous
P. vanterpoolii 4213a	+	-	2.30 1.95 1.75	Filamentous
P. torulosum 4212e	+	-	1.25	Filamentous
P. arrhenomanes 4201c	+	-	1.80	Filamentous
P. arrhenomanes BR607	+	-	2.20 1.80 1.50	Filamentous
P. graminicola/ aristosporum BR608	+	-	2.00 1.75 0.95	Filamentous
P. graminicola/ aristosporum BR166	+	-	1.75	Filamentous
P. aristosporum BR136	+	-	1.55 1.25	Filamentous
Pythium sp. BR147[b]	+	-	2.20 2.10	Filamentous
Pythium sp. BR671[b]	+	-	2.20 1.80	Filamentous

Pythium sp. BR625[b]	+	-	2.10 1.80	Filamentous
P. tardicrescens BR569	+	-	1.90 1.75	Filamentous
P. hypogynum BR635	+	-	2.40 2.10 1.85	Globose
P. hypogynum BR389	+	-	1.90	Globose
P. salpingophorum/ conidiophorum 4331a	+	-	1.95	Globose
P. violae MA2160	+	-	1.75	Unknown
P. violae MA2024	+	-	1.70	Unknown
P. periplocum 4461a	-	-		Filamentous
P. periplocum 4461b	-	-		Filamentous
P. nagae 4321a	-	-		Globose
P. nagae 4321b	-	-		Globose
P. irregulare BR486	-	-		Globose
P. paroecandrum BR574	-	-		Globose
P. mamillatum 4311a	-	-		Globose
P. erinaceous SS78621	-	-		Globose
P. iwayamae 4405g	-	-		Globose
P. rostratum 4329c	-	-		Globose
P. rostratum 4329j	-	-		Globose
P. oligandrum 4410b	-	-		Globose
P. spinosum 4012e	-	-		unknown
P. parvum 4009a	-	-		Unknown
P. parvum 4009b	-	-		Unknown
P. acanthophoron 4000a	-	-		Unknown

P. australe IMI331 762	-	-		Unknown
P. australe IMI332 970	-	-		Unknown
P. ultimum ultimum 4016a	-	-		Unknown
P. ultimum ultimum 4016c	-	-		Unknown
P. ultimum BR471	-	-		Unknown
P. ultimum sporangiferum 4333b	-	-		Unknown
P. ultimum sporangiferum 4333c	-	-		Unknown
P. ultimum sporangiferum 4333d	-	-		Unknown
Phytophthora cryptogea JM10	-	+	1.75 1.55	
Phytophthora cryptogea BR521	-	+	1.55	
Lagenidium giganteum[c]	+	-	1.65	
Pachymetra chaunorhiza	+	-	1.90 1.55	
Verrucalvus flavofaciens	+	-	1.30	
Achlya klebsiana 157a	-	+	1.85	

[a]Isolates with numbers prefixed by "BR" were obtained from the Biosystematics Research Centre, Ottawa, Canada. All other isolates were obtained from the Aquatic Phycomycete Culture Collection, Reading, U.K.
[b]These isolates were not assigned to species due to uncertainties.
[c]*L. giganteum* DNA was a gift from Michael E.S. Hudspeth, University of Northern Illinois.

Fig. 4. Characterization of 5S rRNA sequences in *P. ultimum*. Sizes based on BRL 1kb ladder. A) *Hinc*II+*Eco*RV-digested genomic DNA hybridized to various probes. Lane 1: *P. ultimum* BR600 DNA hybridized to pMF2. Lane 2: Same blot as lane 1 reprobed with primer Y. Lane 3: Same blot as lanes 1 and 2 reprobed with primer N_2. Lane 4: *P. ultimum* BR418 DNA hybridized to pMF2. Lane 5: Same blot as in lane 4 reprobed with primer Y. Lane 6: Same blot as in lanes 4 and 5 reprobed with primer N_2. B) *P. ultimum* BR471 genomic DNA digested with *Hinc*II (lane 1), *Eco*RI (lane 2), *Cla*I (lane 3) and *Kpn*I (lane 4) and hybridized to primer N_2.

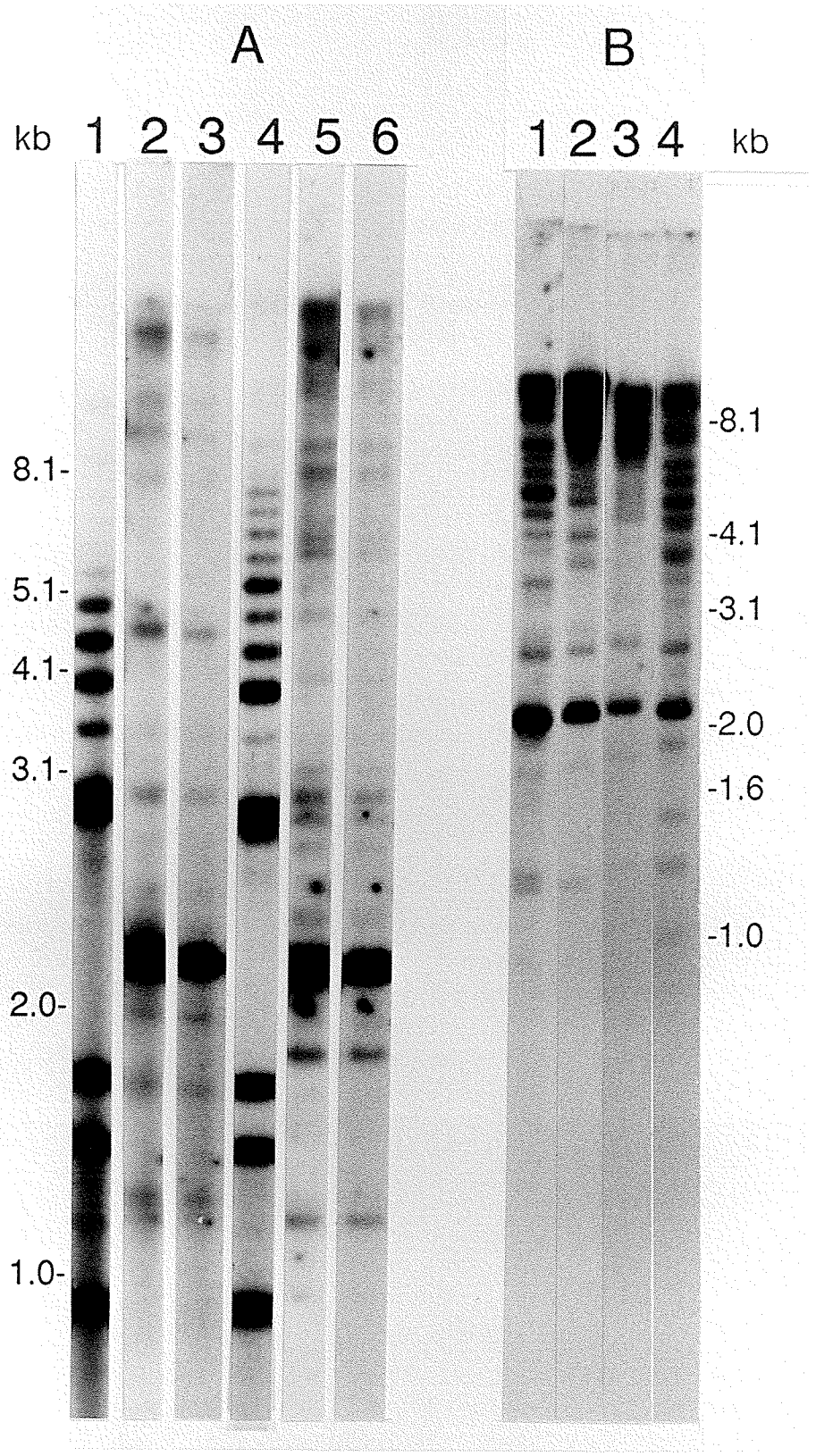

A
B
kb
1 2 3 4 5 6
1 2 3 4
kb
8.1-
5.1-
4.1-
3.1-
2.0-
1.0-
-8.1
-4.1
-3.1
-2.0
-1.6
-1.0

Fig. 5. Amplification and 5S rRNA gene hybridization of the NTS region of 12 *Pythium* species. L: BRL 1 kb ladder. The arrowhead indicates 2.0 kb and bands above it increase in 1 kb increments. Lanes 1 - 10: QP_2 (see Fig. 1) amplification products from *P. periplocum* 4461a (lane 1), *P. periplocum* 4461b (lane 2), *P. australe* 2 (lane 3), *P. paroecandrum* BR574 (lane 4), *P. erinaceous* 78621 (lane 5), *P. iwayamae* 4405g (lane 6), *P. oligandrum* 4410b (lane 7), *P. nagae* 4321a (lane 8), *P. nagae* 4321b (lane 9), and *P. spinosum* 4012e (lane 10). Lane 11: HG amplification product from *P. ultimum* BR471. Lanes 12 & 13: QP_2 amplification products from *P. torulosum* 4212e (lane 12), and *P. graminicola/aristosporum* BR166 (lane 13). A) Ethidium bromide stained gel. B) Gel shown in panel A blotted and hybridized to ribosomal probe pMF2. C) Membrane from panel B stripped of pMF2 and rehybridized with 5S rRNA gene-specific probe Y.

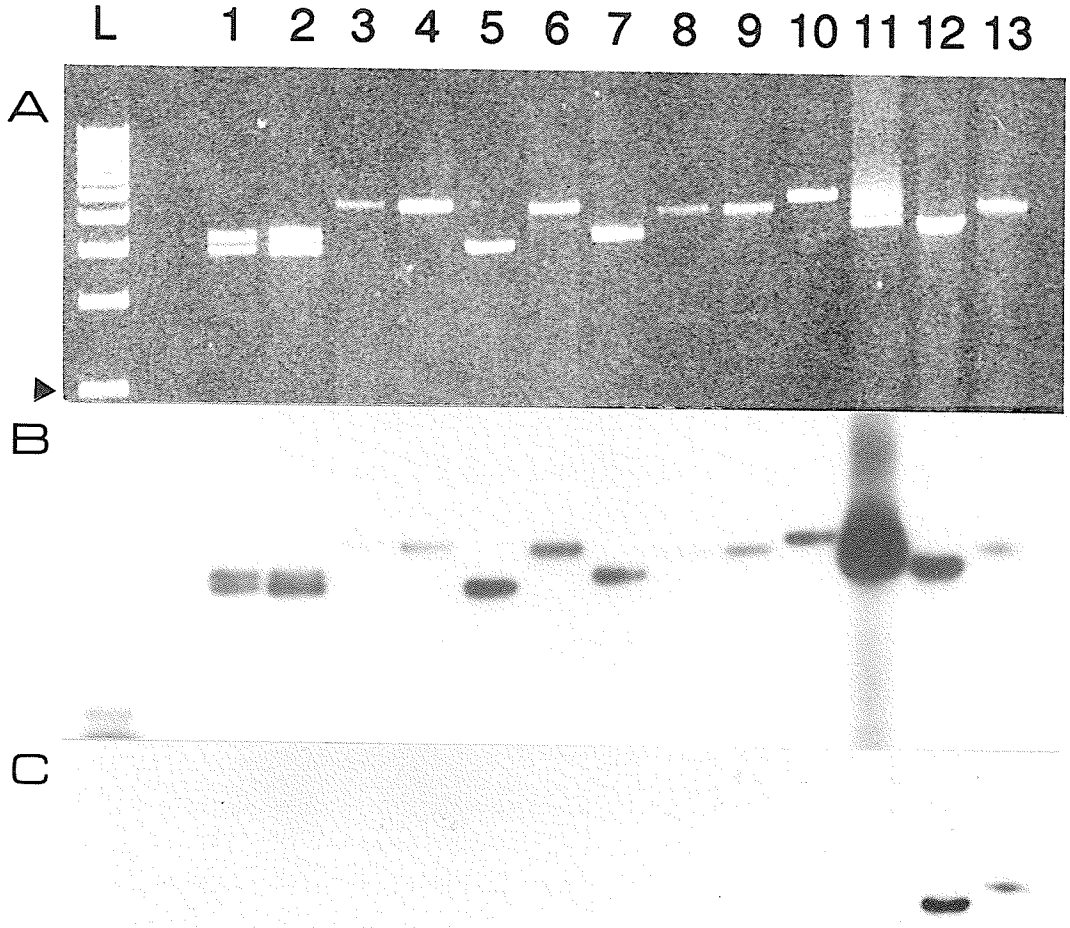
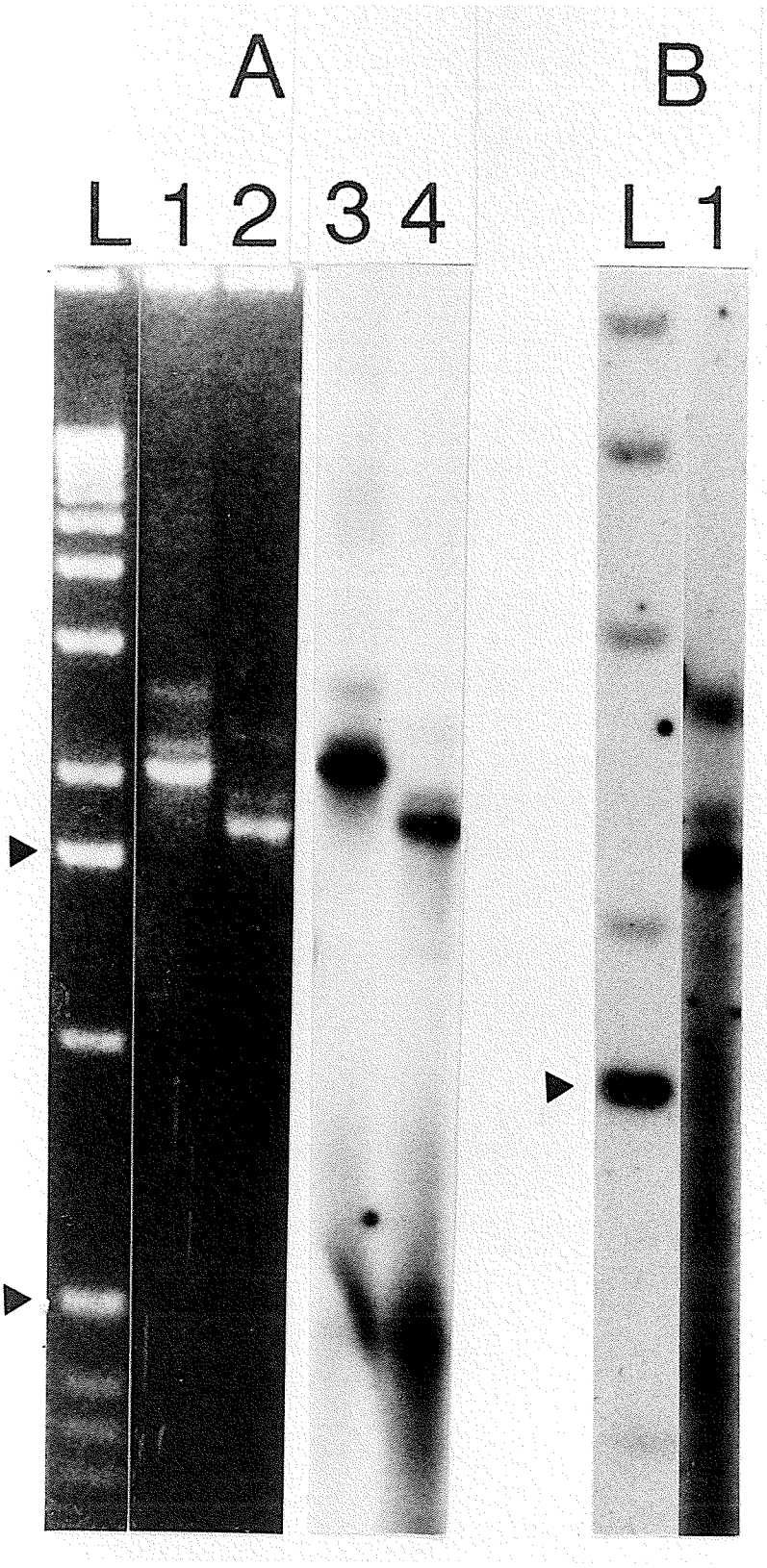

L
1
2
3
4
5
6
7
8
9
10
11
12
13
A
B
C

Fig. 6. Length heterogeneity in NTS1 of *P. vanterpoolii* 4213a. L: BRL 1 kb ladder. Arrowheads in A) indicate 0.5 and 1.6 kb bands, and in B), 1.6 kb. A) Heterogeneous PCR products. Lane 1: Product of amplification with HN_2 primer pair. Lane 2: Product of QN_2 primer pair amplification. Lanes 3 and 4: Hybridization of oligonucleotide Y to blotted gel seen in lanes 1 and 2. B) Heterogeneity in genomic DNA. Lane 1: Hybridization of oligonucleotide Y_3 to AvaI+ClaI-digested genomic DNA.


A
B
L 1 2 3 4
L 1

DISCUSSION

This study has uncovered two fundamentally different genomic locations for the 5S rRNA gene family within the genus *Pythium*. In most *Pythium* species that have filamentous sporangia (Dick 1990), the 5S gene is found in the NTS region of the rDNA repeat unit, and inverted with respect to the other rRNA genes. In most other *Pythium* species, those with globose sporangia or in which sporangia are lacking, the 5S gene family does not appear to be in the rDNA repeat unit. In *P. ultimum*, the 5S gene was shown to be clearly absent from the rDNA repeat, but to occur in 5S gene family arrays which are unlinked to the other rRNA genes; this arrangement is similar to that in plants and animals. However, several invertebrates have also been reported to have 5S genes linked to the rDNA (Drouin *et al.* 1987; Vahidi *et al.* 1988). In *Saccharomyces cerevisiae* a tandem array of variant 5S sequences has been reported, but most of the 5S genes are part of the rDNA repeat unit and the variant cluster, although probably expressed, may be dispensable (McMahon *et al.* 1984).

P. ultimum also appears to have dispersed 5S sequences, perhaps similar to the arrangement reported in *N. crassa* (Selker *et al.* 1981) and other filamentous Ascomycetes (Bartnik *et al.* 1986; Mao *et al.* 1982). Evidence of the dispersed sequences may be seen in Southern blots of genomic

DNA which show complex patterns of bands reproducible by hybridization to two different 5S-specific probes. Whereas the dominant 2.2 kb tandem family is conserved between geographically distant isolates of *P. ultimum*, polymorphism of the minor band patterns indicates that dispersed sequences have been rearranged. This may mean that the dispersed sequences are more mobile within the genome than are the other 5S sequences.

To show that the 5S gene was unequivocally present in the rDNA repeat of *P. torulosum*, PCR products from the region were sequenced. The 5S gene sequence was found in the predicted location and orientation. It was found to differ by two nucleotides from the published 5S sequence of *P. hydnosporum* (Walker and Doolittle 1982) and so it is presumed to be the functional 5S gene. The putative termination region immediately downstream of the 3′ end of the coding region consists of three direct repeats of TTTTTGC followed by TTTTGG. This is very reminiscent of the termination regions in animals (Rubacha *et al.* 1984) and plants (Goldsborough *et al.* 1982). No obvious initiation signals were detected in the 5′ flanking region similar to the "TATA box" found in *N. crassa* (Morzycka-Wroblewska *et al.* 1985), *Drosophila* species (Sharp and Garcia 1988), or mung bean (Hemleben and Werts 1988).

Detection of the rDNA-linked 5S gene by means of amplification with primers specific for the 5S gene paired

with primers specific for the LSrRNA and SSrRNA genes seems to be reliable. Confidence in the method is based on the following: 1) PCR results have been fully confirmed in *P. torulosum* and in *P. ultimum*; 2) different isolates of the same species yield the same qualitative results; 3) different primer pairs produce the same results; 4) PCR results were strictly consistent with sequence orientation (e.g. when amplification with QN is successful, amplification with QY is never successful, and vice-versa); 5) PCR products were recognized by 5S-specific oligonucleotide probes other than the ones used for amplification; 6) amplification of the entire NTS and attempted hybridization with a 5S rRNA gene-specific probe yielded results consistent with the QN/QY amplification results (Fig. 4); and 7) the pattern of presence and absence of 5S RNA sequences in the rDNA repeat unit is with one exception consistent with the form of the sporangium (Dick 1990).

Dick (1990) has arranged *Pythium* species into groups on the basis of zoosporangial morphology, using the criteria and terminology of Plaats-Niterink (1981). Species of *Pythium* with filamentous sporangia have the 5S gene in the rDNA repeat. The one exception to this rule uncovered so far is *P. periplocum*, the only known species with filamentous sporangia. However, this species also has ornamented rather than the smooth oogonia (Plaats-Niterink 1981) that are

found in all other filamentous sporangium species. Thus, the exception to the rule amongst the filamentous zoosporangial forms tested to date is also an atypical member of the group. Species of *Pythium* with globose sporangia tend not to have the 5S RNA gene in the rDNA repeat. The exceptions are *P. hypogynum* and *P. salpingophorum*; and there is no obvious shared character that would distinguish them from the others in this group. *Pythium* species appearing to lack sporangia also tend not to have the 5S gene in the rDNA repeat. The exception is *P. violae*, which is characterized by having relatively large oospores (Dick 1990). Many of the other species in this group (sporangia not known) are morphologically similar to the group of *Pythiums* with globose sporangia (Plaats-Niterink 1981; Dick 1990). In spite of the noted exceptions, these findings appear to be significant particularly since the evolutionary relationships within the genus have not yet been resolved. Zoosporangial form is only one character among many used in *Pythium* taxonomy, and it is surprizing that it should be so useful in predicting the probable 5S rRNA gene location. It should also be kept in mind that in isolated cases the negative PCR results may be due to a failure of the reaction rather than to absence of the 5S gene. It is unlikely that failure to amplify would be due to lack of specificity of the primers because the primers do work with much more distantly related oomycete species such as *Phytophthora*

cryptogea, *Achlya klebsiana* and others (Table 1).

In many cases, amplification of the NTS region yielded multiple bands, especially in NTS1. Although one cannot be sure that these reflect heterogeneity in the template DNA without further investigation of each case, it was shown that for one arbitrarily chosen species (*P. vanterpoolii*), the PCR bands matched the Southern blots both qualitatively and quantitatively. Similar faithfulness of PCR in reproducing template heterogeneity has been demonstrated previously (Buchko and Klassen 1990). The presence of heterogeneity in this region of the rDNA repeat suggests that this region of the repeat is susceptible to rearrangement. In *P. ultimum*, the corresponding region is also highly heterogeneous (Klassen and Buchko 1990) but the 5S gene is absent. Loss (or gain) of the 5S gene in this region of the rDNA repeat may well be related to these sequence characteristics.

This limited survey of the Oomycetes, although limited, shows that the predominant location for the 5S gene may be in the rDNA repeat. *Pythiums* with globose or unknown sporangia are the only exceptions at this time. The distribution pattern of this character suggests that the absence of the gene from the rDNA repeat is a derived character within the Oomycetes; the ancestor of the *Pythiums* with globose sporangia relocated the 5S gene in the course of evolution. Whether or not this was a local phenomenon

confined to oomycete evolution or a general trend also operational in the evolution of plants and animals, is not known at this time. If it is a general phenomenon, Clark's (1987) concept of primitive unlinked rRNA genes would not be supported.

The orientation of the 5S gene, although consistent within the genus *Pythium* ("inverted"), is not conserved for Oomycetes. Of the Oomycetes included in this study, *Phytophthora cryptogea*, it is believed to be most closely related to *Pythium*, has the opposite orientation, while *Lagenidium giganteum*, more distantly related, has the same orientation to the *Pythium* species tested. Similar variation is found within the Saprolegniales (*Achlya klebsiana*, *Pachymetra chaunorhiza*, *Verrucalvus flavofaciens*). In *Coprinus* species both orientations have been reported (Cassidy and Pukkila 1987). The variability found may be another indication that the 5S gene region is susceptible to rearrangement.

These results lead one to the following speculation. If it is assumed that the primordial eukaryotic 5S gene was linked to the other rRNA genes and that this arrangement survived in many fungi, a few invertebrates, and in *Pythiums* with filamentous sporangia and other Oomycetes, two patterns of 5S gene dispersion (unlinking) can be imagined. The fungal pattern would consist of loss of the gene from the rDNA repeat and a general scattering of copies throughout

the genome, as seen in *N. crassa* (Selker *et al.* 1981). The separate ancestors of animals, plants, and Oomycetes would lose the 5S gene from the rDNA repeat, but then assemble copies into tandem arrays. The capacity to form arrays instead of dispersals may be due to the structure of the NTS around the 5S gene before dispersion. Lack of repetitive elements in the fungal NTS may leave the dispersed 5S copies without the potential for forming arrays, while presence of repetitive elements in the NTSs of animals, plants and oomycetes (Klassen and Buchko 1990) may have allowed array formation. Further studies of the 5S sequence arrays in *Pythiums* with globose sporangia are being pursued to determine whether their spacer sequences have repetitive elements similar to those in the NTS of the rDNA repeat, from which they may have been derived.

CHAPTER 2

Organization of the intergenic region in the 5S rRNA tandem arrays of *Pythium ultimum* and related species

INTRODUCTION

The 5S rRNA gene family organization in species of the oomycetous genus *Pythium* is remarkable because some species have their 5S genes in the nontranscribed spacer (NTS) of the rDNA repeat unit, while others have tandem arrays of 5S genes that are unlinked to the rDNA repeat unit (Chapter 1). This is the first report of two fundamentally different 5S gene arrangements occurring in different members of the same genus, and thus may be an opportunity for studying both the nature and direction of the transition from one arrangement to the other. Previously, the only microorganisms in which tandem arrays of functional 5S genes have been reported were species of *Tetrahymena* (Pederson *et al.* 1984), *Trypanosoma* (Hernandez-Rivas 1992) and *Euglena* (Keller *et al.* 1992). The tandem arrays found in certain *Pythium* species appear to be most similar to those in animals (Brown *et al.* 1971) and plants (Hemleben and Grierson 1978). Oomycetes have been considered to be "pseudofungi" (Cavalier-Smith 1981), and on the basis of their small subunit rRNA sequences they appear to be related to golden-brown algae, brown algae, diatoms, and other stramenopiles (Gunderson *et al.* 1987). True fungi do not appear to have their functional 5S arrays arranged tandemly; their 5S genes are linked either to the rDNA repeat, as in *Saccharomyces cerevisiae* (Rubin and Sulston 1973), or are dispersed throughout the genome as in

Neurospora crassa (Selker *et al*. 1981).

Because the genus *Pythium* has species in which the 5S genes are linked to the rDNA repeat unit as well as species in which the 5S genes are found in tandem arrays, an evolutionary study of this divergence might reveal how 5S gene family organization is determined. The clues to gene family rearrangements should be found in the IGRs of the tandem arrays and in the regions flanking the genes in the NTS of the rDNA repeats. This study presents the sequences of the predominant IGRs of the 5S gene IGRs in *Pythium ultimum* and related species, and draws attention to their unusual structure.

RESULTS

Amplification of the 5S rRNA IGR in *P. ultimum*

On the assumption that the 5S rRNA genes of *P. ultimum* are arranged in tandem arrays as direct repeats (Chapter 1), gene to gene amplification was done using primers N_2 and Y (Fig. 1A). At least two products would be expected: the short 50 bp segment within the 5S gene and the longer segment which represents gene to gene amplification. Amplification products representing dimers or trimers of the 5S repeat unit would not be produced because the elongation time step of the PCR was set for a maximum of about 1kb. The 50 bp intergenic product was present, as well as a low abundance product of about 0.9 kb, but the main product was a fragment of about 0.6 kb (Fig. 1B). To show that this product represents the predominant 5S rDNA repeat unit in *P. ultimum*, total genomic DNA was digested with *BstE*II, a restriction enzyme known to cut once in each 5S rRNA gene, and then hybridized to the primer N_2. The main hybridizing band was at about 0.55 kb and there were two additional bands, one at 1.5 and the other at 5.5 kb (Fig. 1C). The *BstE*II to *BstE*II fragment in genomic DNA would be expected to be 50 bp shorter than the N_2Y amplification product because two copies of the 50 bp at the 5′ end of the gene are included in the amplification product. The interpretation depends on the absence of *BstE*II sites in the

IGR; this has been confirmed by subsequent DNA sequencing of the IGR (Fig. 2A). Thus, the genomic evidence is consistent with the results of amplification. The 1.5 and 5.5 kb fragments of genomic DNA which hybridize to the 5S probe have not been identified, but their presence indicates that either the tandem array is complex with a higher order of periodicity than the 0.6 kb pattern or that the 5S genes are in more than one type of array.

Survey of 5S rRNA gene family organization in the genus *Pythium*.

In chapter 1 it was reported that the 5S genes were found in the nontranscribed spacer (NTS) of the rDNA repeat of some *Pythium* species i.e. *P. coloratum*, *P. sulcatum*, *P. pachycaule*, *P. vanterpoolii*, *P. torulosum*, *P. arrhenomanes*, *P. graminicola/aristosporum*, *P. aristosporum*, *P. tardicrescens*, *P. diclinum*, *P. hypogynum*, *P. salpingophorum/conidiophorum*, and *P. violae*. This was shown by amplifying the region from the middle of the 5S gene to the end of the LSrRNA gene (Chapter 1). Next, amplification of the DNA from each of these species was attempted with N_2Y, but in each case it failed except for *P. salpingophorum/conidiophorum* and *P. pachycaule* (Table 1). In the case of *P. pachycaule*, the N_2Y product is one-half the length of the other N_2Y products and there is preliminary evidence that there are two 5S sequences in tandem in the

NTS. These results indicate that when the 5S gene (or genes) is present in the rDNA repeat, it is not found elsewhere in the genome in tandem repeats except in rare cases.

Conversely, the species in which the 5S gene could not be amplified within the NTS of the rDNA repeat i.e. *P. periplocum*, *P. nagae*, *P. irregulare*, *P. iwayamae*, *P. rostratum*, *P. oligandrum*, *P. spinosum*, *P. australe*, *P. mamillatum*, *P. ultimum ultimum*, *P. ultimum sporangiiferum* (Chapter 1), could all be amplified with N_2Y (Table 1). This suggests that those *Pythium* species with 5S genes in tandem repeats outside of the rDNA repeat also generally also lack the 5S genes in the NTS of the rDNA repeat. The two types of 5S gene arrangement appear to be relatively exclusive in the species tested thus far.

A few species failed to amplify with either one of the primer combinations (*P. parvum*, *P. acanthophoron*), but these results may be due simply to a failure of the amplification reactions. Also *Phytophthora* species which have the 5S gene in the rDNA repeat (Chapter 1; Howlett *et al.* 1992), also do not amplify with N_2Y. This further confirms the consistency of the N_2Y amplification method for detecting 5S genes arranged in tandem.

Sequences of the 5S rDNA repeat

The sequences of the N_2Y amplification products from two isolates of *P. ultimum* (BR471, BR638) were determined

using N_2 and Y as sequencing primers (Fig. 2A). The sequences of the two PCR products were found to be identical. The identity of the two sequences from isolates separated by time and geography increases our confidence that the method is valid and that errors due to PCR artifacts are negligible. Isolate BR471 was isolated from soil in Maryland, USA before 1980, and BR638 was isolated from a pea plant in Alberta, Canada more recently (D.J.S. Barr, personal communication). The sequence begins in the upstream 5S gene, three bases downstream of the N_2 primer, and ends in the downstream 5S gene, 6 bases upstream of the end of Y primer. Gene sequences are identical to those reported for 5S rRNA in *Pythium hydnosporum* (Walker and Doolittle 1982) so it is likely that the genes identified in this study are functional 5S rRNA genes and that their termini can be identified by homology with the *P. hydnosporum* sequence. On the basis of these assumptions, the IGR was found to consist of 457 bases.

Immediately downstream of the 3' terminus of the 5S gene is the putative termination region, dominated by two tracts of T. This is followed by 6 direct repeats of a sequence with the consensus AATACAC, followed by a stretch of simple sequence consisting of 10 CA pairs with some interruptions. The central region (bases 161-397) also has some simple sequence stretches (CATT consensus repeated 5 times from base 306 to base 350, and CTG repeated 4 times

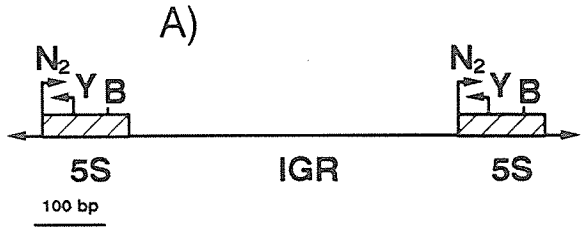
from base 350 to base 387), and a region just upstream of the 5′ terminus of the 5S gene is dominated by GT repeats, interrupted by tracts of T. Embedded in this region is a family of 7 AAG repeats (bases 446-470). The sequence TAGATAT is found 15 bp upstream of the 5′ terminus of the 5S gene and may be significant as a transcription signal.

The most striking deviation from randomness in the IGR sequence is the extreme compositional strand asymmetry at each end (Fig. 3A). At the 5′ end, A and C are segregated predominantly to one strand, and G and T to the other. At the 3′ end, G and T predominate on the first strand, and A and C on the second. The asymmetry for C at the 3′ end is so severe that only two out of the last 128 base positions are occupied by C on the first strand. The central region of the IGR shows no significant asymmetry.

To determine whether the 5S IGRs of other *Pythium* species have similar sequence characteristics, complete sequences of the N_2Y products of *P. spinosum* 4012e and *P. irregulare* BR486 were determined (Fig. 2B,C). In *P. spinosum*, the general pattern of base composition is very similar to that in *P. ultimum* except that CA predominance is extended to the middle of the IGR and beyond. An array of CA repeats occurs in the same relative position as that in *P. ultimum*, but it is more interrupted and irregular. The middle of the sequence also has abundant CA and CAA repeats (Fig. 3B). The GT-rich array near the 3′ end of the sequence

is long with a highly regular GT doublet array (21 repeats) at its center. In *P. irregulare* there are no AC or GT rich regions or simple sequence tracts. Major regional strand asymmetry is also absent (Fig. 3C).

Fig. 1. Amplification of the 5S rRNA intergenic region (IGR) in *P. ultimum*. A, Physical map of 5S rRNA IGR. Symbols above the map indicate oligonucleotide primer sites, with arrows giving 5′-3′ orientation. B, PCR amplification. Lane 1, BRL 1-kb ladder. Lane 2, N_2Y amplification products from *P. ultimum* BR471. C, Hybridization of genomic DNA. Lane 1, BRL 1-kb ladder. Lane 2, *P. ultimum* BR471 genomic DNA digested with *BstE*II and probed with primer N_2.


A)
N2
Y
B
N2
Y
B
5S
IGR
5S
100 bp

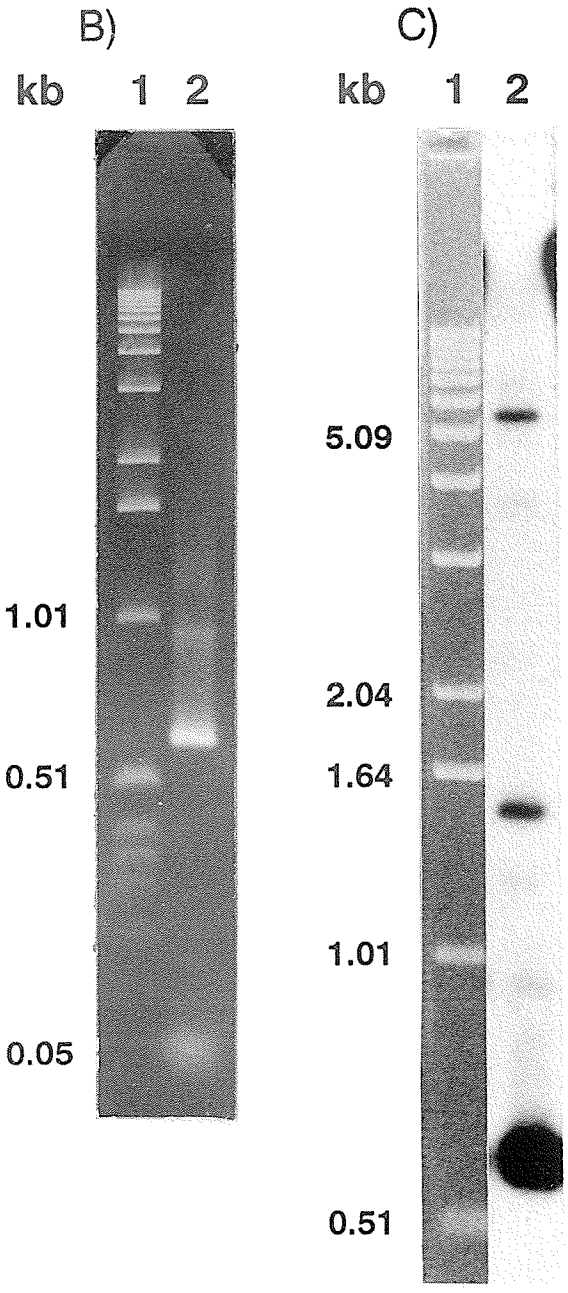

B)
kb
1
2
1.01
0.51
0.05
C)
kb
1
2
5.09
2.04
1.64
1.01
0.51

Table 1. Presence of 5S rRNA sequences in tandem repeats in *Pythium* isolates and related Oomycetes.

Isolate[a]	N_2Y PCR	Band Size(kb)	Sporangial Form
P. coloratum BR483	-		filamentous
P. coloratum BR323	-		filamentous
P. diclinum 4110a	-		filamentous
P. sulcatum BR157	-		filamentous
P. pachycaule 4117b	+	0.3	filamentous
P. vanterpoolii 4213a	-		filamentous
P. torulosum 4212e	-		filamentous
P. arrhenomanes BR607	-		filamentous
P. graminicola/ aristosporum BR608	-		filamentous
P. aristosporum BR136	-		filamentous
P. tardicrescens BR569	-		filamentous
P. hypogynum BR635	-		globose
P. hypogynum BR389	-		globose
P. salpingophorum/ conidiophorum 4331a	+	0.6	globose
P. violae MA2160	-		unknown
P. violae MA2024	-		unknown
P. periplocum 4461b	+	0.7	filamentous
P. nagae 4321a	+	0.8	globose
P. nagae 4321b	+	0.8	globose
P. irregulare BR486	+	0.7	globose
P. paroecandrum BR574	+	0.7	globose

P. mamillatum 4311a	+	0.8	globose
P. mamillatum 4311b	+	0.8	globose
P. erinaceous SS78621	+	0.7	globose
P. erinaceous SS78622	+	0.7	globose
P. iwayamae 4405g	+	0.6	globose
P. rostratum 4329c	+	0.7	globose
P. rostratum 4329j	+	0.7	globose
P. oligandrum 4410b	+	0.8	globose
P. spinosum 4012d	+	0.8	unknown
P. spinosum 4012e	+	0.6	unknown
P. parvum 4009b	-		unknown
P. acanthophoron 4000a	-		unknown
P. australe IMI331 762	+	0.8	unknown
P. ultimum BR471	+	0.6	unknown
P. ultimum sporangiiferum 4333b	+	0.6	unknown
Phytophthora cryptogea JM10	-		
Phytophthora cryptogea BR521	-		

[a]Isolates with numbers prefixed by "BR" were obtained from the Biosystematics Research Centre, Ottawa, Canada. All other isolates were obtained from the Aquatic Phycomycete Culture Collection, Reading, U.K.

Fig. 2. DNA sequence of 5S rRNA intergenic region (IGR). A, *P. ultimum* BR471 and BR638. B, *P. spinosum* 4012e. C, *P. irregulare* BR486. The 5S gene coding sequences are shown in small letters, the IGR sequences are indicated by capital letters. The poly (CA/GT) tracts are underlined.

A)

BstEII

ttaagcagcctcaagctcgggtagtactcgggtgggtgaccaccggggaa -50
gtccgagtgctgtctacTCTTTTTCTTTCCTCCGAATTACACAATCACAC -100
AATCACACAATCACAGAACCACCGACTCAACACCGCAGCACACACACACG -150
CACACACTCAGAACGTCGGCGCGACTCACTGAACCCCCTAAATGACATCG -200
CGACCCCCCGCAATGGAGTGGCATAGGGGTTATGTCACACGGGGAACACG -250
CTCGTGCACTCGTATCAAGTCTCCTGTAAACGCGTCATGGTCAAACGCGC -300
GTTTTCATGCCAAAATGACCATTTCATCCATTTGTGGAGCTTTGTGCATT -350
CTGTTTCGGGCCAAGGAGCCAGGGGGAACTGCTGCTGCAAATATGCAAGG -400
TATGTGGAGAGATGTGTGTTTTGTTTTTGTTGTTTTTGTTGTTGTAAGAG -450
TGGAAGGCAAGAAGAAGAAGTAAGTGGTTGGTGTTTGTAAAATGAATGTG -500
TTGCGGGTGTAGATATGAGTTGNNgtagacggccatcttaggctgagaac -550

B)

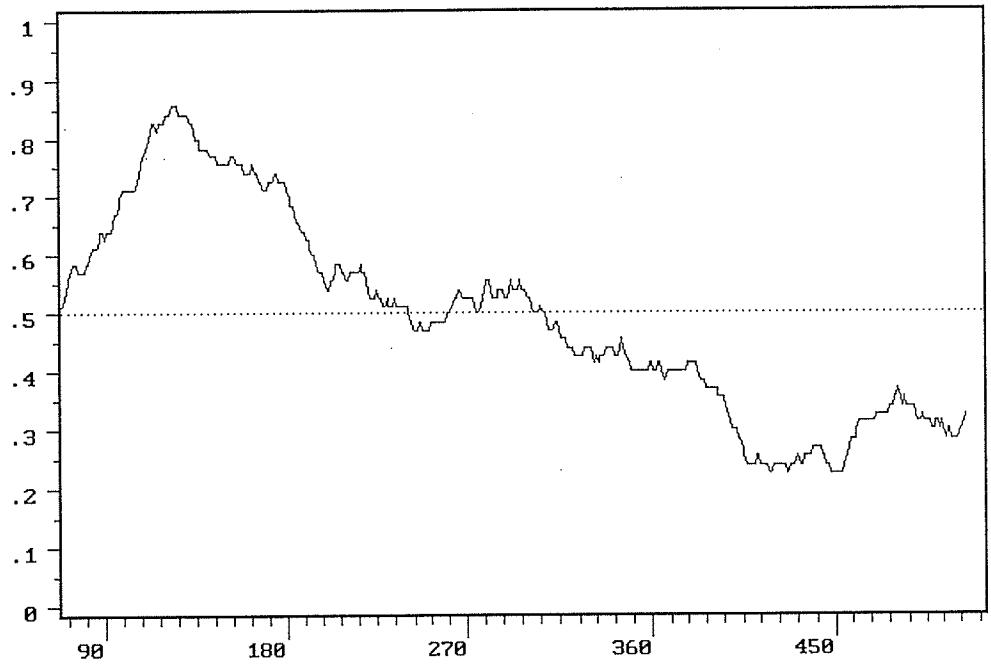
gctcgggtagtactcgggtgggtgaccaccggggaagtccgagtgctgtc -50
tacTCTTTTGCAAATCCTCGCGTGTCATTTCAAATTGCCGATTCATTGTG -100
TGAATCACTTGCTTTACACACATGCACAGTCAGACAGACACAACACATTC -150
GCACATTTCCGTTACACCACACATCGTTGCACATTAAAACACACATGTGC -200
GCATGTGCAAATGCACACACGCATCTTGTTGTGTATACGATCGCAACAAC -250
CAAATGATTTCATAGTCGTTTACCATACACGGCAAGACTGTAGACGTTTC -300
CACAATATGGCGTTACTATATANNACCAAAAACCCAACCACGGTTGTGCT -350
TGCAACGAAAGCGAATACATATGCGTTCAANGGTGCTAACAATCGCACAA -400
TGAACGATTGGTCCGGTGCCAGGTCGTGTTCGCTGTGTACATTGATGTGT -450
GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGAGGCGTGCAAT -500
GGTTGTCTCAAACTAATGAATGTTTTGTTGTGGATGAAAAAGAAGCGTAT -550
GAGTTGGCTGACAGTTTGGTATTNgtagacggccatct -588

C)

gcctcaagctcgggtagtactcgggtgggtgaccaccggggaagtccgag -50
tgctgtctacTCTTTTCTCTTTTTCACCTACTTACTTGTTGCTAACAAAT -100
AAACACATGTTATCACGCTACACTGTAGCGATATAGCTAGTAAATACCGA -150
ATGCTGTCTCGTCTTTATCACTTTTTCCCACTTACCTACTTGTTTGTAAC -200
AGTGTTGTAACGCTACACTGCAATATACCGATATACCAAGTAAATATCGA -250
ATGCTATACAACTTCTTGCTCGTTCGAGGTCTTCTGCAGACACCAAGTGC -300
GCTTTCATATAGAGACTCTCCATACAAAGAGGCGCTTTACGTAAACGCCG -350
ATATCTCGACAACCACAGCAAGCTTTTAGTGCTGTCAACGCAGATCGACG -400
CGTCTTGTCAGCGCCGCACCTCCCTGCCAAGTCTCAGGGCTGGGTCGCGT -450
GTCTCGATGTACTGTCTGCCAGTCTAATGAACGTCATTTCTCTTTTCTGT -500
TTAGCCCAAGTGTTGAGAGTCTCCAAATTGGAAGGAGTTCTTCCATTTCA -550
TCCATAATGATTTGTACTACGGAAAGAACCCGGTCGAGCAGTTGAACACT -600
Agta -604

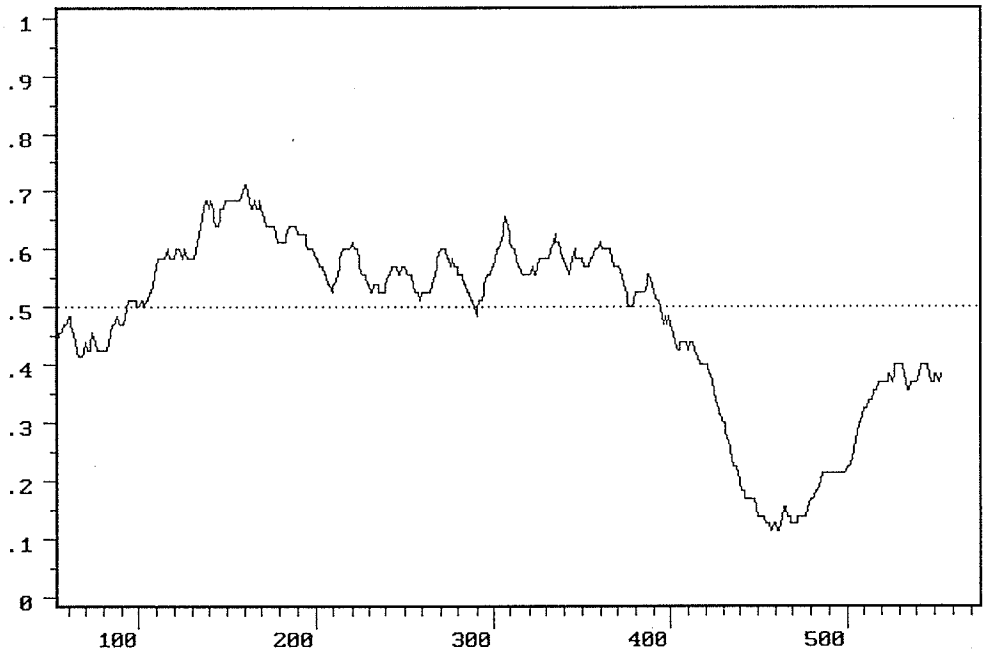
Fig. 3. Compositional strand asymmetry in the IGR sequence. A, *P. ultimum* BR471 and BR638 (refer to Fig. 2A). B, *P. spinosum* 4012e (Refer to Fig. 2B). C, *P. irregulare* BR486 (Refer to Fig. 2C). Plot of AC/ATGC bases was computed using an interval of 70 bases (PBASE program, PC/GENE, IntelliGenetics, Inc.). Base position and frequency are indicated on X and Y axis respectively.

A)



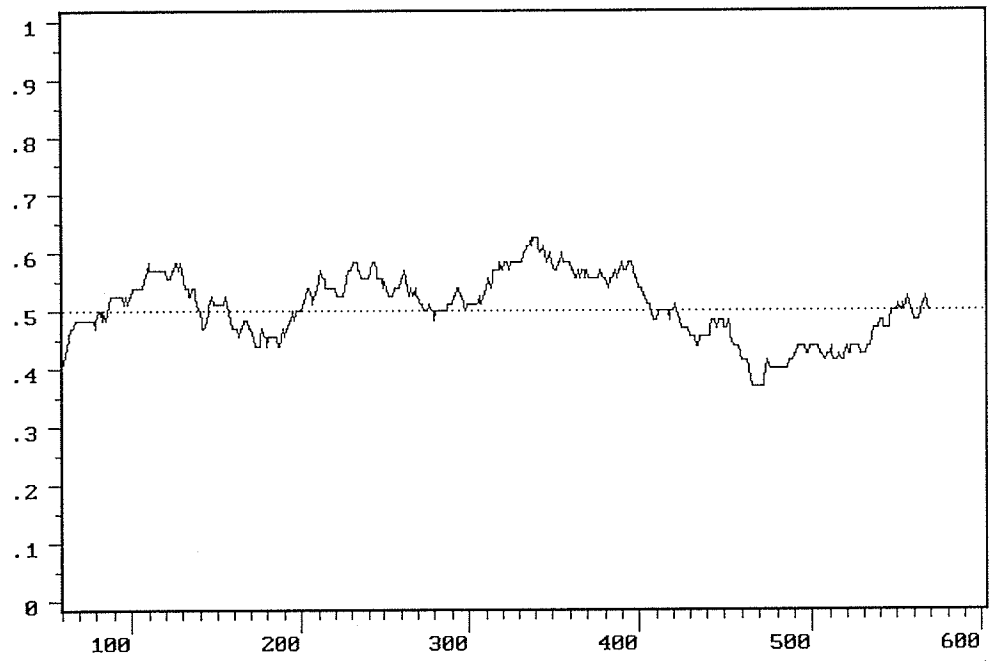
Plot of AC/AIGC in sequence N2V471.
From base 68 to base 524 computed using an interval of 70 bases.

B)



Plot of AC/ATGC in sequence N2Y4012E.
From base 54 to base 574 computed using an interval of 70 bases.

C)



Plot of AC/AIGC in sequence N2V486.
From base 60 to base 602 computed using an interval of 70 bases.

DISCUSSION

This chapter reports on the use of gene to gene amplification (Kolchinski *et al.* 1991) to detect the presence or absence of 5S rDNA tandem repeats in a representative collection of *Pythium* species, and to access the repeat unit for sequence determination.

The results, when taken together with the previous findings (Chapter 1) confirm that there are two kinds of *Pythium* species, with respect to their 5S rRNA gene family organization. Species with filamentous zoosporangia have their 5S genes in the NTS of the rDNA repeat and do not have tandem arrays. The single noted exception is *P. periplocum*, which has filamentous sporangia, 5S genes in tandem repeats, and lacks a 5S gene in the NTS. *P. periplocum* is the only species of *Pythium* which has both ornamented oogonia and filamentous sporangia (Plaats-Niterink 1981), and the former trait would associate it strongly with species that have globose zoosporangia and tandem 5S repeats. A likely explanation is that the filamentous zoosporangia in *P. periplocum* have evolved independently.

Pythium species with globose or unknown zoosporangia have tandem repeats of 5S genes and lack 5S gene in the NTS. The noted exception is *P. salpingophorum/conidiophorum*, which has 5S or 5S-like genes both in tandem arrays and in the NTS. Further work should reveal whether the genes in

both locations are functional and whether this arrangement can be considered transitional.

Gene to gene amplification also can provide the template for the sequencing of both the 5S genes and the intergenic regions. But sequence microheterogeneities within the IGRs of the 5S gene family often preclude complete sequencing and allow only one strand of the template to be partially sequenced. The amplification products for *P. irregulare*, *P. spinosum*, and for both isolates of *P. ultimum* were sufficiently homogenous to allow complete sequencing.

Simple sequences are loosely defined as short regions (less than 100 bases) within which very short sequence motifs (one to a few bases) are repeated in tandem (Tautz and Renz 1984a). Unlike satellite DNA, simple sequence regions are interspersed in the eukaryotic genome (Flavell *et al.* 1977), and may be transcribed (Tautz and Renz 1984b). A simple sequence has been reported in the 5S IGR of pine (Gorman *et al.* 1992). The most obvious cases of simple sequence DNA in the *P. ultimum* 5S IGR are the CA dinucleotide repeat near the 5′ end, and the AAG trinucleotide repeat that is embedded in the GT-rich region near the 3′ end. The GT-rich region itself is too complex to be classified as simple sequence, but it may be related to simple sequence; it could be seen as evolving towards or away from simplicity. It is possible that stretches of AC or GT rich sequences oscillate between simple and complex

states stochastically. In *P. spinosum* the analogous GT-rich region, in fact, has a simple sequence GT array which comprises most of the GT-rich region. On the other hand, where *P. ultimum* has the simple sequence AC array, *P. spinosum* has a complex AC-rich region. The 5S IGR of *P. irregulare* did not show any of these features, however. At this time there is no reason to think that *P. irregulare* is phylogenetically isolated from the other species. Thus, the type of 5S IGR that is dominated by simple sequence is not unique to *P. ultimum*, since it also occurs in *P. spinosum*, but it is not universal in the members of the genus *Pythium*, e.g. it is absent in *P. irregulare*.

The complementary arrangement of AC rich and GT rich regions at either end of the IGRs in *P. ultimum* and *P. spinosum* has not been observed in plants or animals. If further work reveals that most *Pythium* species with tandem 5S genes have this distinctive feature, and if the same feature appears elsewhere in the genome, it may help to explain the way in which the tandem arrays have been assembled.

CHAPTER 3

Diverged 5S rRNA sequences adjacent to the 5S rRNA genes in the rDNA of *Pythium pachycaule*

INTRODUCTION

Diverged copies or pseudogenes of 5S rRNA genes have been found in dispersed families of 5S genes in species of both *Aspergillus* (Bartnik *et al.* 1986) and *Neurospora* (Selker *et al.* 1981), and also within tandem arrays of 5S genes in several animals (Jacq *et al.* 1977; Kay and Gall 1981; Rubacha *et al.* 1984) and one plant (Singh *et al.* 1994). A possible 5S pseudogene has been reported in the NTS (nontranscribed spacer) of the nematode *Meloidogyne arenaira*, but no functional 5S gene was detected there (Vahidi *et al.* 1988). The present study uncovered diverged copies of the 5S gene adjacent to the 5S gene in the NTS of the rDNA repeat of *Pythium pachycaule*. This is the first report of tandemly arranged 5S or 5S-like sequences linked to the rDNA repeat within the NTS.

This discovery in a species of *Pythium* is significant because some species of *Pythium* have the 5S gene in the NTS and others have tandem arrays of 5S genes unlinked to the rDNA repeat (Chapter 1). The linked arrangement can be taken as ancestral to *Pythium* because it is the only arrangement found in species of other oomycete genera such as *Phytophthora*, *Lagenidium*, *Achlya*, *Saprolegnia*, *Pachymetra*, and *Verrucalvus* (chapter 1, Howlett *et al.* 1992). If this is true, *Pythium* species such as *P. ultimum*, in which the 5S gene is absent from the NTS, but is present in tandem arrays

outside the rDNA repeat, must have evolved from a form having a single 5S gene in the NTS. The duplication and divergence of 5S genes in the NTS of *P. pachycaule* may shed light on how tandem arrays could have been generated.

Length heterogeneity in the NTS is very common in *Pythium* species (Klassen and Buchko 1990; Martin 1990; Chapter 1). In *P. pachycaule*, length heterogeneity in the NTS made it possible to isolate two versions of the diverged 5S sequence and to form a hypothesis about their origins.

RESULTS

Location of the 5S and 5S-like sequences in *Pythium pachycaule*

A physical map of the rDNA repeat unit in *P. pachycaule* was constructed by hybridizing pMF2 to multiple digests of genomic DNA, and the gene regions were found to be identical to those in the map for *P. torulosum* (Chapter 1). The NTS region in *P. pachycaule* was also approximately the same size as that in *P. torulosum* but as expected the map was different (Fig. 1).

Amplification of genomic DNA with the primer pair QN yielded three main bands ranging from about 1.0 to 1.5 kb (Fig. 2A, lane 1), whereas amplification with primer pairs QY and NP_2 did not yield any products. These results suggest that 5S genes or 5S-like sequences are present in the NTS within about 1 kb of the 3′ terminus of the LSrRNA gene, that they are in the inverted orientation, and that there may be more than one of them in a tandem arrangement. Primers N_2 and Y were used to determine whether it would be possible to amplify from one 5S sequence to the next in the tandem arrangement and a 300 bp band was obtained (Fig. 2A, lane 2). The 50 bp band is the intragenic amplification which was expected with this primer pair. In *P. torulosum* and other species with filamentous sporangia, no gene-to-gene products were produced by N_2Y amplification; this is

consistent with only one 5S gene being present in the NTS of each rDNA repeat (Chapter 2). In *P. pachycaule*, however, N_2Y amplification yielded a 300 bp fragment which might be a gene-to-gene product of tandem 5S sequences. The size of the fragment is consistent with the difference in size of the two smaller fragments generated by QN amplification (Fig. 2A, lane 1).

Sequence characterization of the 5S gene region in the NTS

To characterize the 5S gene region, the entire NTS was amplified with primers Q and P_2. Two products of 4.5 and 4.7 kb were produced, indicating that two versions of the NTS were present (Fig. 2B). When restriction digests of genomic DNA were probed with the ribosomal gene probe pMF_2, a doublet of bands (0.2 kb apart) was seen whenever the segment from the *Cla*I site near the 3′ end of the LSrRNA gene to the *Sac*II site in the NTS was included between restriction sites (data not shown). The site of the 0.2 kb insertion(s) has not been more precisely located. To determine whether 5S gene sequences occurred on both long and short versions of the NTS, the QP_2 products were blotted and probed with the N_2Y and a strong hybridization signal was observed for both bands (data not shown). The two versions were then eluted separately from the gel and partially sequenced to locate and compare 5S sequences. At least two independently amplified templates were sequenced

to avoid PCR error.

For the short version, the PCR product QN_2 was used as the sequencing template, and to avoid priming of some primers to two sites, QN_2 was cut with *Sac*II and the two resulting fragments used separately as templates. The results of sequencing with primers N_2, Y, Y_3, N, and Y_6 were as follows. Downstream of the N_2 primer site the sequence is identical to the reported 5S gene in *P. torulosum* (Chapter 1) (Fig. 3A) and is thus assumed to be the functional 5S gene because it differs by only two bases from the published sequence of the 5S RNA molecule of *P. hydnosporum* (Walker and Doolittle 1982). This is followed by "spacer 1" (Fig. 3B), which contains the expected *Sac*II site. The 5′ end of the spacer immediately following the end of the gene (TTTTTTGCT) is very similar to the 3′ gene flanking region in *P. torulosum* (TTTTTGCT), which was taken as part of the putative transcription terminator (chapter 1). Downstream of the 'terminator', the spacer sequence is not homologous to the 3′ flanking region in *P. torulosum*. The spacer is followed by the 5S-like sequence designated 5S′ (Fig. 3A), followed by 145 bp of flanking sequence (Fig. 3C). 5S′ is 12 bp shorter than the gene, and the two sequences have been aligned so that 73.6% of positions are homologous. Differences are distributed fairly evenly over the gene sequences except that the N/Y primer region is conserved. *Ava*I and *BstE*II sites, characteristic of 5S genes in species

of *Pythium*, are not conserved, and only N and Y primer sites are intact. The two N primer sites (in 5S and 5S') account for two of the three QN products seen in figure 1 (1.0 and 1.3 kb, lane 1). The sequences between Y_6 and N_2 were determined in both directions. Flanking sequence downstream of Y_6 (Fig. 3C) was determined in only one direction. The 'terminator' at the end of the 5S gene is not repeated after 5S', although there is a tract of 3 T's. The flanking region is not homologous with the spacer region.

For the long version of the NTS, the PCR product QN_2 was used as template, and primers N_2, N_4, Y_3, and Y_6 were used for sequencing. The downstream flanking region could not be sequenced due to heterogeneity of the template. Downstream of the N_2 primer site is another 5S gene sequence identical to the one on the short version, followed by the "spacer 2" region (Fig. 3B) which is highly homologous to "spacer 1" (166 identical positions, 4 base differences, one deletion in spacer 1, and 3 deletions in spacer 2). A 5S-like sequence (5S'') begins at a position precisely homologous to the beginning of the 5S' sequence in the short version. Primer N does not recognize the altered target sequence in 5S'', and so only one band (1.5 kb) can be generated by QN amplification. This accounts for the third band in figure 1 (1.5 kb, lane 1). 5S'' and 5S' differ at only 9 positions, 6 of which are in the N_2 primer region. The last 60% of both sequences are identical.

Fig. 1. A physical map of the *Pythium pachycaule* 4117b rDNA repeat unit. Symbols above the map indicate oligonucleotide primer sites, with arrows giving 5′-3′ orientation. Restriction sites are indicated below the map: V = *EcoRV*; H = *Hind*III; C = *Cla*I; S = *Sac*II; and B = *BstE*II. LSrRNA = large-subunit rRNA gene; SSrRNA = small-subunit rRNA gene; 5S′ and 5S" = diverged copies of 5S rRNA sequences; 5S = 5S rRNA gene; and NTS = nontranscribed spacer. The upper part of the figure represents the short version of the NTS and the lower part shows the relevant details of the long version. Spacer 1 and Spacer 2 refer to the segments between 5S′, 5S and 5S", 5S sequences respectively.

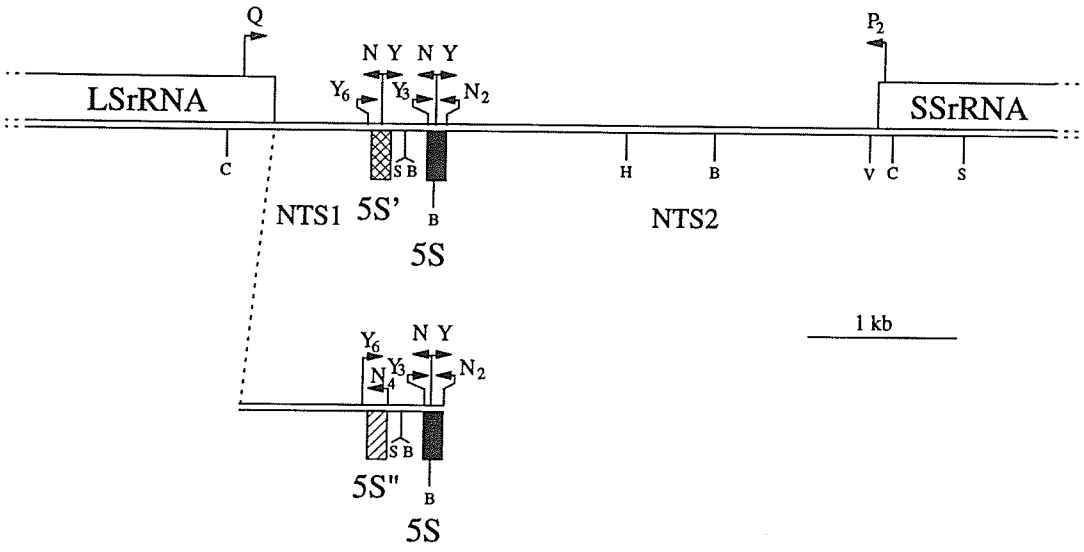

Q
N Y N Y
LSrRNA
Y6
Y3
N2
P2
SSrRNA
C
S B
H
B
V C
S
NTS1
5S'
B
NTS2
5S
1 kb
Y6
N Y
N4
Y3
N2
S B
5S"
B
5S

Fig. 2. PCR amplification of 5S rRNA genes in *P. pachycaule*. A, Lane L, BRL 1-kb ladder. Arrowheads indicate 0.5 and 1.6 kb bands. Lane 1, QN amplification products from NTS1 (refer to Fig. 1). Lane 2, N_2Y amplification product from the 5S spacer region. B, Lane L, 1-kb ladder. The arrowhead indicates the 1.6 kb band. Lane 1, QP_2 amplification products of the entire NTS.

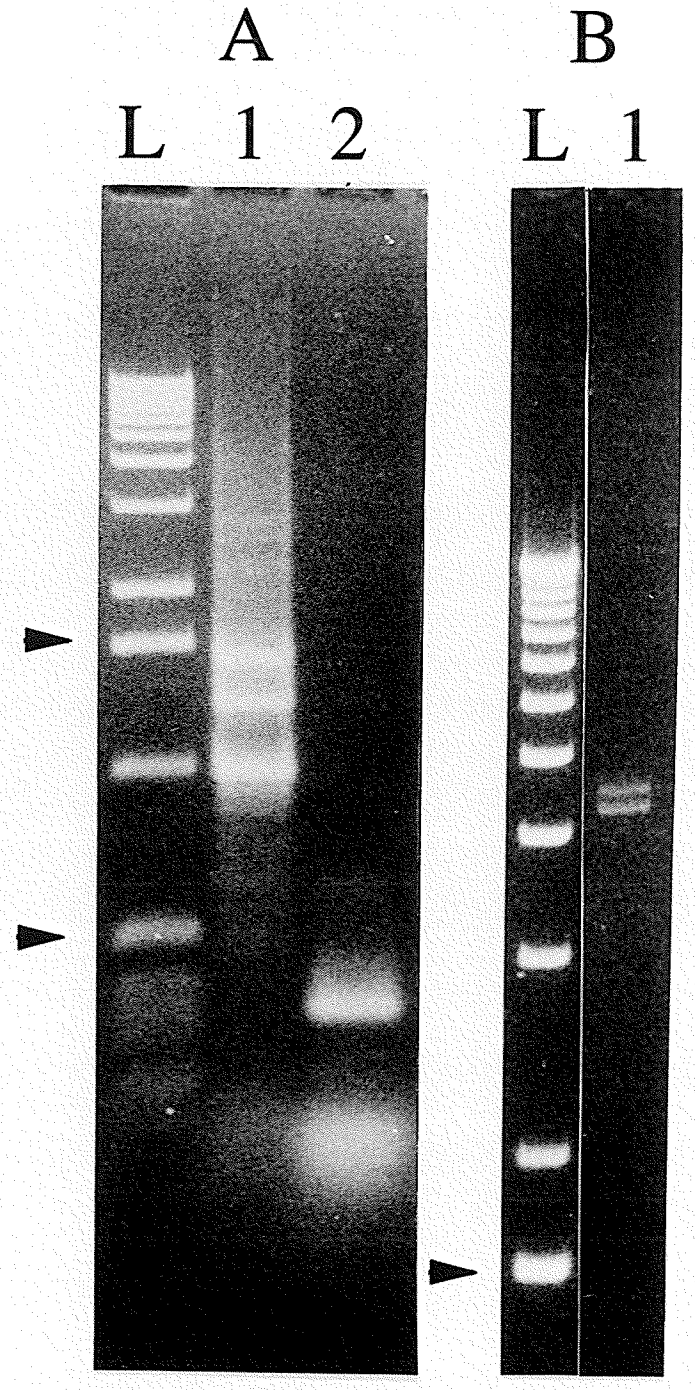
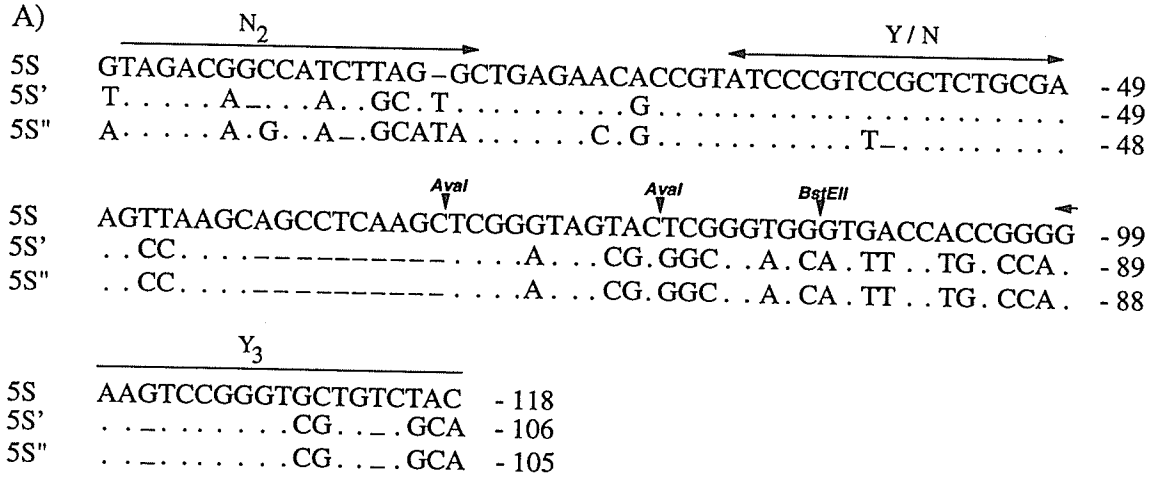
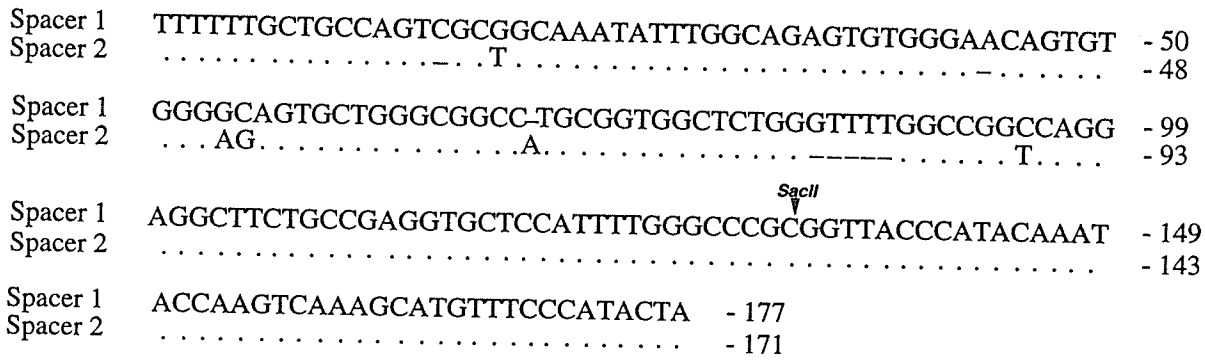

A
B
L
1
2
L
1

Fig. 3. DNA sequence of 5S genes and flanking regions in *P. pachycaule*. A, sequence alignment of the coding gene (5S), the diverged copy from the short NTS version (5S'), and the diverged copy from the long NTS version (5S"). Conserved nucleotides are indicated by dots (.). Deletions are represented by dashes (-). Primer sites are indicated by horizontal arrows and restriction sites are indicated by vertical arrows above the sequences. B, sequence alignment of "Spacer 1", the spacer region between the functional gene (5S) and the diverged copy (5S') in the short version of the NTS, and "Spacer 2", the spacer region between the functional gene (5S) and the diverged copy (5S") in the long version. Conserved nucleotides and deletions are indicated as above. C, sequence alignment of the flanking region downstream from the diverged 5S copy in the short NTS (5S'FS) and the corresponding region in the long NTS (5S"FS).


A)
N2
Y / N
5S GTAGACGGCCATCTTAG–GCTGAGAACACCGTATCCCGTCCGCTCTGCGA - 49
5S' T. . . . . A_. . . A. . GC. T. . . . . G. . . . . - 49
5S" A. . . . . A. G. . A_. GCATA. . . . . C. G. . . . . T_. . . . . - 48
AvaI
AvaI
BstEII
5S AGTTAAGCAGCCTCAAGCTCGGGTAGTACTCGGGTGGGTGACCACCGGGG - 99
5S' . . CC. . . . – – – – – – – – – – – – . . . .A. . . CG. GGC. . A. CA. TT. . TG. CCA. - 89
5S" . . CC. . . . – – – – – – – – – – – – . . . .A. . . CG. GGC. . A. CA. TT. . TG. CCA. - 88
Y3
5S AAGTCCGGGTGCTGTCTAC - 118
5S' . . _. . . . . CG. . _. GCA - 106
5S" . . _. . . . . CG. . _. GCA - 105

B)


Spacer 1
Spacer 2
TTTTTTGCTGCCAGTCGCGGCAAATATTTGGCAGAGTGTGGGAACAGTGT - 50
. . . . . T . . . . . - . . . . . - 48
Spacer 1
Spacer 2
GGGGCAGTGCTGGGCGGCC-TGCGGTGGCTCTGGGTTTTGGCCGGCCAGG - 99
. . . AG. . . . . A. . . . . -----. . . . . T. . . . - 93
SacII
Spacer 1
Spacer 2
AGGCTTCTGCCGAGGTGCTCCATTTTGGGCCCGCGGTTACCCATACAAAT - 149
. . . . . - 143
Spacer 1
Spacer 2
ACCAAGTCAAAGCATGTTTCCCATACTA - 177
. . . . . - 171

C)

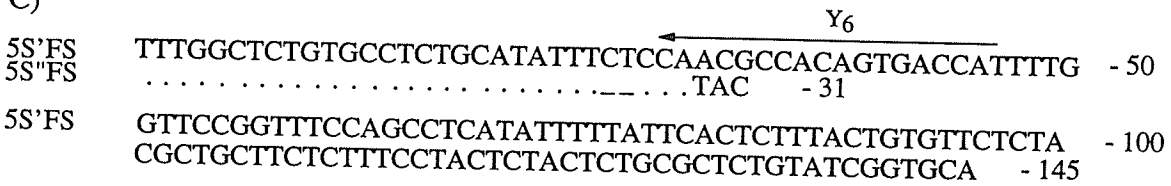

Y6
5S'FS
TTTGGCTCTGTGCCTCTGCATATTTCTCCAACGCCACAGTGACCATTTTG - 50
5S"FS
. . . . . TAC - 31
5S'FS
GTTCCGGTTTCCAGCCTCATATTTTTATTCACTCTTTACTGTGTTCTCTA - 100
CGCTGCTTCTCTTTCCTACTCTACTCTGCGCTCTGTATCGGTGCA - 145

Fig. 4. Secondary structure model for 5S rRNA of *P. pachycaule* showing helices A, B, C, D and E and loops a, b, c, d, and e. The sequences of potential rRNA products of 5S′ and 5S" have been superimposed on the model. When deletions have occurred in both 5S′ and 5S", the position is marked with an asterisk (*). When differences are unique to 5S′, the new base is placed inside a triangle (△) and when unique to 5S", in a square (□). Empty triangles or squares indicate deletions unique to 5S′ and 5S" respectively. Differences shared by 5S′ and 5S" are inside circles (O). An arrowhead indicates an insertion with respect to the functional gene.

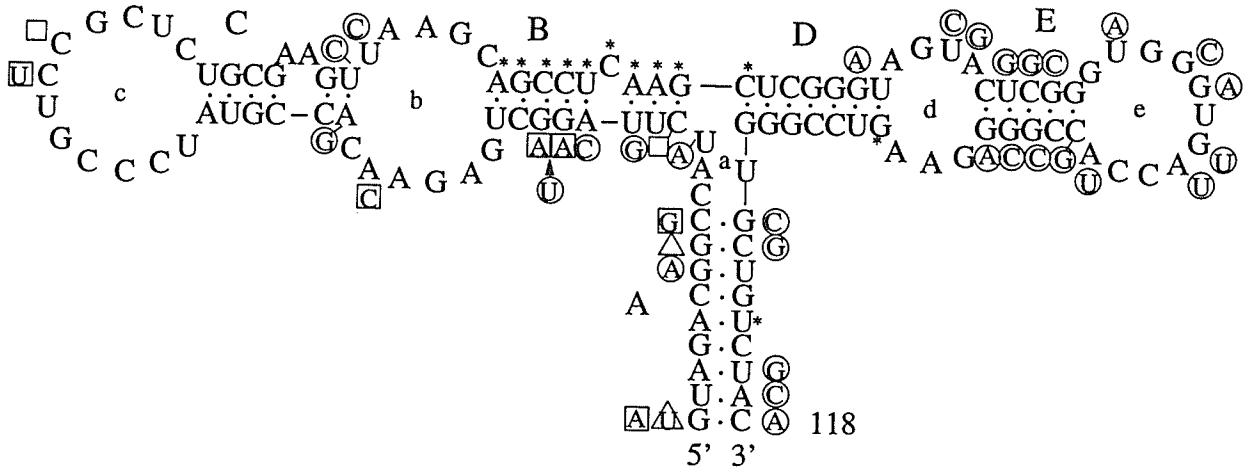
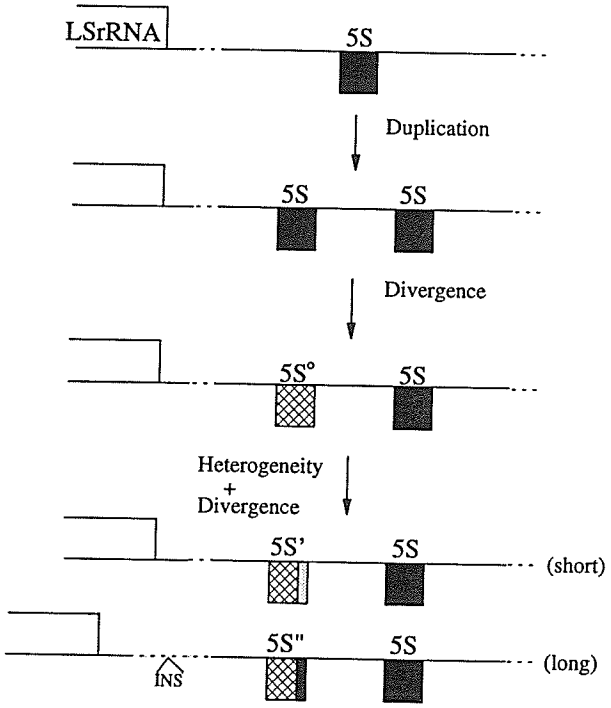

B
D
E
a
b
c
d
e
118
5'
3'

Fig. 5. A model depicting how diverged copies of 5S rRNA sequences in *P. pachycaule* might be generated. 5S° represents a hypothetical 5S RNA ancestor sequence resulting from divergence in a duplicated copy of the functional 5S rRNA gene. The sequence variability of the 5′ end of 5S′ with respect to the corresponding region of 5S", is marked by two distinct shades. "INS" represents the presumed insertion responsible for NTS length difference of the two versions.


LSrRNA
5S
Duplication
5S
5S
Divergence
5S°
5S
Heterogeneity
+
Divergence
5S'
5S
(short)
5S"
5S
(long)
INS

DISCUSSION

Pythium pachycaule has filamentous zoosporangia (Ali-Shtayeh and Dick 1985), and therefore based on previous results (Chapter 1) should have its 5S genes within the NTS of the rDNA repeat. The present studies confirmed this, but the situation is complicated by the apparent presence of 5S pseudogenes and by length heterogeneity. There are two versions of the NTS, each containing two 5S sequences in tandem, one presumed to be the functional 5S gene and the other a possible pseudogene. The presence of 5S pseudogenes adjacent to functional 5S rRNA genes in the NTS is a gene arrangement never reported before.

Sequences 5S′ and 5S′′ are judged to be pseudogenes by comparison of the secondary structures of their potential gene products with a model of functioning 5S rRNA based on that of *X. laevis* (Pieler and Theunissen 1993) (Fig. 4). The model conformed well to the eukaryotic consensus of five helices and five loops (Nishikawa and Takemura 1974). Sequences 5S′ and 5S′′ can also be superimposed on this model, but the central 10-base deletion eliminates the 3′ half of helix B and extends into loop a. In *X. laevis* and *N. crassa*, such a deletion would eliminate a large portion of "box A", which is essential for transcription (Pieler *et al.* 1987; Tyler 1987). The "box A" region in both 5S′ and 5S′′ may also be affected by two U -> C changes. The deletion

would also make it impossible to form helix B. The "box C" region (5′ half of helix E + part of loop e) has 7 differences in the 11-base stretch homologous to "box C" in *X. laevis* and only two out of four differences occurring in helix E are compensatory. Thus, helix E has only two base pairs and thus would probably not exist. Helix D would also be shortened from 7 to 5 bp and helix A would be disrupted to some extent by deletions and several noncompensating changes, although 5S′ and 5S′′ would not be affected in the same way because their sequences differ most significantly in this region. Helix C and loops c and b were the most highly conserved regions of the sequences, accounting for the conservation of N and Y primer sites in 5S′. These results suggest that 5S′ and 5S′′ may not be functional and could be referred to as pseudogenes.

Comparison of the long and short versions of the NTS further supports the idea that 5S′ and 5S′′ are pseudogenes. Whereas the 5S gene has been conserved in the two versions, 5S′ and 5S′′ have diverged significantly. The rate of divergence of the sequence is approximately equal to the rate of divergence of the spacer region between the functional gene and the pseudogene, although the differences are concentrated near the 5′ ends of the pseudogenes as well as the spacers. This similarity between the rates of divergence of the spacers and the pseudogenes supports the idea that natural selection is not maintaining the sequences

of the pseudogenes and that they are no longer functional genes.

The two versions of the rDNA repeat reported here are not merely two random representatives of a heterogeneous repetitive family. The advantage of using PCR products to characterize gene families instead of DNA clones is that what is characterized in PCR analysis represents the predominant form, a consensus that minimizes differences within a family of sequences. Thus, these results show that there are two predominant forms of the rDNA repeat in *P. pachycaule*, each one presumably separately maintained by concerted evolution. The reason for the relative independence of the two closely related gene families may be that either they exist as separate arrays which do not interact during unequal crossing over (Smith 1974), or that the two versions alternate in the array and that concerted evolution operates on the 'dimer' formed by adjacent long and short versions.

A number of explanations for the existence of the two versions of the NTS are possible. The approximate 1:1 ratio of abundance of the two versions might point to heterozygosity due to sexual outcrossing, but it is believed that this species is homothallic, as are the vast majority of species in the genus *Pythium* (Dick 1990). A more likely explanation is that recombination has taken place either during meiosis or sister chromatid exchange. There is

evidence that in other species of *Pythium* the 5′ end of the NTS contains repetitive arrays which would cause a high rate of unequal crossing over (Klassen and Buchko 1990; Martin 1990).

The relationships between the sequences of 5S, 5S′, and 5S′′ suggest that the 5S sequence family of *P. pachycaule* originated by duplication of an ancestral gene, followed by divergence (Fig. 5). The sequence of the functional gene was presumably maintained by natural selection while the duplicate was allowed to accumulate substitutions, single-base deletions, and a large central deletion, giving rise to the ancestral 5S° sequence.

In most species of *Pythium* with globose zoosporangia, 5S genes are not found in the NTS of the rDNA repeat, but are arranged in tandem repeats, similar to the arrangement found in plants and animals (Chapter 1). *P. pachycaule* has filamentous zoosporangia, so the 5S gene is in the NTS as expected, but a rudimentary tandem array has formed consisting of a pseudogene and a functional gene. It is interesting to note that in the 5S array of *X. laevis*, the first such array to be characterized, a truncated pseudogene was found adjacent to each functional 5S gene in the array (Jacq *et al.* 1977). The present report shows that the potential for tandemization of 5S genes exists even in the NTS, and may lead to more insights into the evolutionary event that led to the transition from rDNA-linked 5S genes

to the unlinked tandem arrays, or vice-versa.

CHAPTER 4

5S rRNA gene organization in *Pythium irregulare*

INTRODUCTION

In most fungi, the genes encoding the 5S ribosomal RNA's are either dispersed in the genome or linked to the larger rRNA genes (reviewed by Gerbi 1985). In the oomycetous *Pythium* species with globose or unknown zoosporangia the 5S genes are generally found in tandem arrays independent of the large rRNA genes (Chapters 1 & 2). This gene arrangement is also observed in plants (Kolchinsky *et al.* 1990; Moran *et al.* 1992) and animals (Korn and Brown 1978; Sorensen and Frederiksen 1991). In addition to functional 5S rRNA genes, many species contain pseudogenes and gene variants exhibiting a variable number of deletions and mutations. In species of *Aspergillus* (Bartnik *et al.* 1986; Gniadkowski *et al.* 1991) and *Neurospora* (Selker *et al.* 1981), the 5S pseudogenes are dispersed in the genome. In contrast to this, pseudogenes in species of *Xenopus* (Korn and Brown 1978; Jaq *et al.* 1977), *Notophthalmus* (Kay and Gall 1981), and *Calliphora* (Rubacha *et al.* 1984) are found within the 5S rRNA gene tandem arrays. In *Pythium pachycaule*, however, a 5S pseudogene was located adjacent to the functional 5S rRNA gene in the rDNA repeat (Chapter 3).

In this study, a detailed characterization of a library clone from *Pythium irregulare* which likely contains an intact tandem array of 5S rRNA genes and a 5S pseudogene is reported for the first time.

RESULTS

Genomic organization of 5S rRNA genes in *P. irregulare* BR486

In chapter 2 it was shown that 5S gene-to-gene amplification of *P. irregulare* BR486 genomic DNA with primers N_2 and Y yielded a 0.7 kb product. This product was sequenced to reveal the 3′ end of one 5S gene and the 5′ end of another, with a 541 bp spacer sequence in between (Chapter 2, Fig. 2). The accurate size of N_2Y product as determined by sequencing was 709 bp. These results indicated that the 5S rRNA genes of *P. irregulare* were arranged in tandem arrays. Because this conclusion was based on amplification products only, it was necessary to confirm this finding in genomic DNA. To do this, genomic DNA was digested separately with *Hinc*II, *EcoRV*, and *BstE*II because, according to the sequence of the gene-to-gene amplification product, these cut the 5S repeat unit only once (for site positions chapter 2, Fig. 2, and chapter 4, Fig. 3). The digestion profiles were Southern blotted and hybridized to ^{32}P-labelled N_2Y product (Fig. 1). The probe recognized 0.65 kb fragments from all three digestions and no other significant fragments. These results are in agreement with the results of N_2Y amplification. The specific production of large quantities of 0.65 kb fragments with enzymes which cut once per 5S rRNA gene repeat indicates that *P. irregulare* is likely to have one or more tandem head to tail arrays of 5S

genes and that the arrays are homogeneous, at least with respect to several restriction sites.

Construction of the *P. irregulare* BR486 genomic library

To further characterize the 5S tandem arrays and study their features, a lambda EMBL3 genomic library was constructed (see Materials and Methods) and phage clones containing 5S rRNA genes were screened with 5S gene specific probes and PCR amplification. Phage DNA from clones C1A and C13A was digested with *Sal*I, which cuts the insert out, and hybridized with N_2Y probe. The probe recognized a 12 kbp band in the C1A profile (data not shown) and a 9.2 kbp band in the C13A profile (Fig. 2). Due to its smaller size, the C13A clone was chosen to be analyzed further. To show that the insert contained the 5S array, DNA was digested with *EcoRV*, *Hinc*II, and *Hind*III (also having a single site in the 5S repeat. See Fig. 3 for position), double digested with *Sal*I/*EcoRV*, *Sal*I/*Hinc*II, and *Sal*I/*Hind*III, and partially digested with *Hinc*II. These restriction profiles were then hybridized with N_2Y probe (Figs. 2A & 2B). In addition to the 0.65 kbp band produced with all of the enzymes, partial digestion with *Hinc*II generated a ladder of bands whose sizes were multiples of 0.65 kbp (Fig. 2B, lane 7). Thus digestion data from the clone agrees with data from genomic digestions (Fig. 1A & 1B). The number of steps in the ladder of bands indicates that the 5S array has at least 8 repeats.

Subcloning of C13A fragments into Bluescript plasmid (pM13)

Further characterization of the C13A insert was attempted to define the ends of the array, and to determine its size and homogeneity. Examination of the sequence of N_2Y product revealed the presence of a unique *Pst*I site in the repeat (for position see Fig. 3), which was exploited for subcloning into Bluescript. *Pst*I/*Sal*I fragments were subcloned and resulting recombinants screened using N_2Y PCR amplification. Six positive subclones were obtained, and these were digested with *Sal*I/*Sma*I to reveal inserts of 1-7 kbp. Two subclones (pC13A1, pC13A3) with 4.5 and 3.4 kbp inserts respectively, were selected for mapping. DNA from pC13A1 and pC13A3 was digested with *Pst*I and the restriction profiles hybridized to N_2Y probe. In addition to the 0.65 kbp band which represents the 5S repeat unit, the probe recognized a 1.5 kbp band in the pC13A1 profile (Fig. 4, lane 2). In the case of the pC13A3 profile, however, in addition to the 0.65 kbp band, a 3.6 kbp band, which includes part of the vector, was also detected (Fig. 5, lane 5). The same was true for another subclone (pC13A5), but it seemed to be missing one 5S repeat unit (Fig. 6). This conclusion was based on the number of bands with steps of 0.65 kbp generated from partial digestion of both clones (pC13A3, pC13A5) with *Pst*I, that reacted with the 5S probe. In pC13A3, there were 4 bands in addition to the 3.6 kbp

band (Fig. 5B, lane 6). In the case of pC13A5, however, only 3 bands besides the 3.6 kbp band were noted (Fig. 6B, lane 6). Complete and partial restriction profiles of *BstE*II for both subclones also indicated that the pC13A3 insert was larger by one 5S repeat unit (0.65 kbp) than the one of pC13A5 (Figs. 5&6, lanes 7&8). Based on the fact that *Pst*I is a cloning site and would only cut the insert, and both subclones (pC13A3, pC13A5) share the 3.6 kbp band, although they differ by the number of bands which correspond to a step ladder of 0.65 kb, one would conclude that the two subclones originated from the same end of the original phage clone (C13A). Unlike pC13A1, the results showed that both pC13A3 and pC13A5 lacked the 1.5 kbp *Pst*I band which is not related to the vector, since it is smaller and *Pst*I does not cut in the vector. In fact, the entire vector (2.9 kbp band) in pC13A1 was cut out with *Pst*I (fig. 4A, lane 2); this band did not hybridize with the 5S probe (Fig. 4B, lane 2). The 3.6 kbp *Pst*I band found only in pC13A3 and pC13A5, but not in pC13A1, comprised the vector (2.9 kbp) and a 0.7 kbp 5S related fragment. This was assumed because the 3.6 kbp band hybridized with the 5S probe (Figs. 5&6, lane 5). Based on restriction and Southern profiles described above, it was concluded that the subclones pC13A3 and pC13A5 originated at one end of the C13A clone and the subclone pC13A1 from the other. Partial and complete digestion of DNA with *BstE*II (having a single site in the 5S gene and none in the spacer)

and hybridization to the 5S probe were performed for the three subclones (Fig. 4, lanes 4&5; Figs. 5&6, lanes 7&8). The results suggested that the subclones pC13A3 and pC13A5 comprised 4 and 3 5S genes respectively, and that the subclone pC13A1 had 3 5S genes. Further restriction analyses with *Hinc*II, *EcoR*V, and *Hind*III, and Southern analyses indicated that the pC13A1 insert contained a 2.9 kbp fragment unrelated to the 5S spacer (Fig. 4).

Sequence characterization of C13A 5S genes and flanking regions

The 5S repeating unit of C13A, which contains a spacer and a 5S gene, was amplified using N_2 and Y primers and sequenced with N_2, Y, and Y_4 primers (Fig. 3&7). The sequence was identical to the one obtained from sequencing amplified genomic DNA (Chapter 2). Immediately downstream of the 3′ terminus of the 5S gene is the putative termination region dominated by two tracts of T. This is followed by 2 direct 47 bp repeats with 87.2% identity (bases 48-94 and 143-194); there are 6 nucleotide changes and 5 nucleotide insertions in the second repeat when compared with the first one (Fig. 3). These results indicate that the C13A 5S array may be representative of all the 5S arrays in the genome.

To determine whether the 5S array was homogeneous, it would have been necessary to sequence it completely. Due to time constraints this was not done; instead, the 5S genes

were amplified with primers complementary to the ends of the gene (Nc and Yc) and cloned into the *EcoRI* site of Bluescript (pM13), and sequenced. Twenty-two such clones were sequenced and found to be identical to the expected gene sequence (Fig. 7). Unless amplification did not produce a representative set of products, it appears that the genes in the array are homogeneous in sequence. Confirmation of array homogeneity must await full sequencing of the C13A insert.

One 5S flanking region of pC13A3 was amplified using T3 and N primers, generating a 1 kbp fragment. This PCR product was sequenced with T_3, N, and Y_4c primers. In addition to a conserved 5S spacer and part of the functional 5S gene, the sequence contained a highly diverged copy of a 5S gene located at 360 bp from one end of the insert. This 5S-like sequence had 64% homology with the functional 5S rRNA gene; most of the nucleotide changes occurred in the 3′ end. The 5′ end is flanked by a short direct repeat corresponding to the first five nucleotides of the 5S-like sequence. No putative termination region was detected downstream of the 3′ terminus of the 5S-like sequence (Fig. 7; Fig. 8A). The other flanking region of pC13A3, however, was sequenced with T_7 primer and, as expected, it contained part of the conserved spacer between the *PstI* site and a functional 5S gene (data not shown). The same was true for one flanking region of pC13A1, whereas the opposite region was partially

sequenced with T_3 primer and found to be unrelated to the 5S tandem array (Fig. 8B).

The complete physical map of C13A 5S tandem array

Based on partial and complete restriction profiles, Southern analyses data generated from the phage clone C13A and the three subclones (pC13A1, pC13A3, pC13A5), and sequencing data, it was concluded that the array comprised 9 possible functional 5S rRNA genes interspersed by a conserved 541 bp spacer and flanked at one end by a highly diverged 5S gene (5S') (Fig. 9). The results also indicated that the 5S array was flanked by two regions containing unrelated sequences. This suggests that the C13A array may be intact as one independent unit in the genome.

Fig. 1. The genomic organization of the 5S rRNA genes in *P. irregulare* BR486. Genomic DNA was digested with various restriction endonucleases. Lane 1, BRL high-molecular-weight ladder. Lane 2, *Hinc*II. Lane 3, *EcoR*V. Lane 4, *BstE*II. Lane 5, uncut DNA. Lane 6, BRL 1-kb ladder. A, Ethidium bromide-stained gel. The arrowheads from bottom to top indicate 1.0 kb and 3.0 kb bands. B, Gel shown in panel A, blotted and hybridized to 5S specific random primers probe N_2Y. The arrowhead indicates the 0.65 kb repeat unit.

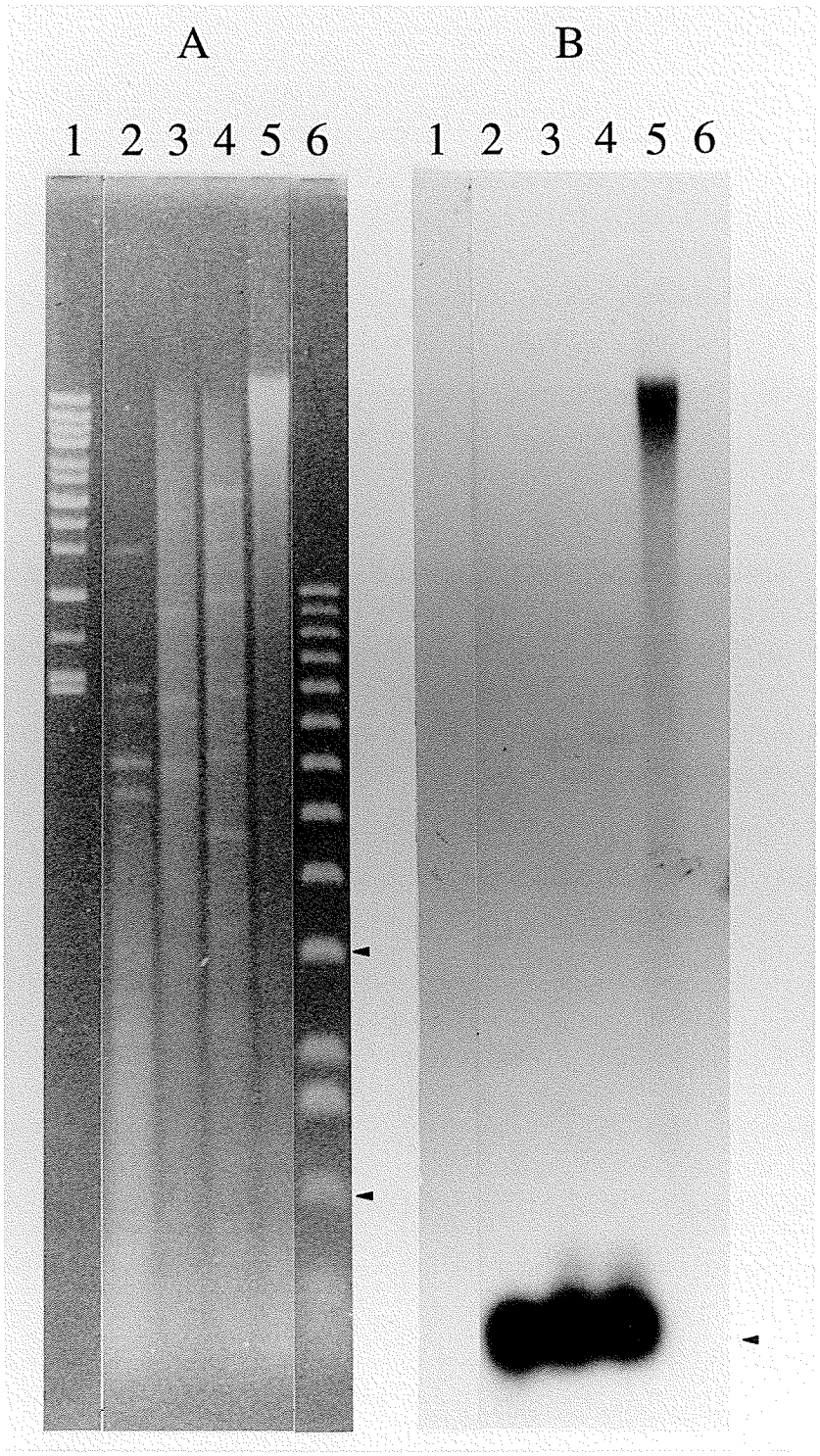

A
B
1 2 3 4 5 6
1 2 3 4 5 6

Fig. 2. The restriction profiles of the C13A clone. Lane 1, BRL high-molecular-weight ladder. Lane 2, BRL 1-kb ladder. Lane 3, *Sal*I. Lane 4, *Sal*I/*EcoRV*. Lane 5, *EcoRV*. Lane 6, *Sal*I/*Hinc*II. Lane 7, *Sal*I (complete)/*Hinc*II (partial). Lane 8, *Hinc*II. Lane 9, *Sal*I/*Hind*III. Lane 10, *Hind*III. A, Ethidium bromide-stained gel. The arrowheads from bottom to top indicate 1.0 kb and 3.0 kb bands. B, Gel shown in panel A, blotted and hybridized to 5S specific probe N_2Y. The arrowhead indicates the 0.65 kb repeat unit.

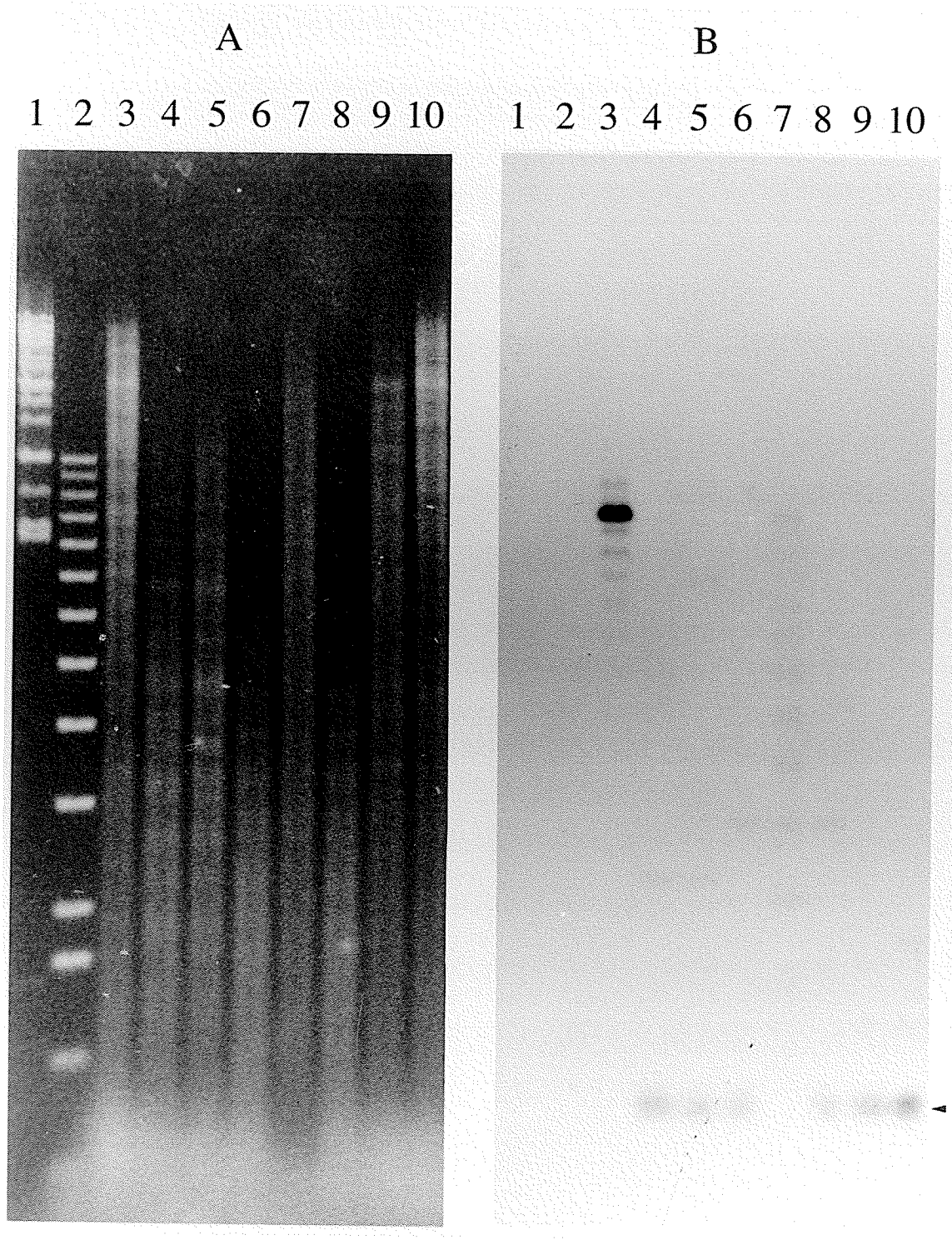

A
1 2 3 4 5 6 7 8 9 10
B
1 2 3 4 5 6 7 8 9 10

Fig. 3. The DNA sequence of the 5S spacer in *P. irregulare*. Direct repeats are indicated by horizontal arrows. Asterisks represent insertions in 'Repeat II' with respect to ' Repeat I', and dots represent differences. Restriction sites are indicated by vertical arrowheads: P = *Pst*I; V = *EcoRV*; D = *Hind*III; H = *Hinc*II.

TCT TT TCT CTT TT TCACCTACTTACTTGTTGCTAACA AATAAACACATGT - 50

Repeat I

TATCACGC TACACTGTAGCGATATAGCTAGTAAATACCGAATGCTGTCTC - 100

GTCTT TATCACTT TT TCCCACT TACCTACT TGT TTGTAACAGTGTTGTAA - 150

Repeat II

CGCTACACTGCAATATACCGATATACCAAGTAAATATCGAATGCTATACA - 200

*****

P

ACT TCTTGCTCGTTC GAGGTCT TCTGCAGAC ACC AAGTGCGCTTTCATAT - 250

V

AGAGACTCTCCATACAAAGAGGCGCTTTACGTAAACGCCGATATCTCGAC - 300

D H

AACCACAGCAAGCTTTTAGTGCTGTCAACGCAGATCGACGCGTCTTGTCA - 350

GCGCCGCACCTCCCTGCCAAGTCTCAGGGCTGGGTCGCGTGTCTCGATGT - 400

ACTGTCTGCCAGTCTAATGAACGTCATT TCT CTT TTC TGT TTAGCCCAAG - 450

TGTTGAGAGTCTCCAAATTGGAAGGAGTTCTTCCATTTCATCCATAATGA - 500

TTTGTACTACGGAAAGAACCCGGTCGAGCAGTTGAACACTA - 541

Fig. 4. The restriction profiles of the pC13A1 subclone. Lane 1, BRL 1-kb ladder. Lane 2, *Pst*I (complete). Lane 3, *Pst*I (partial). Lane 4, *BstE*II (complete). Lane 5, *BstE*II (partial). Lane 6, *Ava*I (complete). Lane 7, *Ava*I (partial). Lane 8, *EcoR*V (complete). Lane 9, *Hinc*II (complete). Lane 10, *Hind*III (complete). A, Ethidium bromide-stained gel. The arrowheads from bottom to top indicate 0.5 kb and 1.6 kb bands. B, Gel shown in panel A, blotted and hybridized to 5S specific probe N_2Y. The arrowhead indicates the 0.65 kb repeat unit.

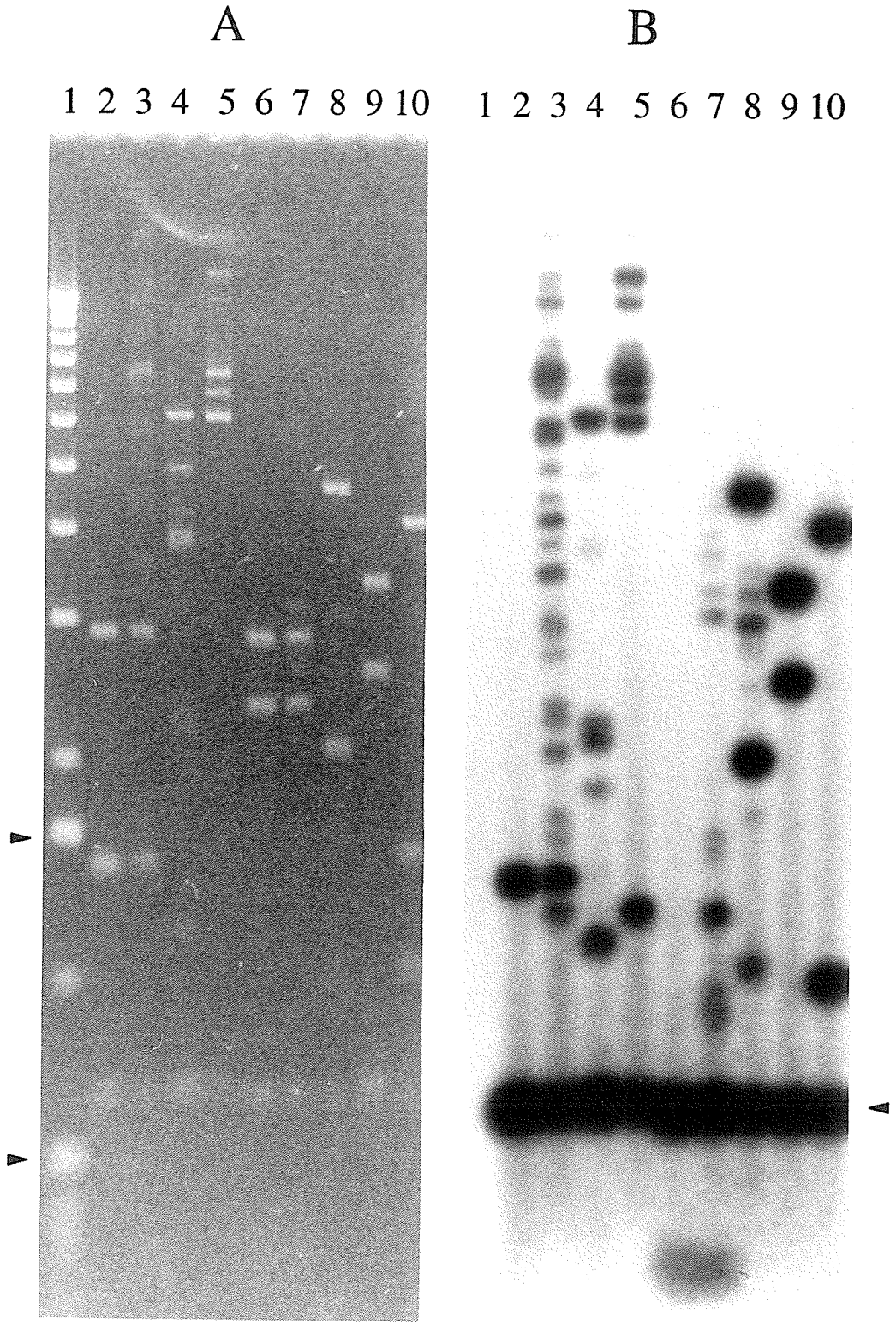

A
1 2 3 4 5 6 7 8 9 10
B
1 2 3 4 5 6 7 8 9 10

Fig. 5. The restriction profiles of the pC13A3 subclone. Lane 1, BRL 1-kb ladder. Lane 2, *Sal*I (complete). Lane 3, *Sma*I (complete). Lane 4, *Sal*I/*Sma*I (complete). Lane 5, *Pst*I (complete). Lane 6, *Pst*I (partial). Lane 7, *BstE*II (complete). Lane 8, *BstE*II (partial). Lane 9, *Hind*III (complete). A, Ethidium bromide-stained gel. The arrowheads from bottom to top indicate 0.5 kb and 1.6 kb bands. B, Gel shown in panel A, blotted and hybridized to 5S specific probe N_2Y. The arrowhead indicates the 0.65 kb repeat unit.

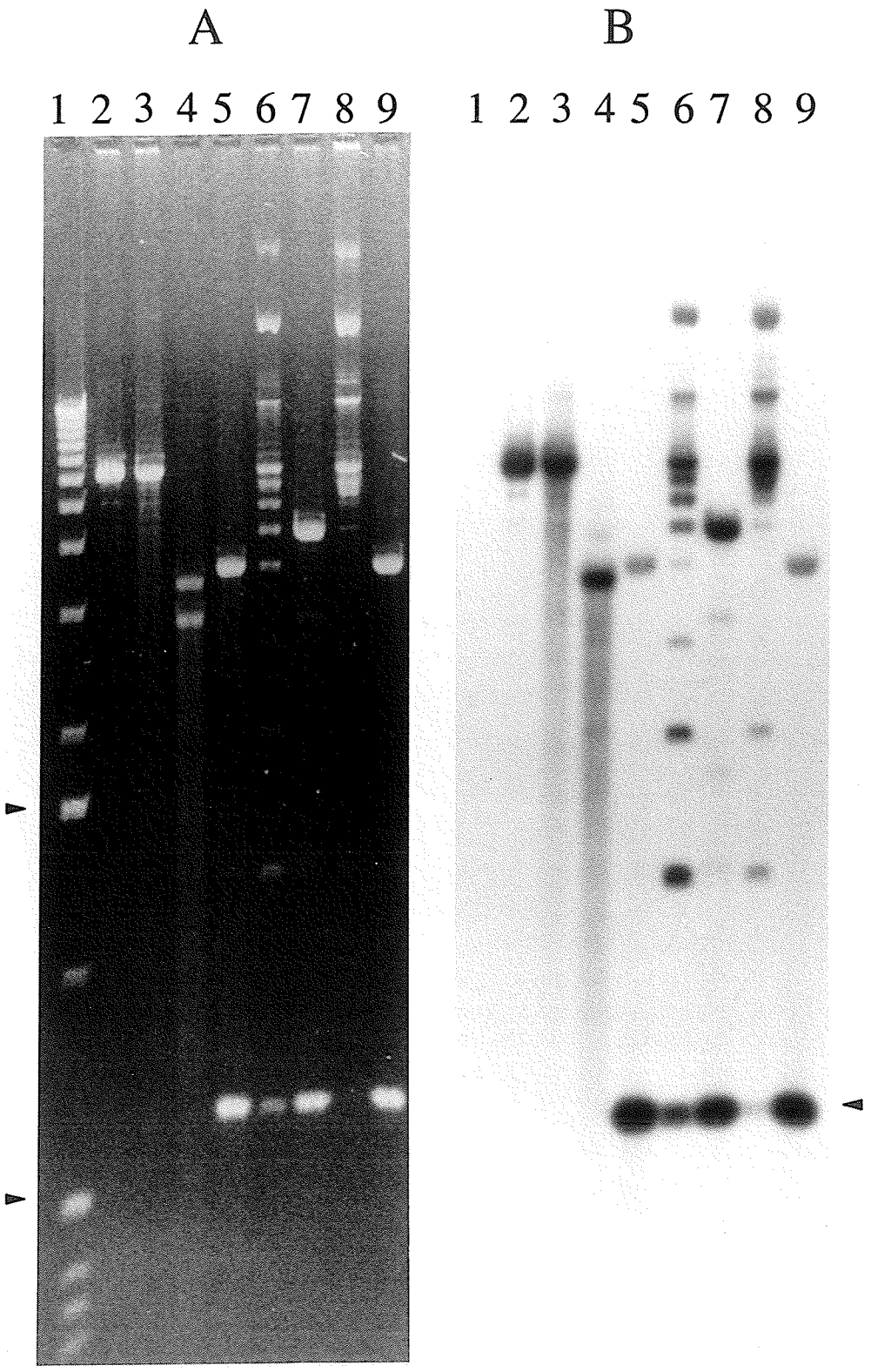

A
B
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9

Fig. 6. The restriction profiles of the pC13A5 subclone. Lane 1, BRL 1-kb ladder. Lane 2, *Sal*I (complete). Lane 3, *Sma*I (complete). Lane 4, *Sal*I/*Sma*I (complete). Lane 5, *Pst*I (complete). Lane 6, *Pst*I (partial). Lane 7, *BstE*II (complete). Lane 8, *BstE*II (partial). Lane 9, *Hind*III (complete). A, Ethidium bromide-stained gel. The arrowheads from bottom to top indicate 0.5 kb and 1.6 kb bands. B, Gel shown in panel A, blotted and hybridized to 5S specific probe N_2Y. The arrowhead indicates the 0.65 kb repeat unit.

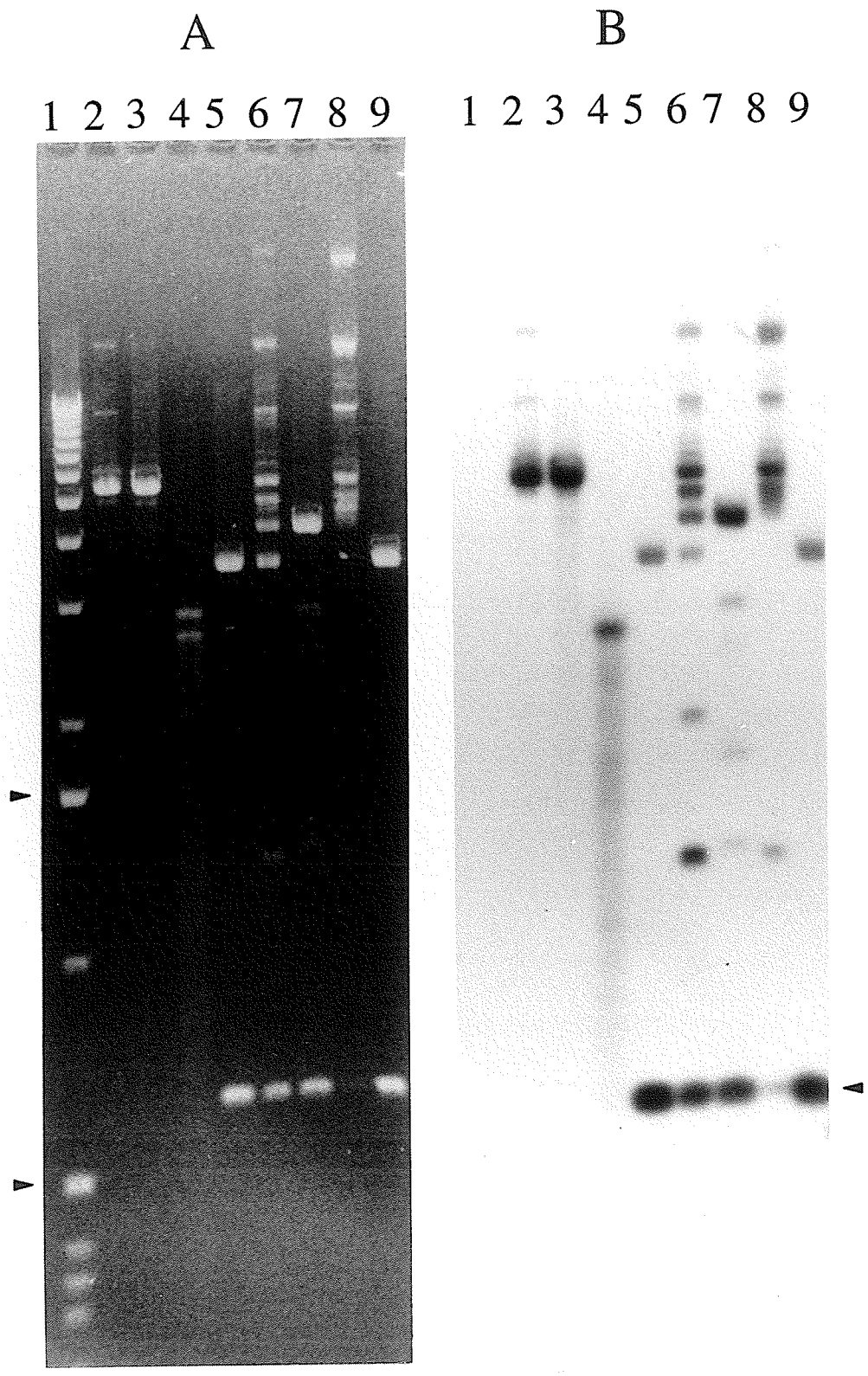

A
B
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9

Fig. 7. The sequence alignment of the coding gene (5S) and the pseudogene (5S′). Conserved nucleotides are indicated by dots (.). Deletions are represented by dashes (-).

```
5S    GTAGACGGCCATCTTAGGCTGAGAACACCGTATC- CCGTTCGCTCTGCGA  -49
5S'   . . . . . . . . . . . . . .G. . . . . . . . . . . . . - . . .GT . . . A. GA.G. GCTCT  -49

5S    AGTTAAGCAGCCTCAA - -GCTCGGGTAGTACTCGGGTGGGTGACCACCGG  -97
5S'   C . A. CTCG . C . T. . . . CG. . . . . A. . . . C. - - - A . A . CTC . - - - . . . . CA  -94

5S    GGAAGTCCGAGTGCTGTCTAC  -118
5S'   . C . - . C. . C . AA . . . CGAA . G  -114
```

Fig. 8. The DNA sequence of flanking regions of C13A clone. A, Sequence of the pseudogene (lower case) and its flanking region (upper case) from the pC13A3 subclone which corresponds to the left end of C13A clone (refer to Fig. 9). The five nucleotide direct repeats are underlined. B, Partial sequence of the flanking region from pC13A1 subclone which corresponds to the right end of C13A clone (refer to Fig. 9).

A)

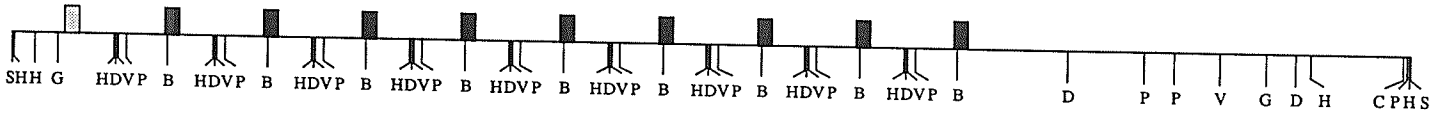
GTAGATgtagacggccatctgaggctgagaacacctatgtccgatgacgc
gctctcgatctcgaccttcaacggctcgagtagcaagatctctccaccca
gcagccccaaagctcgaaagAATCCGCTCCTGCTGACCAACGAACCGAAC
GATGGCTTCACAACTGCGGGTAGTAACAAAAAGAAGCGCTCGCGAAATGA
AGTTGACTTCTCCAATCTCATGATCAAGCAACAGCCGCAGCCTCTCAACG
GAGTCGCCACAGCCAACTACTTTCAAGCGCTCCAGTCGATGGAGGCCAAG
TTCGAGTTGAAGAAAGTGACTGCGGACAAGAAGTACGGAGTTCGCTACCA
CGCGGCCCCCGTCGAC -366

B)

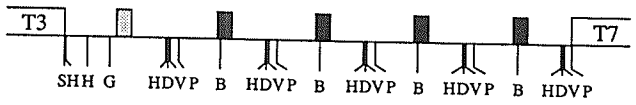
GTCGACCTGCAGGTCAACGGATCACTCTTCTGAACATCGATCAAGCTCGG
ACAGAAGATCCTATCCAAGCNCTTGCAACCCATCCTGCCCAAGCTCCTAC
CCACCGACCAGTATGGCTTTGTTCCCGG -128

Fig. 9. A physical map of the C13A clone. Restriction sites are indicated below the map: P = *Pst*I; V = *EcoR*V; D = *Hind*III; H = *Hinc*II; B = *Bst*EII; G = *Bgl*II; C = *Cla*I; S = *Sal*I. 5S rRNA genes are represented by black boxes and the pseudogene by a dotted box. 5′-3′ transcription is from right to left of the map. The physical maps of pC13A1 and pC13A3 subclones correspond to the right and left part of C13A clone, respectively.

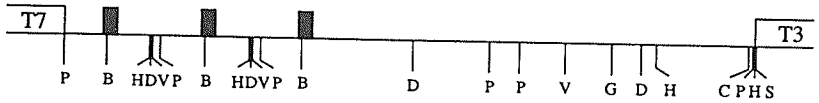
C13A



pC13A3

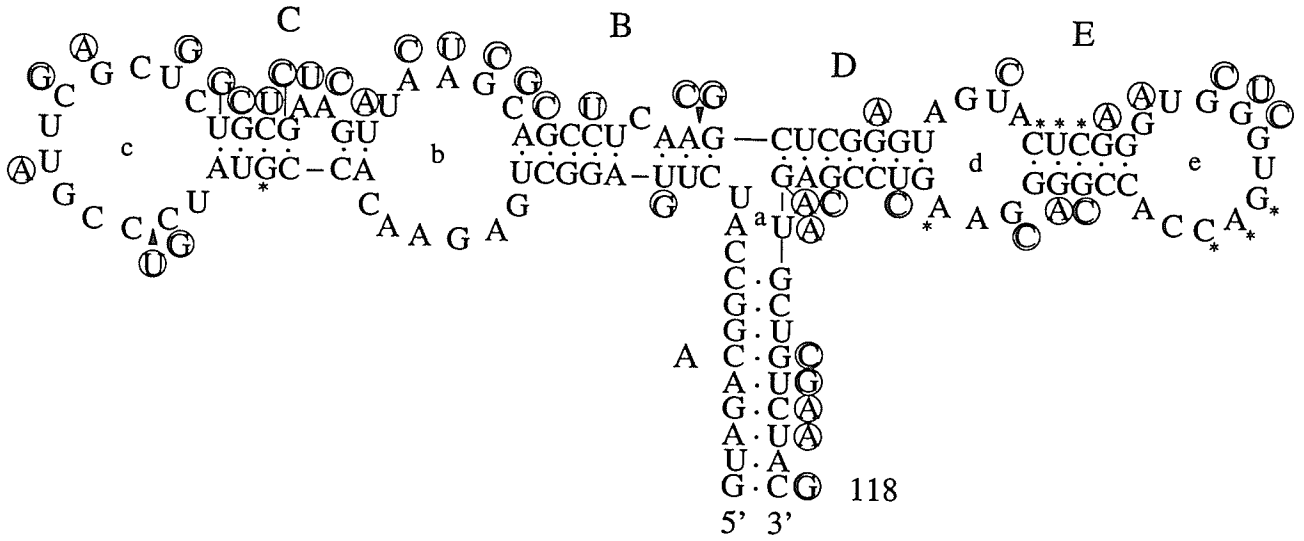


pC13A1



1 kb

Fig. 10. A secondary structure model for the 5S rRNA of *P. irregulare* showing helices A, B, C, D and E and loops a, b, c, d, and e. The sequence of potential rRNA product of the pseudogene (5S') has been superimposed on the model. When deletions have occured in 5S', the position is marked with an asterisk (*). Differences are depicted by placing bases inside circles (O). An arrowhead indicates an insertion with respect to the functional gene.


C
B
D
E
a
b
c
d
e
118
5' 3'

DISCUSSION

Pythium irregulare has globose zoosporangia (Plaats-Niterink 1981), and based on previous results (Chapters 1 & 2) would be expected to have its 5S genes organized in direct tandem repeats unlinked to the rDNA repeat. This prediction has been confirmed, but presence of an apparent 5S pseudogene (5S') has also been detected adjacent to 5S genes in a library clone (C13A). The 5S' sequence is considered to be a pseudogene based on the comparison of the secondary structure of its potential gene product with a model of functioning 5S rRNA based on that of *X. laevis* (Pieler and Theunissen 1993) (Fig. 10). The secondary structure model conformed well to the eukaryotic consensus of five helices A to E, connected by loops a to e (Nishikawa and Takemura 1974). The 5S' sequence can also be superimposed on this model, but due to five noncompensating base changes, part of helix A is disrupted. Helix C, part of "box A", is also affected by a base deletion and four noncompensating changes. The "box A", which is essential for transcription in *Xenopus* species (Pieler *et al.* 1987; Tyler 1987), may also be affected by five base changes in loop b and part of helix B. A deletion of three bases would make it impossible to form helix E. The "box C" region, which is part of loop e, is also highly affected by three base deletion and four changes. These results suggest that 5S'

may not be functional and could be referred to as a pseudogene.

Although due to the limitation of the PCR cloning method employed not all the array 5S genes were fully sequenced and confirmed, restriction data suggest that in addition to the pseudogene, the array contains 9 functional 5S rRNA genes. The fact that the flanking regions are not homologous to the 5S spacer, strongly indicates that the C13A clone comprises an intact 5S tandem array. The size of this array is by no means representative in *P. irregulare*, since preliminary analysis of C1A, a different clone, showed a larger 5S tandem array containing at least 20 genes (data not shown).

A truncated 5S pseudogene was found adjacent to each functional 5S gene in the array of *X. laevis* (Jacq *et al.* 1977). In *P. irregulare*, however, a single pseudogene was located at one end of the array. This may be an isolated case and other 5S tandem arrays may not contain pseudogenes. In contrast to this, in *P. pachycaule* a 5S pseudogene was found adjacent to each functional 5S gene in the NTS of the rDNA repeat. This may be the result of duplication and subsequent accumulation of nucleotide changes (Chapter 3). In the case of *P. irregulare*, the pseudogene is located at one end of the array and is flanked by a direct repeat of 5 nucleotides which correspond to the 5′ end of the 5S rRNA gene. This indicates that the pseudogene may have originated

from a recombination event which would involve two ancestral 5S genes at one end of the array. This would be followed by deletions and mutations due to lack of selective pressure.

Several levels of sequence heterogeneity of the coding regions of 5S genes and their spacers were found in plants (Gerlach and Dyer 1980; Hariharan *et al*. 1987) and animals (Fedoroff and Brown 1978; Wolffe and Brown 1988). Differences in length of the nontranscribed 5S spacer was also detected in plants (Ellis *et al*. 1988; Goldsbourgh *et al*. 1981) and animals (Kay and Gall 1981; Sorensen and Frederiksen 1991). In *P. irregulare*, however, the 5S genes and their spacers revealed neither sequence nor length heterogeneity. This indicates that the formation of 5S tandem arrays in *P. irregulare* may be recent.

Due to the extremely large size of the tandem arrays comprising thousands of 5S gene repeats in plants and animals, only partial characterization have been possible (Brown and sugimoto 1973; Hemleben and Grierson 1978; Gerlach and Dyer 1980). The C13A array is the first known intact tandem array of 5S rRNA genes in plants and fungi, to be cloned and characterized.

CHAPTER 5

Molecular evolutionary studies on *Pythium* species

INTRODUCTION

Pythium is the largest genus in the class Oomycetes, comprising more than 120 described species (Dick 1990). *Pythium* species are economically very important due to their consistent association with root diseases affecting a wide variety of plants such as sugar cane, cereals, and greenhouse plants (Plaats-Niterink 1981). The taxonomy of the genus is mainly based on a limited number of sexual and asexual reproductive structure characteristics, and as a result identification of some morphologically similar *Pythium* species can be difficult. In addition, some species are heterothallic, they need the opposite mating types to be identified, while others lack the sexual stage altogether. These are the most problematic *Pythium* species to identify (Plaats-Niterink 1981).

Recently, molecular techniques have been used extensively to study fungal taxonomy and phylogeny (Taylor 1986; Bruns *et al.* 1991; Kohn 1992). The analysis of restriction fragment length polymorphisms (RFLPs) of PCR amplified nuclear ribosomal DNA and the internal transcribed spacer (ITS) of twenty-five isolates representing five *Pythium* species, revealed three polymorphic groups indicating inter- and intraspecific genetic relationships (Chen *et al.* 1992); the three groupings were *P. arrhenomanes* and *P. graminicola*, and *P. irregulare* and *P. spinosum*, and

P. ultimum. Restriction analysis of the ITS, however, showed that the morphologically similar species of *P. graminicola* and *P. arrhenomanes* were genetically distinct, and intraspecific variation was detected only in *P. arrhenomanes* (Chen and Hoy 1993).

The results from the previous findings (Chapters 1 & 2) show that with respect to 5S rRNA gene organization, there are generally two groups of *Pythium* species. Those with filamentous zoosporangia have their 5S genes in the NTS of the rDNA repeat, and do not have tandem arrays, while *Pythium* species with globose or unknown zoosporangia have tandem repeats of 5S genes and no 5S gene in the NTS. However, a few exceptions were noticed, e.g. *P. hypogynum* has globose zoosporangia and a 5S gene in NTS, contrary to what should be expected.

In this chapter, an attempt was made to confirm the separatness of those *Pythium* species with filamentous zoosporangia from the globose or sporangium missing species by restriction analyses of PCR amplified portions of the large subunit of the rRNA gene. Also the 5S rRNA genes from several *Pythium* species were sequenced and compared to infer phylogenetic relationships. But as most *Pythium* species with filamentous zoosporangia have the 5S gene in NTS, we have been able to amplify and partially sequence the external transcribed spacer (ETS) to reveal inter- and intraspecific relationships within the group.

RESULTS

Restriction fragment length polymorphisms in the nuclear large subunit rDNA

The PCR primers C and D were used to amplify the 5′ end portion of the large subunit rDNA from 24 *Pythium* isolates and *Phytophthora cryptogea*. The amplified CD fragment was about 1.6 kb long and uniform in length among all isolates (Fig. 1). To generate the maximum number of restriction bands, DNA was digested with the 4-base-cutter endonucleases *Mbo*I, *Rsa*I, *Hha*I, *Hae*III, *Alu*I, and *Taq*I. Restriction fragments were separated using polyacrylamide gel electrophoresis, and sizes were determined.

A binomial matrix of RFLP profiles from all isolates for each endonuclease was constructed to represent the variations in restriction sites. The presence or absence of a band was coded by a G or an A, respectively (Tables 1, 2, 3, 4, 5 & 6) (Smith and Anderson 1989). Due to the fact that the number of bands generated by each endonuclease separately was fairly small (2-7 bands), restriction profiles of the six enzymes represented in the matrices for each isolate were combined in the same order as above, and treated as nucleotide sequences. The combined profiles for all the isolates were initially aligned using CLUSTAL (PC-gene; IntelliGenetics, Inc., Mountain View, Cal.) (Fig. 2). The READSEQ program was used to reformat the file

originating from CLUSTAL in order to transfer the alignment to PHYLIP (Version 3.4; Felsenstein 1991). As used herein, PHYLIP was a component of BIRCH (Biological Research Computer Hierarchy), a collection of programs set within the framework of the SUN Unix system at the University of Manitoba (Fristensky 1991). Divergence (or distance) between two profiles was calculated by DNADIST using Kimura's (1980) two parameter model. The NEIGHBOR program (UPGMA option) was used to generate a distance dendrogram based on a data set of 17 profiles (the data set of 24 profiles was reduced to 17 by removal of identities). The results were drawn to scale as a network (Fig. 3). As expected, the network indicated that *Phytophthora cryptogea* was the most distant from all *Pythium* isolates. The *Pythium* isolates with filamentous zoosporangia clustered together at close proximity at node 11. *Pythium hypogynum* which has globose zoosporangia was also included in the cluster. *Pythium* isolates with globose or unknown zoosporangia, however, seemed to be separated by greater distances. Three isolates of *Pythium paroecandrum*, two isolates of *Pythium irregulare*, and *Pythium mamillatum* had identical profiles, and as a result they shared the same branch. Surprisingly, however, other *P. irregulare* and *P. paroecandrum* isolates appeared to group separately. *P. acanthophoron*, *P. hydnosporum*, and *P. oligandrum* also appeared to share identical profiles, grouping at a considerable distance from the other *Pythium*

isolates. *P. spinosum* branched out close to the *P. irregulare* and *P. paroecandrum* cluster, but away from *P. ultimum*.

Signature positions in the 5S rRNA gene sequence of *Pythium* species

To go beyond the RFLP data to a more definitive approach, several regions of the rRNA gene family were sequenced. First of all, the 5S rRNA gene from the isolates listed in Table 7 was sequenced and compared. Depending on its location with respect to the large rRNA genes, the 5S rRNA gene was amplified with either primer pairs QN_2 or N_2Y (Chapters 1 & 2). All *Pythium* species with filamentous zoosporangia were amplified with QN_2 except *P. periplocum*, which was amplified with N_2Y due to the fact that it has the 5S gene organized in tandem arrays outside the rDNA repeat unit. However, the *Pythium* species with globose or unknown zoosporangia were amplified with N_2Y except *P. hypogynum* which was amplified with QN_2 because it has the 5S gene in NTS. The 5S gene of *Phytophthora cryptogea* was amplified with the primer pair P_2N_2 because the 5S gene is located in NTS, and is transcribed in the same orientation as the other rRNA genes, contrary to the case in *Pythium* species (Chapter 1). The 5S rRNA gene of each isolate in Table 7 was then sequenced in both directions with primers N_2, Y, and Y_3 (Materials and Methods).

The 5S rRNA sequence in all isolates studied comprised 118 bp with only two variable positions 39 and 107 (Table 7). Generally *Pythium* isolates with filamentous zoosporangia had C at position 39 and G at position 107. *P. graminicola*, however, differed at position 39; it had a T instead of a C residue. The other exception was *P. periplocum*, a species with filamentous zoosporangia; it had a T and an A. This was expected since the latter has the 5S genes tandemly repeated outside the rDNA repeat unit. The same was true for *Pythium* isolates with globose or unknown zoosporangia. A few exceptions, however, were noticed. *P. hypogynum* which has globose zoosporangia had C and G residues, agreeing with the group of *Pythium* species with filamentous zoosporangia. This was also expected because it has the 5S gene in the NTS. *P. anandrum* differed at position 107 with respect to the globose and unknown zoosporangial group; it had a G instead of an A residue. The same was true for *Phytophthora cryptogea*, although it had the 5S gene in the NTS.

The phylogeny of *Pythium* species with filamentous zoosporangia

The presence of the 5S rRNA gene in the NTS made it easier to access the external transcribed spacer (ETS) for phylogenetic analysis because it made amplification of NTS2 possible (Fig. 1). The NTS2 was amplified in 25 *Pythium* isolates including *P. hypogynum* with P_2 and Y_3 primers, and

the ETS was partially sequenced with primer P_2 (Materials and Methods).

The sequences of 135 positions from all isolates were initially aligned using CLUSTAL and then the alignment was improved by eye with MASE (Multiple aligned sequence editor; Faulkner and Jurka 1988). Six duplicate sequences were excluded from the alignment (Fig. 4). The READSEQ program was used to reformat the file originating from MASE, thus the alignment could be transferred to PHYLIP. The divergence (or distance) between two sequences was calculated by DNADIST using Kimura's (1980) two paramater model, generating a distance matrix. The KITSCH program was used to carry out Fitch and Margoliash's least-square method for estimating phylogenies from the distance matrix, and unrooted phylogenetic distance networks were produced (Fig. 5). The KITSCH program was used to determine the outgroup member and initial clusters based solely on distance. The two isolates of *P. vanterpoolii* seemed to be the most distant with respect to the other isolates. One isolate of *P. aristosporum*, one of *P. tardicrescens*, two of *P. arrhenomanes*, two of *P. graminicola/aristosporum*, and two undescribed *Pythium* species formed a very tight cluster at node 9. The other tight grouping at node 17, was represented by two isolates of *P. hypogynum*, one of *P. coloratum*, one of *P. sulcatum*, and two of *P. dissimile*. Three isolates of *P. graminicola* were positioned between the latter grouping and

a cluster of three *P. torulosum* isolates. Another *P. torulosum* isolate (BR489), however, was closely linked to *P. graminicola*. This indicated intraspecific variation within *P. torulosum*. *P. volutum*, *P. pachycaule*, and *P. myriotylum* appeared to branch out separately at a considerable distance with respect to the other *Pythium* isolates.

In order to estimate the confidence in groupings comprised of more than one species, bootstrap analysis was carried out. SEQBOOT was used to generate 1000 bootstrap replicates, and distance matrices generated by DNADIST for each bootstrap replicate were analyzed by the NEIGHBOR program (neighbor-joining option; Saitou and Nei 1987), another distance method. This was chosen instead of KITSCH due to its relatively short execution time. A majority-rule consensus tree was constructed by the CONSENSE program (Fig. 6). The overall topologies and branching patterns were maintained with respect to the distance networks obtained from the KITSCH analysis. The DNAPARS program was used to carry out unrooted parsimony on the partial ETS sequences for estimating phylogenies by minimizing the number of mutational events necessary to account for the sequence differences. In order to estimate the confidence in groupings based on parsimony criteria, bootstrap analysis was carried out by generating 1000 bootstrap replicates with SEQBOOT. Each bootstrap replicate was then analyzed by DNAPARS. The *P. vanterpoolii* 4213a sequence was chosen as

the outgroup based on the distance method analysis, and a majority-rule consensus tree was generated by CONSENSE. A consensus tree from a bootstrap analysis can be considered as an overall estimate of the phylogeny (Felsenstein 1985). Only clusters which correspond to those generated by the distance method are indicated by two numbers of level of confidence (Fig. 6). The phylogenetic trees constructed by both distance and parsimony methods had overall similar topologies, although bootstrap support for nodes varied somewhat. The split of *P. vanterpoolii* from the other *Pythium* species at node 1 was supported by a 100% confidence level in both NEIGHBOR and DNAPARS analyses. The tight cluster which included *P. graminicola/aristosporum*, *P. tardicrescens*, *P. arrhenomanes*, *P. aristosporum*, and the two undescribed *Pythium* isolates appeared to be monophyletic due to their substantial bootstrap support at node 5 (NEIGHBOR 98.2%, DNAPARS 99.6%). The branching of *P. volutum* from the latter cluster at node 3 had bootstrap confidence levels of 48.6% (NEIGHBOR) and 91.3% (DNAPARS). Node 4, however, grouped all the other *Pythium* isolates, with confidence levels of 54.1% (NEIGHBOR) and 75.7% (DNAPARS). Within the large group, *P. pachycaule* might form a potential monophyletic subgroup with three isolates of *P. torulosum*, supported by 68.2% (NEIGHBOR) and 75.4% (DNAPARS) at node 11. The other *P. torulosum*, however, also could form another monophyletic subgroup with three isolates of *P. graminicola*,

with confidence levels of 78.2% (NEIGHBOR) and 68.1% (DNAPARS) at node 10.

Fig. 1. A physical map of the rDNA repeat unit of *Pythium* species. Symbols above the map indicate oligonucleotide primer sites with arrows giving 5′-3′ orientation. LSrRNA: large subunit ribosomal RNA gene, SSrRNA: small subunit ribosomal RNA gene, NTS: nontranscribed spacer, ETS: external transcribed spacer.

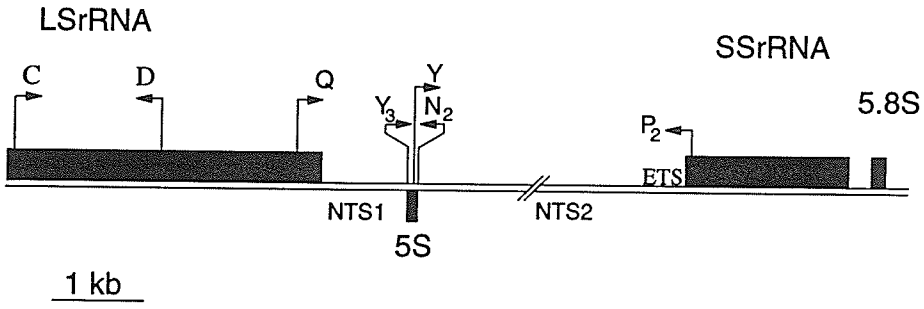

LSrRNA
SSrRNA
C
D
Q
Y
Y_3
N_2
P_2
5.8S
ETS
NTS1
NTS2
5S
1 kb

Table 1. A binomial matrix of the restriction fragments from the amplified CD region of the large subunit rDNA digested with MboI. Fragment present (G), fragment absent (A).

Molecular size (bp)	Isolate																								
	4205a	BR389	4212e	4201c	4216a	4204e	4214a	4213a	BR146	BR483	BR706	BR568	4012a	BR163	BR601	BR419	BR174	BR486	4311a	BR479	BR406	4000a	4006a	4410b	JM10
MboI restriction fragments																									
850	A	A	A	A	A	A	A	A	A	A	G	G	G	G	G	G	G	G	G	G	A	A	A	A	A
680	G	G	G	G	G	G	G	G	G	G	A	A	A	A	A	A	A	A	A	A	G	G	G	G	G
510	A	A	A	A	A	A	G	G	A	G	A	A	A	A	A	A	A	A	A	A	A	G	G	G	G
270	G	G	G	G	G	G	A	A	A	A	G	G	G	G	G	G	G	G	G	G	G	A	A	A	A
260	G	G	G	G	G	G	A	A	A	A	G	G	G	G	G	G	G	G	G	G	G	A	A	A	A
230	A	A	A	A	A	A	A	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
200	A	A	A	A	A	A	A	A	G	A	A	A	A	A	A	A	A	A	A	A	G	A	A	A	A
155	G	G	G	G	G	G	G	G	G	G	A	A	A	A	A	A	A	A	A	A	A	G	G	G	G
150	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G

Table 2. A binomial matrix of the restriction fragments from the amplified CD region of the large subunit rDNA digested with RsaI. Fragment present (G), fragment absent (A).

Molecular size (bp)	Isolate																								
	4205a	BR389	4212e	4201c	4216a	4204e	4214a	4213a	BR146	BR483	BR706	BR568	4012a	BR163	BR601	BR419	BR174	BR486	4311a	BR479	BR406	4000a	4006a	4410b	JM10
RsaI restriction fragments																									
720	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	A
580	G	G	G	G	G	G	G	G	G	G	A	A	G	G	G	G	G	G	G	A	A	G	G	G	A
490	A	A	A	A	A	A	A	A	A	A	G	G	A	A	A	A	A	A	A	A	G	A	A	A	A
450	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G
300	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	A	A	A	A	A
170	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
150	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G
130	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	A
110	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G

Table 3. A binomial matrix of the restriction fragments from the amplified CD region of the large subunit rDNA digested with HhaI. Fragment present (G), fragment absent (A).

Molecular size (bp)	Isolate																									
	4205a	BR389	4212e	4201c	4216a	4204e	4214a	4213a	BR146	BR483	BR706	BR568	4012a	BR163	BR601	BR419	BR174	BR486	4311a	BR479	BR406	4000a	4006a	4410b	JM10
HhaI restriction fragments																										
1300	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	G	G	A
680	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	A	A	A
610	A	A	A	G	G	A	G	G	G	G	G	G	G	A	A	A	A	A	A	A	G	A	A	A	A
510	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G
470	A	A	A	A	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
400	G	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
350	A	A	A	A	A	A	A	A	A	A	A	A	A	G	G	G	G	G	G	G	A	A	A	A	A
260	A	G	A	A	A	A	A	A	A	A	A	A	A	G	G	G	G	G	G	G	A	A	A	A	G
200	A	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
195	G	G	A	A	A	A	A	A	A	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
175	A	G	G	G	A	A	A	A	A	G	G	G	G	G	G	G	G	G	G	G	A	A	A	A	G
150	G	A	A	A	G	G	G	G	G	G	A	A	A	A	A	A	A	A	A	A	G	A	A	A	A
140	A	A	A	A	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
125	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G
110	G	A	G	G	G	G	G	G	G	A	A	A	A	G	G	G	G	G	G	G	G	G	G	G	A

Table 4. A binomial matrix of the restriction fragments from the amplified CD region of the large subunit rDNA digested with HaeIII. Fragment present (G), fragment absent (A).

Molecular size (bp)	Isolate																								
	4205a	BR389	4212e	4201c	4216a	4204e	4214a	4213a	BR146	BR483	BR706	BR568	4012a	BR163	BR601	BR419	BR174	BR486	4311a	BR479	BR406	4000a	4006a	4410b	JM10
HaeIII restriction fragments																									
1100	A	A	G	G	G	G	G	G	G	A	A	A	A	A	A	A	A	A	A	A	A	G	G	G	A
950	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G
750	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
720	A	A	A	A	A	A	A	A	A	G	G	G	G	A	A	A	A	A	A	G	G	A	A	A	A
470	A	A	A	A	A	A	A	A	A	A	G	G	G	G	G	G	G	G	G	G	G	A	A	A	A
400	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
390	A	A	A	A	A	A	A	A	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
240	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	A	A	A	A
220	A	A	A	A	A	A	A	A	A	A	A	A	A	G	G	G	G	G	G	A	A	A	A	A	A
200	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	A	A	A	G
115	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G

Table 5. A binomial matrix of the restriction fragments from the amplified CD region of the large subunit rDNA digested with AluI. Fragment present (G), fragment absent (A).

Molecular size (bp)	Isolate																								
	4205a	BR389	4212e	4201c	4216a	4204e	4214a	4213a	BR146	BR483	BR706	BR568	4012a	BR163	BR601	BR419	BR174	BR486	4311a	BR479	BR406	4000a	4006a	4410b	JM10
AluI restriction fragments																									
400	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	G	G	G	G
335	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	A	A	A	A
300	A	A	A	A	A	A	A	A	A	A	G	G	A	A	A	A	A	A	A	A	A	G	G	G	G
270	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
230	G	G	G	G	G	G	G	A	G	G	A	A	A	A	A	A	A	A	A	A	G	A	A	A	A
210	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	A	G	G	G	G
195	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	G	G	G	G
175	G	G	G	G	G	G	G	G	G	G	A	A	G	G	G	G	G	G	G	G	G	A	A	A	A
155	A	A	A	A	A	A	A	G	A	A	A	A	G	G	G	G	G	G	G	G	A	A	A	A	A
150	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	A	A	A	A	A
110	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G

Table 6. A binomial matrix of the restriction fragments from the amplified CD region of the large subunit rDNA digested with TaqI. Fragment present (G), fragment absent (A).

Molecular size (bp)	Isolate																									
	4205a	BR389	4212e	4201c	4216a	4204e	4214a	4213a	BR146	BR483	BR706	BR568	4012a	BR163	BR601	BR419	BR174	BR486	4311a	BR479	BR406	4000a	4006a	4410b	JM10
TaqI restriction fragments																										
700	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G
600	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	A	A	A	A	A	A
580	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	A	A	A	A
480	G	A	A	G	A	A	A	A	A	G	G	G	G	G	G	G	G	G	G	A	A	G	G	G	A	
430	A	G	G	A	G	G	G	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G
300	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	G	G	A	
250	G	G	G	G	G	G	G	G	A	G	A	A	A	A	A	A	A	A	A	A	A	G	G	G	A	
200	A	A	A	A	A	A	A	A	G	A	G	G	G	G	G	G	G	G	G	A	G	G	G	G	A	
170	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	
145	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	

Fig. 2. An alignment of the combined restriction profiles obtained from the six matrices (Tables 1, 2, 3, 4, 5, 6). *Pythium* isolates followed by >, >>, or >>> share identical profiles, with one, two, or three other isolates, respectively.

```
     1                                                  50
 1-  AGAGGAAGGGGAAAGAGAAGAAAGAAAGAGAAGAAGAAGAGAAGGAAGGG
 2-  AGAGGAAGGGGAAAGAGAAGAAAGAGAGGAAAAAAGAAGAGAAGGAAGGG
 3-  AGAGGAAGGGGAAAGAGAAGGAAAAAAAGAAAGGAAAAAAGAAGGAAGGG
 4-  AGAGGAAGGGGAAAGAGAAGGAAAAAAAAGAAGGAAAAAAGAAGGAAGGG
 5-  AGAGGAAGGGGAAAGAGAAGAAAGAAGAGAAAGGAAAAAAGAAGGAAGGG
 6-  AGAGGAAGGGGAAAGAGAAGAAGAAAAAAGGAGGAAAAAAGAAGGAAGGG
 7-  AGGAAAAGGGGAAAGAGAAGGAAAAAAAAGAAGGAAAAAAGAAGGAAGGG
 8-  AGGAAAAGGGGAAAGAGAAGGAAAAAAAAGAAGGAAAAAAGAAGGAAGAG
 9-  AGAAAGGGGGGAAAGAGAAGGAAAAAAAAGAAGGAAAAAAGAAGGAAGGG
10-  AGGAAAAGGGGAAAGAGAAGGAAAAAAAGGAAAAAAGAAGGAAGGAAGGG
11-  AGGAAAAGGGGAAAGAGAGAAAAAAAAGAAAAGGAAAAAAAAAGGAGGAG
12-  AGGAAAAGGGGAAAGAGAGAAAAAAAAGAAAAGGAAAAAAAAAGGAGGAG
13-  AGGAAAAGGGGAAAGAGAGAAAAAAAAGAAAAGGAAAAAAAAAGGAGGAG
14-  GAAGGAAAGGAGAAGAGAAGGAAAAAAGGAAAAAAAGGAAGAAGGAGGAG
15-  GAAGGAAAGGAGAAGAGAAGGAAAAAAGGAAAAAAAGGAAGAAGGAGGAG
16-  GAAGGAAAGGGAAAGAGAAGGAAAAAAGGAAAAAAAGGAAGAAGGAAGAG
17-  GAAGGAAAGGGAAAGAGAAGAAAAGGAGGAAAGAAAAGAAGGAGGAAGAG
18-  GAAGGAAAGGGAAAGAGAAGAAAAGGAGGAAAGAAAAGAAGGAGGAAGAG
19-  GAAGGAAAGGGAAAGAGAAGAAAAGGAGGAAAGAAAAGAAGGAGGAAGAG
20-  GAAGGAAAGGGAAAGAGAAGAAAAGGAGGAAAGAAAAGAAGGAGGAAGAG
21-  GAAGGAAAGGGAAAGAGAAGAAAAGGAGGAAAGAAAAGAAGGAGGAAGAG
22-  GAAGGAAAGGGAAAGAGAAGAAAAGGAGGAAAGAAAAGAAGGAGGAAGAG
23-  GAAGGAAAGGAAAGGAGAAGAAAAGGAGGAAAGAAAGGAAGAAGGAAGAA
24-  AGAGGAGAGGAGAAGAGAAGGAAAAAAGAGAAGAAAGGAAAAGGAGAGGA
25-  AGGAAAAGGAAAGAGGAGAAAGAAAGAGGAAGAAGAAAAAAAGGGAGGAG
```

```
   51              65
 1- GGAAGAAGGAAGAAG Pythium graminicola 4205a
 2- GGAAGAAGAGAGAAG Pythium hypogynum BR389
 3- GGAAGAAGGAAGAAG Pythium arrhenomanes 4201c
 4- GGAAGAAGAGAGAAG Pythium myriotylum 4216a
 5- GGAAGAAGAGAGAAG Pythium torulosum 4212e
 6- GGAAGAAGAGAGAAG Pythium dissimile 4204e
 7- GGAAGAAGAGAGAAG Pythium volutum 4214a
 8- GGGAGAAGAGAGAAG Pythium vanterpoolii 4213a
 9- GGAAGAAGAGAAGAG Pythium sulcatum BR146
10- GGAAGAAGGAAGAAG Pythium coloratum BR483
11- GAAAGAAAGAGGGAG Pythium acanthophoron 4000a>
12- GAAAGAAAGAGGGAG Pythium hydnosporum 4006a>
13- GAAAGAAAGAGGGAG Pythium oligandrum 4410b>
14- GAAAGAAGGAAAGAG Pythium irregulare BR706>>
15- GAAAGAAGGAAAGAG Pythium paroecandrum BR568>>
16- GGGAGAAGGAAAGAG Pythium spinosum 4012a
17- GGGAGAAGGAAAGAG Pythium paroecandrum BR163>>>
18- GGGAGAAGGAAAGAG Pythium paroecandrum BR601>>>
19- GGGAGAAGGAAAGAG Pythium paroecandrum BR419>>>
20- GGGAGAAGGAAAGAG Pythium irregulare BR174>>>
21- GGGAGAAGGAAAGAG Pythium irregulare BR486>>>
22- GGGAGAAGGAAAGAG Pythium mamillatum 4311a>>>
23- GGGGGAGGAAAAAAG Pythium paroecandrum BR479
24- AGAAGAAGAAAAGAG Pythium ultimum BR406
25- GAAAGGAAAGAAAGG Phytophthora cryptogea JM10
```

Fig. 3. An unrooted UPGMA network illustrating groupings of *Pythium* species and *Phytophthora cryptogea*. The network was generated by the analysis of RFLPs profiles from the CD fragment (65 positions) with the NEIGHBOR program (UPGMA option). The numbers along the branches represent the nodes.

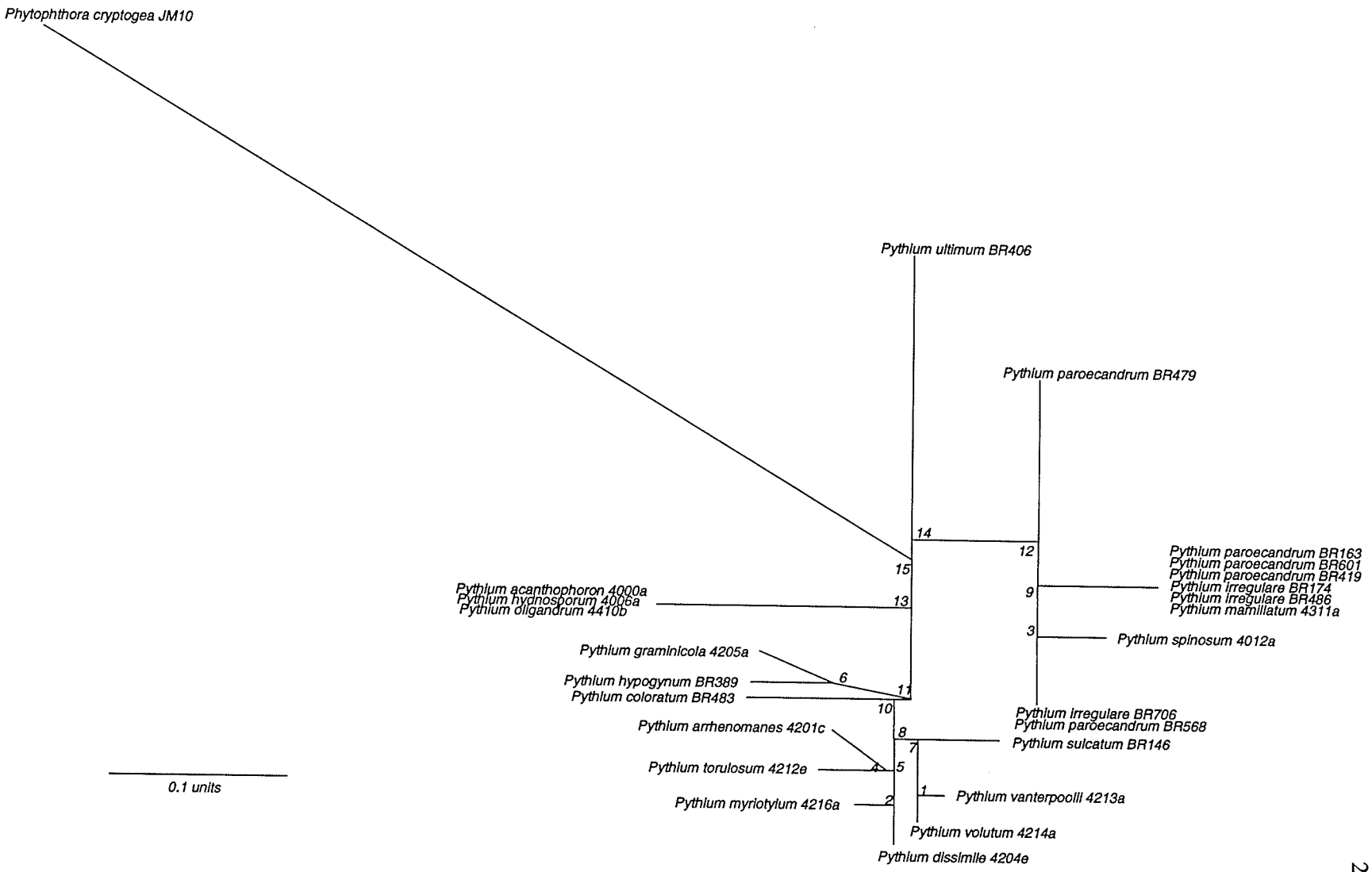

Phytophthora cryptogea JM10
Pythium ultimum BR406
Pythium paroecandrum BR479
Pythium paroecandrum BR163
Pythium paroecandrum BR601
Pythium paroecandrum BR419
Pythium irregulare BR174
Pythium irregulare BR486
Pythium mamillatum 4311a
Pythium spinosum 4012a
Pythium acanthophoron 4000a
Pythium hydnosporum 4006a
Pythium oligandrum 4410b
Pythium graminicola 4205a
Pythium hypogynum BR389
Pythium coloratum BR483
Pythium irregulare BR706
Pythium paroecandrum BR568
Pythium arrhenomanes 4201c
Pythium sulcatum BR146
Pythium torulosum 4212e
Pythium vanterpoolii 4213a
Pythium myriotylum 4216a
Pythium volutum 4214a
Pythium dissimile 4204e
14
12
15
9
13
3
6
11
10
8
7
4
5
1
2
0.1 units

Table 7. 5SrRNA gene signature positions in *Pythium* species. Nucleotide residues at positions 39 and 107 represent the only differences in the entire 118 bp 5SrRNA gene sequence.

Isolate	Position 39*	Position 107*	Zoosporangial form
Pythium diclinum 4110a	C	G	filamentous
Pythium sulcatum BR653	C	G	filamentous
Pythium aphanidermatum BR206	C	G	filamentous
Pythium volutum 4214a	C	G	filamentous
Pythium tardicrescens BR569	C	G	filamentous
Pythium coloratum BR483	C	G	filamentous
Pythium arrhenomanes BR140	C	G	filamentous
Pythium torulosum 4212e	C	G	filamentous
Pythium pachycaule 4117b	C	G	filamentous
Pythium aristosporum BR136	C	G	filamentous
Pythium myriotylum 4216a	C	G	filamentous
Pythium graminicola 4205d	T	G	filamentous
Pythium salpingophorum/ conidiophorum 4331a	T	A	globose
Pythium periplocum 4461a	T	A	filamentous
Pythium periplocum 4461b	T	A	filamentous
Pythium hypogynum BR635	C	G	globose
Pythium hypogynum BR389	C	G	globose
Pythium nagae 4321a	T	A	globose
Pythium oligandrum 4410b	T	A	globose
Pythium iwayamae 4405g	T	A	globose
Pythium paddicum 480a	T	A	globose
Pythium ultimum BR471	T	A	unknown
Pythium paroecandrum BR163	T	A	globose
Pythium paroecandrum BR574	T	A	globose
Pythium irregulare BR486	T	A	globose
Pythium spinosum 4012e	T	A	unknown
Pythium spinosum 4012d	T	A	unknown
Pythium hydnosporum 4006a	T	A	unknown
Pythium mamillatum 4311b	T	A	globose
Pythium rostratum 4329j	T	A	globose
Pythium anandrum 4401d	T	G	globose
Phytophthora cryptogea JM10	T	G	

* Refer to figure 7.

Fig. 4. An alignment of the partial ETS sequences from *Pythium* species that have the 5S rRNA gene in the NTS. Numbers above the sequences indicate nucleotide positions (1-135) within the alignment. Symbols following *Pythium* isolates indicate sequence identity with other isolates: * = *Pythium* sp BR620; # = *P. graminicola/aristosporum* BR166, *Pythium* sp BR667; > = *P. graminicola* 4205b, *P. graminicola* 4205g; >> = *P. torulosum* 4212d, *P. torulosum* BR158.

```
    1              15 16             30 31             45 46             60 61             75 76             90
 1  GGCAAA-TGATAGCA GAGATGTGTCTGTAA GACCTG-CTGCCGCT GAGTA-TTCGGTGGA T-TGTG-G--GTTGA -CGGATGCAGTTGAC
 2  GACAA-CTGATAGCA GAAATGTGTCTGTAA GGTTTG-CCGCCCTC GAGTTCTTTGGGGGA T-TGTGTG-AGCTAT GCGGATGC-GTTGAC
 3  GGCAAA-TGATAGCA GAAATGCGTCTGTAA GGTTTG-CTGCCTCT GAGTA-TTCGGAGGA TGTGTG---AGCTGG ACGGATGC-GTTGAC
 4  GGCAAA-TGATAGCA GAAATGCGTCTGTAA GGTTTG-CTGCCTCT GAGTA-TTCGGGGGA TGTGTG---AGCTGG ACGGATGC-GTTGAC
 5  GACAA-CTGATAGCA GAAATGTGTCTGTAA GGTTTG-CCGCCCTC GAGTTCTTTGGGGGA T-TGTGTG-AGCTAT GCGGATGC-GTTGAC
 6  GACAA-CTGATAGCA GAAATGTGTCTGTAA GGTTTG-CCGCCCTC GAGTTCTTTGGGGGA T-TGTGTG-AGCTAT GCGGATGC-GTTGAC
 7  GACAA-CTGATAGCA GAAATGTGTCTGTAA GGTTTG-CCGCCCGC GAGTTCTTTGGGGGA T-TGTGTG-AGCTAT GCGGATGC-GTTGAC
 8  GGCAAA-TGATAGCA GAAATGCGTCTGTAA GGTTTG-CTGCCTCT GAGTA-TTCGGGGGA TGTGTG---AGCTGG ACGGATGC-GTTGAC
 9  GACAAA-TGATAGCA GAAATGCGTCTGTAA GGTTTG-CTGCCTCT GAGTA-TTCGGGGGA TGTGTG---AGCTGG ACGGATGC-GTTGAC
10  GGCAAA-TGATAGCA GAAATGCGTCTGTAA GGTTTG-CTGCCTCT GAGTA-TTCGGGGGA TGTGTG---AGCTGG ACGGATGT-GTTGAC
11  GACAAA-TGATAGCA GAAGTGTGTCTAGTT GATCCG-CTGCCT-C GAGTTCTTCGGGTGA T--GTGTG--GTTGA TCGGATGCGCTATAT
12  GACTTTCTGATAGCA GAAATGTGTCTGTAA GGTTTG-CTGCCTTC GAGTTATTCGGAGGA T-TGTGCG-AGCTGG ACGGATGC-GTTGAC
13  GACAA-CTGATAGCA GAAATGTGTCTGTAA GGTTTG-CCGCCCTC GAGTTCTTTGGGGGA T-TGTGTG-AGCTAT GCGGATGC-GTTGAC
14  GGCAAA-TGATAGCA GAAACGCAGCCAGAA AGTTTG-CTGCCTCT GAGTA-TTCGGAGGA TATGCG---AGCCGG ACGGAGGC-GTTGAC
15  GGCAAA-TGATAGCA GAAGTGTGTCTGTAA TGCTTG-CTGCCTCT GAGTA-TTCGGAGGA TGTGTG---AGTGGC GCGGGTGC-ATTGAC
16  GGCAAA-TGATAGCA GAGATGTGTCTGTAA GACCTG-CTGCCTCT GAGTA-TTCGGGGGA TGTGTG-G--GTTGG ACGGATGC-GTTGAC
17  GGCAAA-TGATAGCA GAAGCGTGTCTGTAA GGTTTG-CTGCCTCT GAGTA-TTCGGAGGA TGTGTG---AGCTGG ACGGACGC-GTTGAC
18  GGCAAA-TGATAGCA GAGATGTGTCTGTAA GGCTTG-CTGCCTCT GAGTA-TTCGGAGGA TTTGTG---AGCTGG ACGGGTGC-ATTGAC
19  GACAAA-TGATAGCA GAAGTGTGTCTAGTT GATCCG-CTGCCTC- GAGTTCTTCGAGTGA T-TGTGTG-GTTTGA CCGGATGCGCTATAT
```

```
    91          105 106         120 121         135
 1  GTTTGAAATTGTTGG TTCAATTTCTAGTAT TTTTT-AAAGATATC P.torulosum BR489
 2  GTTTGAGATTGTTGG TTCAATTTCTAGTAT TTTT-GAAAGATATC P.tardicrescens BR569*
 3  GTTTGAAATTGTTGG TTCAATTTCTAGTAT TTTTT-AAAGATATC P.coloratum BR483
 4  GTTTGAAATTGTTGG TTCAATTTCTAGTAT TTTGT-AAAGATATC P.dissimile BR160
 5  GTTTGAGATTGTTGG TTCAATTTCTAGTAT TTTT-GAAAGATATC P.arrhenomanes BR140#
 6  GTTTGAGATTGTTGG TTCAATTTCTAGTAT TTTT-GAAAGATATC P.aristosporum BR136
 7  GTTTGAGATTGTTGG TTCAATTTCTAGTAT TTTT-GAAAGATATC P.graminicola/aristosporum BR608
 8  GTTTGAAATTGTTGG TTCAATTTCTAGTAT TTTT-GAAAGATATC P.hypogynum BR389
 9  GTTTGAAATTGTTGG TTCAATTTCTAGTAT TTTTTTAAAGATATC P.sulcatum BR653
10  GTTTGAAATTGTTGG TTCAATTTCTAGTAT TTTTT-AAAGATATC P.hypogynum BR635
11  GTTTGAGATTGTGTG -TCAATTTCTAGTAT TTTT-GAAAGATATC P.vanterpoolii BR488
12  GTTTGAGATTGTTGG TTCAATTTCTAGTAT TTTT-GAAAGATATC P.volutum 4214a
13  GTTTGAGATTGTTGG TTCAATTTCTAGTAT TTTT-GAAAGATATC P.arrhenomanes 4201b
14  GTTTGAAATTGTTGG TTCAATTTCTAGTAC TTTTT-AAAGATATC P.myriotylum 4216a
15  GTTTGAAATTGTTGG TTCAATTTCTAGTAT TTTCTGAAAGATATC P.pachycaule 4217b
16  GTTTGAAATTGTTGG TTCAATTTCTAGTAT TTT--GAAAGATATC P.graminicola 4205a>
17  GTTTGAAATTGTTGG TTCAATTTCTAGTAT TTTT-GAAAGATATC P.dissimile 4204b
18  GTTTGAAATTGTTGA TTCAATTTCTAGTAT TTTCT-AAAGATATC P.torulosum 4212e >>
19  GTTTGAGATTGTGTG TTCAATTTCTAGTAT TTTT-GAAAGATATC P.vanterpoolii 4213a
```

Fig. 5. An unrooted Fitch-Margoliash network showing the phyletic relations among *Pythium* species with filamentous zoosporangia. The network was generated by the KITSCH program based on partial ETS sequences (135 positions). The nodes are represented by numbers. *Pythium* isolates preceded by *, #, >, or >> indicate ETS sequence identity.

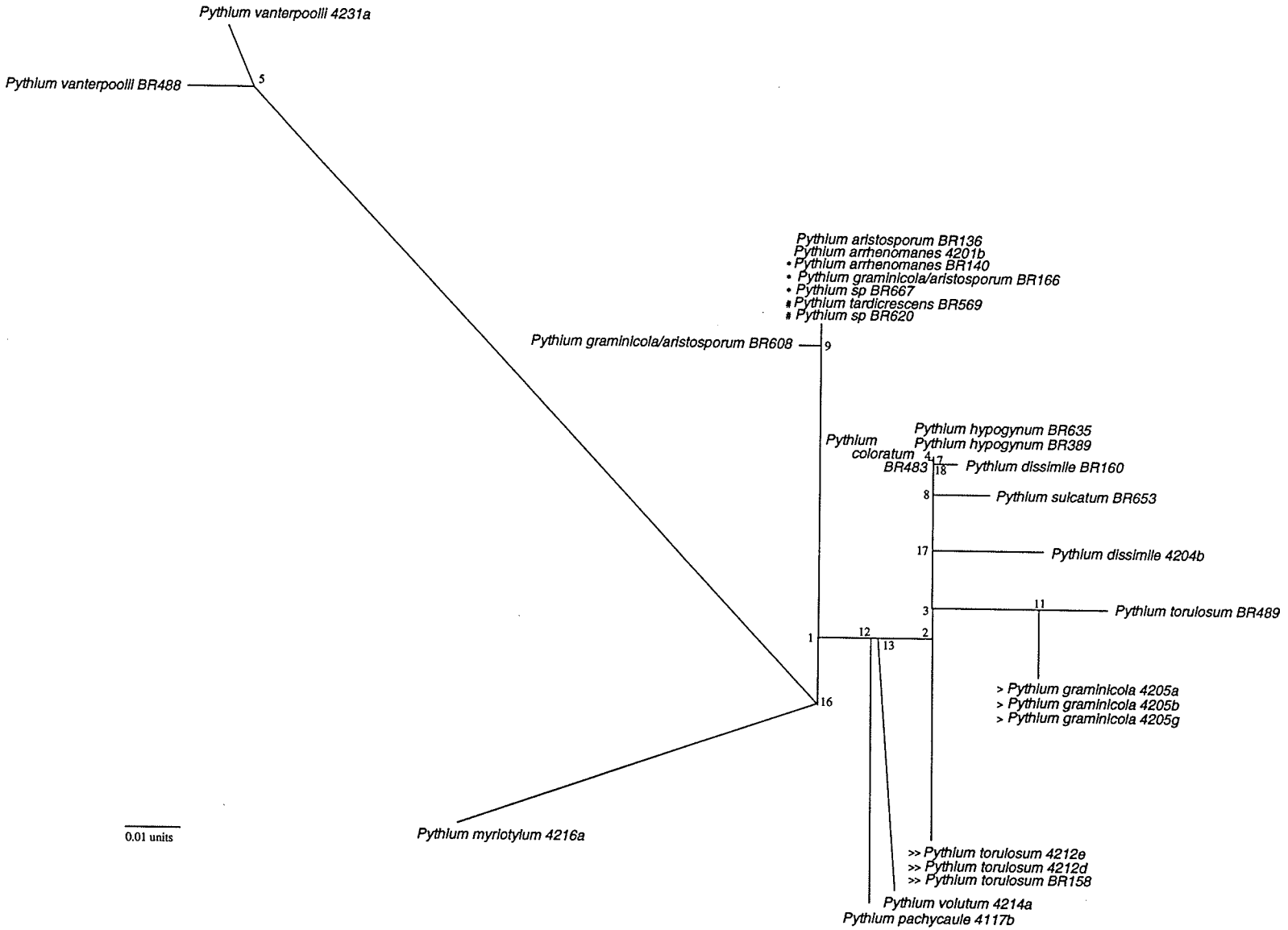
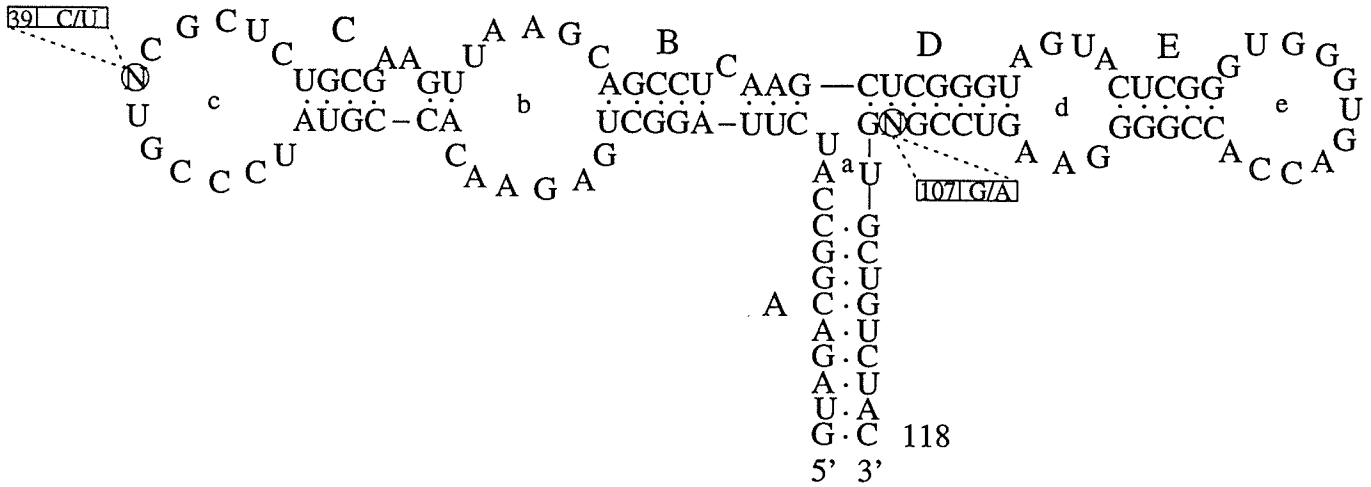

Pythium vanterpoolii 4231a
Pythium vanterpoolii BR488
5
Pythium aristosporum BR136
Pythium arrhenomanes 4201b
• Pythium arrhenomanes BR140
• Pythium graminicola/aristosporum BR166
• Pythium sp BR667
Pythium tardicrescens BR569
Pythium sp BR620
Pythium graminicola/aristosporum BR608
9
Pythium hypogynum BR635
Pythium hypogynum BR389
Pythium coloratum BR483
4
17
18
Pythium dissimile BR160
8
Pythium sulcatum BR653
17
Pythium dissimile 4204b
3
11
Pythium torulosum BR489
1
12
2
13
16
> Pythium graminicola 4205a
> Pythium graminicola 4205b
> Pythium graminicola 4205g
0.01 units
Pythium myriotylum 4216a
>> Pythium torulosum 4212e
>> Pythium torulosum 4212d
>> Pythium torulosum BR158
Pythium volutum 4214a
Pythium pachycaule 4117b

Fig. 6. A majority-rule consensus tree illustrating the phyletic relationships between *Pythium* species with filamentous zoosporangia. The tree was constructed using the bootstrap procedure (SEQBOOT) in PHYLIP (Felsenstein 1991) and the DNA distance matrix contained in the NEIGHBOR program (neighbor-joining option) based on partial ETS sequences; The upper percentages at the nodes are the frequences with which a given branch appeared in 1000 replications. The bootstrap percentages positioned underneath the upper ones at the nodes represent the common branches also found in another majority-rule consensus tree constructed using SEQBOOT and DNAPARS (parsimony method). Members of each cluster of *Pythium* isolates preceded by a vertical line share the same partial ETS sequence.


Pythium hypogynum BR635
Pythium sulcatum BR653
Pythium dissimile BR160
Pythium hypogynum BR389
Pythium coloratum BR483
Pythium dissimile 4204b
Pythium myriotylum 4216a
Pythium torulosum 4212d
Pythium torulosum 4212e
Pythium torulosum BR158
Pythium pachycaule 4117b
Pythium torulosum BR489
Pythium graminicola 4205a
Pythium graminicola 4205b
Pythium graminicola 4205g
Pythium graminicola/aristosporum BR608
Pythium sp BR620
Pythium tardicrescens BR569
Pythium sp BR667
Pythium graminicola/aristosporum BR166
Pythium arrhenomanes BR140
Pythium arrhenomanes 4201b
Pythium aristosporum BR136
Pythium volutum 4214a
Pythium vanterpoolii BR488
Pythium vanterpoolii 4213a
25.6%
17.6%
17
33.5%
15.6%
16
49.8%
15
30.2%
14
40.9%
12
54.2%
13
54.1%
75.7%
4
68.2%
75.4%
11
46.9%
9
78.2%
68.1%
10
100%
100%
2
100%
100%
1
98.2%
99.6%
5
96.9%
47.9%
8
96.3%
31.3%
7
48.6%
91.3%
3
97.8%
29.8%
6

Fig. 7. A secondary structure model for the 5S rRNA of *Pythium* species and *Phytophthora cryptogea* showing helices A, B, C, D, and E and loops a, b, c, d, and e. Variable residues at positions 39 and 107 are indicated by a circle containing N. Boxes contain possible nucleotide changes (Table 7).


39 C/U
107 G/A
5' 3'
118

DISCUSSION

The phylogenetic relationships among the species of *Pythium* have not yet been resolved, although several preliminary studies have been done (Chen and Hoy 1993; Chen *et al.* 1992; Martin and Kistler 1990). The main implication of this research is that most *Pythium* species fall into one of two groups- those with the 5S rRNA gene in the NTS of the rDNA repeat, and those with 5S genes in tandem repeats unlinked to the rDNA repeat. The former almost invariably have filamentous zoosporangia and the latter do not. Confirmation of this primary division of the genus was attempted by comparison of RFLP patterns of a variable region near the 5′ end of the LSrRNA, by sequence comparison of the 5S rRNA genes, and by partial sequencing of the ETS region just upstream of the SSrRNA gene, although the last of these projects is incomplete.

Pythium species with filamentous zoosporangia form a tight monophyletic cluster distant from those species with globose or unknown zoosporangia based on a distance analysis of combined RFLPs profiles of the CD fragment (LSrRNA). It would be ideal, of course, to analyze each RFLP profile separately (Smith and Anderson 1989; Taylor and Natvig 1989), but in the case of this study it was not practical since each profile consisted of fewer than 10 bands. Although this method was prone to some degree of error;

nevertheless, it did show the basic groupings of *Pythium* species. The clustering of *Pythium* species with filamentous zoosporangia separately from those species with globose or unknown zoosporangia, seems to correlate with the physical location of the 5S gene with respect to the other rRNA genes. Most filamentous *Pythium* species have the 5S gene in NTS (Chapter 1). The fact that *P. hypogynum*, which has globose zoosporangia, was found among the filamentous group, is not surprising since it also has the 5S gene in NTS (Chapter 1).

The results indicated that the *Pythium* species with globose or unknown zoosporangia formed a polyphyletic network with individuals and several groups separated by considerable distances. First of all, *P. ultimum* is significantly isolated from the other species. This species is also unusual in that it is the only known species of *Pythium* with dramatic length heterogeneity in the NTS (Buchko and Klassen 1990; Klassen and Buchko 1990). It also has smooth-walled oogonia and typical monoclinous sac-like antheridia (Plaats-Niterink 1981). *P. acanthophoron*, *P. hydnosporum*, and *P. oligandrum*, had identical profiles, and form a group also well separated from the others. The three species have ornamented oogonia with slight variations in the shape and size of projections. The third group consisted of *P. paroecandrum*, *P. irregulare*, *P. mamillatum*, and *P. spinosum*. The fact that some *P. irregulare* and *P.*

paroecandrum isolates shared the same RFLP profiles and others did not, suggests that differentiation of the two species is difficult on morphological grounds. Based solely on morphology, *P. paroecandrum* is related to *P. irregulare* but differs from it by the more regular and slightly larger, mostly intercalary oogonia which also lack the typical finger-like projections; moreover, in *P. irregulare* no intercalary antheridia occur (Plaats-Niterink 1981). In *P. mamillatum*, the oogonial projections are rather irregular in shape, length and number, whereas in *P. spinosum*, they are longer and cylindrical (Plaats-Niterink 1981) . This indicates that oogonial ornamentation may not be adequate to discriminate between clusters of *Pythium* species. These groupings are in agreement with groupings obtained from RFLP analysis of the ITS from 25 isolates representing 5 *Pythium* species (Chen *et al.* 1992).

Further evidence for the monophyly of each of the two postulated subgroups of *Pythium* species was sought in the sequences of the 5S rRNA genes. Two "signature positions" were found which correlate well with the two groups. The importance of the two positions can be inferred from a consideration of the possible secondary structure of the deduced 5S rRNA product of the gene. The secondary structure of the potential 5S gene product for all isolates studied was based on the *Xenopus laevis* model (Pieler and Theunissen 1993) (Fig. 7). The secondary structure model conformed well

to the eukaryotic consensus of five helices A to E, connected by loops a to e (Nishikawa and Takemura 1974). The signature positions at residues 39 and 107 are part of loop c and helix D, respectively. Structural constraints may be imposed on residue 107 to maintain the base-paired helix which would preserve the 5S rRNA function, by allowing only compensating substitutions to occur. Residue 39, however, may not be subject to any constraints since its substitution will not impair the secondary structure of the 5S rRNA molecule. Based on this assumption, one would predict that the substitution rate of residue 39 would be much higher than that of residue 107. Thus, the latter residue would be more informative in revealing phylogenetic relationships between various *Pythium* species than the former residue. Based on residue 107, there seems to be a phylogenetic split between *Pythium* species with filamentous zoosporangia and those with globose and unknown zoosporangia. *P. periplocum*, a species with filamentous zoosporangia, was an exception; it grouped with globose and unknown zoosporangial *Pythium* species. In *P. periplocum*, the 5S gene was found organized in tandem repeats outside the NTS (Chapter 2), a feature that puts the species together with the globose and unknown zoosporangial group. *P. periplocum* is also the only species of *Pythium* which has filamentous zoosporangia and ornamented oogonia (Plaats-Niterink 1981), a trait that would associate it strongly with species that have globose zoosporangia and

5S tandem repeats. A likely explanation is that the filamentous zoosporangia in *P. periplocum* have evolved independently. The other exception, as expected, was *P. hypogynum*. This is reciprocal to *P. periplocum; P. hypogynum* has globose zoosporangia, but appears to cluster with the filamentous zoosporangial group. Residue 107 surprisingly puts *P. anandrum* together with the filamentous zoosporangial group, whereas residue 39 indicates its position with the globose and unknown zoosporangial group. The fact that *Phytophthora cryptogea* shares the same residue (G) at position 107 as the filamentous zoosporangial *Pythium* group, and the 5S gene location in the NTS, makes it possible to assume that the residue 107 (G) may be ancestral. This leads to the possibility that the ancestor of *P. anandrum* diverged from the main lineage after the loss of the 5S rRNA gene from the NTS but before the change from G -> A at position 107.

The ultimate solution to resolving the phylogeny of *Pythium* species would be to find a region or regions of DNA sequence with a suitable amount of variability for this purpose. An attempt was made to find such a region within the rDNA repeat unit. The most variable sequence in the rRNA genes is likely to be one of the known variable regions in the LSrRNA. Our CD region includes one such variable region (Hausner *et al.* 1992; Hausner *et al.* 1993), but sequencing of part of this region in *P. graminicola/aristosporum* BR166,

P. torulosum 4212e, *P. muriotylum* 4216a and *P. irregulare* BR174 revealed no differences. It was concluded that it was unlikely to find sufficient variability in the genes, and so attention was turned to the intergenic regions.

The presence of the 5S rRNA gene in the NTS of *Pythium* species with filamentous zoosporangia allowed us to amplify the region downstream of the 5S gene and to use the product as a template for sequencing of the ETS. Thus we were able to sequence and compare 135 bp from the ETS of all of the filamentous zoosporangial species (Figs 4-6). The only ETS sequence available from a *Pythium* species that does not have the 5S gene in the NTS is that of *P. ultimum* BR471 (John Buchko, personal communication). An attempt to align this sequence with the consensus of sequences in Figure 4 was unsuccessful. This may mean that the ETS sequence will be useful for phylogenetic analysis only within the filamentous zoosporangial group, but it also further supports the separatness of the filamentous zoosporangial group from other *Pythium* species. This conclusion must remain tentative until more ETS sequences become available; it may be that *P. ultimum* alone is atypical. *Pythium hypogynum* is the only species, to date, which has globose zoosporangia and an ETS that aligns with the corresponding sequence from the filamentous zoosporangial group. This again indicates its phylogenetic position within the latter group and the likelihood that its zoosporangial form is not homologous

with that of the globose zoosporangial group. Due to the fact that most *Pythium* species with globose and unknown zoosporangia do not have the 5S gene located in the rDNA repeat, the ETS region was not as easily accessible by PCR amplification and subsequently no ETS sequences have yet been obtained from the group. Amplification of the entire NTS (4-5 kb) is possible and is the next logical step in the extension of the ETS database to species which lack the 5S rRNA gene in the NTS.

Relationships within the filamentous zoosporangial group were clarified to some extent by phylogenetic analysis of the ETS sequences. Distance and cladistic analyses of ETS sequences indicate that there is a monophyletic group comprising two isolates of *P. graminicola/aristosporum* (BR166, BR608), two isolates of *P. arrhenomanes* (4201b, BR140), one isolate of *P. tardicrescens* BR569, one isolate of *P. aristosporum* BR136, and two undescribed *Pythium* isolates (BR667, BR620) (Fig. 6). The undescribed isolates were isolated from maize and barley (Canada). Although their morphological taxonomy has not been thoroughly determined, the molecular data assign them to the same group. *P. tardicrescens* has filamentous hyphal swellings, and the loss of its ability to produce zoospores does not seem to affect its close phylogenetic relation with *P. arrhenomanes* and *P. aristosporum* within the group. In addition, based solely on morphology, *P. tardicrescens* is closely related to *P.*

aristosporum. The main differences are the somewhat larger oogonia and oospores of *P. aristosporum*, and the slow growth of *P. tardicrescens* (Plaats-Niterink 1981). The results show that isolates of *P. graminicola* group separately from isolates of *P. graminicola/aristosporum*. This indicates that the latter isolates may actually be phylogenetically closer to *P. aristosporum* (Fig. 6). *P. volutum*, however, seems to be the most distant species within the monophyletic group (Fig. 6). It is characterized by its diclinous long antheridial stalks which coil around the oogonial stalk and the oogonium (Plaats-Niterink 1981). Although *P. graminicola* and *P. arrhenomanes* are morphologically very similar and mainly separated by differences in oogonial diameter and number of antheridia per oogonium (Plaats-Niterink 1981), they do not cluster together and subsequently should be maintained as distinct species. This is in agreement with the conclusion based on analysis of PCR-RFLPs of the ITSs and portions of the nuclear large-subunit rDNA from several isolates of *P. graminicola* and *P. arrhenomanes* (Chen and Hoy 1993). The larger group, which includes *P. graminicola* and the other *Pythium* species, is not substantially supported statistically as is the smaller monophyletic group described above (Fig. 6). The overall topology of the large group indicates inter- and intraspecific variation between species. The low bootstrap numbers suggest lack of resolution and limitation of the data. They may also reflect

the pattern of sampling of *Pythium* isolates. *P. vanterpoolii* appears to be phylogenetically the most distant from the other *Pythium* species, although morphologically it is similar to *P. torulosum* (Plaats-Niterink 1981).

CONCLUSIONS

This thesis represents the beginning of an evolutionary analysis of a repetitive gene family in the genus *Pythium*. Major novelties have been uncovered, including an evolutionary switch from one type of gene organization to another, the presence of 5S rRNA pseudogenes in the NTS of the rDNA repeat and in a tandem array. It reports the first characterization of a 5S tandem array in microorganisms, and is the first to exploit the ETS region of the rDNA repeat for phylogenetic purposes. The survey of 5S gene location needs to be extended to all available *Pythium* species and the few unusual species that do not conform to expectation need to be fully investigated. It is possible that true intermediates may be found, in which the switch from "linked" to "unlinked" 5S genes is in progress.

The data analysis has uncovered two possible cases of convergent evolution with respect to zoosporangial form (*P. periplocum*, *P. hypogynum*) and has provided evidence for the correct taxonomic placement of two species that do not sporulate (*P. ultimum*, *P. tardicrescens*).

The reconstruction of *Pythium* phylogeny is in its infancy. An appropriate target sequence has not yet been found in the rDNA repeat or in the 5S arrays. The ETS may prove to be useful for the resolution of the filamentous zoosporangial group and some others, but it will not likely

be useful accross the entire genus, since the *P. ultimum* ETS cannot be aligned with the ETS of the filamentous zoosporangial species, although we have investigated only the 130 bp upstream of the SSrRNA gene. Perhaps a sequence outside of the ribosomal gene families will have to be exploited.

The main contribution of the study at this time is the hypothesis, with some supporting evidence, that the genus is made up of at least two major groups, one of which (filamentous zoosporangial) is more cohesive than the other. Two "signature" positions in the highly conserved 5S gene provide independent support for the hypothesis. These findings may lead eventually to the erection of a new genus name for the group with globose zoosporangia because the type species of the genus, *P. monospermum*, has filamentous zoosporangia.

Further analysis of the ribosomal gene family in *Pythium* may reveal more of the principles governing the evolution of such repetitive families and may eventually resolve the phylogenetic relationships within this large and important genus of Oomycetes.

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