RIBOSOMAL GENE SPACERS IN *PYTHIUM*: EVOLUTION OF SPECIES BOUNDARIES

by

Malgorzata Balcerzak

A thesis submitted in partial fulfillment of
the requirements for the degree of

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ABSTRACT

Pythium species are pathogenic to many plants and can cause severe losses in cereals and other crops, as well as ornamental plants. Traditional taxonomy in *Pythium* is based primarily on growth characteristics and morphology and has always been difficult. These difficulties result from the limited number of morphological characters available for species identification and the significant variability of these characters. Rapid, simple and reliable identification of *Pythium* species may be possible using molecular techniques. The major objective of this study was to use ribosomal DNA spacer variation to develop molecular markers for identification and classification of *Pythium* species.

The spacer region between the 5S rRNA genes diverges rapidly after speciation and was used as a species-specific hybridization probe for identification of twelve *Pythium* species. Development of probes was based on the amplification of the intergenic spacer (IGS) between tandemly repeated 5S rRNA genes with primers complementary to the conserved ends of the genes. The species-specific probes recognized the genomic DNA of the isolates from which the probe had been derived but not the genomic DNA from 92 other tested *Pythium* species. Furthermore, probes derived from one mating type also

recognized the opposite mating type and reacted satisfactorily with other available isolates from the same species.

Probing of genomic DNA with the 5S spacer probe was useful not only for identification of *Pythium* isolates to the species level but also allowed for detection of close relationships between species. The cross-hybridization signal observed between seven species – *P. irregulare*, *P. mamillatum*, *P. spinosum*, *P. kunmingense*, *P. paroecandrum*, *P. cylindrosporum* and *P. polymorphon* – that share similarity in morphology, indicated that these species are very closely related. 5S rRNA spacers from seven species that form the "*P. irregulare* 5S spacer homology group" were cloned and sequenced. The spacers of members of the group were highly homologous with each other, but could not even be aligned with the spacers found in closely related species that were not members of the homology group. This sharp discontinuity may correspond to a natural species boundary. The common structure of the 5S spacers and a high level of sequence similarity indicate that gene exchange occurs between the IGS regions of these seven species. Both neighbor-joining and parsimony analysis of 5S spacer variability support the assignment of a close relationship between the species of the *P. irregulare* cluster and suggest that they may be conspecific.

The interspecific restriction site variation in the intergenic region between the large and the small rDNA gene subunit of 42 isolates representing 5S spacer homology group was also investigated and the results permitted clear

identification of individual strains. Inferred relationships based on RFLP analysis of the IGS region were generally consistent with our grouping based on the 5S spacer tree. These results provide evidence that the IGS regions are useful for investigating *Pythium* taxonomy and systematics and determining interspecific relatedness in this genus.

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LIST OF ABBREVIATIONS

A	adenine
ATP	adenosine 5' -triphosphate
bp	base pairs
C	cytosine
CTAB	hexadecyltrimethyl amonium bromide
DIG	digoxigenin
DNA	deoxyribonucleic acid
dATP	2' –deoxyadenosine 5' -triphosphate
dNTP	2' –deoxyribonucleoside 5' –triphosphate
dUTP	2' –deoxyuridine 5' -triphosphate
EDTA	ethylenediamine-tetra-acetic acid
ETS	external transcibed spacer
G	guanidine
IGR	intergenic region
IGS	intergenic spacer
IPTG	isopropylthiogalactoside
ITS	internal transcribed spacer
kb	kilobase pairs
lsrRNA	large subunit ribosomal RNA

NTS	nontranscibed spacer
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
rDNA	ribosomal DNA
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
SSC	sodium saline citrate
ssrRNA	small subunit ribosomal RNA
T	thymine
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume
X-Gal	5–bromo-4-chloro-3-indolyl-β-D-galacoside

Chapter 1

INTRODUCTION

There have been over 130 species described for *Pythium*. Species from this genus can cause a wide variety of diseases on many host plants. Identification and classification of *Pythium* species is mainly based on morphology of reproductive structures, which are typically examined in pure culture. For many *Pythium* species, however, variability in morphology within and between species is often too large to allow reliable identification. Many taxonomic characteristics have continuous rather than discrete variation, and a large proportion of isolates in a species can differ considerably from the type species. Many important taxonomic characteristics also have ranges that overlap between species. Sets of characters that define species may be incomplete for any particular isolate due to loss of reproductive structures, which creates additional problems in identification. Morphological plasticity and overlap in phenotype among species make classification difficult and complicate the use of morphology for estimating interspecific relatedness. Species with similar morphology but independent lineages may be incorrectly grouped together or, in other cases, inconsistent morphological characters can be used to infer relationships in more than one way. As a result, several nonmorphological

approaches have been used to identify *Pythium* species and to investigate relationships between them.

Molecular approaches provide a new set of characters likely to produce greater phylogenetic accuracy than that obtainable by weighted analysis of morphological criteria and allow for more reliable, simple and faster identification of isolates. The major objective of this study was to find a DNA sequence that would be appropriate for species identification and setting species boundaries. We were trying to find a DNA sequence that contains sufficient inter-specific variability to be informative, but with minimal intra-specific variation to be conclusive. Ribosomal gene sequences are not appropriate because they change too slowly and because their rate of change is continuous, reflecting evolution over the long term. The spacers between genes, however, can be more useful. This is related to the fact that rDNA spacers are relatively free of structural and functional constraints, which allow them to evolve relatively rapidly. Moreover, the effect of molecular drive and concerted evolution generally act to minimize the degree of intra-specific variation.

In a number of *Pythium* species, 5S rRNA genes are organized into tandem arrays unlinked to the rDNA repeat unit (Belkhiri et al. 1992, Belkhiri et al. 1996). The intergenic regions between repeated, conserved genes diverge rapidly after speciation and therefore can be used as species-specific probes for *Pythium* species in routine identification by nonisotopic dot blot hybridization.

Development of probes was based on the amplification of the 5S spacer with primers complementary to the ends of the 5S rRNA genes.

The another purpose of this study was to use 5S spacer sequence comparisons to define species boundaries in the genus *Pythium*. Because spacers in tandem arrays diverge rapidly after speciation but are subject to homogenization before speciation, they give rise to a sharp discontinuity at species boundaries. We report one such discontinuity which may indicate that seven morphological *Pythium* species are in group that exchanges genes.

The next goal of this project was to utilize restriction fragment length polymorphism (RFLP) analysis of the IGS region between the large and the small subunit of rDNA to develop species-specific fingerprints for identification of individual strains of closely related species that share similar morphological characteristics. The data from these studies can be useful not only for species identification but can also provide insight into phylogenetic relationships among *Pythium* species.

Chapter 2

LITERATURE REVIEW

Organization of nuclear rRNA genes

The organization and expression of the nuclear genes encoding ribosomal RNAs have been extensively studied. In eukaryotes the rRNA genes coding for 18S, 28S and 5.8S rRNA are organized in operons and are transcribed by RNA polymerase I forming a large rRNA precursor. During rRNA maturation two internal transcribed spacers (ITS 1 and ITS 2) are discarded. Each rRNA operon is separated from the next by an intergenic spacer (IGS), also called a nontranscribed spacer (NTS). Multiple copies of rDNA units are typically found clustered in long direct tandem arrays. The 5S rRNA genes are transcribed by RNA polymerase III and are also often found clustered in direct tandem repeats consisting of the 120 bp gene sequence and the intergenic spacer (reviewed in Gerbi 1985). Studies of 5S rRNA genes in many plants and animals have shown that the genes are present in multiple copies and they are unlinked to the genes coding for 18S, 5.8S and 28S rRNA.

The rDNA repeats provide useful regions for analysis of DNA variation within a species, and between species and genera, due to the varying levels of conservation of the DNA (Sastri et al. 1992). While the intergenic spacer region has diverged over evolutionary time, the transcribed region of the gene has been relatively highly conserved, presumably reflecting its function as a component of ribosomes (Appels and Honeycutt 1989). The less conserved spacer regions contain information for transcription termination and contribute to the formation of the transcription initiation complex (Lassar et al. 1983).

5S rRNA gene arrangements in diverse taxonomic groups

Given that 5S rRNA genes and the other three rRNA genes are transcribed by different RNA polymerases, the observation that 5S rRNA genes of many eukaryotic species are not linked to their rDNA units is not surprising. What is surprising is that 5S genes have in fact often been found within the rDNA locus in the genome of some fungi and protozoa (Gerbi 1985) and several "higher" eukaryotic species, such as nematode and arthropod species (Drouin and de Sa 1995). In these cases where the 5S gene is linked to the rDNA units, it can be transcribed from the same strand and thus in the same direction as the other ribosomal genes, such as in *Coprinus cinereus* (Cassidy et al. 1984) and in *Dictyostelium discoideum* (Maizels 1976, Hofmann et al. 1993) or it

can be transcribed from the opposite strand and thus in the opposite direction, such as in *Saccharomyces cerevisiae* (Maxam et al. 1977, Kramer et al. 1978).

The linkage of 5S rRNA genes to the rDNA units of some "lower" eukaryotic species was previously interpreted as representing a primitive condition. Similarly, the observation of the linkage of 5S genes to the rDNA units of crustaceans was also initially thought to represent a primitive condition in this group (Drouin et al. 1987). However, a later study showed that this arrangement, although present in several crustacean species, was not conserved in all related species (Drouin et al. 1992). This is also true for a diverse collection of oomycetes and fungi. Both linked and unlinked 5S genes exist in the oomycete genus *Pythium* (Belkhiri et al. 1992). In most *Pythium* species that have filamentous sporangia (Dick 1990), the 5S gene was found in the IGS region of the rDNA repeat unit, inverted with respect to the other genes. In most other *Pythium* species, with globose or absent sporangia, 5S genes were unlinked to the other rRNA genes and they were organized in tandem arrays, similar to the arrangement in plants and animals (Belkhiri et al. 1992). This may suggest either that the common ancestor to all *Pythium* species had linked 5S genes and that such linkage was lost in species with globose zoosporangia or that this ancestor did not have such linkage and it occurred later in the lineage.

The linkage relationship of 5S genes to rDNA repeat units is also not conserved in fungi (Drouin and de Sa 1995). Although most ascomycetes have

5S genes linked to the noncoding strand of their rDNA repeat units, *Schizosaccharomyces pombe*, an ascomycete considered to be primitive, and *Yarrowia lipolytica*, do not have their 5S genes linked to their rDNA repeat units (Mao et al. 1982, Clare et al. 1986). Finally, in most filamentous yeasts, the 5S genes are dispersed throughout the genome in a complex manner (Metzenberg et al. 1985, Labat et al. 1985, Lockington et al. 1982). Thus, the linkage of 5S rRNA genes to the rDNA repeat units of fungi species was established and lost repeatedly during their evolution.

In protozoan species, 5S genes have been found linked either to the rDNA repeat units or to the repeat unit of trans-spliced leader sequences (Drouin and de Sa 1995). Phylogenetic distribution of 5S rRNA gene linkages in the trans-spliced leader (TSL) and rDNA repeat unit was also studied in nematode species. 5S genes were found linked to the rDNA unit in the *Meloidogyne arenaria* (Vahidi et al. 1988) whereas *Spiruria* and *Ascaridina* have their 5S genes linked to the TSL repeat units (Zeng et al. 1990, Nilsen et al. 1989). This linkage relationship was then lost in *Strongylida* and some *Rhabditida* species (Bektesh et al. 1988, Joshua et al. 1991, Nelson and Honda 1989). Phylogenetic analysis of 5S rRNA gene linkages in the rDNA repeat units of arthropod species suggests that several gain and loss events of 5S gene linkages must have occurred during the evolution of this group. The 5S gene linkage to the rDNA repeat units of the spider *Araneus* species was lost in the lineage leading to the

other arthropod groups, such as hexapods and some species of branchipods (Drouin et al. 1992, Crease 1993). The above results clearly show that 5S rRNA gene linkages were repeatedly established and lost during the evolution of some eukaryotic genomes.

In higher plants and animals the unlinked 5S genes are typically arranged into arrays of tandem repeats (Singer and Berg 1991, Sastri al. 1992). Most angiosperm studies have shown that the 5S DNA units are located at multiple chromosomal sites (Appels and Honeycutt 1989). The units at a given locus are very similar in DNA sequence but marked differences have been observed between loci. This suggests that arrays at a particular locus evolve in unison and little exchange, if any, occurs between units at different chromosomal loci (Appels et al. 1992).

The structure of the 5S DNA repeat unit

5S DNA units have been isolated from a range of higher plants, including corn (Mascia et al. 1981), wheat (Gerlach and Dyer 1980, Appels et al. 1992), wild wheats (Scoles et al. 1988), rice (Hariharan et al. 1987), flax (Goldsborough et al. 1982), lupin (Rafalski et al. 1982), soybean (Gottlob-McHugh et al. 1990), rye (Reddy and Appels 1989, Scoles et al. 1988), pea (Ellis et al. 1988), and animals, including insects (Sharp and Garcia 1988), frogs (Korn 1982) and mammals (Leah et al. 1990). In considering evolutionary changes in

the 5S DNA unit it is convenient to divide it into smaller regions. The broad delineation of these regions is mostly based on studies of the 5S DNA units from species in the *Triticeae* after aligning the available databases (Sastri et al. 1992). The 5S DNA unit consists of the conserved 5S RNA gene itself, a 3'-downstream (3'-ds) spacer region, a middle spacer (ms) region and a 5'-upstream (5'-up) spacer region (Fig 1). The division of the spacer region is based on the distribution of variation found in the aligned sequence database.

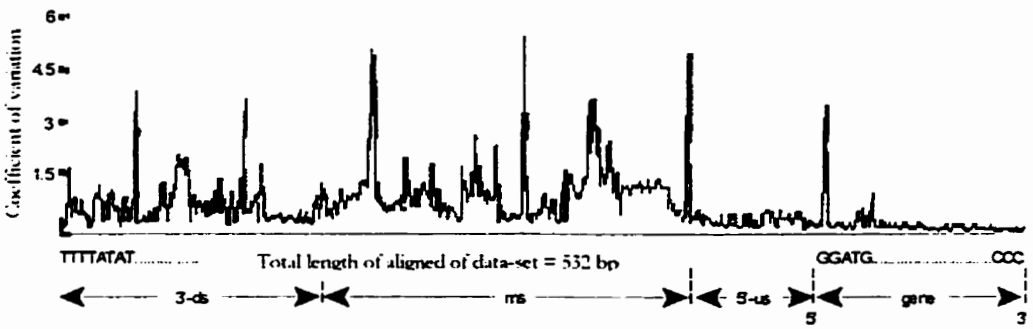


Figure 1. The diagram of the 5S DNA unit according to Sastri et al. 1992.
The conserved 5S rRNA gene, a 3'-downstream (3'-ds) spacer region, a middle (ms) region and a 5'-upstream (5'-us) spacer region are indicated on X-axis. The coefficient of variation for each position of an aligned data set of *Triticeae* 5S DNA sequences is shown on the Y-axis.

The 5S RNA gene region.

The 5S RNA gene region has been extensively studied in plants and animals by sequencing the RNA genes, and updates of the sequence data-base are regularly published (Erdmann and Wolters 1986). The gene is generally well

conserved in sequence. Therefore probes derived from wheat and its relatives (Scoles et al. 1988) have been used to isolate 5S DNA units from *Acacia* spp. (Playford et al. 1992), *Pinus radiata* (Moran et al. 1992), *Oryza* spp. (McIntyre et al. 1992) and *Arachis* spp., *Bromus inermis*, and *Zea mays* (Sastri et al.1992). However some length variation can occur in the 5S gene region. It has been suggested that the region near the 3' end of the 5S gene is prone to an error in replication (Scoles et al. 1988) and can generate a great deal of variability in the DNA. The significance of this mechanism is enhanced by the observation of length variation in the TCTCTC tract in 5S DNA units from *Pinus*, the length variation in the T tracts, especially in *Arachis*, and the common occurrence of a duplication in the gene region, centred on the CGAGAG sequence located 48 bp from the 3' end of the gene in some species of the *Triticeae* as well as *Oryza brachyantha* (Sastri et al. 1992). Inspection of these type of changes suggests that slippage during replication may generate observed duplications and deletions (Efstradiatis et al. 1980).

In addition to the genes coding for 5S rRNA found in ribosomes, most species contain gene variants and pseudogenes differing from the gene by a variable number of substitutions and deletions. Both gene variants and pseudogenes have been found within the genomes of *Xenopus* (Korn 1982), *Drosophila* (Sharp and Garcia 1988), and mammalian cells (Emerson and Roeder 1984, Reddy et al. 1986, Sorensen and Frederiksen 1991). In general, the

pseudogenes are considered transcriptionally inactive, whereas the gene variants, some of which differ from the genes in only one or a few positions, are usually expressed in vivo (Lassar et al. 1983, Sorensen and Frederiksen 1991). For example, human cells have been found to contain three pseudogenes and two gene variants (Doran et al. 1987). Pseudogenes of 5S rRNA genes have been also found in dispersed families of 5S genes in *Aspergillus* (Bartnik et al. 1986) and *Neurospora* (Selker et al. 1981) and also adjacent to 5S rRNA genes in the rDNA repeat unit of *Pythium paclycaule* (Belkhiri and Klassen 1996).

The 3'-downstream spacer region.

The 3'-ds spacer region is defined in broad terms by a simple polymer sequence characterized by tracts of T of varying length immediately following the 3'-end of the gene and, in many cases with a "TATAT" motif further downstream. The cluster of T residues is recognized by RNA polemerase III as a signal of transcription termination. The "TATAT" motif is recognizable in most of the plant 5S DNA units examined but in some, such as *Matthiola, Vigna, Pisum,* and *Glycine* it is found within 20 bp of the start of the 5' end of the gene. The variable position of the "TATAT" motif relative to the gene suggests that it is not equivalent to the "TATA" box found in the 5' upstream regions of genes transcribed by RNA polymerse II.

The middle spacer region.

The ms region is defined as the region that separates the 3'-ds and 5'-us spacer regions. It is the most variable of all the spacer regions and comparisons between plant species indicate that few generalities can be drawn about its structure. The changes include point mutations, deletions and duplications. Length variation in this region can arise from numerous deletions or variable lengths of a simple sequence such as the *Pinus* TCTCTC tract (Cullis et al. 1988, Moran et al.1992).

The 5'-upstream spacer region.

The 60-90 bp region upstream from the 5'-end of the gene is defined as the 5'-us spacer region. Within a tribe such as the *Triticeae* the 5'-us region is relatively highly conserved compared with the rest of the spacer (Scoles et al. 1988). It has been suggested that this region may carry information involved in forming of the transcription complex. Therefore, it is surprising that in the data base available from a broader spectrum of species, conserved regions in the spacer have not been found. This may suggest that the primary recognition sequence for forming the transcription initiation complex for RNA polymerase III lies within the gene region as shown for *Xenopus* 5S DNA, where the TFIIIA and TFIIIC proteins bind to the gene (Lassar et al. 1983). The spacer region would be expected to bind TFIIIB and modify the activity of the transcription

complex (Kassavetis et al. 1990, Sorensen and Frederiksen 1991), but it is clear that the expected target sequences are not conserved.

Chromosomal location of 5S RNA genes

The genes coding for 5S RNA rarely occupy only a single chromosomal location. In *Xenopus laevis* many sites have been reported (Pardue et al. 1973) although most of the 5S genes are located at the telomeres of most of the chromosomes. In humans most of the 5S rDNA is located on the long arm of chromosome 1, but several other minor locations have been reported. The 5S RNA genes in *Drosophila melanogaster* occur in a single cluster of about 160 genes on the second chromosome at location 56F (Glover et al. 1975). In the angiosperms, arrays of RNA genes tend to be localized to one or a few sites on one to three homologous pairs of chromosomes. A dramatic exception to this rule is flax (Schneeberger et al. 1989) where the genes are dispearsed. In the tomato, tandemly repeated 5S rRNA genes have a single chromosomal location on the short arm of chromosome 1, in a region close to the centromere (Lapitan et al. 1991). Studies on the 5S DNA loci in species of the Triticeae have shown that, within a species, two separate loci for the 5S DNA can coexist and that these loci are characterized by different sizes of the 5S DNA unit. In *Secale* (Reddy and Appels 1989), *Triticum tauschii* (Lagudah et al. 1989), *T. monococcum* (Dvorak et al. 1989) and *T. aestivum* (Cox et al. 1992) the long 5S DNA units (469-

496 bp long) are located on group 5 chromosomes, whereas the short units (327-469 bp long) are located on group 1 chromosomes.

An interesting exception to the presence of the 5S rDNA sequences on *Triticeae* chromosomes 1 and 5 occurs in barley. In this species the short length class of 5S DNA units was mainly found on chromosome 2 (Kolchinsky et al. 1990). The additional minor locations of 5S genes were identified on the short arm of chromosome 1 and the long arm of chromosomes 3 and 4 (Leitch and Heslop-Harrison 1993). In rice, the 5S rDNA gene complex was located at the end of the short arm of chromosome 9. This clearly indicates that the chromosomal location of genes from species with homologous genomes can not be assumed without experimental proof.

The survey of Triticeae species with 5S DNA probes suggests that in addition to major locations of 5S genes on homologous chromosome groups 1 and 5, minor locations of 5S DNA units may also be distributed throughout the genome (Reddy and Appels, 1989). This raises the possibility that these minor sites represent an ancestral state and that major locations at a particular locus (resulting from the amplification of a few units) are of recent origin. If this model is correct, the observation of a major location for 5S DNA units on chromosome 2 on barley suggests that the immediate ancestor to barley amplified 5S DNA units on chromosome 2. The alternative models propose the possibility of a small translocation in the formation of minor sites of 5S rDNA or occasional deletions.

An ancestral array of repetitive 5S DNA units may undergo a large deletion of many units as a result of an unequal sister chromatid exchange (Koebner et al. 1986) or intrachromosomal recombination event.

It is of interest that other plants show more chromosomal sites for 5S DNA than the Triticeae species. The 5S rRNA genes in flax (up to 58,500 copies per haploid genome) are distributed over many chromosomes (Schneeberger et al. 1989). The 5S genes in the gymnosperm, *Pinus radiata*, have also been shown to be dispersed over many chromosomes and in multiple positions per chromosome (Gorman et al. 1992). Similar dispersed gene arrangements are also characteristic of *Neurospora crassa* and several other ascomycetous fungi (Bartnik et al. 1986). The number of 5S DNA units in the genomes of a range of plants is quite variable. In the flowering plants it ranges from 3600 copies/1C in *Matthiola incana* (Hemleben and Werts 1988) to about 100,000 copies/1C in flax (Goldsborough et al. 1982). Furthermore, while *Matthiola* has 1.5 pg/1C, flax contains only 0.75 pg/1C and hence contains about 56 times as many 5S genes on a pg basis. These and other data on 5S rRNA copy number suggest that 5S gene number is unrelated to genome complexity. The study of 5S rDNA in species of the *Triticeae* (Vakhitov et al. 1986) shows that changes in the numbers of 5S DNA units at a given locus can occur relatively quickly within an evolutionary time frame. In a survey of 415 accessions of *Triticum tauschii* (Lagudah et al. 1989) it was found that

the numbers of 5S DNA units (short and long) at 5S DNA loci varied at least 10-20 fold.

Evolution at the 5S DNA loci

It is of interest to ask if the extended DNA sequence variability at 5S DNA loci in different species reflects the taxonomic relationships between the respective species. The 5S rRNA gene region has been used to study higher order relationships in a diverse range of organisms (Hori and Osawa 1987) but is too highly conserved to be informative at the level of genus or species. Comparisons between widely diverse genera based on the gene region (Specht et al. 1991) have found that the 120 bp region contains too small a number of phylogenetically informative sites to allow for analysis of close relatives, and it evolves too rapidly for reliable comparisons among distantly related taxa (Steele et al. 1991, Halanych 1991). Therefore the 5S rRNA gene sequence database has been suggested to have limited power in resolving phylogenetic relationships at any taxonomic level, unless a very small number of taxa are involved. The spacer region, on the other hand, has diverged at a faster rate and appears to be phylogenetically useful for the study of relationships between species. Rapidly evolving intergenic spacers between 5S genes were used in studies of the Triticeae (Baum and Appels 1992), *Pinus* (Moran et al. 1992), *Acacia* (Playford et al. 1992), *Oryza* (McIntyre et al. 1992) and *Eucalyptus* and *Angophora* (Udovicic et al. 1995).

Within a tribe such as the Triticeae the spacer region of the 5S DNA unit has been found to be suitable for phylogenetic analysis (Baum and Appels 1992). The trees obtained from the neighbour-joining method using the distances computed separately from the "short" and "long" 5S DNA units, yielded results supportive of traditional taxonomy of Triticeae. However, the tree obtained from all the sequences aligned together produced confusing results. The combination of the two size classes in the alignment generated many unknown sites in the data due to gaps. When the "short" and "long " units were analysed separately, the gaps were minimized in each new data set. This indicates that the "long" and "short" 5S DNA units should be treated as separate characters. It assumes that different classes of 5S DNA units evolve independently. Phylogenetic analysis of *Acacia* species based on 5S DNA unit sequences also suggests that at least three classes of 5S DNA units that exist in *Acacia* do not evolve at the same rate (Playford et al. 1992). This multiple chromosomal location for the 5S DNA loci is a complicating factor for the study of relationships between species. However, this problem is not only limited to the 5S DNA units but applies to a range of sequences currently being used for phylogenetic analysis.

The relatively rapid evolution of the 5S DNA spacer region has also been observed in rice (McIntyre et al. 1992). The widely divergent nature of the 5S DNA spacer in rice probably reflects the great biologic diversity of *Oryza* species. The second possibility is that independent lineages of 5S DNA loci exist in *Oryza*

species (as is the case in the Triticeae) and that extensive sequence differences occur between them. The nucleotide sequence divergence was sufficient to allow the 5S DNA clones to be used as genome specific probes to distinguish between many wild and cultivated rice species. The taxonomic relationships between *Oryza* species based on the 5S DNA sequences agreed well with relationships based on morphological characters and cytogenetic studies (McIntyre et al. 1992). The 5S rDNA repeat has been also used to examine the relationships of *Eucalyptus* and *Angophora* (Udovicic et al. 1995). Parsimony analysis supported taxonomic revision of *Eucalyptus* based on studies of morphology.

The expression of 5S RNA genes

The expression of 5S RNA genes have been intensively investigated in the African clawed toad, *Xenopus laevis* (Wolffe and Brown 1988). The *Xenopus* genome contains two major types of genes (termed oocyte and somatic type) encoding similar 5S ribosomal RNAs that are differentially regulated during development. The somatic type, comprising only 2% of the animal's 5S DNA, encode more than 95% of the 5S RNA synthesized in somatic cells in all stages of development (Peterson et al. 1980). *Xenopus* oocytes synthesize and accumulate mainly oocyte type 5S RNA. Because there are only 400 somatic 5S genes but over 20,000 oocyte 5S genes per haploid genome, this is a final differential gene transcription of over 1000-fold in somatic cells. After fertilization and

development of the embryo, the oocyte-specific 5S RNA genes are repressed, while the somatic 5S RNA genes remain active. This is an example of what may be a common developmental mechanism: two gene family have similar controlling elements that are recognized by the same factors but are nonetheless controlled differently. What then accounts for the oocyte 5S RNA genes activation in oocytes and their repression in somatic cells when the somatic 5S genes function in both cell types? One model for this developmental regulation explains that differences in the stability of transcription complexes assembled on major oocyte and somatic 5sRNA genes account for differential transcription.

A region of about 50 nucleotides within the 5S RNA gene, called the "Internal Control Region"(ICR), is analogous in function to the promoter of a procaryotic gene, because it is required for RNA polymerase III to initiate transcription at the start site of the gene (Bogenhagen et al. 1980, Pieler et al. 1987). However, the RNA polymerase does not recognize this DNA sequence directly. It recognizes a transcription complex that includes DNA in the ICR and three or more other molecules (Lassar et al. 1983). The ICR is the binding site for a 38,000 Dalton protein called TFIIIA. Transcription factor TFIIIA along with at least two other factors (TFIIIB and TFIIIC) form a transcription complex on the ICR that directs RNA polymerase III to initiate transcription (Fig. 2). The importance of transcription complex stability lies in its role in the control of gene expression (Wolffe and Brown 1987).

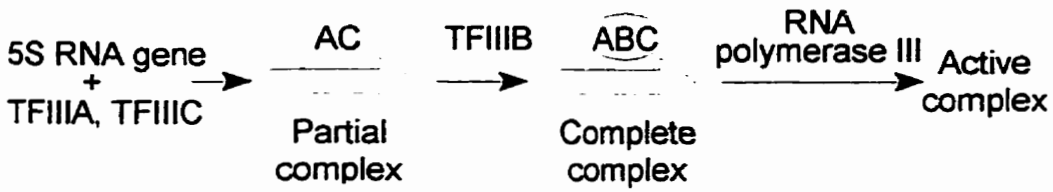


Figure 2. The transcription complex of 5S RNA genes of *Xenopus laevis* according to Wolffe and Brown 1988.
TFIIIA and TFIIIC bind rapidly to a 5S RNA gene forming a TFIIIA/TFIIC/5S DNA complex. The TFIIIA/TFIIIC/5S DNA complex matures as TFIIIB is sequestered, forming a complete transcription complex. The complex is then competent to bind RNA polymerase III and transcription initiates.

Oligonucleotide-directed mutagenesis within the internal control region identified three distinct sequence elements that regulate transcription activity: box A, containing the common, conserved class III promoter domain, and two 5S gene specific segments, termed the intermediate element and box C (Pieler et al. 1987). Analysis of individual steps in the formation of the stable initiation complex revealed that the two 5S gene specific elements are the main determinants for the stable binding of TFIIIA. In contrast, TFIIIC binding appears to be dependent on interactions with TFIIIA and on direct DNA interactions in box A as well as in box C.

The somatic and oocyte 5S genes differ by six nucleotides. Three of the six base differences between oocyte and somatic 5S RNA genes contribute to

differential transcription complex stability. It was demonstrated that somatic 5S genes bind four times tighter to TFIIIA than oocyte 5S genes (Wormington et al. 1981). It has been also suggested that the stabilization of the binding of TFIIIA by TFIIIC is involved in this differential stability. When oocyte 5S RNA genes become unoccupied by transcription factors, a repressed chromatin structure forms that excludes the subsequent binding of these factors (Wolffe and Brown 1988). Therefore dissociation of transcription factors from oocyte 5S RNA genes during development allows them to be repressed by chromatin assembly. In the same cells, somatic 5S RNA genes remain active because their transcription complexes are stable.

Both oocyte and somatic 5S DNA repeats are associated with nucleosomes (Young and Carroll 1983); however, differences exist in the organization of chromatin over the oocyte and somatic 5S RNA genes. It has been shown that nucleosomal arrays containing histone H1 are responsible for maintaining the oocyte genes in a repressed state (Schlissel and Brown 1984). Promoter elements of the repressed oocyte genes are inaccessible to transcription factors because they are packed into nucleosomes containing histone H1. Histone 1 is shown to have a role both in determining the organization of nucleosomes over the oocyte 5S DNA repeat and in repressing transcription of the oocyte 5S RNA genes (Chipev and Wolffe 1992).

Information concerning expression of 5S RNA genes in other systems is limited. The human 5S rRNA genes are found in clusters of tandem repeated units. The number of genes and closely related gene variants is found to be 300 - 400 per haploid human genome (Sorensen and Frederiksen 1991). Comparative study with the *X. laevis* somatic 5S gene showed that the human gene deviates in eight positions. One of them (at position 53) is located within the A box of the internal control region (Pieler et al. 1987). The time-course of transcription and optimal concentrations of template and transcription factors were found to be similar for both genes (Wingender et al. 1988). It has been shown that if 5'-flanking sequences are removed from the human genes, transcription is reduced by a factor 10 or more, suggesting that sequences upstream of the coding region are important for the level of transcription (Sorensen and Frederiksen 1991). In contrast, the entire 5'-flanking region could be deleted from a *X. borealis* somatic 5S gene without affecting the transcription in vitro (Sakonju et al. 1980). An absolute requirement for 5'-flanking sequences has been reported for the transcription of *D. melanogaster, B. mori* and *N. crassa* 5S rRNA genes (Sharp and Garcia 1988, Morton and Sprague 1984, Tyler 1987). Transcriptional studies on *Neurospora crassa* indicate that sequences outside of the gene, in the spacer region, are important for accurate and efficient transcription, including TATA box-like sequences (Tyler 1987). Moreover, because *Neurospora* 5S genes differ in their coding regions, the possibility of differential expression of individual genes or

sub-classes was raised. However, deletion and substitution mutations introduced into variable regions showed that these variable sequences play little role in transcription (Tyler 1987).

In plants a large amount of information is available concerning organization and sequences but not much concerning gene expression. Studies conducted on rice 5S rRNA gene expression showed that dormant seeds contained one species of 5S RNA while 48 h germinated seedlings contained at least 2 species of 5S rRNA (Hariharan et al. 1987). Information regarding sequences required for specific transcription is limited to sequence comparisons rather than functional assays. Comparison of a number of 5S rRNA sequences indicates that the region corresponding to the Internal Control Region (ICR) of the *X. laevis* genes is highly conserved in plants (Hemleben and Werts 1988, Gorman et al. 1992).

The structure and function of the intergenic spacer

Each rRNA transcription unit is separated from the next by several kilobases of DNA called an intergenic spacer. The IGS spacer shows great diversity among different species. Such variability raised the question of whether the spacer carries out any functions. Currently we know that elements of the spacer can have a strong enhancer effect on RNA polymerase I transcription.

Length variability and sequence repetition in the IGS

The IGS structure, which has been studied in many organisms by restriction enzyme digestion and sequencing, often shows internal repetitions. In *Xenopus laevis* the repeating units are 60 or 81 bp, which begin 260 bp upstream from the rRNA initiation site and extend about 1.5 - 5 kb further upstream (Boseley et al. 1979, Sollner -Webb and Reeder 1979, Moss et al. 1980). Similarly, the mouse IGS has a 135-bp repeat, which begins about 170 bp upstream of the initiation site for transcription and extends 650-2700 bp upstream (Arnheim and Kuehn 1979). Internal repetition in the IGS is also found in various *Drosophila* species, where 250-bp repeats begin about 50-80 bp upstream of the initiation site (Long et al. 1981, Rae et al. 1981, Renkawitz-Pohl et al. 1980).

A variable number of repeat units within IGS results in different overall length of the entire rDNA unit both between organisms and even in one individual. The spacer of *X. laevis* varies in length from 2.7 to 5.5 kb (Wellauer et al. 1976) and this variation contributes directly to length heterogenity within and between individuals. Botchan et al. 1977 divided the spacer into four regions (A, B, C, and D), differing with respect to their overall variability. Comparison of four cloned fragments revealed the conservative nature of region A relative to the length variation observed in regions B and D. Region B consists of multiple *Sma*I sites and varies in length from 1 to 1.2 kb. Region D varies in length from 0.89 to 3.8 kb and is characterized by a regular distribution of *Bam*HI sites. This region

accounts for most of the spacer length variation. The length variation that resulted from the insertion or deletion of units of a repeating DNA sequence was also found in Chinese hamster (Stambrook 1978), and mouse and man (Arnheim and Southern 1977). Mapping of mouse IGS shows that length variation is localized to a specific region defined by *SalI*, which varies from 1 to 7 kb and as many as 15 discrete fragment classes have been identified in wild and inbred mice (Arnheim 1979, Grummt et al. 1979). Among *Triticum* species, as well as among individuals of a population of a single species, considerable spacer length variation occurs (Dvorak and Appels 1982, Appels and Dvorak 1982). Within the wheat varieties, specific size classes could be assigned to either chromosomes 1B, 6B, or 5D. In Chinese Spring, for example, the 9.0-9.1 kb spacer lengths derive almost entirely from chromosome B, while the 9.4 kb variant derives from chromosome 1B. The cloned samples of 9.0 and 9.1 kb variants differ in length due to a single 133 bp spacer repeat unit.

Length heterogeneity is rarely seen among fungi. In yeast (Meyerink and Retel 1976, Nath and Bollon 1977), sequence analysis showed that the larger repeated stretches do not occur, although shorter direct repeats of 4 - 16 nucleotides were found in variable quantities (Skryabin et al. 1984). Length variability reported in the ascomycete *Yarrowia lipolytica* does not appear to be due to variations in the number of subrepeats (van Heerikhuizen et al. 1985). Length heterogeneity in the IGS seems to be more common in oomycetes than in other

fungi. Spacer length variability was reported in several *Pythium* species. IGS region of *Pythium ultimum* includes two regions of heterogeneity (Klassen and Buchko 1990). A region located about 1 kb downstream of the 3' end of the large subunit rRNA gene contains a segment which is present in multiple versions differing in size by as much as 0.9 kb. Each of 14 examined isolates showed a unique pattern of heterogeneity in this region. The other region, located near the centre of the IGS, consists of a 385-bp repeat. The size of the repeat segment is identical in all *P. ultimum* isolates, but the number of subrepeats (6 - 12) is highly polymorphic. Length heterogenity in the IGS was also reported for *P. padycaule* (Belkhiri and Klassen 1996). Two versions of IGS differ by about 200 bp. Both versions contained a conserved 5S gene and pseudogene as a tandem repeat. For many other *Pythium* species having the 5S gene in the IGS, the region between the large subunit rRNA gene and the 5S gene appeared to be also variable (Belkhiri et al. 1992). Restriction analysis of the IGS of other *Pythium* species (*P. paroecandrum, P. spinosum, P. sylvaticum,* and *P. irregulare)* revealed the presence of polymorphic forms in the same isolate, variation in the number or polymorphic forms in different isolates of the same species, and insertions-deletions in multiples of approximately 60 bp adjacent to the 3' end of the large subunit rRNA gene (Martin 1990).

Functional regions within the IGS

The length and sequence variations in the IGS suggest that the IGS may lack a functional role, however some regions with specific function have been identified within the IGS. The structure of a typical *X. laevis* intergenic spacer is shown diagrammatically in the Figure 3 (Reeder 1984). On the right site, the spacer is bounded by the gene promoter, which extends from about -142 to +6 (Moss 1982, Sollner-Webb et al. 1983). On the left side, the spacer is bounded by a transcription termination signal at the 3' end of each gene (Bakken et al. 1982). Every spacer is punctuated by two to seven or more imperfect duplications of the gene promoter (the so-called Bam island, because it contains a *Bam*HI restriction site). These spacer promoters are separated from each other and from the gene promoter by 60 and 81 bp tandem repeating elements. The 81 bp repeats are essentially the same as the 60 bp elements with an additional 21 bp added on. A 42 bp stretch of sequence found in the gene promoter at -73 to -114 can also be found within each 60/80 bp repeat as well as within each spacer promoter.

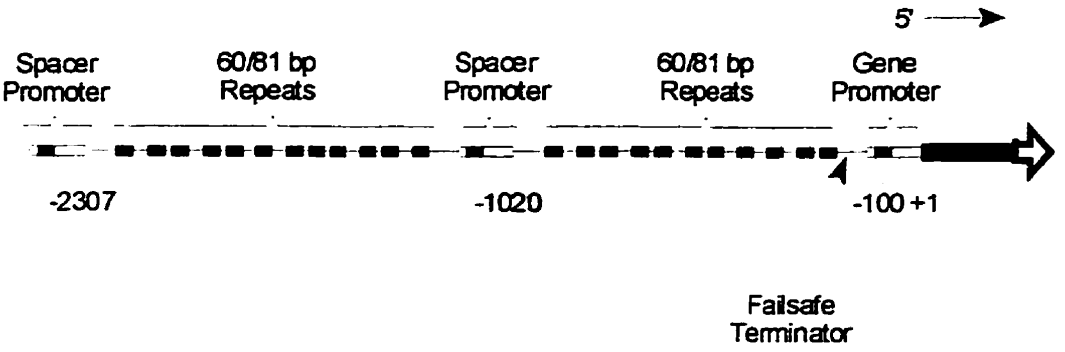


Figure 3. Organization of the IGS of *Xenopus laevis* according to Reeder 1984. The white boxes represent the gene promoter and duplicated spacer promoters; the black boxes represent the conserved 42 bp region found in the promoters and in the 60/81 bp repeats.

The IGS duplication has been used to explain the enhancer-like properties of the IGS in DNA transcription. It has been proposed that the 60/81 bp repeats contain sequences that act as enhancers on the gene promoter, and it is likely that the active core of the enhancer elements is the 42 bp element. Reeder 1984 has proposed that the enhancers act by attracting a transcription factor or factors which bind to the gene promoter and activate it for transcription. This model affords an explanation for the nucleolar dominance of transcription of *X. laevis* rDNA over *X. borealis* rDNA, since the former has at least 24 and the latter has only 4 of the 42 bp repeats in the IGS (La Volpe et al. 1985). However, this model is not a general explanation for all cases of nucleolar dominance, since the dominance of mouse over human rDNA transcription in cell hybrids appears to

be due to inactivation or loss of a gene for a species specific transcription factor needed to activate human rDNA (Miesfield and Arnheim 1984, Onishi et al. 1984). No enhancer activity has been reported for the very large spacers of either mouse or human ribosomal genes. Nucleolar dominance has also been observed in several genera of plants (Wallace 1982) and in crosses between *Drosophila melanogaster* and *Drosophila simulans* (Durica and Krider 1978). However, genetic experiments have shown that a locus outside of the ribosomal gene locus is also involved in nucleolar dominance in *Drosophila* (Durica and Krider 1978).

If spacer promoters are truly important for enhancement of rDNA transcription, one might expect to find them in a vide variety of species. The existence of spacer promoters is not obvious in yeast, since they have diverged in sequence, but a counterpart of the rDNA initiation region can be found (Verbeet et al. 1984). The region 2230 to 2420 of the yeast IGS has been shown to have enhancer-like activity for rRNA transcription (Warner et al. 1985). Even *E. coli* has four duplicated rDNA promoters (Boros et al. 1983). It is also known that control of transcription resides at the level of the individual rDNA unit, since during developmental activation of the rDNA cluster some units may be fully active while others are entirely inactive in transcription (Scheer et al. 1976).

The signals for termination of transcription appear to be highly conserved in evolution, both among different RNA polymerase gene families and also among widely divergent species. This signal includes a region of dyad symmetry

followed by the cluster of T-residues. Such a signal for termination is even recognized by *E. coli* RNA polymerase, and is found in the rDNA genes in *E. coli* (Young 1979, Brosius et al. 1981). There is no fixed termination site in the IGS of *Drosophila melanogaster*. A similar termination signal consisting of a short palindrome followed by an oligo-T tract was found at -225 of the *Xenopus* IGS and acts as a "failsafe" terminator (Morgan et al. 1983). In humans, four possible termination sites were identified (Safrany 1989). Lang et al. 1994 proposed a model for termination involving a pause element mediated by a protein factor and a 5' flanking release element.

Molecular approaches in the study of phylogeny

The study of molecular phylogeny began at the turn of the century. Immunochemical studies showed that serological cross-reactions were stronger for closely related organisms than for distantly related ones. In the 1960's and 1970's, protein sequence data was extensively used to infer phylogenetic relationships among organisms. Presently DNA analysis has had a great impact on molecular phylogeny. It has been used to study both phylogenetic relationships among closely related species and very ancient evolutionary occurrences, such as the divergence of phyla and kingdoms.

At present three basic approaches are available to obtain information at the DNA level which can be used to characterize similarities and differences

between species. I will discuss these in increasing order of resolution, namely restriction enzyme analysis, hybridization analysis, and finally sequencing studies. Each approach has its advantages and disadvantages in an evolutionary study of repeated sequence of DNA such as that found at the rDNA locus. Ideally, such a study should combine these three techniques.

Restriction enzyme analyses

Digestion of DNA samples with restriction endonucleases and separating the DNA fragments on a size basis using electrophoresis is the first step in characterizing the rDNA of an organism. In evolutionary study the restriction enzyme analyses serve to map the rDNA unit and thus locate regions of variability within the unit. However, RFLPs do not provide useful information on the relationships between species. The reason for this is that in such analyses, lengths of DNA fragments are measured and a single mutation can lead to loss of a restriction site and a dramatic change in length of the DNA fragment being assayed. It has been estimated that sequence divergences of 3 to 10% are usually correlated with much higher divergences of fragment patterns (40 to 80%) (Rae et al. 1981). In addition, it is true for the rDNA system that the length variation within the species can be just as extensive as the variation observed between species (Dvorak and Chen 1984, Appels and Dvorak 1982, Arnheim et al. 1982). The internally repeated sequence nature of the rDNA spacer region means that

length variation is not always due to inactivation of a restriction enzyme site, but could also result from variation in the number of repeating units in the spacer.

In a study of members of the Triticeae to identify species which are closely related to the genomes of hexaploid wheat, *Triticum aestivum*, (Peacock et al.1981) *Eco*RI and *Bam*HI digests were utilized to characterize the rDNA unit. Dvorak and Appels (1982) found extensive variation in the length of the IGS fragments defined by *Taq*I in populations of *T. speltoides*. The application of a restriction enzyme analysis of the rDNA region in relation to phylogeny has also been carried out in *Drosophila* sp. (Coen et al. 1982). Examination of published data indicates that the extensive variation for the spacer length exists within lines of *D. melanogaster* derived from a natural population (Coen et al. 1982 a). Therefore, phylogeny of this type can not be considered reliable because adequate sampling of populations of the species was not done. Restriction analyses at the rDNA locus have been conducted on *Chlamydomonas* species (Marco and Rochaix 1981), *Tetrahymena* species (Din and Engberg 1979), and ciliated protozoans (Swanton et al. 1982). In the case of *Tetrahymena* the phylogenetic relationships determined by rDNA restriction analyses were different from those in existing phylogenies. rDNA mapping was also used to study evolution in five yeast genera (Verbeet et al. 1984a). RFLP analysis of mitochondrial and nuclear rDNA has been used to estimate intra- and interspecific relatedness in *Phytophthora* (Förster et al. 1988, 1990, Förster and Coffey 1991, Mills et al. 1991), *Neurospora*

(Russell et al. 1984), and *Fusarium oxysporum* (Kistler et al. 1987). The comparison of restriction maps of the rDNA repeating units was used to determine taxonomic affinities of some *Pythium* species which lack definitive morphological characters and thus were not amenable to traditional taxonomic techniques (Klassen et al. 1987).

The development of the polymerase chain reaction (PCR) and its automation have revolutionized molecular biology and phylogenetic analysis (Mullis and Faloona 1987, Saiki et al. 1988). PCR is a process of DNA amplification that allows for the detection of a single molecule of target DNA. The technique has wide application in studies of population biology (Arnheim et al. 1990). It was used to detect bacteria directly from environmental samples and has provided a powerful means to examine the diversity of natural microbial populations, avoiding reliance on cultivatability.

White et al. 1990 designed a series of PCR primers based on highly conserved regions of rDNA. These primers are applicable to a variety of organisms and can be used as "universal" primers to amplify the DNA regions encoding the nuclear small subunit rRNA and the transcribed spacer region between the small and the large rDNAs. Because these two regions of rDNA evolve at different rates, analysis of spacer and coding regions provides different sensitivities and resolution for taxonomic and phylogenetic studies.

RFLP analysis of PCR amplified 16S rRNA genes was used to identify bacterioplankton samples collected from the Sargasso Sea which do not resemble any previously known bacterial species (Giovannoni et al. 1990). The same approach has been also used to dissect and characterize the evolutionary relationships of a complex epibiotic microbial community, avoiding difficulties in culturing these organisms (Haddad et al. 1995). RFLP analysis of PCR amplified small subunit ribosomal RNA (ssrDNA) and the internal transcribed spacer (ITS) was used to study the genetic variability in twenty five isolates representing five *Pythium* species collected from diverse host and geographic origins (Chen et al. 1992). Restriction digestion revealed little intraspecific variation in the ssrDNA and ITS, and species could be reliably distinguished. However, two morphologically similar species, *P. arrhenomanes* and *P. graminicola* which were easily separated from the other species under study, could not be differentiated from each other. Data presented suggest that *P. arrhenomanes* and *P. graminicola* are not distinct species and need to be combined. These results indicate that PCR-RFLPs can be used as a simple and speedy taxonomic tool for ecological study of *Pythium* species. However, Martin and Kistler 1990 indicated the possibility that comigrating bands from different species may not be homologous and that comparison of fragment size alone may not be useful for estimation of evolutionary relatedness. The plant, insect and fungal examples presented here indicate that length of a given sequence as defined by restriction endonucleases is

not a useful taxonomic character unless many restriction endonucleases are examined.

Hybridization and hybrid melting point analyses

Utilization of rRNA as a probe in melting point analyses has provided a valuable parameter to quantify relationships between groups of bacteria (Klipper-Balz and Schleifer 1981, Mordarski et al. 1981, Stackebrandt et al. 1981). Melting point analyses using probes from a well-defined region of the genome such as the rDNA region allows hundreds of species to be examined within a few weeks for a quantitative assignment of the degree of relatedness with respect to the sequence assayed. A classic example, where melting point analysis was utilized, is the study of *X. laevis* and *X. borealis* (Brown et al. 1972). Brown et al 1972 used hybridization to show that the 18S and 28S coding regions are highly homologous between two *Xenopus* species, unlike the dissimilarity between their IGS sequences. They found small numbers of heterologous hybrids which melted approximately 7° C lower than homologous hybrids. This Tm suggested 10% mismatch among cross-hybridizing sequences. It is interesting to examine these early studies in relation to the structure of rDNA as it is known today. The overall structure of the IGS of *X. laevis* and *X. borealis* is quite different at the restriction enzyme level. Sequence studies have demonstrated an overall mismatch of approximately 47% with certain stretches showing complete homology. In relating this sequencing information to the early Tm data, the

limitation of the Tm type analysis can be demonstrated. The analysis underestimates the degree of difference between two DNA sequences being compared. If the regions being compared are too dissimilar, they will not cross-hybridize and thus will not be assayed with a given probe.

The use of the ΔTm parameter in an evolutionary study was adapted by Appels and Dvorak (1982) in their analysis of the rDNA in species of the tribe Triticeae. They used as probes 11 different parts of the cloned rDNA region of wheat, which were 100 to 400 bp in length. Five *Triticum* species were compared to *T. aestivum*, the source of the probe. Tm values for each probe allowed the species to be ranked with respect to their relatedness to *T. aestivum*.

Hybridization analysis has been used in some systematic studies of ascomycetous and basidiomycetous yeast (Lachance et al. 1986, Kurtzman and Phaff 1987, Kurtzman 1990) and with some limitation for filamentous fungi (Kurtzman 1985, Vilgalys 1988). These studies demonstrated that the percentage of DNA that cross-hybridizes between closely related species is very low, less than 20%, while the percentage cross-hybridizing between members within a species is generally greater than 90% (Bruns et al. 1991).

Sequence analyses

In principle, the DNA sequence of a region of interest is the "ultimate" for an evolutionary study. DNA sequence analysis can solve many of the problems associated with RFLP and hybridization analysis. rDNA sequence

comparison can generate a large number of characters for phylogenetic inference and provide a more accurate measurement of genetic distances between species. rDNA nucleotide sequence comparisons provide a means for analyzing phylogenetic relationships over taxonomic levels ranging from species (Jorgensen and Cluster 1988) to kingdoms (Bruns et al. 1991, Hendriks et al. 1991, Schlegel 1991). rDNA consists of highly conserved regions interspersed with variable regions, making it an ideal candidate for molecular evolutionary studies, both for close and distantly related organisms (White et al. 1990). The phylogenetic analysis of nucleotide sequences has been generally based on two main categories of methods for inferring trees. In the distance matrix methods, evolutionary distances are computed for all pairs of taxa, and phylogenetic tree is constructed by using an algorithm based on some functional relationships such as the distance value (Felsenstein 1988). The principle of maximum parsimony methods involves the identification of a tree that requires the smallest number of evolutionary changes to explain the differences among the taxa under study (Eck and Dayhoff 1966, Fitch 1977). The general assumption of parsimony analysis is that evolution takes the shortest route, so that the maximum parsimony tree should have the minimum of number of changes.

Ribosomal RNA gene sequences proved useful as an "evolutionary clock" for studying relationships of fungi species. Sequencing of SrDNA demonstrated that the fungi-like protoctists such as the Oomycetes and the

acellular slime molds are not directly related to fungi but that they originated before the fungi (Förster et al. 1990). The Chytridiomycetes, however, were included with the true fungi (Bowman et al. 1992, Li and Heath 1992). Large subunit DNA sequence comparison gave good results in a study of phylogeny of some *Fusarium* species (Guadet et al. 1989). Partial sequences of more variable regions of SrDNA (D2 domain) from 23 species of the Pythiaceae were compared to assess their phylogenetic relationships (Briard et al. 1995). In contrast to a high level of diversity found within *Pythium*, *Phytophthora* appeared to be very homogenous, with small phylogenetic distances among all 15 species investigated. Therefore to establish filiations within *Phytophthora*, it is necessary to investigate other sequences such as ITS, shown to be useful by Lee and Taylor (1992). ITS regions evolve fast and vary among species within a genus or among populations (Gonzales et al. 1990).

Characterization of the genus *Pythium*

The oomycete genus *Pythium* is a common inhabitant of terrestial and aquatic ecosystems. In addition to being saprophytes on fresh organic substrates, species in this genus also may be important pathogens on a number of economic crop plants. *Pythium* species can cause rot of fruit, roots or stems, damping of seeds and seedling. There have been over 130 species described for this genus (Dick 1990); many of which may differ by minor morphological features. A

taxonomic account of *Pythium* has been given by Middleton (1943) and keys to species and original descriptions by Waterhouse (1967, 1968). The revision of the species of *Pythium* and dichotomous key to species identification was published by Van der Plaats-Niterink (1981) and Dick (1990).

Morphology and reproduction

The mycelium of *Pythium* is coenocytic but delicate, colourless, often with hyphal swelling, rarely with chlamydospores. Haustoria are not formed. The walls are fibrillar in organization, with a complex chemical structure consisting of polysaccharide, protein and lipid (Hunsley 1973, Sietsma et al. 1975). Glucans constitute about 90% of the wall and cellulose, a β -(1-4) linked glucan, makes up about 36% of whole glucan.

The asexual reproduction takes places by means of zoosporangia and zoospores. The sporangium is separated from the rest of the mycelium by a cross wall. The sporangia can be filamentous or more or less spherical. In some species, filamentous sporangia are hardly distinguishable from vegetative hyphae or they are inflated and may form dendroid structures. In *Pythium* the zoospores are not formed in the sporangium itself but in a vesicle outside it. Sporangia form a tube through which the sporangial contents move and form a vesicle at the tip with an undifferentiated mass of protoplasm. This mass differentiates into a number of zoospores. The zoospores are bean or pear-shaped with two laterally attached flagella (Colt and Endo 1972, Kobayashi and Akai 1974, 1974a).

Sexual reproduction takes places by means of oogonia and antheridia. The oogonia, the female organs, are terminal or intercalary spherical swellings with a smooth or ornamented wall. The antheridia, the male organs, consist of an antheridial cell which can be sessile on a hypha, intercalary, or formed terminally on an antheridial stalk. The antheridial cell touches the oogonium and penetrates it by means of a fertilization tube. If antheridia originate from the oogonial stalk, they are termed monoclinous. Antheridia are called diclinous if they originate from a different hypha not connected with the one supporting the oogonium. After fertilization, a single oospore is usually produced inside the oogonium. The oospore secretes a double wall which is smooth except in *P. dictyosporum* where they are reticulate. Oospores need several weeks of rest before they are capable of germinating (Van der Plaats-Niterink 1981).

Most species of *Pythium* are homothalic, however heterothalism can also occur in *Pythium* (Campbell and Hendrix 1967). For example, *P. sylvaticum, P. heterothallicum* and *P. splendens* are heterothallic, although homothallic isolates are sometimes encountered (Hendrix and Campbell 1968, Van der Plaats-Niterink 1968, 1969, 1972).

Taxonomy of the genus *Pythium*

The genus *Pythium* in the present sense was introduced by Pringsheim (1858) and was placed in the Saprolegniaceae. Pringsheim based his genus on two fungi which he named *P. monospermum* and *P. enthopythium*. The latter one was

transferred to *Lagenidium* (Zopf 1890), so that *P. monospermum* remained as lectotype species. Presently *Pythium* is regarded as type genus of the family *Pythiaceae* in the Peronosporales.

Identification and classification of *Pythium* species is mainly based on the morphology of reproductive structures, which are typically examined in a pure culture. Important characteristics include sporangium presence, shape and size; zoospore production; oogonium location, ornamentation, size and shape; oospore size and wall thickness; and the number, shape, and source of antheridia (Van der Plaats-Niterink 1981). Certain species are differentiated by quantitative differences, such as the size of oogonia and oospores, and the number of antheridia per oogonium. Identification of *Pythium* species, which do not produce all the reproductive structure, can be very difficult (Hendrix and Papa 1974). *Pythium* isolates that do not reproduce sexually and heterothalllic cultures that require the opposite mating types are especially difficult to identify. Therefore the isolates that fail to produce oogonia and oospores in culture were grouped into five group based only on zoosporangia criteria: *Pythium* F - zoosporangia strictly filamentous, *Pythium* T - zoosporangia with inflated elements, *Pythium* G - zoosporangia spherical or ellipsoid but not proliferous, *Pythium* P - zoosporangia spherical or ellipsoid and proliferous, *Pythium* HS - sporangia not known, hyphal bodies are present or absent (Van der Plaats-Niterink 1981, Dick 1990).

Species-specific probes

For many *Pythium* species, variability in morphology within and between species is often too large to allow reliable identification. Many taxonomic characteristics have continuous rather than discrete variation, and a large number of isolates can differ considerably from the type species. Many important taxonomic characteristics have ranges that overlap between species, which creates additional problems in identification. As a result, several nonmorphological approaches have been used to identify *Pythium* species.

One relatively new approach is the construction of highly specific DNA probes for the identification of plant pathogenic fungi. Species identification with a DNA probe can be relatively rapid and provides a degree of objectivity not always possible in traditional methods of species identification. Cloned random DNA fragments have been used as probes to identify species of fungi, such as *Fusarium oxysporum* and *Phoma tracheiphila* (Manicom et al. 1987, Rollo et al. 1987). For *Phytophthora* species, DNA probes selected from random clones have been developed for *P. parasitica* (Goodwin et al. 1989, 1990) and *P. citrophtora* (Goodwin et al. 1990a). These cloned probes were used to detect the fungus in root tissue and soil sample as well as to distinguish genetic differences among isolates. Serological assay kits also have been tested for their ability to detect *P. cinnamomi* (Benson 1991). A mitochondrial DNA probe cloned from *Gaumannomyces graminis* was developed for identification of *Gaumannomyces* species, the causative agent of

take-all disease of wheat and barley (Henson 1989). However, this mt DNA clone hybridized also weakly to DNA from *Phialophora* spp. and *Neurospora crassa*.

The selection of species-specific probes could be simplified by identification and directed cloning of specific regions of the genome that are variable among species but conserved among isolates of the same species. This approach was used to select probes specific for several species of *Phytophthora* (Lee and Taylor 1992, Lee et al. 1993). Twenty base pair oligonucleotide probes constructed from the transcribed spacer region between the 17S and 5.8S rRNA genes were species specific for *P. megakarya, P. palmivora, P. citrophora, P. capsici,* and *P. cinnamomi*.

Many DNA probes have been devised for the identification of parasitic protozoa such as *Leishmania* (Barker et al. 1986, Barker 1989), *Plasmodium* (Delves et al. 1989, Lal et al. 1989), and diverse species of trypanosomes (Hide et al. 1990, Masiga and Gibson 1990). The intergenic region between 5S rRNA genes has been used for identification of species of the protozoan parasite *Eimeria* which causes coccidiosis in a variety of domestic animals.

DNA probes have also allowed identification of several *Pythium* isolates to species level. DNA fragments from regions of the mitochondrial chromosome showing interspecific variation provided probes for *P. oligandrum* and *P. sylvaticum* (Martin 1991). A DNA probe recognizing a repeated sequence was isolated from a genomic library of *P. irregulare* (Matthew et al. 1995). This probe reacted only

with *P. irregulare* and *P. spinosum*. The restriction fragment probes from the ITS spacer had a high degree of species specificity for *P. ultimum* when tested against 24 other *Pythium* species (Lévesque et al. 1994).

Chapter 3

MATERIALS AND METHODS

Cultivation of strains

Mycelial isolates of *Pythium* species were obtained from the CBS (Centraalbureau voor Schimmelcultures, Baarn) collection in the Netherlands and from the Aquatic Phycomycete Culture Collection in Reading, England (Table 1). Cultures were first subcultured on cornmeal agar (occasionally on V8 or V8-sea water agar) at 25°C. After 2 to 3 days of incubation (slow growing cultures up to 7 days), the colonies were transferred to petri dishes filled with pea-broth medium, containing filtered decantation of 200 g of frozen peas boiled for 20 min in 1 liter of water to which 5 g of glucose was added, and incubated at 25°C for 2 to 3 days. The incubation of slow growing species was prolonged up to 10 days. Mycelia were harvested by vacuum filtration through Whatman No. 1 filter paper, washed twice with distilled water and frozen at -20°C. Frozen mycelia were freeze-dried overnight, and dry mycelia were stored at -20°C when not used immediately.

The *E. coli* strain JM109 was used in all cloning protocols.

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

Species	Culture ID[a]	Status[b]	MT[c]	Ref. No.[d]	Origin[e]
acanthicum[R]	CBS 284.31	TYPE		71	U.S.
	CBS 227.94			151	France
	CBS 431.68			148	Neth.
acanthophoron[un]	CBS 337.29	AU		52	U.S.
acrogynum[R]	CBS 549.88			69	China
adhaerens	CBS 520.74			25	Neth.
amasculinum[R]	CBS 552.88	AU		180	China
anandrum[R]	CBS 285.31	TYPE		1	?
angustatum	CBS 522.74	PN		27	Neth.
aphanidermatum	CBS 216.46			72	?
aplerotictum	CBS 772.81			70	Neth.
aquatile	CBS 215.80	NEOTYPE		21	U.K.
aristosporum	CBS 263.38	TYPE		2	Canada
arrhenomanes	CBS 324.62	TYPE		3	U.K.
ascophallon	APCC 4004a			A	Russia
boreale[R]	CBS 551.88			79	China
buismaniae[un]	CBS 288.31	TYPE		63	Neth.
capillosum	CBS 222.94	AU		73	France
catenulatum	CBS 842.68	PN	a	81	U.S.
	CBS 843.68	PN	b	80	U.S.
chamaehyphon[R]	CBS 259.30	TYPE		82	?
chondricola	CBS 203.85	TYPE		87	Neth.
coloratum	CBS 154.64	TYPE		22	Australia
conidiophorum[R]	CBS 223.88			83	U.K.
cylindrosporum[R]	CBS 218.94	TYPE		74	Germany
	CBS 219.94			155	?
debaryanum[R]	CBS 752.96			336	?
deliense	CBS 314.33	NEOTYPE		66	?
diclinum	CBS 664.79	NEOTYPE		30	Neth.
dimorphum[R]	CBS 406.72	TYPE		31	U.S
dissimile	CBS 155.64	TYPE		32	Australia
dissotocum	CBS 166.68	PN		4	U.S.
P. sp. (P. drechsleri)	CBS 221.94	TYPE		75	Algeria
echinulatum[R]	CBS 281.64	PN		33	Australia
erinaceus[R]	CBS 505.80	TYPE		34	N.Z.
flevoense	CBS 234.72	TYPE	f	84	Neth.
	CBS 236.72	PN	m	85	Neth.

Table 1. Extended

Species	Culture ID[a]	Status[b]	MT[c]	Ref. No.[d]	Origin[e]
folliculosum	CBS 220.94	TYPE		76	Switz.
graminicola	CBS 327.62	NEOTYPE		5	Jamaica
grandisporangium[g]	CBS 286.79	TYPE		54	U.S.
helicandrum[g]	CBS 393.54	AU		40	U.S.
helicoides[g]	CBS 286.31	PN		50	U.S.
heterothallicum[g]	CBS 451.67	TYPE	f	24	Canada
	CBS 450.67	TYPE	m	23	Canada
hydnosporum[un]	CBS 253.60	PN		90	Germany
hypogynum[g]	CBS 692.79			55	Canada
inflatum	CBS 168.68	PN		86	U.S.
insidiosum	CBS 574.85	TYPE		65	C.R.
intermedium[g]	CBS 221.68	PN	+	142	Neth.
	CBS 223.68		+	190	Neth.
	CBS 222.68		-	234	Neth
	CBS 266.38	PN	-	6	Neth.
	CBS 268.38		-	141	Neth.
	CBS 380.34		-	191	?
	CBS 136.87		?	233	Norway
	adc 94.24		?	244	?
irregulare[g]	CBS 250.28	NEOTYPE		67	Neth.
	CBS 269.38			97	Neth.
	CBS 461.48			98	Australia
	CBS 469.50			99	?
	CBS 493.86			100	Poland
	CBS 263.30			101	U.S.
	CBS 494.86			102	Poland
	adc 94.02			105	Neth.
	adc 94.05			106	Neth.
	CBS 287.31			127	Germany
	CBS 265.38			128	U.S.
	CBS 492.86			131	Poland
	CBS 733.94			174	Canada
	adc 94.10			245	Neth.
	adc 94.13			246	Neth.
iwayamae[g]	CBS 156.64	PN		29	Australia
	CBS 697.83			64	Japan
kunmingense[g]	CBS 550.88	TYPE		60	China
lutarium	CBS 222.88	TYPE		36	U.K.

Table 1. Extended

Species	Culture ID[a]	Status[b]	MT[c]	Ref. No.[d]	Origin[e]
macrosporum[R]	CBS 574.80	TYPE	+	7	Neth.
	CBS 575.80	TYPE	-	8	Neth.
	CBS 579.80		-	143	Canada
mamillatum[R]	CBS 251.28	PN		9	Neth.
	CBS 381.34			112	?
	CBS 212.68			113	Neth.
	CBS 213.68			114	Neth.
	APCC 4311c			4311c	Russia
marinum	CBS 312.93			94	U.S.
marsipium[R]	CBS 773.81			96	Neth.
mastophorum[R]	CBS 375.72	PN		57	U.K.
middletonii[R]	CBS 528.74	PN		35	Neth.
minus[R]	CBS 226.88	TYPE		37	U.K.
monospermum	CBS 158.73	NEOTYPE		10	U.K.
multisporum[R]	CBS 470.50	TYPE		53	U.S.
myriotylum	CBS 254.70	NEOTYPE		11	Israel
nague	APCC 4321c			4321c	U.K.
oedochilum[R]	CBS 292.37	AU		38	U.S.
	CBS 738.94			211	S. Africa
okanoganense[R]	CBS 315.81	TYPE		59	U.S.
oligandrum[R]	CBS 382.34	PN		12	U.K.
	CBS 530.74			162	Neth.
orthogonon[R]	CBS 376.72	TYPE		56	Lebanon
ostracodes[R]	CBS 768.73	PN		49	Spain
pachycaule	APCC 4117a			4117a	U.K.
paddicum[R]	CBS 698.83			46	Japan
paroecandrum[R]	CBS 157.64	PN		68	Australia
	CBS 203.79			115	Neth.
	CBS 651.79			116	Neth.
	BR 163			163	?
	BR 559			559	?
	BR 568			568	Canada
	BR 635			635	U.K.
	BR 637			637	U.K.
parvum[R]	CBS 225.88	TYPE		42	U.K.
periilum	CBS 169.68	PN		26	U.S.
periplocum[R]	CBS 289.31	TYPE		91	U.S.
pleroticum[R]	CBS 776.81			51	Neth.

Table 1. Extended

Species	Culture ID[a]	Status[b]	MT[c]	Ref. No.[d]	Origin[e]
polymastum[g]	CBS 811.70	PN		93	Neth.
polymorphon[g]	CBS 751.96			334	U.K.
porphyrae	CBS 369.79	PN		215	Japan
prolatum[g]	CBS 845.68	TYPE		62	U.S.
pyrilobum[g]	CBS 158.64	TYPE		43	Australia
radiosum	CBS 217.94	TYPE		77	France
rostratum[g]	CBS 172.68			192	U.K.
	CBS 383.34			193	Neth.
	CBS 533.74	NEOTYPE		88	Neth.
salpingophorum[g]	CBS 471.50	PN		39	Germany
scleroteichum[un]	CBS 294.37	AU		89	U.S.
spinosum[g]	CBS 275.67	PN		13	Neth.
	CBS 290.31			117	S. Africa
	CBS 274.67			118	U.S.
	CBS 276.67			119	Neth.
	CBS 377.72			120	Japan
	adc 85.058			194	Neth.
	APCC 4012b			4012b	U.K.
	APCC 4012d			4012d	U.K.
	APCC 4012h			4012h	U.K.
splendens[g]	CBS 266.69	PN	+	61	Belgium
	CBS 462.48	PN	-	14	U.S.
sulcatum	CBS 603.73	TYPE		44	U.S.
sylvaticum[g]	CBS 226.68		m	167	Neth.
	CBS 228.68		m	132	Neth.
	CBS 230.68		f	133	Neth.
	CBS 232.68		f	134	Neth.
	CBS 233.68		f	121	Neth.
	CBS 452.67	TYPE	m	15	U.S.
	CBS 453.67	TYPE	f	16	U.S.
	CBS 633.67			168	U.K.
	CBS 720.94		m	225	Canada
	CBS 721.94		m	226	Canada
	CBS 722.94		m	223	Canada
	CBS 723.94		?	175	Canada
	adc 94.11		?	216	?
	adc 94.12		?	217	?
	adc 94.15		?	218	?

Table 1. Extended

Species	Culture ID[a]	Status[b]	MT[c]	Ref. No.[d]	Origin[e]
sylvaticum[g]	adc 94.16		?	219	?
tardicrescens	APCC 4215a			4215a	Canada
torulosum	CBS 316.33	PN		17	Neth.
tracheiphilum[g]	CBS 323.65	TYPE		92	Italy
tumidum	CBS 223.94	TYPE		78	France
ultimum var. *spor.*[g]	CBS 656.68			202	Neth.
	CBS 111.65			145	Lebanon
	CBS 114.79			146	Spain
	CBS 171.68			147	?
	CBS 219.65	TYPE		19	U.S.
ultimum var. *ult.*[g]	CBS 114.19			122	?
	CBS 249.28			198	Neth.
	CBS 264.38			199	Neth.
	CBS 291.31			200	?
	CBS 296.37			201	U.K.
	CBS 305.35			130	Neth.
	CBS 378.34			144	?
	CBS 398.51	NEOTYPE		18	Neth.
	CBS 488.86			103	Poland
	CBS 489.86			228	Poland
	CBS 490.86			229	Poland
	CBS 491.86			230	Poland
	CBS 725.94			224	Canada
	CBS 726.94			176	Canada
	CBS 728.94			177	Canada
	CBS 729.94			178	Canada
	CBS 730.94			173	Canada
	CBS 656.68			202	Neth.
	adc 94.06			220	Norway
uncinulatum[g]	CBS 518.77	TYPE		203	Neth.
undulatum[g]	CBS 157.69	NEOTYPE		48	U.S.
vanterpoolii	CBS 295.37	TYPE		58	U.K.
vexans[g]	CBS 119.80	PN		20	Iran
violae[g]	CBS 159.64	PN		28	Australia
	CBS 178.86			126	Neth.
volutum	CBS 699.83			47	Japan
zingiberis	CBS 216.82			41	Japan

[a] CBS = accession number of strains obtained from Centraalbureau voor Schimmelcultures, Baarn, Netherlands; APCC = accession number of strains obtained from the Aquatic Phycomycete Culture Collection, Reading, England; BR = accession number of strains from Biosystematics Research Centre, Ottawa, Canada; adc = strains not maintained.
[b] Type = strain from which the type material was derived; AU = authentic strain, identified by the author of the species; PN = strain used for description in Van der Plaats-Niterink monograph, NEOTYPE = strain designated as neotype by Van der Plaats-Niterink because all type material is missing; and ? = uncertain or unknown.
[c] MT = mating type; a, b, or +, - = opposite mating type; f = female; and m = male.
[d] Ref. No. = number by which the isolate is referred to in text and figure captions.
[e] Neth. = Netherlands; ? = unknown; Switz. = Switzerland; C. R. = Costa Rica; S. Africa = South Africa.
[g] Species with globose sporangia and/or hyphal swellings.
[un] Species of which no sporangia or hyphal swelling are known.

Isolation of DNA

DNA was extracted from the *Pythium* isolates according to the method of Möller et al. 1992 with some modification. Freeze-dried mycelium (30 mg) was extracted in 1 ml of TES buffer (100 mM Tris, pH 8.0, 10 mM EDTA, and 2% sodium dodecyl sulfate) with 100 to 200 mg of proteinase K at 55°C for 30 min with occasional gentle stirring. 280 µl of 5M NaCl and 138 µl of hexadecyltrimethyl ammonium bromide (CTAB)/NaCl (10% w/0.7 M) were added, and the mixture was incubated for 10 min at 65°C. Next the lysed suspension was centrifuged (16,000 x g) for 10 min at 4°C to remove cell debris, and the supernatant (1 ml) was extracted with an equal volume of chloroform/isoamyl alcohol (24:1, v/v) for 30 min on ice. The CTAB-protein complex and SDS were removed by centrifugation (16,000 x g) for 10 min at 4°C. The supernatant was re-extracted by adding 450 µl of 5M ammonium acetate and incubating for at least 30 min on ice. This was followed by centrifugation (16,000 x g) for 10 min at 4°C. The DNA was precipitated by adding 715 µl of isopropanol to the aqueous phase (1,300 µl) and centrifugation (16,000 x g) for 15 min. The DNA pellet was washed for 15 min with 1 ml of 70% ethanol at room temperature. After drying of the pellet, the DNA was resuspended in 100 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and stored frozen at -20°C.

Plasmid DNA isolation was performed using the alkaline lysis method (Maniatis et al. 1982). A 20 ml culture of *E. coli* JM109 containing the plasmid was

grown overnight at 37°C on LB-amp broth (10 g tryptone, 5 g yeast extract, 5 g NaCl in 1 L water, containing 100 μg/ml ampicillin) with vigorous shaking. Cells were pelletted down by high speed centrifugation for 2 min and then resuspended in 200 μl of TEG buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris HCL, pH 8.0) containing 0.1 mg/ml RNase. 400 μl of freshly prepared 0.2M NaOH-1% SDS was added slowly and then the solution was mixed by gently inversion. Next, 300 μl of potassium acetate (pH 4.8) was added, mixed gently and the solution was incubated in ice for 10 min. Cell debris were pelletted down by high speed centrifugation for 15 min and then the supernatant was recentrifuged for 15 min again. The supernatant was extracted with an equal volume of phenol/chloroform (1:1, v/v) and next with an equal volume of chloroform/isoamyl alcohol (24:1, v/v). DNA was precipitated by adding of two volumes of ice cold absolute ethanol and incubating at -60°C for 30 min. The nucleic acids were recovered by centrifugation for 15 min at 4°C and washed two times in 1 ml of 70% ethanol. The DNA pellet was air dried at 50°C for 10 min. Purified plasmid DNA was resuspended in 100 μl of TE buffer.

DNA digestion and electrophoresis

Endonuclease digestions were performed using enzymes obtained from Gibco BRL (Burlington, ON) according to the manufacturer's recommendations. 2 μl of loading buffer (0.25% bromophenol blue and 40%, w/v sucrose) was

added to the DNA restriction reactions (10 μl) after the recommended incubation period.

Electrophoresis was carried out in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 7.6) on 0.8 to 2.0% agarose submarine gels at 2 to 10 V/cm. The DNA size marker 1 Kb Ladder (Gibco BRL) was used as a fragment size standard. DNA was visualized by the addition of ethidium bromide (Sigma) to the agarose gel (final concentration 0.5 μg/ml) and exposure to ultraviolet light (310 nm) on a transluminator (Fotodyne Incorporated, Mississauga, ON). The gels were photographed under ultraviolet light using polaroid 667 film.

Amplification of DNA

Polymerase chain reaction (PCR) was performed in a volume of 50 μl with the following reagents: 5 μl of 10 x *Taq* DNA polymerase reaction buffer (Promega, Madison, WI), 3 μl of 25 mM $MgCl_2$ (final concentration 1.5 mM), 4 μl of dNTP mixture (final concentration of each nucleotide 200 μM), 1 μl (30 pmol) of each of the relevant oligonucleotide primers, 0.25 μl of *Taq* DNA polymerase (1.25 units) (Promega), 2 μl of template DNA solution, and 33.5 μl of ultrapure water. The DNA template solution consisted of 20 ng of a crude nucleic acid extract per microliter. All oligonucleotide primers used for PCR are listed 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). The amplification was performed in a programmable thermal controller (PTC-100, MJ Research, Watertown, MA), using the step cycle program, including denaturation at 93°C for 1 min, annealing at 50°C for 1 min, and polymerization at 72°C for 7 min with Q and P_2 primers, 2 min with N_2 and Y, 1 min with SL and SR, or 0.5 min with N_2 and Y_3. The PCR cycle was repeated 30 times. 10 μl DNA from amplification reactions was directly electrophoresed on a 1.5% agarose gel.

Table 2. Oligonucleotides used for amplification rDNA and oligonucleotide probes.

Primer	Location	Sequence (5' to 3')
$d(GC)_9$		GCGCGCGCGCGCGCGCGC
$d(GT)_9$		GTGTGTGTGTGTGTGTGT
N_2	2-19[a]	TAGACGGCCATCTTAGGC
P2	80-98[b]	ATACTTAGACATGCATGGC
Q	3110-3128[c]	ACGCCTCTAAGTCAGAATC
Q1	3350-3369	CGATCTGCTGAGATTTAGC
SBL	7-25[a] +*Bam*HI sites	GGATCCGGATCCTTCTCAGCCTAAGA~ ~TGGCC
SBR	96-112[a] +*Bam*HI sites	GGATCCGGATCCGGGGAAGTCCGAG~ ~TGCT
SEL	7-27[a] up	AATTCTCAGCCTAAGATGGCC
SER	91-112 down	AATTCGGGGAAGTCCGAGTGCT
SL	1-20[a] up	AGCCTAAGATGGCCGTCGAC
SR	99-118[a] down	GAAGCCCGGGTGCTGTCTAC
T_3	Bluescript M13	ATTAACCCTCACTAAAG
T_7	Bluescript M13	AATACGACTCACTATAG
Y	34-52[a]	TCGCAGAGCGAACGGGAT
Y_3	98-118[a]	GTAGACAGCACCCGGACTTC

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

Isolation of DNA fragments from agarose gels

The whole PCR product or one DNA band was eluted from the agarose gel by the freeze-squeeze method (Tautz and Renz 1983). Bands were cut out of ethidium bromide-stained gels and frozen at -60°C for 30 min. The gel plug was placed between two layers of parafilm (American National Can, Greenwich, Conn.) and thawed by steady finger pressure. The resulting liquid containing DNA was collected and 2M NaCL/2% CTAB was added to final concentration 1M/1%. The mixture was incubated at 55°C for 10 min and extracted two times with chloroform/isoamyl alkohol (25:1, v:v). DNA was precipitated by the addition of two volumes of absolute ethanol.

Hybridization

PCR probes were labeled with digoxigenin (DIG)-11-dUTP, alkali-labile by the random primed method according to the supplier's instructions (Boehringer Mannheim GmbH, Germany). The whole PCR product or one DNA band eluted from the agarose gel was used as a template for the labeling reaction. Oligonucleotide primers were 3'-end labeled with DIG-ddUTP. The labeling reaction was performed using the DIG Oligonucleotide 3'-End Labeling

Kit according to the manufacturer's recommendation (Boehringer Mannheim GmbH, Germany).

Dot blots were prepared by spotting 1 μl of denatured (heated at 100°C for 5 min) genomic or amplified DNA target on a Hybond-N nylon membrane (Amersham Corp., Arlington Heights, IL). DNA was fixed to the membrane by cross-linking with UV light for 4 min.

Amplified DNA which was digested with restriction endonucleases and run on 2% agarose gels was transferred onto Hybond-N membranes (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions.

Membranes were prehybridized at 65°C for 1 h in 15 ml of hybridization solution (1% SDS and 1 M NaCl) with constant agitation. The PCR probes (random labeled) or $d(GC)_9$ (3'-end labeled) were denatured by boiling for 5 min before adding to the prehybridization solution. Hybridization was performed with gentle agitation overnight at 65°C with SL-SR or $d(GC)_9$ probes, at 55°C with the 5S gene probe, or at 42°C with $d(GT)_9$ probe. Probe concentrations were from 5 to 10 ng/ml hybridization solution. Once hybridization was completed, the membranes were washed twice, 5 min per wash, in 2x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) and 0.1% SDS at room temperature, then twice for 20 min in 0.1x SSC and 0.1% SDS at 68°C or 55°C for the gene probe and 42°C for the $d(GT)_9$ probe.

Chemiluminescent detection was performed with anti-digoxigenin antibody conjugated to alkaline phosphatase and CDP-Star substrate (Boehringer) according to the manufacturer's instructions as follows. After posthybridization washes, membranes were washed briefly in buffer 1 (100 mM maleic acid and 150 mM NaCl, pH 7.5) and incubated in buffer 2 (1% blocking reagent in buffer 1) for 1 h with gentle agitation. Anti-DIG-alkaline phosphatase was added to fresh buffer 2 to achieve a dilution of 1:10,000, followed by incubation with gentle agitation for 30 min. Membranes were washed twice for 15 min in buffer 1 and then equilibrated in buffer 3 (100 mM Tris-HCl, pH 9.5, 100mM NaCl, and 50 mM $MgCl_2$) for 2 min. Membranes were placed on plastic sheets, and 100 μl of a 1:200 dilution of a 25 mM solution of CDP-Star was spread over each 15 cm^2 of membrane. Membranes were sealed in the plastic sheets and exposed to Kodak X-Omat X-ray film (Eastman Kodak Co., Rochester, NY) for 1 to 15 min to record chemiluminescence.

Cloning amplified 5S intergenic spacers

The amplicons produced by SBL-SBR amplification of the IGS spacer were cloned into the *Bam*HI site of Bluescript M13ks+ (Stratagene, La Jolla, CA). For *P. spinosum* and *P. cylindrosporum*, 5S spacers were amplified with the primers SEL and SER and the products were cloned into the *Eco*RI site of Bluescript M13ks+ (Stratagene).

Insert preparation

The SBL-SBR amplicons were eluted from the agarose gel by the freeze-squeeze method and digested with 2 units *Bam*HI restriction endonuclease at 37°C for 2 h. The digest was brought to a volume of 100 μl with water and extracted with an equal volume of phenol/chloroform (1:1, v:v) and ethanol precipitated using 0.1 volume of 3M sodium acetate and two volumes of ice cold absolute ethanol. DNA was resuspended in 10 μl of TE buffer and 1 μl was run on the agarose gel to estimate the insert concentration.

The amplicons produced by SER-SEL amplification were subjected 3'-end exonuclease activity of T4 DNA polymerase as follows. The reaction mixture consisted of: 5 μg of PCR product purified by ethanol precipitation, 4 μl T4 DNA polymerase buffer, 1 μl dGTP (10mM) and 1 μl T4 DNA polymerase (Pharmacia). This was brought to a volume of 20 μl and incubated at 37°C for 30 min. The reaction was stopped by heating at 65°C for 15 min and the following reagents were added: 5 μl One-Phor-All buffer (Pharmacia), 2 μl ATP (10 mM), 1 μl T4 kinase (Pharmacia) and 22 μl of water. The reaction mixture was incubated at 37°C for 30 min, followed by heating at 65°C for 15 min. DNA was purified by phenol/chloroform extraction and ethanol precipitation.

Vector preparation

Bluescript M13ks+ digested with *Bam*H II or *Eco*RI was dephosphorylated using calf intestinal alkaline phosphatase (Pharmacia) according to the supplier's instruction. The reaction was brought to a volume of 125 μl with TE buffer and extracted with an equal volume of LTSPC buffer (10 μl 8M LiCl, 5 μl 1M Tris, pH 9.5, 4 μl 10% SDS, 125 μl phenol/chloroform, 1:1, v:v). The recovered upper aqueous layer was next extracted with an equal volume of chloroform/isoamyl alcohol (24:1, v:v) and the ethanol precipitated as described under insert preparation.

Vector-insert ligation

Ligation was performed using T_4 DNA ligase (Pharmacia) as recomended by manufacturer (Maniatis et al. 1982). The ligation reaction mixture of cohesive ends consisted of: 1 μg vector DNA, 3 μg insert DNA, 2 μl 5 x dilution buffer and 1 μl T_4 DNA ligase. The reaction mixture was brought to a final volume of 10 μl and incubated at room temperature for 1 h. The reaction was stopped by heating to 65°C for 10 min.

Calcium chloride transformation

Calcium chloride transformation of *E. coli* JM109 cells was conducted according to the procedure given in Short Protocols in Molecular Biology (Ausubel et al. 1992). Cells were grown in 10 ml LB media overnight at 37°C with

shaking, from which 1 ml was used to inoculate 100 ml LB medium and grown to early log phase (approximately 2 h) at 37°C with shaking. These cells were chilled 10 min on ice and subsequently centrifuged 5 min at 1600 x g at 4°C. After the cells were pelleted, they were resuspended in 10 ml cold $CaCl_2$ solution (60 mM $CaCl_2$, 10 mM PIPES, pH 7.0, 15 % glycerol) and placed on ice for 15 min. The competent cells were spun down again and resuspended in 2 ml cold $CaCl_2$ solution. Aliquots of 200 μl were placed at -60°C for long term storage.

Approximately 5 μl of ligation reaction was added to an aliquot of competent cells. The mixture was incubated on ice for 10 min. The cells were then heat shocked at 42°C in a water bath for 2 min. 0.5 ml of LB medium was added and the mixture was incubated for 1 h at 37°C on a roller drum. The entire mixture was spread-plated on LB + ampicillin (100 μg/ml) plates containing 32 μg/ml X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) (BRL) and 6.4 μg/ml IPTG (isopropyl-β-D-galactopyranoside)(Sigma). The plates were incubated at 37°C overnight and white colonies were picked and replated on LB+ ampicillin plates.

Each transformant on the new plates was then spotted with 2 μl of 100 mM IPTG/2% X-Gal (10:75, v:v) and grown overnight. White colonies were screened for the possession of an insert. Plasmid DNA was isolated from white colonies and digested with *Bam*HI or *Eco*RI and electrophoresed on a 1.5% agarose gel.

Sequencing reactions

Plasmid DNA containing *Pythium* inserts was sequenced on both directions with primers developed from the vector (T3 and T7). Sequencing was carried out on a Perkin Elmer Cetus DNA Thermal Cycler using the Applied Biosystems Prism Ready Reaction Dyedeoxy Terminator Cycle Sequencing Kit (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, SK).

Sequence analysis and construction of phylogenetic trees

Sequences were manually aligned using "Gene-Runner" program for multiple aligning. Gaps were introduced into the sequences to increase their alignment similarities. The location of gaps was always chosen to obtain minimal nucleotide differences between all sequences.

The READSEQ program was used to reformat the file originating from "Gene-Runner", after which the alignment could be transferred to the PHYLIP (Version 3.57) package for phylogeny construction. Distances were inferred from sequences by DNADIST using Kimura's (1980) two-parameter model, generating the distance matrix. Topology and branch lengths of 5S spacer phylogenetic trees were calculated by using the Neighbor-Joining (NJ) method (Saitou and Nei 1987) which is included in the PHYLIP package. The maximum parsimony

method (DNAPARS) (Felsenstein 1988) was also used to investigate the tree topology. Confidence limits for branches of the most parsimonious tree were estimated by bootstrap analysis with 1,000 iteractions (DNABOOT).

In cases when a data matrix was constructed from the restriction fragments of amplified IGS region, the presence or absence of a restriction site was coded by a 1 or 0, respectively by using (RESTDIST). The KITSCH program was used to carry out Fitch and Margoliash's least-square method for estimating phylogenies from the distance matrix.

Trees were processed for figures using the TREETOOL tree editor. All computer programs for construction DNA phylogeny were run from the Genetic Data Environment (GDE 2.3).

Chapter 4

RESULTS AND DISCUSSION

Development of species-specific probes for *Pythium*

Traditional taxonomy in *Pythium* is based primarily on morphology and growth characteristics and has always been difficult. These difficulties result from the limited number of morphological characteristics available for species identification and the significant variability of these characteristics. The development of species-specific probes can greatly enhance the ability to identify species.

The target sequence for generation of a species-specific probe must exhibit sufficient inter-specific variability to be informative, but minimal intraspecific variation to be conclusive. Nuclear ribosomal DNA spacers have been shown to serve this purpose well (Drouin and de Sa 1995). This is related to the fact that rDNA spacers are relatively free of structural and functional constraints, which allow them to evolve relatively rapidly. Moreover, the effect of molecular drive (Dover 1982) and concerted evolution (Smith 1976) generally act to minimize the degree of intra-specific variation.

It was previously shown that species of *Pythium* with globose sporangia generally have their 5S rRNA genes arranged into tandem arrays (Belkhiri et al. 1992). The intergenic region between repeated, conserved genes diverges rapidly and therefore may be useful to develop species-specific probes for genus *Pythium*. PCR primers based on a consensus sequence of the conserved 5S rRNA coding regions can be used to selectively amplify the much more variable non transcribed spacer. Gene-to-gene amplification has been done previously to gain access to the 5S spacer in plants (Appels et al. 1992, Cox et al. 1992, Gorman et al. 1992, Playford et al. 1992) and for genome fingerprinting of plants (Kolchinsky et al. 1991). Probes derived from the intergenic region of 5S rRNA unit were also used for species identification of the protozoan parasite *Eimeria* (Stucki et al. 1993). A similar approach was used in this project to develop probes for identification of *Pythium* species. This approach has a great advantage over other tedious and costly methods for construction of highly specific DNA probes such as random cloning and sequencing variable regions. PCR- generated 5S spacer fragments can be used for rapid production of species-specific probes that does not require sequencing or construction of a genomic DNA library. A disadvantage of this method is that this approach is limited to species having tandem repeats of 5S genes and excludes *Pythium* species with filamentous sporangia that lack such repeats.

Probe construction

The probes were derived from the type cultures (or other recognized representatives) of *Pythium* species having 5S rRNA gene arranged in tandem repeats. Developing a probe was based on the amplification of the IGS region with primers SL and SR (Fig.4).

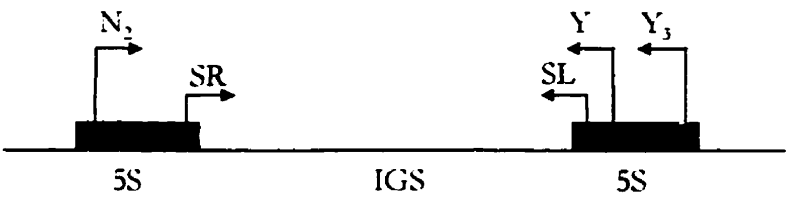


Figure 4. *Pythium* 5S ribosomal RNA tandem genes showing positions of primers used for amplification.
IGS = intergenic spacer.

Amplification of genomic DNA from different species of *Pythium* with the primers SL and SR yielded DNA fragments ranging in size from approximately 0.3 to 1.0 kb fragments (Fig. 5). In some cases, more than one amplification product was generated, although there was usually a major fragment near 0.5 kb. This indicates that the spacer region between 5S rRNA genes is approximately 0.5 kb in length. In a number of cases, the second most prominent band, about twice the size of a main band, was produced, which appears to be the

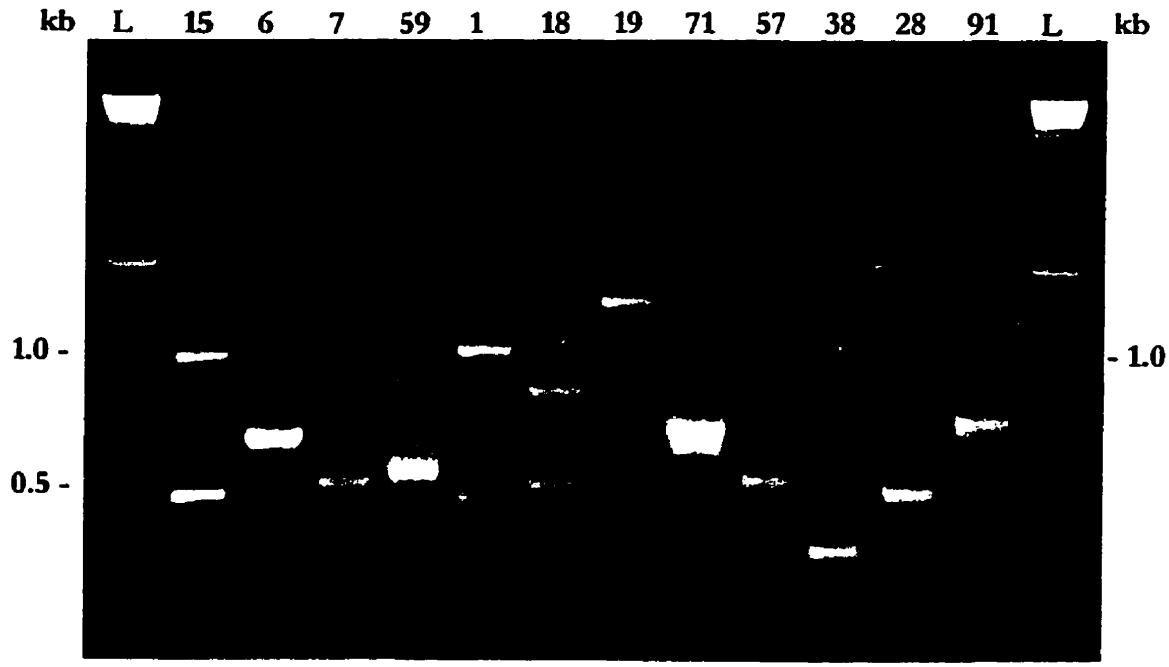
dimer generated by amplification across two spacer regions. Furthermore, other additional weak bands were also present in DNA profiles after amplification of the IGS region. These additional bands hybridized with a probe derived from the most abundant amplicon, suggesting that these products were *bona fide* IGS amplification products, representing other variants of the 5S rRNA gene array, differing in the length of the spacer region.

For all of the species in this study except *P. anandrum*, *P. ultimum* var. *sporangiiferum* and *P. mastophorum*, the SL-SR products were labelled and used as probes without further purification, but for the latter three species, the most prominent band was eluted from the gel and labeled separately. Purification and labeling of the 0.45-kb band for *P. anandrum*, the 0.3-kb band for *P. ultimum* var. *sporangiiferum*, and the 0.5-kb band for *P. mastophorum* greatly improved the specificity of these probes.

To further confirm that the labeled products originated from the 5S rRNA spacer, the genomic DNA from each species was also amplified with the other set of primers. Amplification of the IGS region with primers N2 and Y led to the production of amplicons which were about 130 bp larger than the SL-SR product as was expected from the map. The N_2-Y amplification yields the product that includes 120 bp of the first gene, the spacer, and 50 bp of the second gene whereas the SL-SR amplicon includes 20 bp of the first gene, the spacer, and 20 bp of the second gene.

Figure 5. SL-SR amplicons of the 5S intergenic spacer used for preparation of *Pythium* species-specific probes.

Lane L, 1 kb DNA ladder. Numbers correspond to reference numbers of *Pythium* isolates provided in Table 1: **15**, *P. sylvaticum* (CBS 452.67); **6**, *P. intermedium* (CBS 266.38); **7**, *P. macrosporum* (CBS 574.80); **59**, *P. okanoganense* (CBS 315.81); **1**, *P. anandrum* (CBS 258.31); **18**, *P. ultimum* var *ultimum* (CBS 398.51); **19**, *P. ultimum* var. *sporangiferum* (CBS 219.65); **71**, *P. acanthicum* (CBS 284.31), **57**, *P. mastophorum* (CBS 375.72); **38**, *P. oedochilum* (CBS 292.37), **28**, *P. violae* (CBS 159.64); **91**, *P. periplocum* (CBS 289.31).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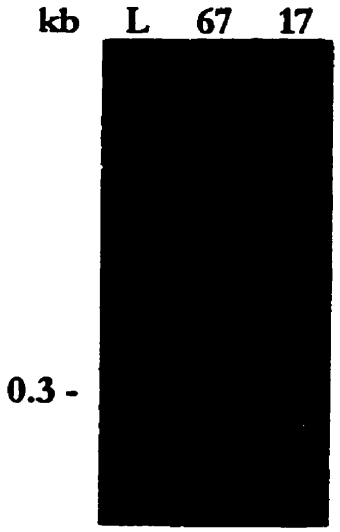
- 1.0

In order to normalize the amount of target genomic DNA from *Pythium* species the 5S rRNA gene probe was constructed. The 5S gene probe was obtained by amplification of *P. irregulare* and *P. tolumsum* genomic DNA with primers N_2 and Y_3 (Fig. 6) and isolation of the 120-bp product which represents amplification of the entire 5S rRNA gene. These two products purified from the gel were mixed in equal proportion and used as the template for the labeling reaction. The two species were chosen because it is known (Belkhiri et al. 1992) that *Pythium* species with globose sporangia have 5S genes that differ in two bases from the 5S genes of *Pythium* species with filamentous sporangia. *P. irregulare* represents the former, and *P. torulosum* , the later.

Figure 6. 5S rRNA genes of *P. irregulare*, 67, and *P. torulosum*, 17, amplified with primers N_2 and Y_3, used for the preparation of the 5S rRNA gene probe.

Lane L, 1 kb DNA ladder.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0.3 -

Detection of *Pythium* species by hybridization to genomic spot blots

Probes representing the entire IGS region were tested for specificity against 92 *Pythium* species (including seven mating pairs of heterothallic species and the two varieties of *P. ultimum*). Genomic DNA was applied in subsets to five hybridization membranes in concentrations determined by preliminary hybridization with the 5S rRNA gene probe on the basis of the assumption that the number of 5S genes is equal the number of gene spacers. Therefore the amount of target DNA in each spot was nearly uniform between species. The results of hybridization with the 5S gene probe are shown in Figure 7. Although some differences in target amounts were still present in the final spot blot, the specificity of 5S spacer probes tested in this study was high enough to eliminate the possibility that unequal target amount would alter the degree hybridization of a probe to the target DNA. Spots contain from 0.05 to 1.0 μg of DNA.

Of 42 probes tested for specificity, 13 recognized the genomic DNA of the isolate from which the probe had been derived but not the genomic DNA from other *Pythium* species. Furthermore, probes derived from one mating type also recognized the opposite mating type within the same species. When tested for specificity against other *Pythium* spp. the probe derived from the type culture of *P. sylvaticum* (male) recognized only itself (Fig. 7B, spot 15) and the type culture of *P. sylvaticum* (female) (spot 16). The *P. intermedium* probe (CBS 266.38, mating type MT-) derived from an isolate used by Van der Plaats-Niterink for the

description of this species (Van der Plaats-Niterink 1981) recognized only itself (Fig. 7C, spot 6) and the opposite mating type, *P. intermedium* CBS 221.68 MT+ (spot 142). The *P. macrosporum* probe, derived from the type culture (MT+), hybridized strongly only to its target DNA (Fig. 7D, spot 7) and weakly to *P. macrosporum* MT- (spot 8). The *P. okanogenense* probe was derived from the type culture. This probe distinguished only itself from all other species tested (Fig. 7E, spot 59). The *P. anandrum* probe was also derived from the type culture and recognized only itself and not the target DNA of other *Pythium* species (Fig. 7F, spot 1). The probe derived from the type culture of *P. ultimum* var. *ultimum* hybridized strongly to itself (Fig. 7G, spot 18) and very weakly to the type culture of *P. ultimum* var. *sporangiiferum* (spot 19) and two isolates of *P. splendens* (CBS 462.48 MT-, spot 14 and CBS 266.69 MT+, spot 61). The weak level hybridization in these trials was very faint and detectable only after prolonged exposure.

Figure 7. Hybridization of the *Pythium* 5S rRNA gene and 5S spacer probes against spot blots of genomic DNA.

Positions of spots are given in the schematic diagram; numbers correspond to reference numbers provided in Table 1. **A**, 5S rRNA gene probe (primer pair N_2-Y_3), **B**, *P. sylvaticum*, 15; **C**, *P. intermedium*, 6; **D**, *P. macrosporum*, 7; **E**, *P. okanogenense*, 59; **F**, *P. anandrum*, 1; **G**, *P. ultimum* var. *ultimum*, 18: **H**, *P. ultimum* var. *sporangiiferum*, 19; **I**, *P. acanthicum*, 71; **J**, *P. mastophorum*, 57; **K**, *P. oedochilum*, 38; **L**, *P. rioiae*, 28; **M**, *P. periplocum*, 91.

								80	92
3	61	1	29	38	49	59	70	81	93
6	15	4	64	39	50	60	71	82	94
142	16	5	30	40	51	62	72	83	96
7	17	22	31	41	52	63	73	84	162
8	18	23	32	42	53	65	74	85	203
9	19	24	33	43	54	66	75	86	211
10	20	25	34	44	55	67	76	87	215
11	21	26	35	46	56	68	77	88	4004a
13	22	27	36	47	57	115	78	89	4215a
14	23	28	37	48	58	69	79	90	4117a
								91	4321c

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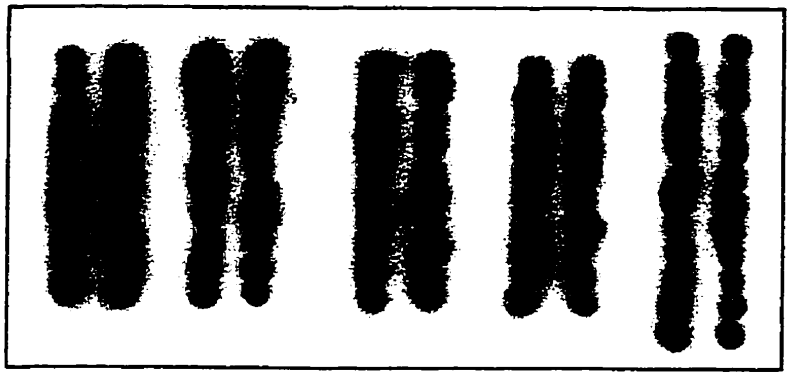

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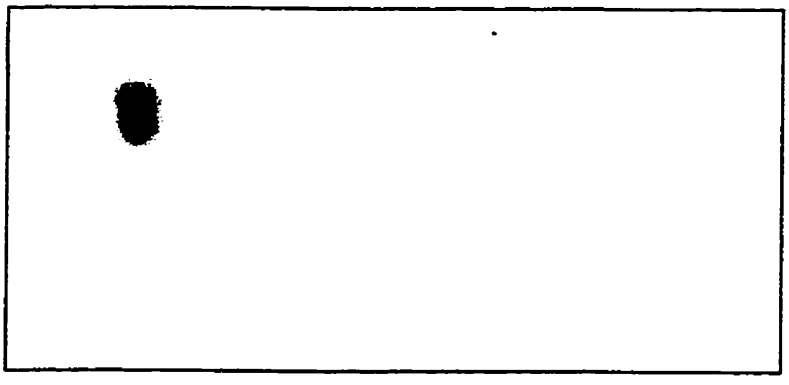

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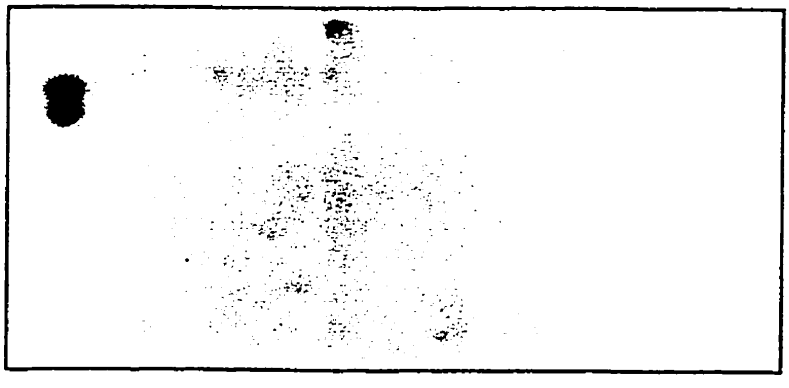

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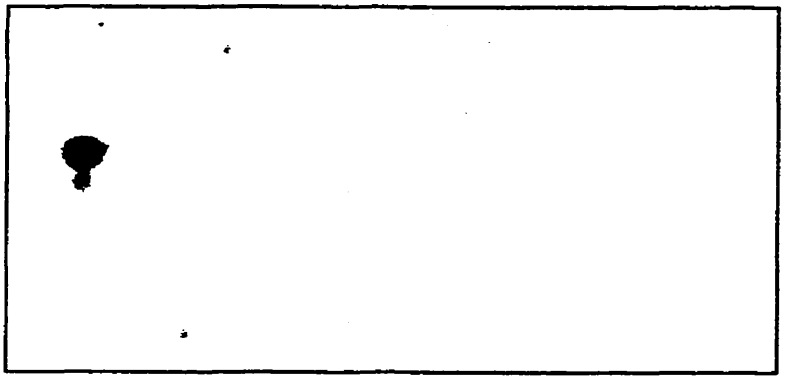

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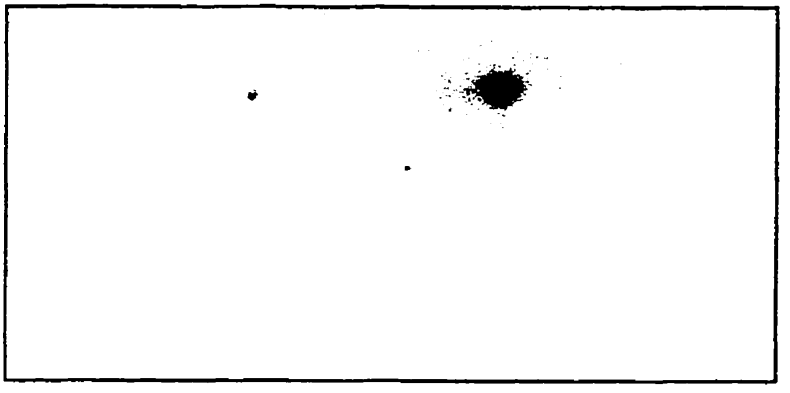

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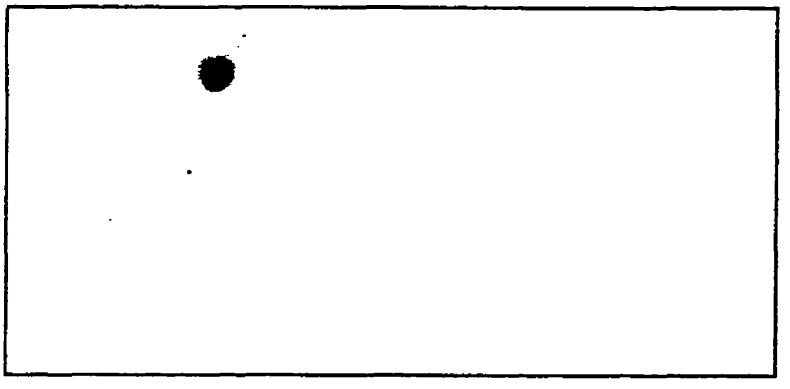

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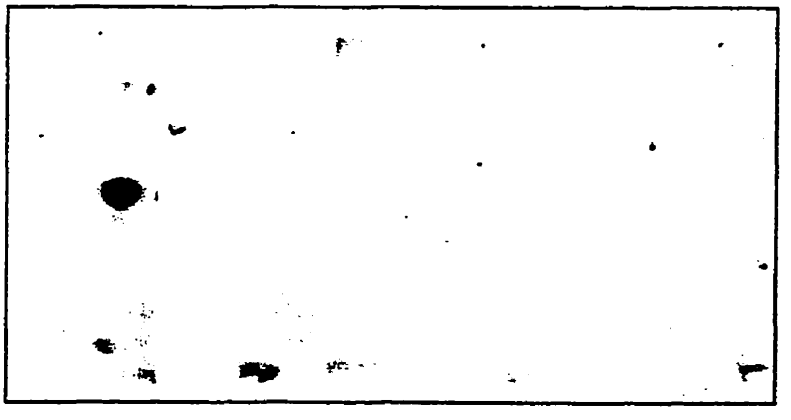

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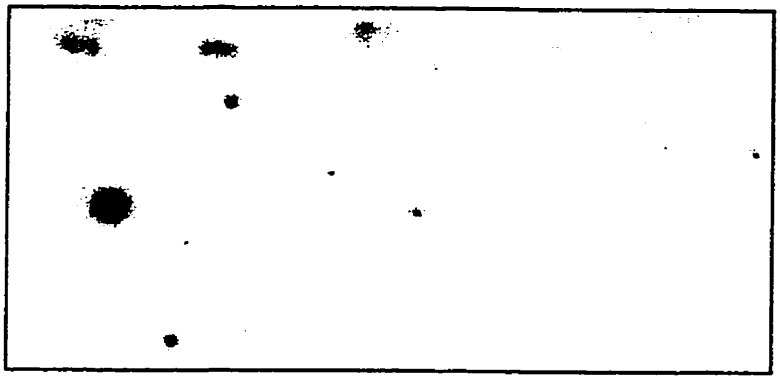

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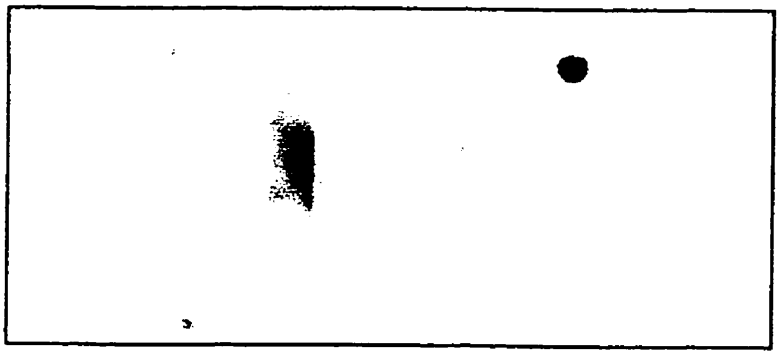

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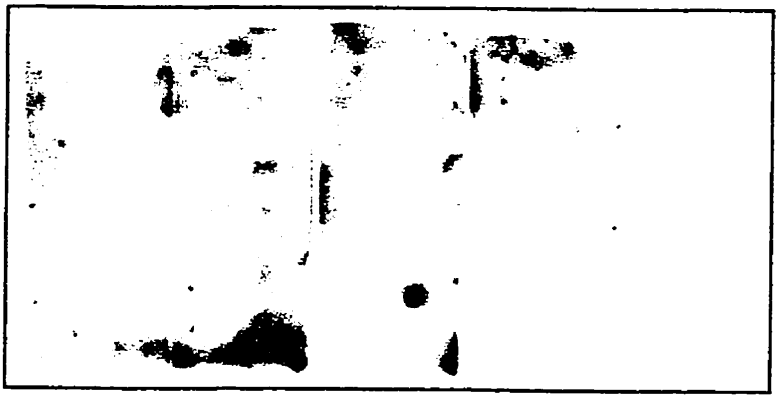

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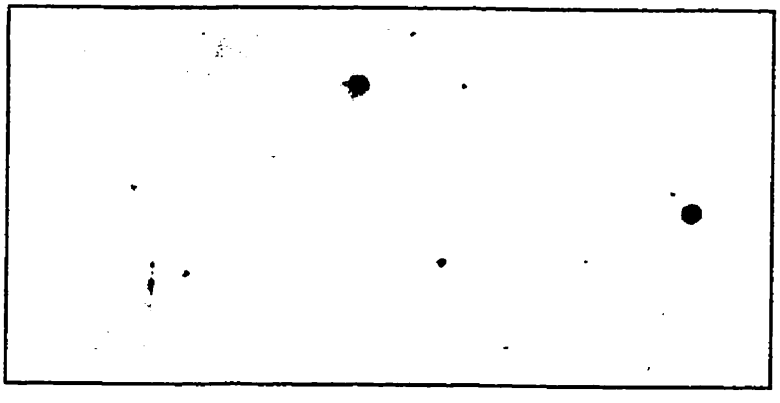

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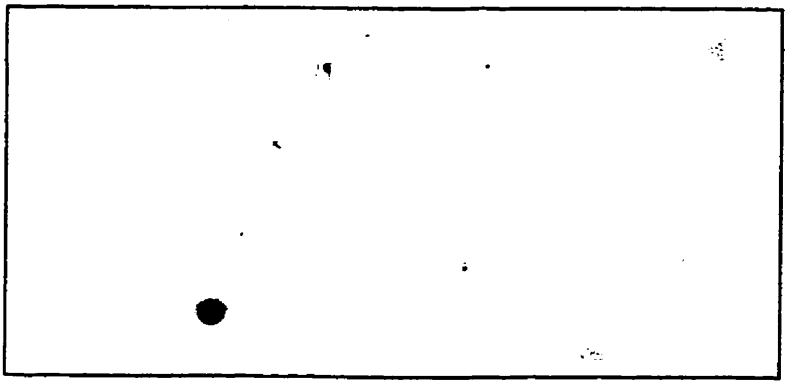

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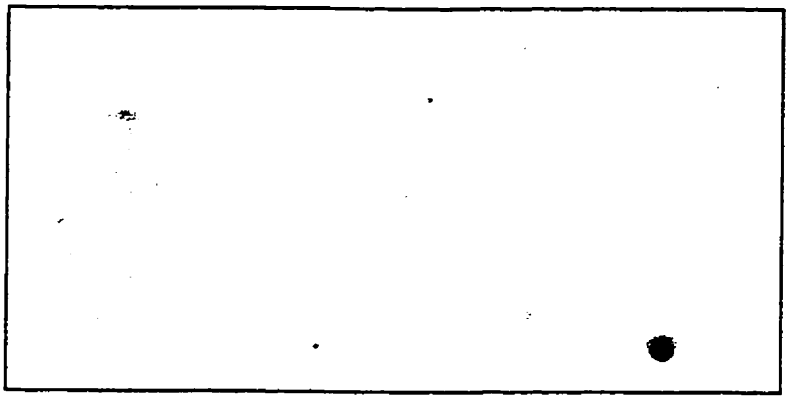


The 0.3-kb IGS spacer of the type culture of *P. ultimum* var. *sporangiferum* hybridized only to its target DNA (Fig. 7H, spot 19). The probe derived from the type culture of *P. acanthicum* also recognized only itself (Fig. 7I, spot 71). The *P. mastophorum* (CBS 375.72) probe derived from an isolate used by Van der Plaats-Niterink in her monograph of the genus *Pythium* (Van der Plaats-Niterink 1981) hybridized only to itself (Fig. 7J, spot 57). The 5S spacer of *P. oedochilum* (CBS 292.37) recognized itself and another isolate of this species (CBS 738.94) included in this study (Fig. 7K, spot 38 and 211). The probe of *P. violae* (CBS 159.64) derived from the neotype strain proposed by Van der Plaats-Niterink (Van der Plaats-Niterink 1981) was also species specific (Fig. 7L, spot 28). It recognized itself primarily and exhibited faint reaction with *P. iwayamae* (spot 29) and *P. mstratum* (spot 88). Finally, the probe derived from the type culture of *P. periplocum* recognized itself (Fig. 7M, spot 91), reacted weakly with *P. polymastum* (spot 93) and gave faint signal in reaction with *P. pleroticum* (spot 51), *P. multisporum* (spot 53) and *P.* sp. (Type of P. "*drechsleri*" Paul) (spot 75).

Intraspecific hybridization with species-specific probes

Evaluation of the specificity and the range of probes was performed by screening a variety of isolates within the same species. To see whether other isolates within the same species are also recognized by species-specific probes, 5S spacer probes were hybridized against as many isolates as were available. Negative controls were included with each hybridization.

Probe *P. intermedium* recognized all eight isolates of *P. intermedium*, however two of the isolates (CBS 266.38 and CBS 221.68) had significantly weaker reactions than the others (Fig. 8A). Sixteen tested isolates of *P. sylvaticum* were recognized by the probe, however weak hybridization was observed with six of them (Fig. 8B). This indicates that the 5S spacer of *P. sylvaticum* demonstrates extensive intra-specific sequence variability. The 5S spacer of *P. sylvaticum* hybridized also to three *P. irregulare* isolates (CBS 733.94, adc 94.10 and adc 94.13) and one isolate of *P. paroecandrum* (CBS 651.79) (data not shown). As reported above, the *P. macrosporum* probe derived from the MT+ hybridized weakly with the isolate of the opposite mating type (CBS 575.80 MT-). When tested for specificity with another isolate of *P. macrosporum* (CBS 579.80 MT-), the probe recognized the DNA target with high intensity (Fig 8C). The *P. ultimum* var. *ultimum* probe hybridized strongly to DNA from all nineteen isolates of this species (Fig 8D). Of the four isolates of *P. ultimum* var. *sporangiiferum* used in this study, only two was recognized by the *P. ultimum* var. *sporangiiferum* probe (CBS 219.65 and CBS 111.65); the other two reacted with the *P. ultimum* var. *ultimum* probe (CBS 114.79 and CBS 171.68) (Fig. 8E) The *P. acanthicum* probe recognized two additional isolates of *P. acanthicum* (CBS 227.94 and CBS 431.68) (Fig. 8F).

Figure 8. Hybridization of *Pythium* species-specific probes to intraspecific targets.

A, Probes: first column, *P. intermedium* CBS 266.38; second column, 5S rRNA gene. Targets (top to bottom, both columns) (Table 1 provides reference numbers): *P. intermedium* 6, 141, 142, 190, 191, 233, 234, 244, *P. macrosporum* 7 and *P. acrogynum* 69 (control).

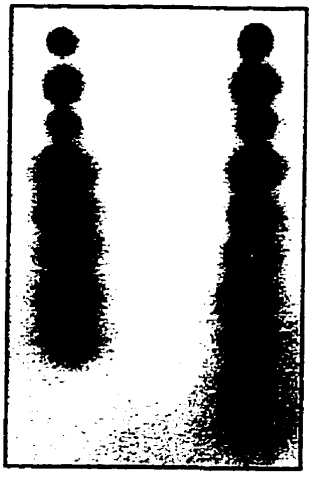
B, Probes: first and second columns, *P. sylvaticum* CBS 452.67; third and fourth columns, 5S rRNA gene. Targets (top to bottom): first and third columns, *P. sylvaticum* 15, 16, 121, 132, 133, 134, 167, 168; and second and fourth columns, 175, 216, 217, 218, 219, 223, 225, 226.

C, Probes: first column, *P. macrosporum* CBS 574.80; second column, 5S rRNA gene. Targets (top to bottom, both columns): *P. macrosporum* 7, 8, 143.

D, Probes: first and second columns, *P. ultimum* var. *ultimum* CBS 398.51; third and fourth columns, 5S rRNA gene. Targets (top to bottom): first and third columns, *P. ultimum* var. *ultimum* 18, 103, 122, 130, 144, 173, 176, 177, 178, 198; and second and fourth columns, 199, 200, 201, 202, 220, 224, 228,229,230 and *P. mamillatum* 9 (control).

E, Probes: first column, *P. ultimum* var. *ultimum* CBS 398.51; second column, *P. ultimum* var *sporagiiiferum* CBS 219.65; third column, 5S rRNA gene. Targets (top to bottom, all columns): *P. ultimum* var *sporagiiiferum* 19, 145, 146, 147, *P. mamillatum* 9 and *P. sylvaticum* 15 (control).

A



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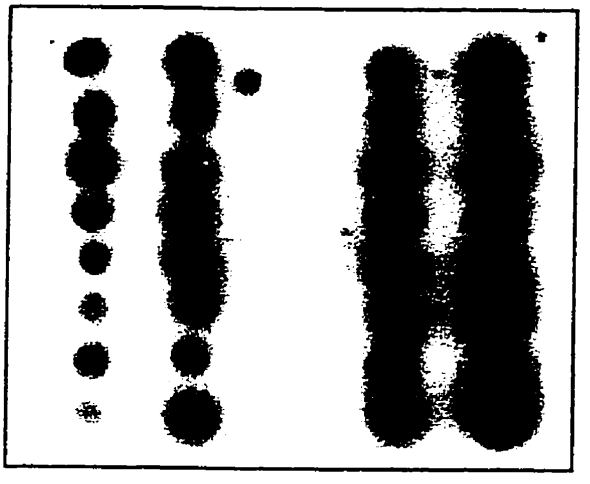
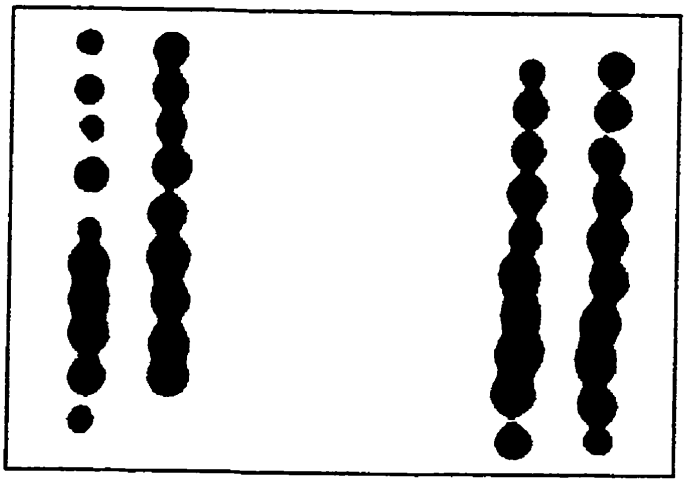


C

F, Probes: first column, *P. acanthicum* CBS 284.31; second column, 5S rRNA gene.

Targets (top to bottom, both columns): *P. acanthicum* 71, 148, 151.

D



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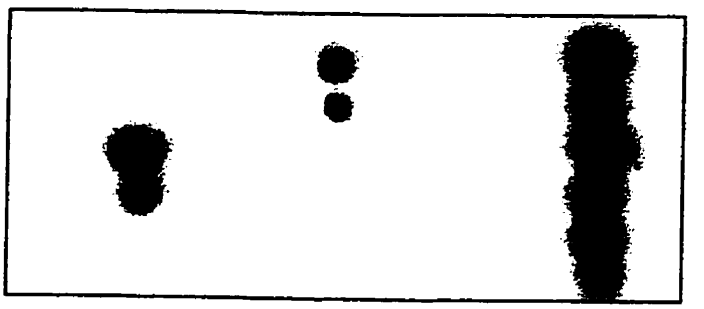

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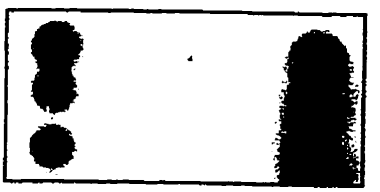
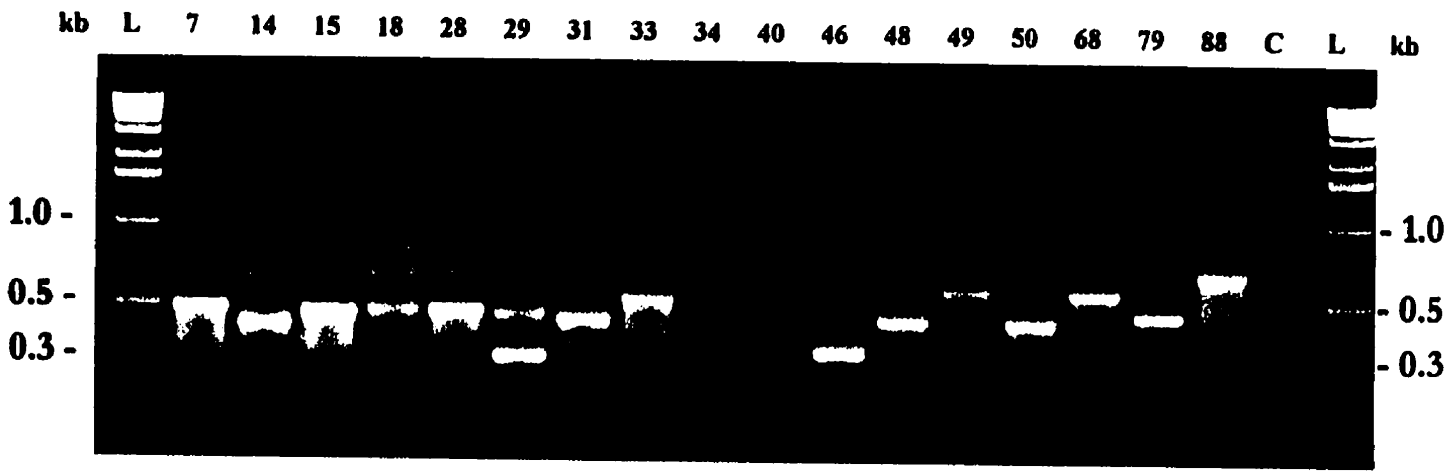


Masking of simple sequence repeat motifs in the 5S rRNA spacer

5S spacer probes derived from more than 15 *Pythium* species hybridized with high intensity with a wide range of *Pythium* isolates (data not shown). Lack of species specificity for those probes may be caused by the presence of short simple repeats that are randomly distributed within the 5S rRNA spacer. To test this hypothesis and show that the IGS regions of some *Pythium* species share similar sequence characteristics, the 5S spacers amplified with the primers SL and SR (Fig. 9) were probed with the $(GT)_9$ simple repeat probe. As it is shown in Fig. 10, the $(GT)_9$ probe hybridized strongly to the 5S rRNA spacers of P. splendens, *P. iwayamae*, *P. echinulatum* , *P. erinaceus*, and *P. rostratum*. A weaker signal was observed in the reaction with 5S spacers of *P.ultimum* var. *ultimum*, *P. paddicum*, *P. ostracodes and P. boreale*. The faint signal, visible after prolonged exposure, was also detectable in the reaction with $(GT)_{40}$ DNA control target.

Figure 9. Amplification of *Pythium* 5S spacers with the primers SL-SR.

Lanes L, 1 kb ladder. Numbers correspond to reference numbers of *Pythium* isolates provided in Table 1: **7**, *P. macrosporum*, **14**, *P. splendens*, **15**, *P. sylvaticum*, **18**, *P. ultimum*, **28**, *P. violae*, **29**, *P. iwayamai*, **31**, *P. dimorphum*, **33**, *P. echinulatum*, **34**, *P. erinaceus*, **40**, *P. helicandrum*, **46**, *P. paddicum*, **48**, *P. undulatum*, **49**, *P. ostracodes*, **50**, *P. helicoides*, **68**, *P. paroecandrum*, **79**, *P. boreale*, **88**, *P. rostratum*, **C**, control without DNA.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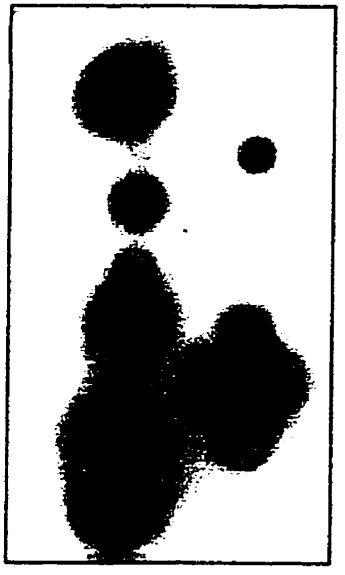
- 1.0
- 0.5
- 0.3

Figure 10. Hybridization of the $(GT)_9$ probe against 5S spacer amplicons. Position of spots are given in the schematic diagram. . Numbers correspond to reference numbers of *Pythium* isolates provided in Table 1: **7**, *P. macrosporum*, **14**, *P. splendens*, **15**, *P. sylvaticum*, **18**, *P. ultimum*, **28**, *P. violae*, **29**, *P. iwayamai*, **31**, *P. dimorphum*, **33**, *P. echinulatum*, **34**, *P. erinaceus*, **40**, *P. helicandrum*, **46**, *P. paddicum*, **48**, *P. undulatum*, **49**, *P. ostracodes*, **50**, *P. helicoides*, **68**, *P. paroecandrum*, **79**, *P. boreale*, **88**, *P. rostratum*.

A



7	46
14	48
15	49
18	50
28	68
29	79
31	88
33	$(GT)_{40}$
34	$(AT)_9$
40	$(GC)_9$

Reaction of 5S rRNA spacers with the $(GT)_9$ probe indicates that the lack of species specificity for some probes may be a reflection of the presence of shared simple repeat motifs. To improve the specificity of those probes, the attempt was made to mask a simple sequence interspersed within the genomic DNA using $(GT)_{40}$ microsatellite sequence cloned from the genome of *Aspergillus flavipes* (Fernandez 1997). Genomic DNA of *Pythium* species was also hybridized with the *P. boreale* probe together with the $(GT)_{40}$ repeat motif. The specificity of the *P. boreale* probe when used together with the $(GT)_{40}$ repeat in the hybridization reaction was greatly improved in comparison to significant amounts of cross reactivity observed with the *P. boreale* probe alone (Fig. 11).

A similar attempt at masking of simple repeats was made with the *P. rostratum* probe .The probe derived from *P. rostratum* CBS 533.74 neotype strain proposed by Van der Plaats-Niterink (Van der Plaats-Niterink 1981), hybridized not only to itself but also to target DNA of many other *Pythium* species. Using the $(GT)_{40}$ repeat together with the *P. rostratum* probe also improved the specificity of this probe. The *P. rostratum* probe recognized itself strongly although it still hybridized weakly to some other *Pythium* species (Fig. 12).

Figure 11. Hybridization of the 5S spacer *P. boreale* CBS 551.88 probe against spot blots of genomic DNA.

Positions of spots are given in the schematic diagram provided in Fig. 7. **A**, *P. boreale* probe alone; **B**, *P. boreale* probe with $(GT)_{40}$.

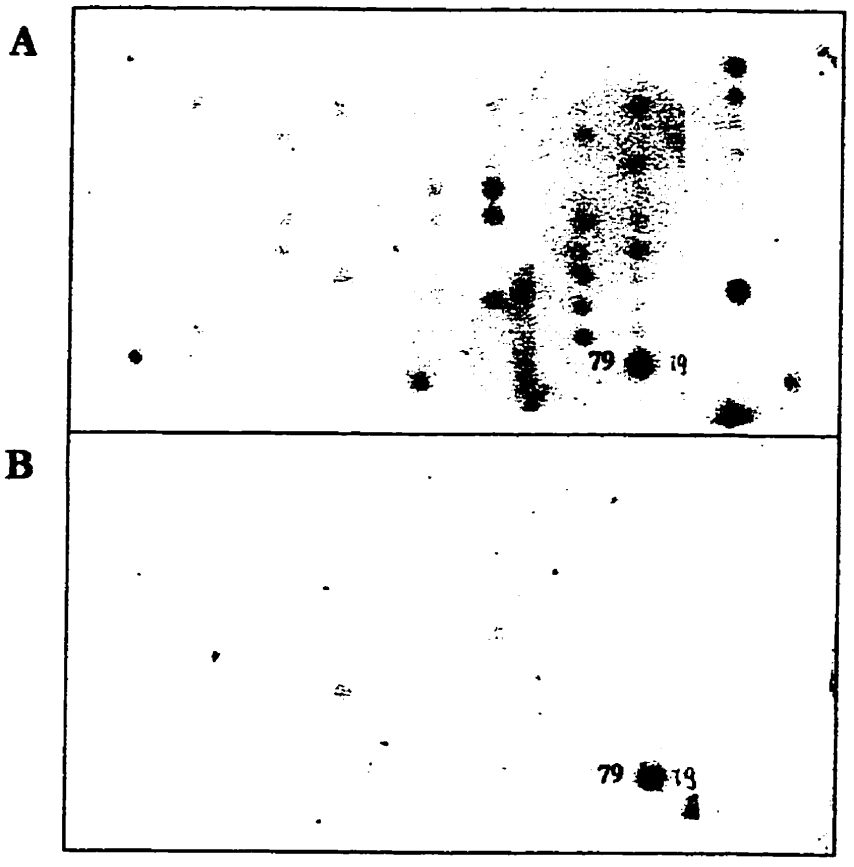
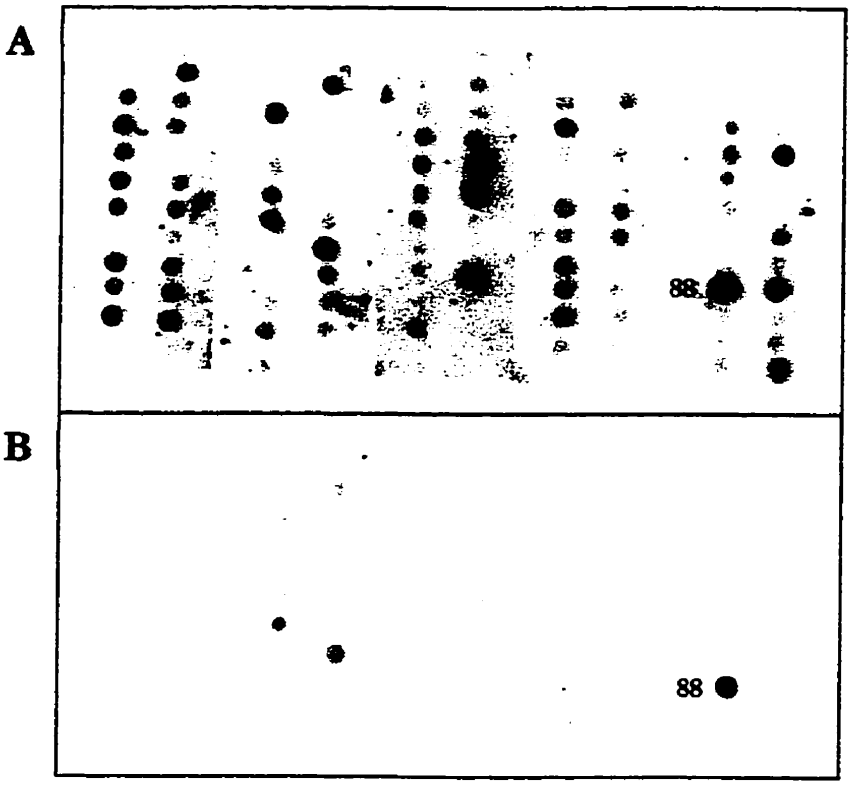

A
B
79
79

Figure 12. Hybridization of the 5S spacer *P. rostratum* CBS 533.74 probe against spot blots of genomic DNA.

Positions of spots are given in the schematic diagram provided in Fig. 7. **A**, *P. rostratum* probe alone; **B**, *P. rostratum* probe with $(GT)_{40}$.


A
88
B
88

Cross-hybridization between 5S spacers from closely related *Pythium* species

During probing of genomic DNA with the 5S rRNA spacer a strong cross-hybridization signal was observed between the following species: *P. buismaniae, P. polymastum and P. uncinulatum.* The *P. buismaniae* probe derived from the type culture recognized itself (Fig 13A, spot 63) and two other species (Fig. 13A, spot 93 and 203) with the same intensity. The *P. polymastum* probe (CBS 811.70) was derived from an isolate used by Van der Plaats-Niterink (Van der Plaats-Niterink 1981) to portray the species. Its hybridized strongly to itself (Fig. 13B, spot 93), annealed more weakly to *P. buismaniae* (Fig. 13B, spot 63) and gave a faint signal in reaction with *P. uncinulatum* (Fig. 13B, spot 203). Similarly, the *P. uncinulatum* probe derived from the type culture recognized itself strongly (Fig 13C, spot 203), *P. buismaniae* moderately (Fig. 13C, spot 63) and *P. polymastum* weakly (Fig. 13C, spot 93).

The strong cross-hybridization signal was also observed between three other species: *P. oligandrum*, *P. amasculinum* and *P. hydnosporum.* The *P. oligandrum* probe (CBS 530.74) hybridized strongly to itself (Fig. 14A, spot 162), less intensely to *P. hydnosporum* and even more weakly to *P. amasculinum* (Fig 14A, spot 90 and 180). The *P. amasculinum* probe, derived from isolate CBS 552.88 (only one representative strain in the CBS collection), recognized itself and the DNA targets of *P. oligandrum* and *P. hydnosporum* but not the target DNA of other *Pythium* species (Fig. 14B, spots 180, 90, 162). Similarly *P. hydnosporum* probe derived from

an isolate CBS 253.60 chosen by Van der Plaats-Niterink to describe the species in her monograph (Van der Plaats-Niterink 1981) recognized all three targets (Fig. 14C, spots 90, 162, 180) but also DNA of *P. tracheiphilum* (Fig. 14C, spot 92). However the *P. tracheiphilum* probe, when tested for specificity, did not recognize *P. hydnosporum* target DNA (data not shown).

When tested for specificity, probes of *P. irregulare*, *P. mamillatum*, *P. spinosum*, *P. kunmingense*, *P. paroecandrum* and *P. cylindrosporum* recognized not only genomic DNA of the isolate from which the probe had been derived but also the genomic DNA of the other five *Pythium* species (data not shown). Each one of the six probes recognized all six targets and no others. The probes also hybridized to 9 other isolates of *P. irregulare*, 4 of *P. mamillatum*, 5 of *P. spinosum* and 2 of *P. paroecandrum* (data not shown). Three isolates of *P. irregulare* (CBS 733.94, adc 94.10 and adc 94.13) and one of *P. paroecandrum* (CBS 651.79) were not recognized by the *P. irregulare* group probes but were recognized by a species-specific *P. sylvaticum* probe. Another *P. paroecandrum* isolate (CBS 203.79) was not recognized by the *P. irregulare* group probes nor by any other available probes.

Figure 13. Hybridization of the 5S spacer *P. buismaniae, P. polymastum, P. uncinulatum* probes against spot blots of genomic DNA.

Positions of spots are given in the schematic diagram provided in Fig. 4. Probes: **A**, *P. buismaniae* 63 (CBS 288.31); **B**, *P. polymastum* 93 (CBS 811.70); **C**, *P. uncinulatum* 203 (CBS 518.77).

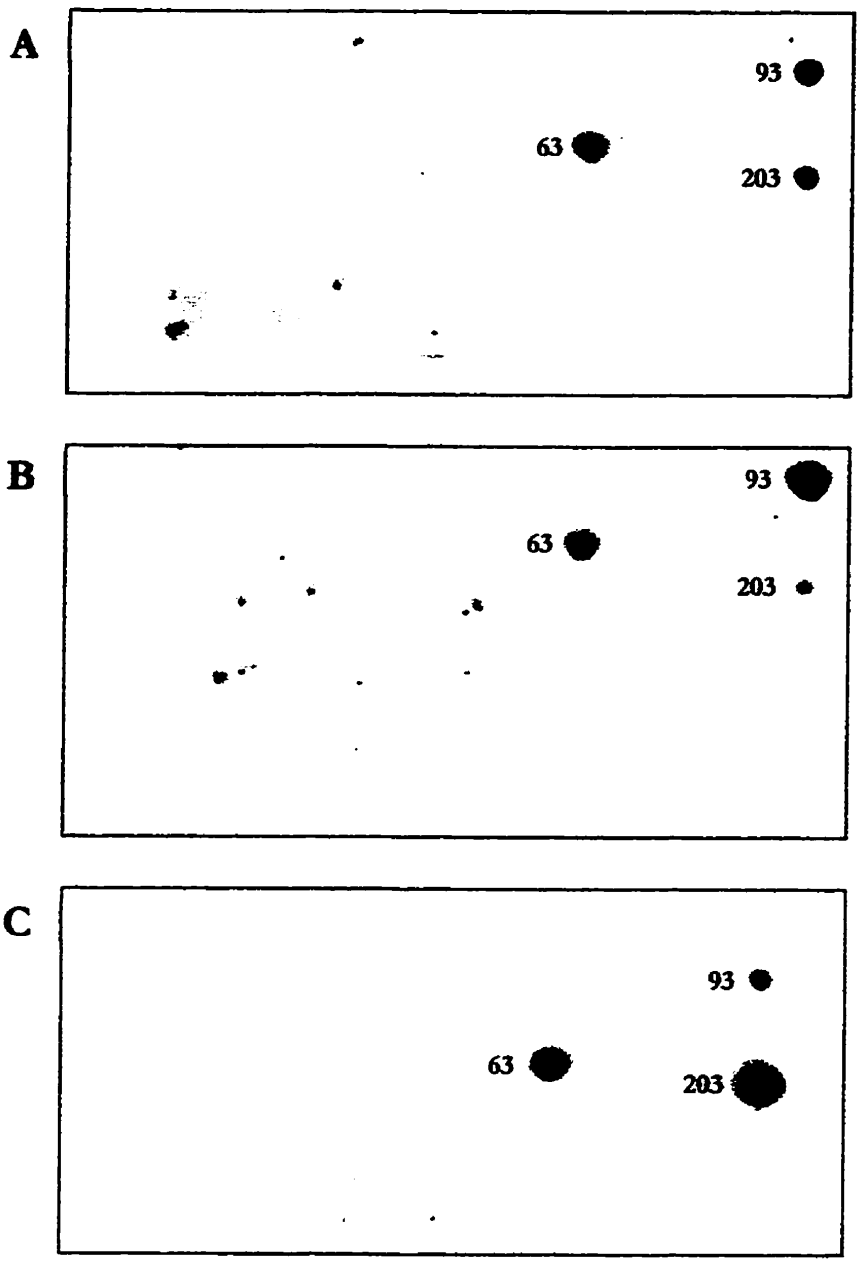
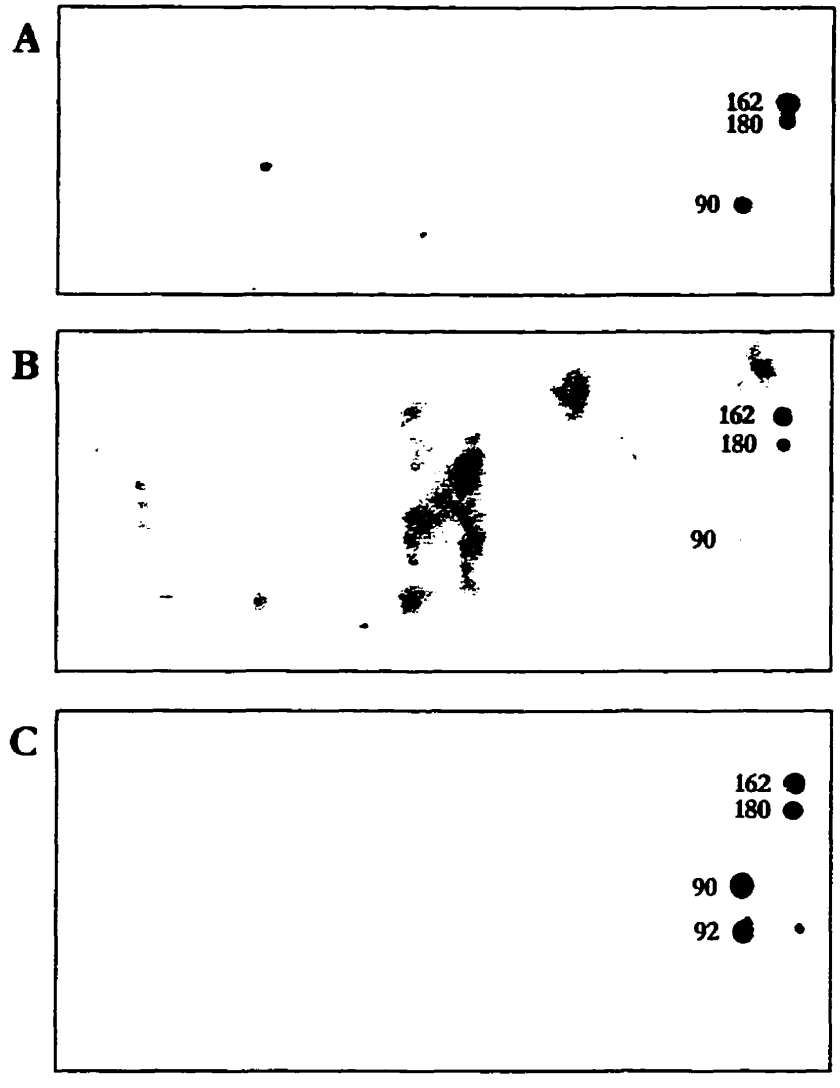

A
93
63
203
B
93
63
203
C
93
63
203

Figure 14. Hybridization of the 5S spacer *P. oligandrum, P. amasculinum, P. hydnosporum* probes against spot blots of genomic DNA.

Positions of spots are given in the schematic diagram provided in Fig. 4. Probes: **A,** *P. oligandrum* 162 (CBS 530.74); **B,** *P. amasculinum* 180 (CBS 552.88); **C,** *P. hydnosporum* 90 (CBS 253.60).


A
162
180
90
B
162
180
90
C
162
180
90
92

Discussion

The potential uses of species-specific DNA probes in plant pathology and systematics are many. Unambiguous and reliable detection of fungal pathogens such as *Pythium* spp. is critical in disease diagnosis and in ecological and epidemiological researches. A number of approaches have been tried in the search for *Pythium* species-specific probes. DNA restriction fragments of the mitochondrial chromosome of *P. oligandrum* and *P. sylvaticum* provided probes with varying levels of isolate and species specificity (Martin 1991). The restriction fragment probes derived from the amplified of the internal transcribed spacer (ITS1) of *P. ultimum* var. *ultimum*, tested against 24 *Pythium* species, had a high degree of species specificity, however recognized not only *P. ultimum* var. *ultimum* but also *P. ultimum* var. *sporangiiferum* and several strains of *Pythium* group G (Lévesque 1994). A DNA probe, representing a repeated sequence, isolated from a genomic library of *P. irregulare* was tested for reactivity with 13 *Pythium* species and recognized *P. irregulare* and *P. spinosum* (Matthew et al. 1995).

The results presented in our study indicate that amplifing the 5S rRNA intergenic spacer is a good source of probes for rapid identification of species that have 5S genes in tandem arrays. Even though the presence of SL and SR sequences, which comprise both ends of 5S rRNA genes, in the probe and genomic target did not lead to background hybridization when conditions of maximum stringency were used. Sometimes amplification of the 5S spacer

produced more than one product. In these cases, specificity of the probes was achieved by separation and labeling of the most prominent DNA band. Many SI-SR amplification products indicate the existence of more than one version of the 5S spacer and therefore further analysis of PCR products is necessary before conclusions can be drawn about evolutionary relationships among isolates.

13 5S spacer probes presented in this study were species-specific because they recognized strongly the genomic DNA of the isolate from which the probe had been derived but not the genomic DNA from more than 90 other *Pythium* species. This suggests that 5S rRNA spacers between tandem gene repeats diverged rapidly after speciation.

Our experiments also tested a number of conspecific isolates for six probes. Screening more isolates from the species where only one was available for our study is necessary for a better evaluation of the specificity and the range of those probes. The *P. intermedium* probe recognized equally well 8 isolates of this species. The *P. sylvaticum* probe was sufficiently sensitive to detect 16 isolates of this species, however the degree of hybridization was highly variable, suggesting extensive intra-specific variation. In addition, the *P. sylvaticum* probe also recognized three isolates of *P. irregulare* and one isolate of *P. paroecandrum*. Because there are no major morphological differences between those three species, this finding indicates much doubt about the true identity of these isolates and suggests that their classification should be revised. This case will be discussed in more

detail when the isolate identification by the RFLP analysis of the large IGS region is presented.

In the case of *P. macrosporum*, only two additional isolates were available; one reacted weakly, and the other reacted strongly with the probe. When the 5S spacer of *P. macrosporum* were amplified and used as a target for the probe, the isolate that reacted weakly when its genomic DNA was used as a target, reacted strongly when its PCR product was used. This result can be partially explained by the lower abundance of the 5S spacer target in the genomic DNA because this isolate hybridized more weakely with the 5S gene probe. We can also not exclude the possibility that there are many more 5S genes than spacers in this isolate or that there are different variants of 5S spacer sequence which do not hybridize with our *P. macrosporum* probe.

The case of *P. ultimum* var. *ultimum* and *P. ultimum* var. *sporangiferum* is also complex. The *P. ultimum* var. *ultimum* probe strongly recognized 19 isolates of this species and two isolates of *P. ultimum* var. *sporangiiferum*. The other two isolates of *P. ultimum* var. *sporangiferum* were recognized by the *P. ultimum* var. *sporangiiferum* probe derived from the type culture. These results, although they can be useful for the identification of certain isolates, may not eliminate the existence of the close relationship between the two varieties of *P. ultimum* because the *P. ultimum* var. *sporangiferum* probe was derived from one particular band in the PCR

product. Further analysis of the other versions of the 5S rRNA spacer may reveal sequence elements common to these two varieties of *P. ultimum*.

Generally, our results demonstrate that the 5S spacer region is usually sufficiently homogenous within a species to allow recognition of examined species by one probe. Such homogeneity in repeated gene families can be attributed to concerted evolution acting within species (Coen et al. 1982, Long and David 1980).

Although the intergenic spacers between 5S genes can serve as species-specific hybridization probes, the presence of microsatellite DNA that consists of stretches of monotonously repeated short nucleotide motifs can cause lack of species specificity for some probes. Simple sequences are widely interspersed in the genomes of all eukaryotes including fungi (Tautz and Renz 1984). These types of sequences consist of mono, di, or tri-nucleotide motifs, which are tandemly repeated (Edwards et al. 1991). The occurrence of simple sequences in eukaryotes may depend on the frequency of accidental amplifications and deletions and the mechanisms that spread the sequences in the genome. The mechanism of concerted evolution can allow simple sequences to spread from one spacer to the neighboring spacers of ribosomal RNA multigene families. Of the many permutations of simple sequences one can generate, the $(GT/CA)_n$ repeats, in particular, have been thought to be the most abundant in eukaryotic genomes (Dietrich et al. 1992, Wu et al. 1993).

Probing of 5S spacers from more than 15 *Pythium* species with the $(GT)_9$ simple repeat probe demonstrated the presence of the (GT/CA) arrays within the IGS region from species which probes demonstrated lack of specificity. In the case of the *P. boreale* and *P. rostratum* probes, the masking of a simple sequence interspersed within the genomic DNA by using the probe together with the $(GT)_{40}$ repeat motif in the hybridization process greatly improved the specificity of those probes. The presence of weak cross hybridization, especially in the case of *P. rostratum*, may be a reflection of the presence of different types of simple sequences that were not masked by the $(GT)_{40}$ array. DNA sequence analysis of the IGS region of *P. rostratum* and identification of other microsatelites can help in further enhancing of the probe specificity.

Probing of genomic DNA with the 5S rRNA spacer is useful not only for identification of *Pythium* isolates to species level but also allows for detection of close relationships between species. Molecular based relationships should be consistent with species affinities that can be inferred from morphology.

A strong cross-hybridization signal was observed between the following species: *P. buismaniae, P. polymastum and P. uncinulatum*, which also share similarity in morphology. These three species develop globose, terminal oogonia on short side branches. The oogonial wall is ornamented with conical spines. Development of sporangia seems to be the most significant difference. *P. buismaniae* has never been observed to produce sporangia (Drechsler 1939). *P.*

polymastum and *P. uncinulatum* develop globose sporangia, elipsoidal or irregularly shaped, terminal or occasionally intercalary. The presence of antheridia is another common characteristic for these three species. *P. buismaniae* and *P. polymastum* form 1-4 antheridia per oogonium, which are mostly diclinous, variously shaped and borne on one antheridial stalk, which has a tendency to form lobate or diverticulate projections with hyphal knots. *P. uncinulatum* produces 1-8 antheridial cells per oogonium. Antheridia are also diclinous and form a complicated knot consisting of swollen branches and diverticles around the oogonium (Van der Plaats-Niterink 1981). Thus, the three species share not only the same oogonial ornamentation but also diclinous antheridia, lobate or with diverticulate projections.

On the basis of cross-reactivity, three other species - *P. oligandrum*, *P. amasculinum* and *P. hydnosporum* - appear to be related. Moreover, these species have a important morphological characteristic in common - oogonia with similar ornamentation of long slender spines. The development of sporangia and antheridia appears to be the only significant difference. Zoospore production has only been observed in *P. oligandrum*. This species develops "contiguous" sporangia, irregularly globose structures connected with hyphal parts. Although *P. amasculinum* has never been observed to produce zoospores (Yu 1989), it develops hyphal swellings very similar to sporangia produced by *P. oligandrum*. It has been believed that *P. hydnosporum* does not produce zoospores,

sporangia or hyphal swellings (Butler 1907, Middleton 1943, Van der Plaats-Niterink 1981). However, an illustration of this species presented by Van der Plaats-Niterink (1981) clearly shows contiguous structures with hyphal swellings which are called "sporangia". Thus, the three species seem to share the important character of contiguous structures, which may or may not produce zoospores. Another major difference among these species seems to be the presence or absence of antheridia. No antheridia have been observed in *P. amasculinum*. *P. oligandrum* very rarely produces antheridia which are diclinous, adhering lengthwise to the oogonium and appearing lobate. In *P. hydnosporum*, normally hypogynous antheridia are present (Butler 1907, Middleton 1943, Van der Plaats-Niterink 1981). However, the first illustration of this species (Barry 1876) does not show the presence of hypogynous antheridia. It is also possible that the hypogynous antheridia of *P. hydnosporum* is a misinterpretation of the oogonial stalk (de Cock, personal communication).

The hybridization results indicate the close relationship of *P. irregulare*, *P. mamillatum*, *P. spinosum*, *P. kunmingense*, *P. paroecandrum* and *P. cylindrosporum*. The strong level of cross-hybridization between those species allowed us to segregate them to one *P. irregulare* 5S spacer homology group. There are no major morphological differences that would preclude a close relationship between those species. *P. spinosum* and *P. kunmingense* have not been observed to produce zoospores, however zoospore production does not seem to be the important

taxonomic characteristic. Sporangia are not or only rarely produced, except in *P. mamillatum*, where they normally have been reported. If sporangia are present, they are always globose and non-proliferous. If they are not present, globose hyphal swellings have been observed, which are morphologically similar to the sporangia but do not produce zoospores. A major difference among those species seems to be the amount of ornamentation on the oogonium. *P. spinosum* and *P. mamillatum* have many papillae on each oogonium, however in both species the number of spines per oogonium is variable and they may be completely absent on some oogonia in *P. mamillatum* (Van der Plats-Niterink 1981). *P. irregulare* and *P. kunmingense* have one or a few spines; in some isolates spines can be absent. Oogonium ornamentation has not been reported for *P. paroecandrum* and *P. cylindrosporum*. However, one or a few digitate spines may be present on a few oogonia in those species (de Cock, personal communication).

The use of PCR-based identification of plant pathogenic fungi has many advantages over the use of traditional taxonomy and other molecular identification schemes. PCR is primer directed, and as such, the primers can be designed to recognize highly conserved sequences in the 5S rRNA genes and amplify 5S spacers from distantly related organisms. PCR can be performed on very small biological samples. In addition, nonradioactive methods of detection make the identification safer and easier to use. Finally, the use of 5S rRNA spacer probes may provide sensitive tools that can be useful aids in rapid, reliable

identification of organisms having tandem repeat families, including plants and animals. This approach may lead to a more objective and quantitative criterion for species delimitation.

Pythium irregulare 5S spacer homology group

One of the challenges of trying to use molecular approaches to improve plant and fungal systematics is to find a molecular system that gives just the right level of discrimination between genotypes when comparisons are made within or between taxa. For a system to be useful it should not be too sensitive, otherwise it is difficult to interpret the results because of the difficulty in recognizing homologies. Alternatively, if it is not sensitive enough, then it may not produce sufficient numbers of useful markers. Consequently, different systems are suitable for different purposes. For instance, the use of ribosomal RNA sequences is appropriate for the study of relationships between kingdoms and phyla. However, the 5S gene sequence data base has been suggested to have a limited use for phylogenetic estimation (Halanych 1991, Steele et al. 1991). This conclusion was based on a small number of phylogenetically informative sites over a diverse range of genera and a high rate of change at those positions that were free to vary. On the other hand, the 5S spacer region has been found to be suitable for phylogenetic analysis of the *Triticeae, Rosidae, Poaceae* and *Brassicaceae* (Steele et al. 1991). The spacer sequences vary much more than gene sequences because there are many fewer functional constraints conferring selective disadvantage upon them. Some areas of the spacer were identified as hot spots for variation whereas other areas were more conserved (Scoles et al. 1988, Appels and Baum 1991, Sastri et al. 1992).

We showed that in a number of *Pythium* species that have 5S rRNA genes in tandem arrays, the spacers between those genes diverge very rapidly after speciation. Therefore, in many cases the spacer can serve as a species specific hybridization probe. In general, the spacer sequence is variable between species, but it is only conserved within species. Lack of the spacer divergence within species is assumed to be due to processes such as unequal crossing over (Smith 1976) and gene conversion (Jeffreys 1979), which tend to homogenize multigene families, such as the 5S gene family, as long as gene flow or gene exchange is maintained in a population. This form of sequence conservation allows for the transfer of mutations among the members of the family and for the spread of mutations to all individuals in the population. Thus, observed homogenity of 5S rRNA spacers could be an indicator of gene flow or gene exchange and could be used to set species boundaries.

In *Pythium* there been over 130 described species, most of them homothallic, or self-fertilizing. Isolates are assigned to species mainly on the basis of morphological characters, most of which are zoosporangial or oogonial features (Van der Plaats-Niterink 1981, Dick 1990). Sets of characters that define species often overlap between species, or may be incomplete for any particular isolate due to loss of reproductive structures. Obviously, species boundaries need to be confirmed or redrawn on the basis of genetic characters. Because the 5S rRNA spacers diverge rapidly after speciation but are subject to homogenization

before speciation, they can give rise to a sharp discontinuity at species boundaries. Probing of genomic DNA with the 5S spacer allowed us to detect groups of morphologically related species that cross-hybridized together, indicating that those species are in the group that exchanges genes.

Comparison of 5S spacer sequences

To further explore the degree of relationship among species of the *P. irregulare* cluster, their 5S rRNA spacer sequences were analyzed. The 5S spacers of *P. spinosum* CBS 275.67 and *P. cylindrosporum* CBS 218.94 were amplified with primers SEL and SER and then the products were cloned into the *Eco*RI site of Bluescript M13 (Stratagene) and sequenced. The 5S IGS region of *P. irregulare* CBS 250.28, *P. mamillatum* CBS 251.28, *P. kunmingense* CBS 550.88, *P. paroecandrum* CBS 157.64 and *P. polymorphon* CBS 751.96 were amplified with primers SBL and SBR and amplicons were cloned into the *Bam*HI site of Bluescript M13 (Stratagene). The inserts were sequencing in both directions. The 5S spacers of *P. sylvaticum* CBS 252.67 and *P. intermedium* CBS 266.38 were also amplified with primers SBL and SBR and the products cloned into the *Bam*HI site of Bluescript M13 (Stratagene). These two species were chosen as outgroups because phylogenetic analysis inferred from the partial sequences of 28S RNA genes showed that *P. irregulare* is related most closely to *P. sylvaticum* and *P. intermedium* (Briard 1995).

For *P. spinosum, P. kunmingense* and *P. sylvaticum,* amplification of the 5S rRNA spacer produced two prominent amplicons. In each case both amplicons were cloned and sequenced to yield "long" and "short" versions of the 5S spacer. For *P. spinosum* and *P. kunmingense*, the main structural difference between long and short versions was that the short version lacked the "A repeat" region (Fig 15). For *P. sylvaticum*, the difference was more profound.

The sequences were manually aligned as shown in Fig. 15 The "termination block" immediately downstream of the 5S gene is well conserved but variable in length. It contains a long T-tract, presumably the information for transcription termination. The two repeat regions, A and B, that follow the termination block, are separated by a variable region very rich in tracts of T, similar to the termination block. Repeat A, together with its termination-like block, is missing in the "short" versions of *P. spinosum* and *P. kunmingense.* The large central block is well conserved but its end is more variable and cannot be aligned. The "insertion" region that follows is absent in *P. irregulare, P. polymorphon, P. paroecandrum* and *P. cylindrosporum.* Length variation present, particularly in the first part of this region, is mainly caused by a variable length of tracts of T. The 90 bp region upstream of the 5'end of the gene, called the "initiation block", is relatively well conserved. Its last 11 bp are identical. There is no evidence that it functions in the initiation of transcription.

Figure 15. 5S IGS sequence alignment for seven *Pythium* species.

Dots indicate identities and dashes indicate introduced gaps.

5S gene

Termination Block

Repeat A

Repeat B

Central Conserved Block

Variable Regions

Central Insertion

Initiation Block

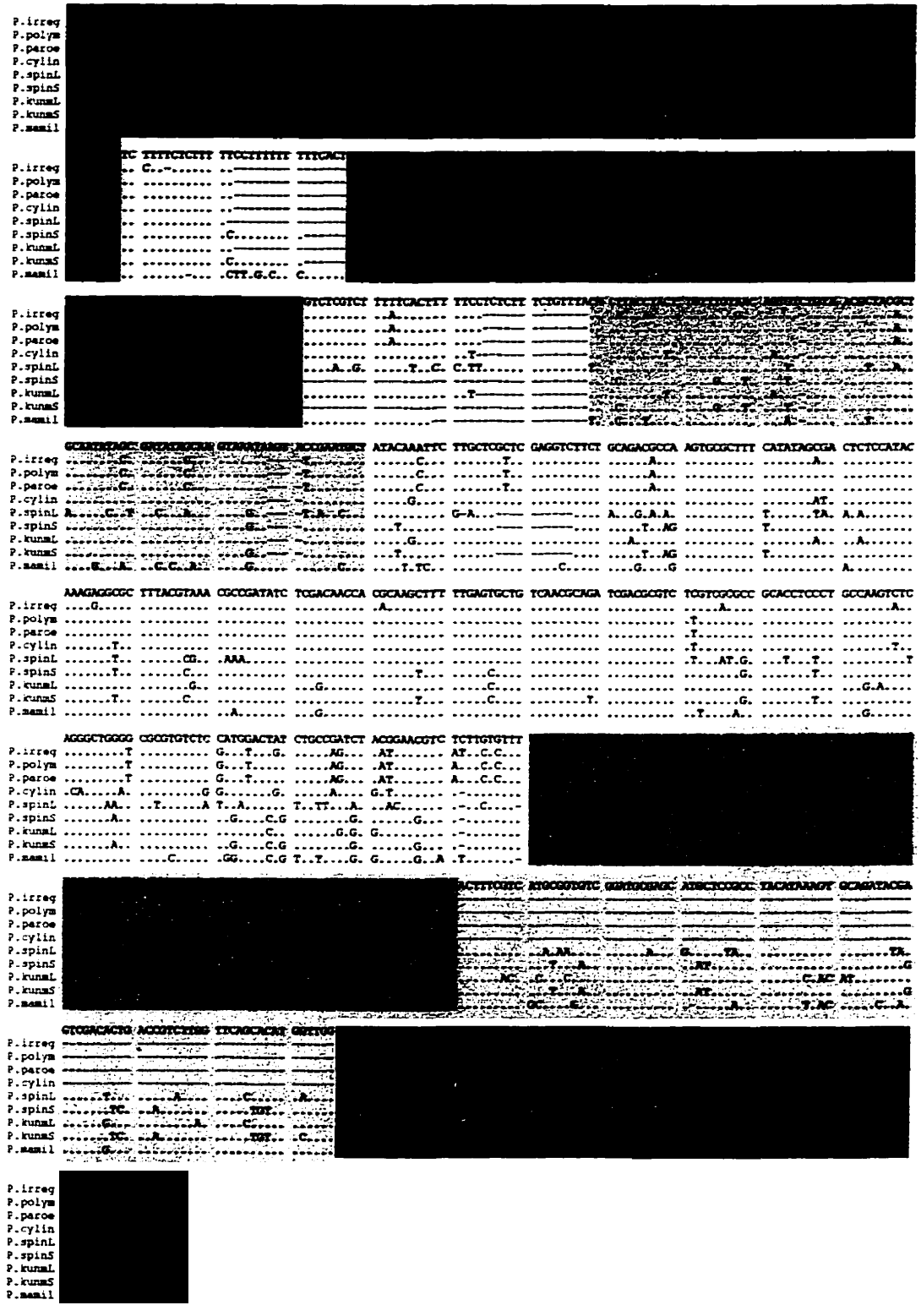
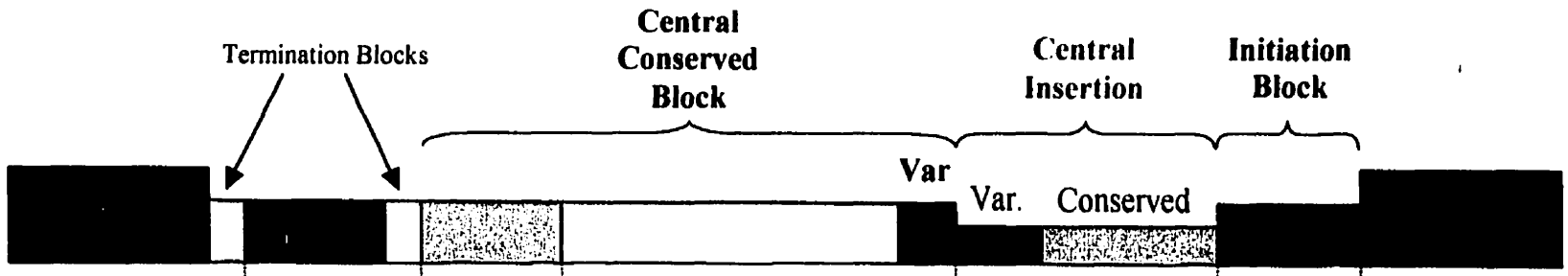

P.irreq
P.polym
P.paroe
P.cylin
P.spinL
P.spinS
P.kunmL
P.kunmS
P.mamil

Figure 16. The structure of the 5S rRNA spacer of *P. irregulare* homology group.



P. irregulare	YES	YES	YES	NO	YES
P. polymorphon	YES	YES	YES	NO	YES
P. paroecandrum	YES	YES	YES	NO	YES
P. cylindrosporum	YES	YES	YES	NO	YES
P. spinosum (long)	YES	YES	YES	YES	YES
P. spinosum (short)	NO	YES	YES	YES	YES
P. kunmingense (long)	YES	YES	YES	YES	YES
P. kunmingense (short)	NO	YES	YES	YES	YES
P. mamillatum	YES	YES	YES	YES	YES

The DNA sequence data presented above shows that the major size difference in the 5S IGS of *P. irregulare* complex is the absence of the 104 bp repeat A and the presence of the "insertion" region (Fig 16). The absence of the repeat A accounts for the size difference between the "short " and "long" versions of *P. spinosum* and *P. kunmingense*. In contrast the "central insertion" is present only in the "short " and "long" versions of *P. spinosum* and *P. kunmingense* and in *P. mamillatum*.

The 5S spacer sequences of 7 species of *P. irregulare* homology group were compared with the 5S spacer sequences of *P. sylvaticum* and *P. intermedium* using "CLUSTALW" multiple sequence alignment analysis. *P. sylvaticum* and *P. intermedium* have only 40 and 39 score of homology with other members of the *P. irregulare* cluster, respectively. In contrast, the homology score for the members of the *P. irregulare* group is 78, whereas in the conserved regions they have 89 homology score. When the consensus sequence of the 5S spacer homology group was compared with the 5S spacer sequences of *P. sylvaticum* and *P. intermedium* using "PRDF" analysis that compares a test sequence to a shuffled sequence, unshuffled optimum score was 84 and 65 respectively, whereas shuffled score ranged from 43 to 82 and from 50 to 108, respectively. For comparison, the unshuffled optimum score for the 5S spacers of *P. irregulare* and *P. polymorphon*, which demonstrate 97 homology score, was as high as 3152, whereas the shuffled score ranged from 48 to 242. This results indicate that 5S

spacers of *P. sylvaticum* and *P. intermedium* are unalignable to the 5S IGS region of the *P. irregulare* group. This lack of DNA sequence homology between the members of the *P. irregulare* cluster and *P. sylvaticum* and *P. intermedium,* shows a sharp discontinuity between the *P. irregulare* group and other species that are consider to be closely related to them.

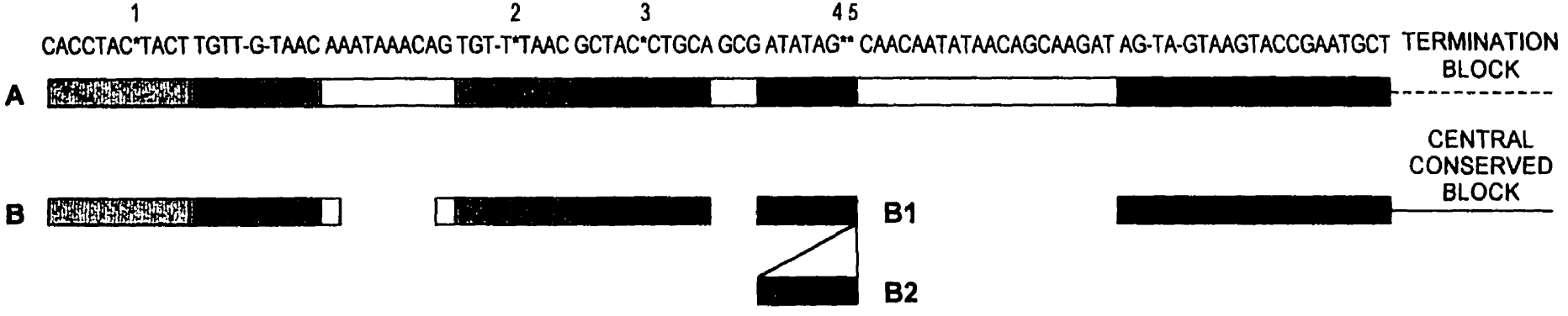
Structure of the repeated regions

Segments A and B are repeats within the 5S IGS repeats. Comparison of A and B reveals at least one more level in the hierarchy of repeats (Fig. 17). The second block (TGTT-G-TAAC) is an imperfect repeat, which occurs twice in tandem in B and is separated by an insertion in A. The fourth block (ATATAG**) occurs once in A and twice in B. The first block (CACCTAC*TACT) could be a triple repeat with a consensus of TAC*. The last insertion (CAACAATATAACAGCAAGAT), present only in A, contains three copies of the CAA motif, suggesting a lower level in the hierarchy of repeats within repeats. These results suggest that the repeat region of the 5S spacer is subject to frequent recombination events or replication slippage which result in internal duplications and deletions.

The consensus sequence for the repeat regions contain 5 positions that are informative with respect to the relationships between the repeats and between internal repeated regions. These informative sites show that consistencies between species are greater than consistencies between A and B versions within a

species. For example, the first informative site represents an almost consistent difference between the A and B repeat. The repeat A has "T "in all eight sequences, except *P. parvicandrum,* where C is present, but repeat B has "C "in all seven sequences in this position. The fourth informative site, which is the first informative site in the fourth block (ATATAG**) has "T" in the repeat A in all eight sequences, except *P. mamillatum,* which has "C", and consistently "C" in both repeats B1 and B2. The fifth informative site, which is the second informative site in the fourth block consistently has "A" in the repeat A, "G" in the B1 repeat, and again "A" in the B2 repeat.

Figure 17. Structure of the repeated regions within the 5S IGS of the *P.irregulare* group based on the alignment in Fig. 15.


1
2
3
4 5
CACCTAC*TACT TGTT-G-TAAC AAATAAACAG TGT-T*TAAC GCTAC*CTGCA GCG ATATAG** CAACAATATAACAGCAAGAT AG-TA-GTAAGTACCGAATGCT
TERMINATION BLOCK
A
CENTRAL CONSERVED BLOCK
B
B1
B2

Phylogenetic analysis of the *P. irregulare* cluster

The topology and branch lengths of the phylogenetic tree relating 7 *Pythium* species in the 5S spacer homology group to each other was evaluated by using the Neighbor-Joining (NJ) method (Saitou and Nei, 1987). Parsimony analysis (DNAPARS, PHYLIP 3.1) was also used to investigate the tree topology. In the most parsimonious tree, confidence limits for branches were estimated by bootstrap analysis with 100 iteractions (SEQBOOT, PHYLIP 3.1) For NJ, the distance matrices of Kimura were used (Table 3). Phylogeny was inferred from sequence data of the central conserved block plus the initiation block. A total 439 nucleotide positions were included in the analysis.

In the 5S spacer tree, *P. polymorphon* and *P. irregulare* are very closely related (the bootstrap value = 100) (Fig 18 and 19). Moreover, these two species have very low nucleotide distance values equal to 0.026 (Table 3). Phylogenetic analysis also indicates close relationship between *P. cylindrosporum* and the *P. polymorphon* - *P. irregulare* cluster (the bootstrap value = 96) (Fig 19). Significant intra-specific sequence variation was present in *P. spinosum* and *P. kunmingense*. Distance values were estimated between the long and short version of *P. spinosum* and *P. kunmingense* (distance values are equal 0.171 and 0.103 respectively) (Table 3). In both cases, the single isolate has two versions that are not closely related to each other compared with spacers in other species. Both short versions of *P.*

spinosum and *P. kunmingense* are very similar; they have the smallest nucleotide distance value: 0.0025 (Table 3).

Table 3. Nucleotide distances between seven *Pythium* species of the *P. irregulare* homology group.

Distance values were inferred from sequences of the central conserved block plus the initiation block using the two parameter model of Kimura (1980). Values are expressed as $d \times 100$.

	P. irreg	P. polym	P. paroe	P. cylin	P. spin(L)	P. spin(S)	P. kunm(L)	P. kunm(S)	P. mamil
P. irreg	0.00	2.65	20.24	12.72	23.00	17.87	13.18	18.22	20.75
P. polym	2.65	0.00	18.13	11.28	20.50	15.56	11.43	15.90	19.03
P. paroe	20.24	18.13	0.00	16.76	21.91	15.43	14.56	15.78	18.93
P. cylin	12.72	11.28	16.76	0.00	21.57	13.66	7.51	13.99	17.55
P. spin(L)	23.00	20.50	21.91	21.57	0.00	17.07	19.54	17.41	21.12
P. spin(S)	17.87	15.56	15.43	13.66	17.07	0.00	9.95	0.25	13.96
P. kunm(L)	13.18	11.43	14.56	7.51	19.54	9.95	0.00	10.25	13.70
P. kunm(S)	18.22	15.90	15.78	13.99	17.41	0.25	10.25	0.00	14.28
P. mamil	20.75	19.03	18.93	17.55	21.12	13.96	13.70	14.28	0.00

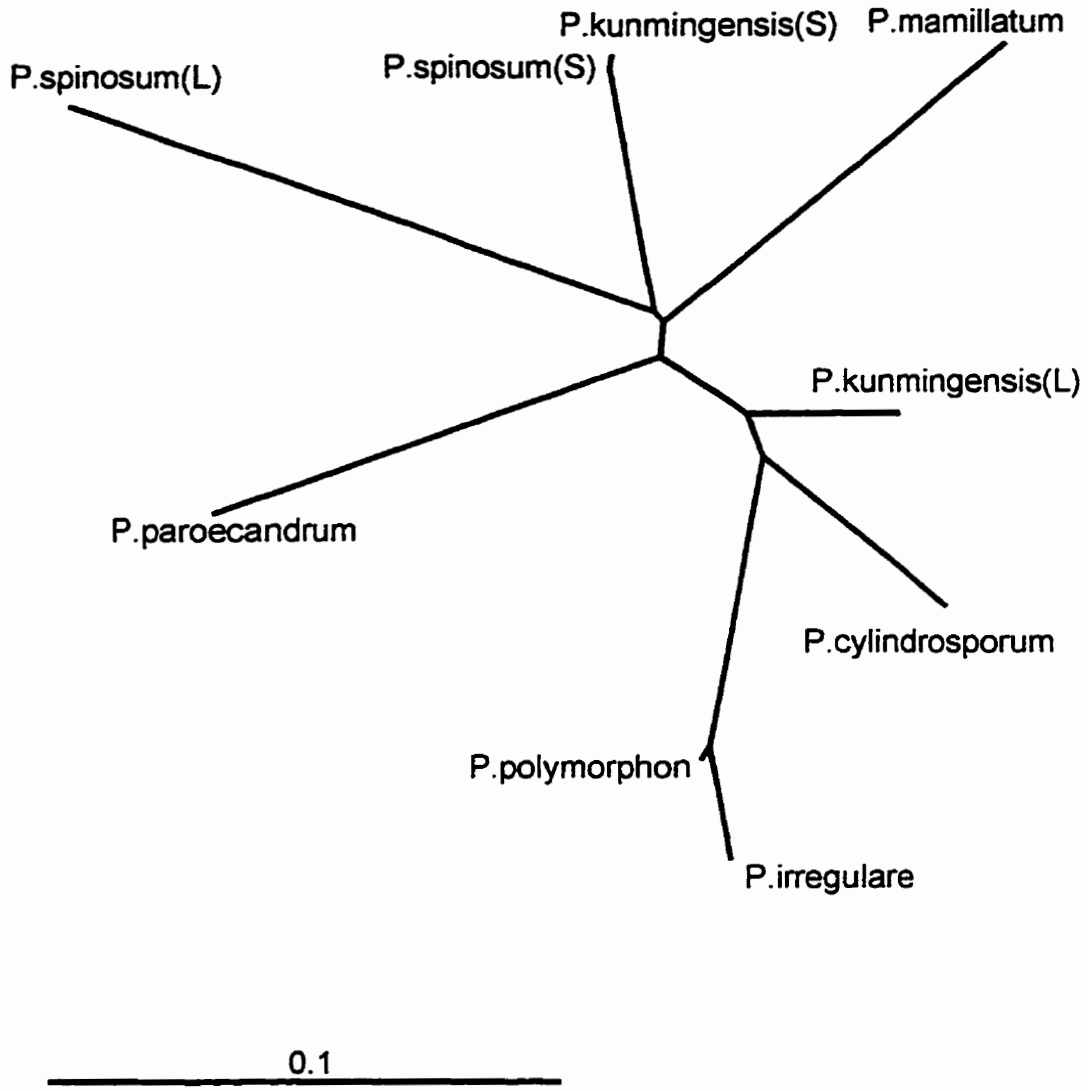


Figure 18. Unrooted phylogenetic tree for the seven *Pythium* species aligned in Fig 15.
The tree was inferred from the sequence data of the central conserved block plus the initiation block (439 bp). The relative genetic distances were estimated by the N-J method using the Kimura distance values.

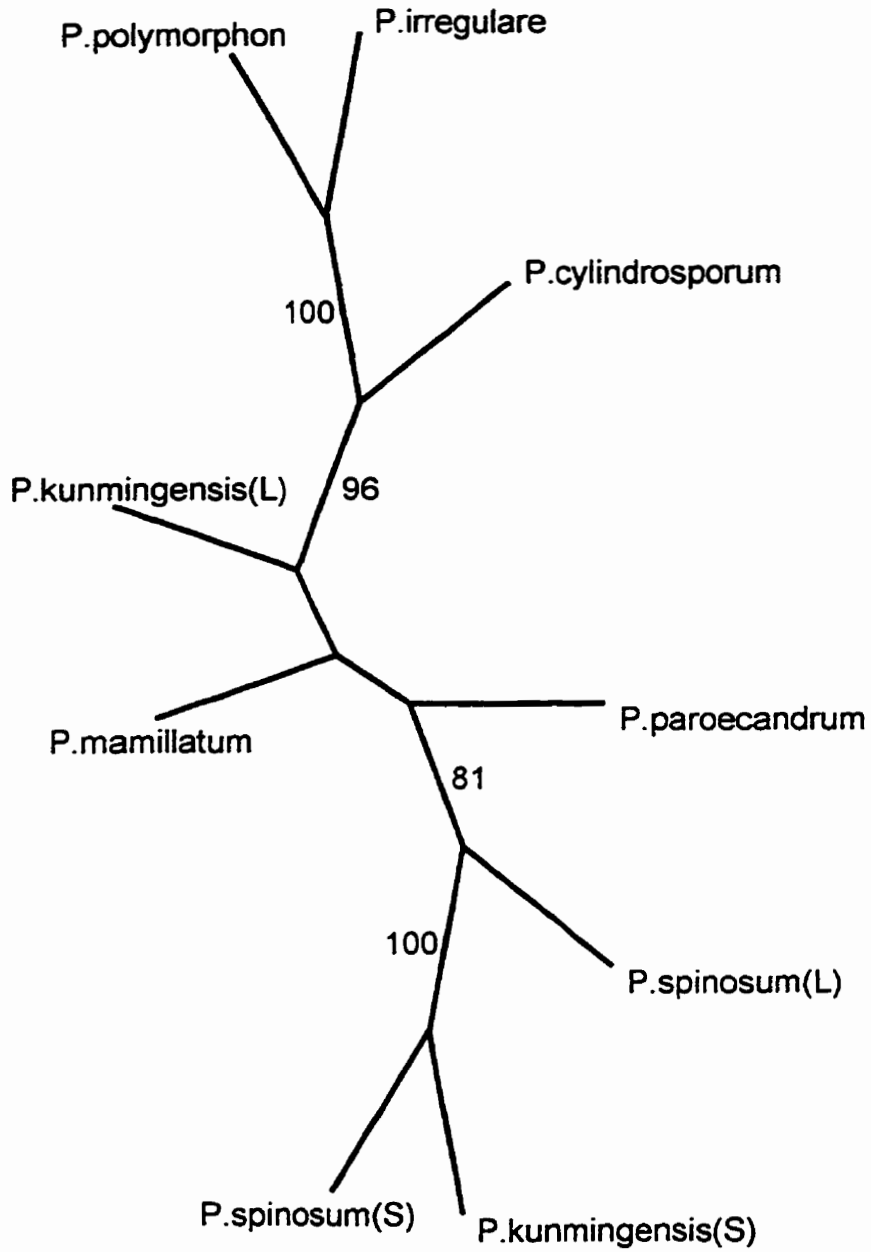


Figure 19. Most parsimonious phylogenetic tree for the seven *Pythium* species aligned in Fig. 15.
The tree was inferred from the sequence data of the central conserved block plus the initiation block (439 bp). Confidence limits of branches were estimated by using bootstrap analysis. Bootstrap values were not indicated when less than 60% (not significant).

Discussion

The 5S spacer sequences in a group of 7 *Pythium* species, known here as the *P. irregulare* group, are organized in the same way and have a high level of sequence homology in conserved areas. Interspersions of highly conserved regions with divergent regions suggest that some areas are subject to stronger selective pressures than others. The organization of the 5S spacer region of the *P. irregulare* cluster is similar to that of the 5S rDNA units from Triticeae species. Our "termination block" that follows the 3' end of the 5S gene is similar to the 3'-downstream spacer region, a simple polymer sequence characterized by tracts of T's of varying length (Sastri et al. 1992). Our "TATA" motif which is present in both A and B repeats is also recognizable in most of the plant 5S spacers. However, the variable position of the "TATA" motif relative to the gene suggests that it is not equivalent to the "TATA" box found in the 5'-upstream regions of genes transcribed by RNA polymerase II (Sastri et al. 1992). The 90 bp region upstream from the 5'-end of the gene was defined in the Triticeae as the 5'-us region which is relatively more conserved than the rest of the spacer. Our "initiation block" is also well conserved and provides a clear cut-off for the occurrence of deletions. Although it has been suggested that this region may carry information for transcription initiation, conserved regions in the 5S spacer have not been found after aligning the available database from a broader spectrum of species. This indicates that the 5S rRNA gene alone contains the recognition

sequence for forming the transcription initiation complex for RNA polymerase III.

The changes that occur in the 5S spacer region sequences include point mutations, deletions and duplications. Inspection of these types of changes, especially the length variation in the T tracts of both "termination blocks" suggests that "slippage" during replication may be a common occurrence. The significance of this mechanism in the generation of a great deal of variability in the DNA is enhanced by the observation of length variation in the TCTCTC tract in the 5S spacer from *Pinus*, the length variation in the T tract in *Arachis* and a duplication in the gene region (CGAGAG) in the *Triticeae* as well as in *Oryza brachyntha* (Efstradiatis et al. 1980, Scoles et al 1988).

The degree of homogenization of 5S IGS populations appears to vary from species to species. Our results demonstrate that significant intra-specific variation exists in *P. spinosum* and *P. kunmingense*. Comparison of the sequence variation in two different versions (long and short) of the intergenic spacer in the same species strongly suggests occasional deletion and amplification events in the evolution of the 5S rRNA genes arrays. An ancestral 5S intergenic spacer may be subjected to a large deletion or duplication as a result of an unequal sister chromatid exchange or intrachromosomal recombination event. Subsequent amplification of the new version of the spacer would create a new tandem array of 5S DNA units differing from the original. Changes would then accumulate at

random in the tandem array by a number of mechanisms (discussed above) and these changes could be the source of the new version of the repetitive 5S unit array. The large distance value estimated between the "long" and "short" version of the 5S spacer from *P. spinosum* and *P. kunmingense* supports this hypothesis. Moreover, the sequence analysis of "long" and "short" 5S DNA units in the Triticeae also suggests that these different classes that occupy two different chromosomal locations have independent spacer evolution although they have concerted gene evolution. Phylogenetic analysis of *Acacia* species based on 5S DNA unit sequences also suggests that at least three classes of 5S DNA units that exist in *Acacia* do not evolve at the same rate (Playford et al. 1992). Although we do not have any information about the chromosomal location of the 5S gene arrays in *Pythium*, we can assume that the "long" and "short" version of the 5S spacer from *P. spinosum* and *P. kunmingense* have separate loci which do not evolve in concert.

It is of interest that although in each isolate of *P. spinosum* and *P. kunmingense* two versions are not closely related to each other compared with spacers in other species, both short versions are almost identical. This indicates that 5S repeat units are exchanged between examined isolates of *P. spinosum* and *P. kunmingense* faster than they can be homogenized within one isolate. This means that variation in the 5S spacer should be introduced into species at a level

high enough to partially counteract the effects of concerted evolution. Our hypothesis suggests that interspecific gene flow is required.

Generally our results show that 5S spacer sequence variation was low enough between species of the *P. irregulare* complex to cluster them together but was high between this group and the other *Pythium* species. The DNA element shared by the *P. irregulare* complex does not appear to be present in the genomic DNA of 85 other isolates representing 85 other species, judging from the failure of such DNA samples to cross-hybridize with probes made from the different versions of the common element. A few other species, such as *P. sylvaticum* and *P. intermedium*, that have sometimes been confused with members of the *P. irregulare* group, have 5S spacer sequences that are so different from those within the group that they cannot be aligned for comparison. There is thus a sharp discontinuity between the *P. irregulare* group and other isolates that could be closely related to them on basis of their morphology.

Molecular-based relationships between species of the *P. irregulare* group are consistent with species affinities that can be inferred from morphology. The seven species share several significant characteristics. They only rarely produce zoosporangia. Only in *P. mamillatum* are sporangia normally present. Zoosporangia, when produced, are always globose and non-proliferous. There is much variability within the group with regard to the amount of ornamentation on the oogonium, as discussed in a previous chapter.

A close relationship indicated between *P. irregulare* and *P. polymorphon* is consistent with their traditional morphological grouping. Several authors have concluded that *P. polymorphon* is identical with *P. irregulare* (Kouyeas 1964, Vaartaja 1967, Ahrens 1971, Van der Plaats-Niterink 1981). In the original description of *P. polymorphon*, oogonia are reported to be smooth, whereas oospores are either smooth or "echinulate" with a variable number of spines. This is most certainly a misinterpretation because the photographs show oogonia with digitate spines exactly like those in *P. irregulare* (de Cock, personal communication).

5S spacer distances support a close relationship among *P. cylindrosporum* and *P. irregulare* that is also consistent with their morphology. The original description of *P. cylindrosporum* is in excellent agreement with the description of *P. irregulare* by Van der Plaats-Niterink (1981). This is also in accordance with information from Arthur de Cock (personal communication) that the morphology of *P. cylindrosporum* is very similar to that of *P. irregulare*, including the presence of a few digitate spines on some oogonia.

The comparison of relationships based on the 5S spacer variability with those based on morphological characters provides evidence that the IGS regions are useful for investigating interspecific relatedness in the genus *Pythium*. There is no argument on morphological grounds that would make our grouping of seven *Pythium* species untenable. On the other hand, we have found no morphological

character state exclusive to our group, probably due to the limited number of such characters being used in taxonomy.

There are various definitions of species. The biological species concept emphasizes reproductive isolation. The taxonomic species concept defines species as a group of organisms resembling each other more than they resemble organisms outside the group. According to the evolutionary species concept, species are defined as lineages evolving separately from others. Molecular analysis that clusters organisms mainly on the basis of sequence data has tended to favor the evolutionary species concept. In many cases, reproductive isolation is difficult to confirm. Also for many species, variability in morphology within and between species and lack of discrete species characteristics do not allow for reliable identification. For *Pythium* taxonomy, the species concept is purly defined and depends essentially on authority. The sharp discontinuity seen in our molecular analysis may reflect the reproductive isolation of our *P. irregulare* 5S spacer homology group and may provide a way of testing the biological species concept with molecular data.

Species-specific polymorphism in the IGS of the *P. irregulare* rDNA cluster

In searching for the molecular characters that can be utilized in systematic studies, the appropriate molecular markers should be used to infer relationships among organisms at the desired taxonomic level. Restriction fragment length polymorphism (RFLP) analysis of the mitochondrial small subunit ribosomal RNA gene and the nuclear rDNA region containing the internal transcribed spacer (ITS) regions and the 5.8S rRNA gene have been used in determining relationships between fungal genera and species (Carder and Barbara 1991, Chen 1992, Chen et al. 1992, Manicom and Baayen 1994, Martin and Kistler 1990, Matsumoto and Fukumasa-Nakai 1995). Species-specific polymorphism in transcribed ribosomal DNA of eight *Pythium* species was reported and little intraspecific variation was observed (Chen 1992, Chen et al. 1992). The variation in restriction sites within the ITS regions was also determined for 36 *Pythium* species (Wang and White 1997). Inferred relationships based on RFLP analysis compared with those based on sporangial forms supported the division of the species with filamentous sporangia and those with globose sporangia into two main branches.

RFLP analysis of the ITS region, however, is unlikely to yield adequate resolution for closely related species due to its limited size and due to inclusion of highly conserved gene regions. We have also attempted to use the spacer between

5S rRNA genes in the tandem arrays as a source of markers for species definition (data not shown). Here, too, the size is limited (about 600 bp), and comparisons were frustrated by a high degree of intra-isolate heterogeneity in the size and sequence of the spacer, and the survey was aborted in favor of the approach described below.

The large intergenic spacer, extending for about 6000 bp from the 3' end of the large subunit gene to the 5' end of the small rDNA subunit gene appeared to contain a lower degree of heterogeneity (Fig. 20). Use of this region permitted the clear identification of individual strains after digestion with restriction endonucleases.

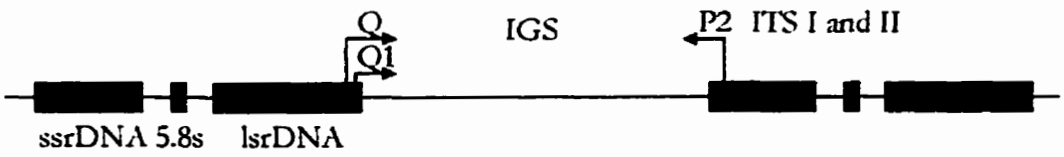


Figure 20. The rDNA repeat showing the location of the intergenic spacer region (IGS) amplified by primers Q or Q1 and P2.
ssrDNA = small subunit rRNA gene; lsrDNA = large subunit rRNA gene; ITS = internal transcribed spacer.

RFLP analysis of the large IGS region

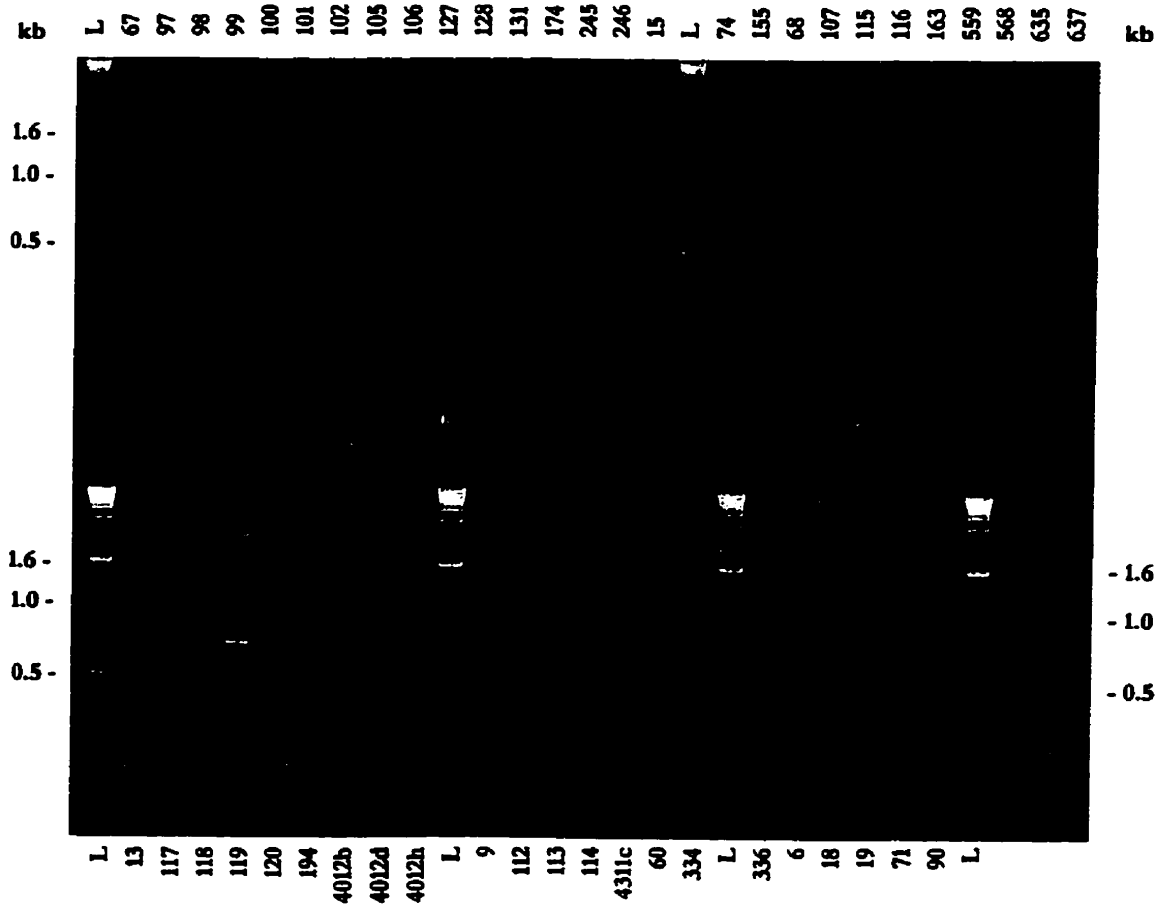
Amplification of IGS with primers Q and P2 was successfully carried out for each of the tested isolates of 7 species which belong to *P. irregulare* homology group, giving an amplification product of approximately 6000 bp that varied somewhat in size depending on the species. Isolates of *P. sylvaticum* and the type cultures for *P. intermedium*, *P. ultimum* var. *ultimum* and *P. ultimum* var. *sporangiiferum* were also included in the analysis. Five of the 45 isolates studied yielded two amplification products. In case of *P. sylvaticum* 115 (CBS 203.79) the additional amplification product was approximately 2800 bp long. For five isolates of *P. spinosum* (CBS 275.67, CBS 276.67, CBS 377.72 and APCC 4012a) the additional amplification products were approximately 800 bp long. The presence of multiple amplification products might indicate that additional priming sites are located within the intergenic spacer region. When the IGS region was amplified with primers Q1 and P2, no additional amplification products were observed.

Therefore, it is most likely that regions homologous to the Q region of the large subunit rRNA gene were present in IGS and accounted for the production of two amplification products. No length variation of the major 6000 bp amplicon was observed after running products on the 2% agarose gel. However, we can not exclude the presence of slightly shorter or longer versions of the IGS spacer that could not be separated on the agarose gel.

The IGS regions amplified with primers Q and P2 were subjected to digestion with 5 restriction endonucleases, namely *Hin*dII, *Taq*I, *Hin*fI, *Hpa*II and *Alu*I. Restriction enzymes *Rsa*I and *Hae*III were used for digestion of the IGS region amplified with primers Q1 and P2. Restriction digestion analysis of the amplified IGS region produced very similar restriction patterns for isolates of the same species that allowed them to be identified in cluster analysis. Restriction profiles for the 45 isolates of 11 species for seven enzymes assayed are shown in Figures 21 – 27.

Figure 21. Restriction digestion of *Pythium* IGS spacers (Q-P2 amplicons) with *Hin*dII.

Numbers correspond to reference numbers of *Pythium* isolates provided in Table1: *P. irregulare* 67, 97, 98, 99, 100, 101, 105, 106, 127, 131, 174, 245, 246, *P. sylvaticum* 15, *P. cylindrosporum* 74, 155, *P. paroecandrum* 68, 107, 115, 116, 163, 559, 568, 635, 637, *P. spinosum* 13, 117, 118, 119, 120, 194, 4012b, 4012d, 4012h, *P. mamillatum* 9, 112, 113, 114, 4311c, *P. kunmingense* 60, *P. polymorphon* 334, *P. debaryanum* 336, *P. intermedium* 6, *P. ultimum* var. *ultimum* 18, *P. ultimum* var. *sporangiiferum* 19, *P. acanthicum* 71, *P. hydnosporum* 90. Lane L, 1 kb ladder.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L 13 117 118 119 120 194 4012b 4012d 4012h L 9 112 113 114 4311c 60 334 L 336 6 18 19 71 90 L

Figure 22. Restriction digestion of *Pythium* IGS spacers (Q-P2 amplicons) with *Taq*I.

Numbers correspond to reference numbers of *Pythium* isolates provided in Table1: *P. irregulare* 67, 97, 98, 99, 100, 101, 105, 106, 127, 131, 174, 245, 246, *P. sylvaticum* 15, *P. cylindrosporum* 74, 155, *P. paroecandrum* 68, 107, 115, 116, 163, 559, 568, 635, 637, *P. spinosum* 13, 117, 118, 119, 120, 194, 4012b, 4012d, 4012h, *P. manillatum* 9, 112, 113, 114, 4311c, *P. kunmingense* 60, *P. polymorphon* 334. Lane L, 1 kb ladder.

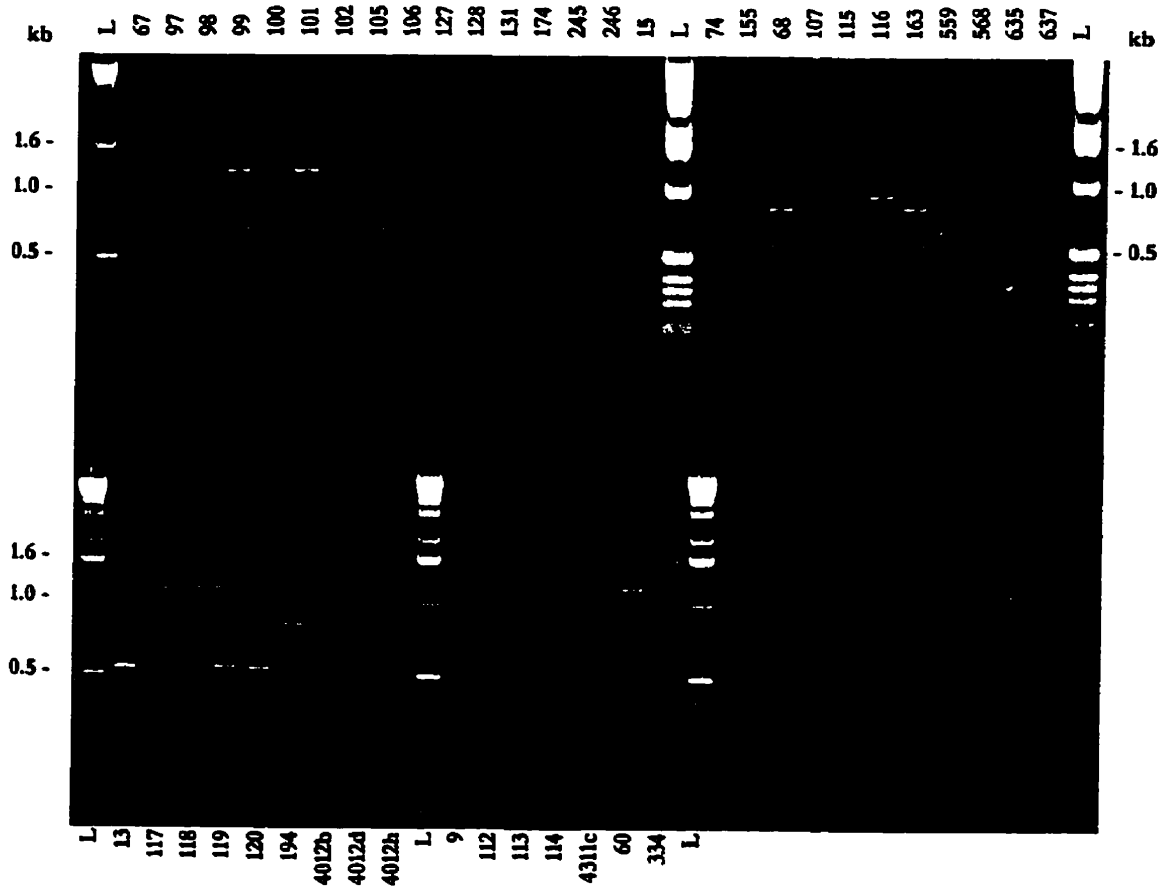
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Figure 23. Restriction digestion of *Pythium* IGS spacers (Q-P2 amplicons) with *Hinf*I.

Numbers correspond to reference numbers of *Pythium* isolates provided in Table1: *P. irregulare* 67, 97, 98, 99, 100, 101, 105, 106, 127, 131, 174, 245, 246, *P. sylvaticum* 15, *P. cylindrosporum* 74, 155, *P. paroecandrum* 68, 107, 115, 116, 163, 559, 568, 635, 637, *P. spinosum* 13, 117, 118, 119, 120, 194, 4012b, 4012d, 4012h, *P. mamillatum* 9, 112, 113, 114, 4311c, *P. kunmingense* 60, *P. polymorphon* 334, *P. intermedium* 6, *P. ultimum* var. *ultimum* 18, *P. ultimum* var. *sporangiiferum* 19, *P. acanthicum* 71, *P. hydnosporum* 90. Lane L, 1 kb ladder.

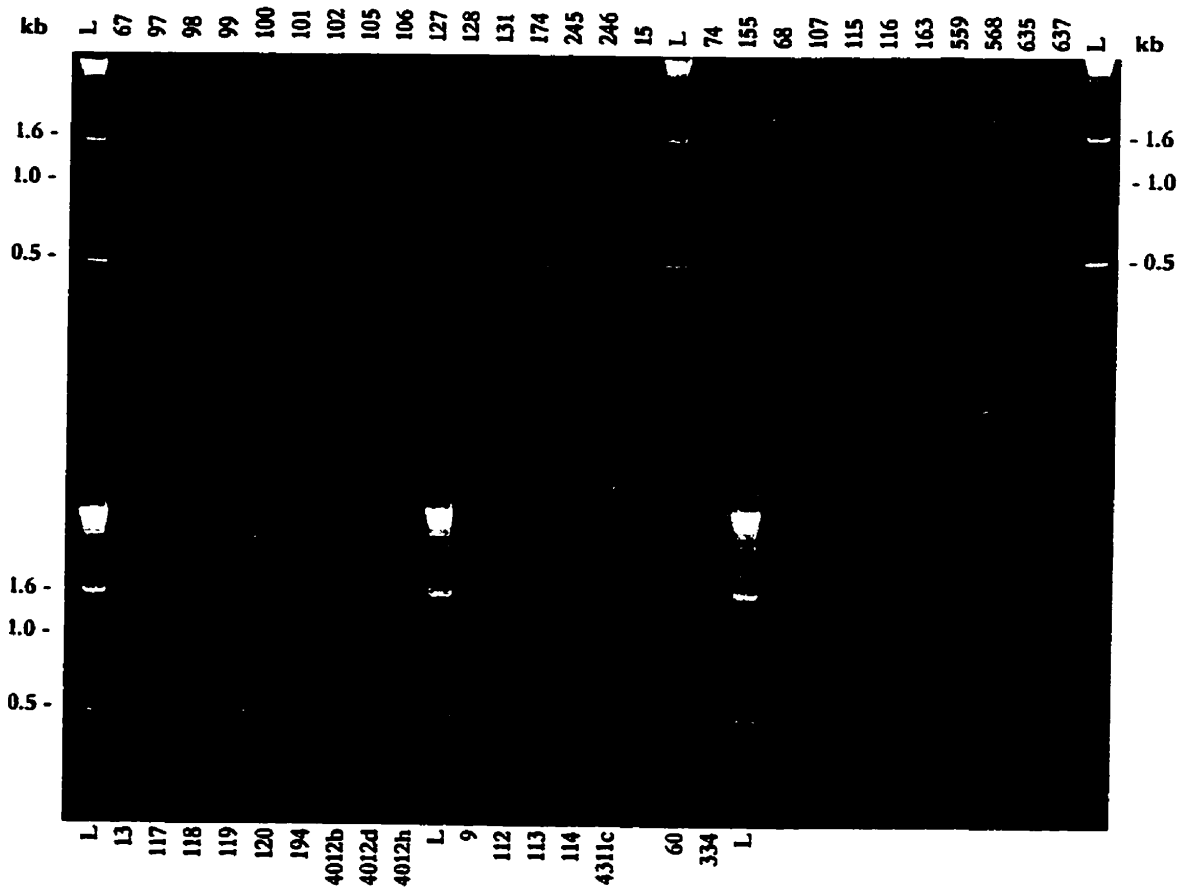
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Figure 24. Restriction digestion of *Pythium* IGS spacers (Q-P2 amplicons) with *Hpa*II.

Numbers correspond to reference numbers of *Pythium* isolates provided in Table1: *P. irregulare* 67, 97, 98, 99, 100, 101, 105, 106, 127, 131, 174, 245, 246, *P. sylvaticum* 15, *P. cylindrosporum* 74, 155, *P. paroecandrum* 68, 107, 115, 116, 163, 559, 568, 635, 637, *P. spinosum* 13, 117, 118, 119, 120, 194, 4012b, 4012d, 4012h, *P. mamillatum* 9, 112, 113, 114, 4311c, *P. kunmingense* 60, *P. polymorphon* 334. Lane L, 1 kb ladder.

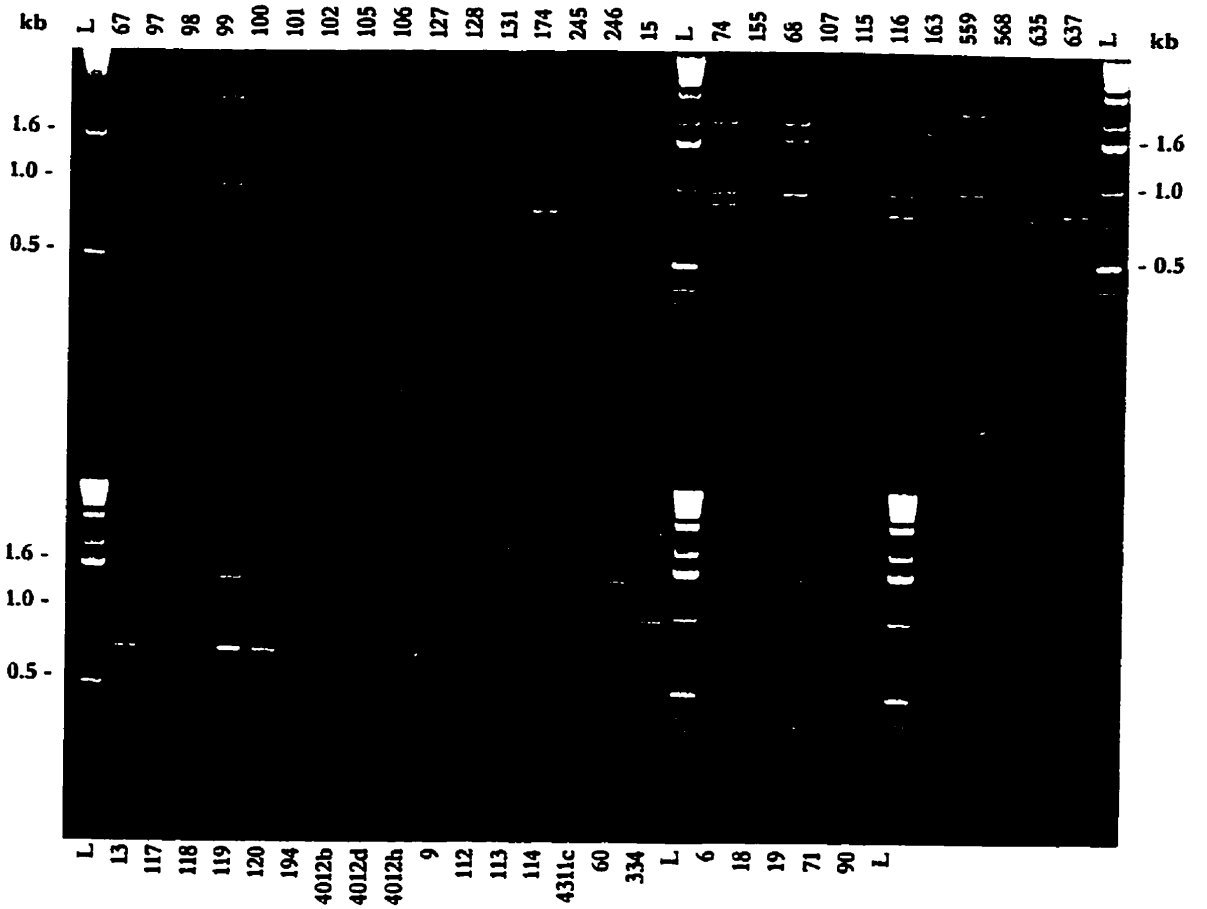
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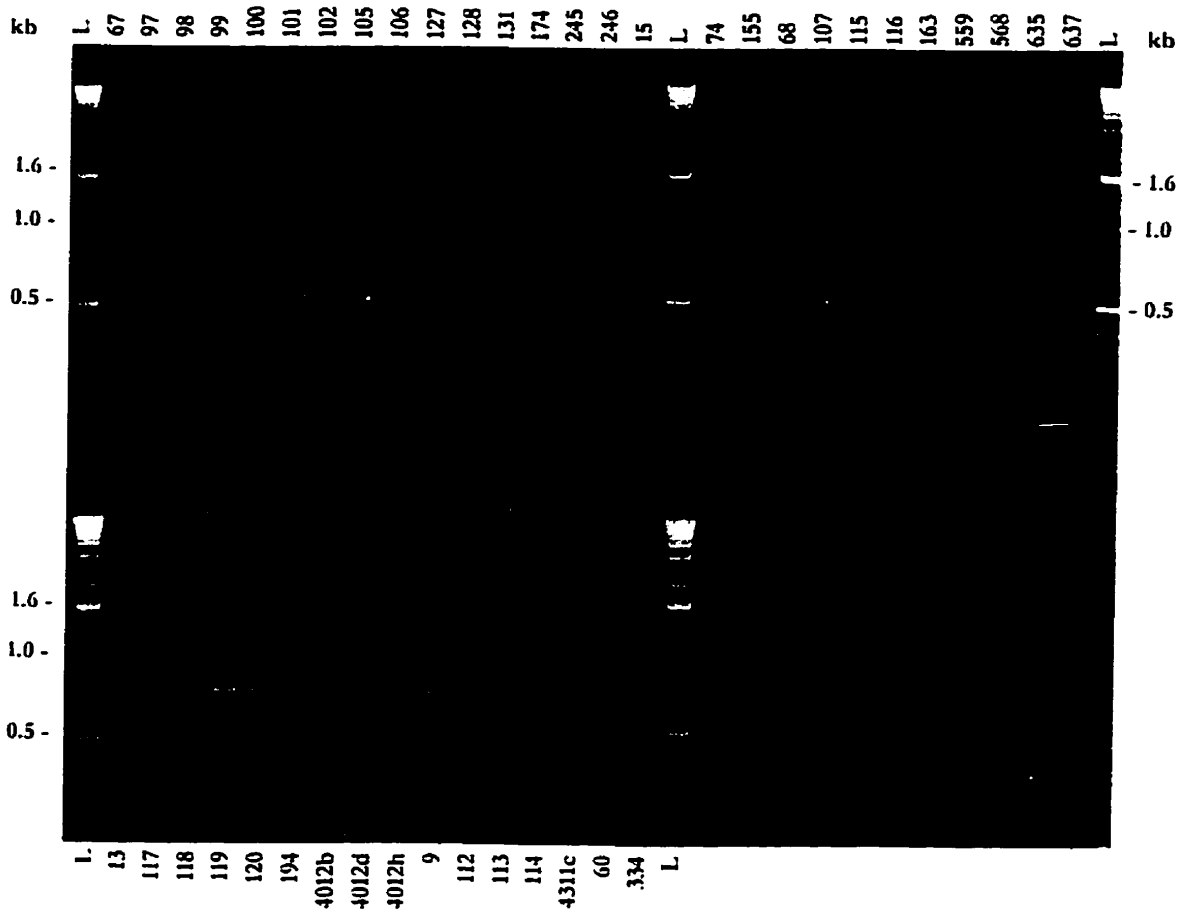
Figure 25. Restriction digestion of *Pythium* IGS spacers (Q-P2 amplicons) with *Alu*I.

Numbers correspond to reference numbers of *Pythium* isolates provided in Table1: *P. irregulare* 67, 97, 98, 99, 100, 101, 105, 106, 127, 131, 174, 245, 246, *P. sylvaticum* 15, *P. cylindrosporum* 74, 155, *P. paroecandrum* 68, 107, 115, 116, 163, 559, 568, 635, 637, *P. spinosum* 13, 117, 118, 119, 120, 194, 4012b, 4012d, 4012h, *P. mamillatum* 9, 112, 113, 114, 4311c, *P. kunmingense* 60, *P. polymorphon* 334. Lane L, 1 kb ladder.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4311c
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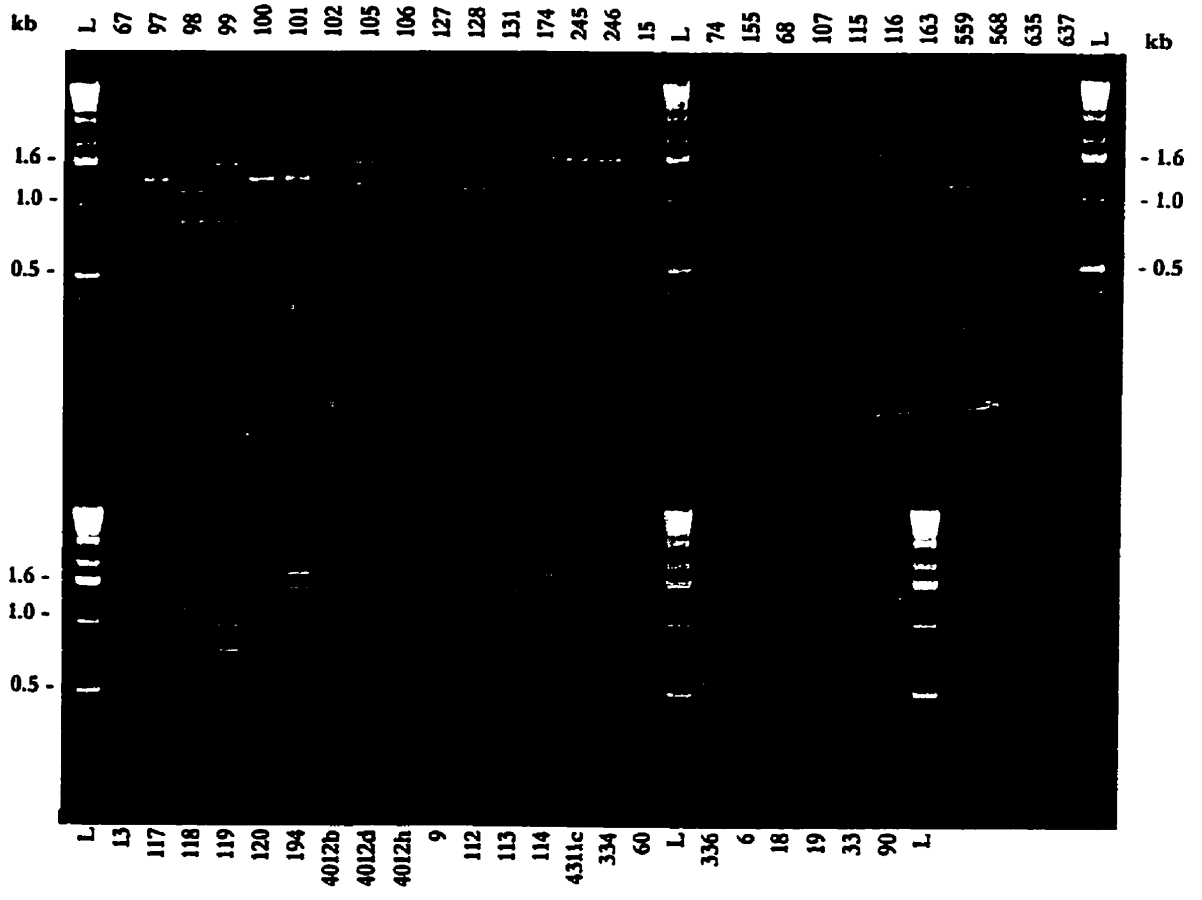
L

Figure 26. Restriction digestion of *Pythium* IGS spacers (Q1-P2 amplicons) with *Rsa*I.

Numbers correspond to reference numbers of *Pythium* isolates provided in Table1: *P. irregulare* 67, 97, 98, 99, 100, 101, 105, 106, 127, 131, 174, 245, 246, *P. sylvaticum* 15, *P. cylindrosporum* 74, 155, *P. paroecandrum* 68, 107, 115, 116, 163, 559, 568, 635, 637, *P. spinosum* 13, 117, 118, 119, 120, 194, 4012b, 4012d, 4012h, *P. mamillatum* 9, 112, 113, 114, 4311c, *P. kunmingense* 60, *P. polymorphon* 334, *P. deburyanum* 336, *P. intermedium* 6, *P. ultimum* var. *ultimum* 18, *P. ultimum* var. *sporangiiferum* 19, *P. echinulatum* 33, *P. hydnosporum* 90. Lane L, 1 kb ladder.


kb
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4012d
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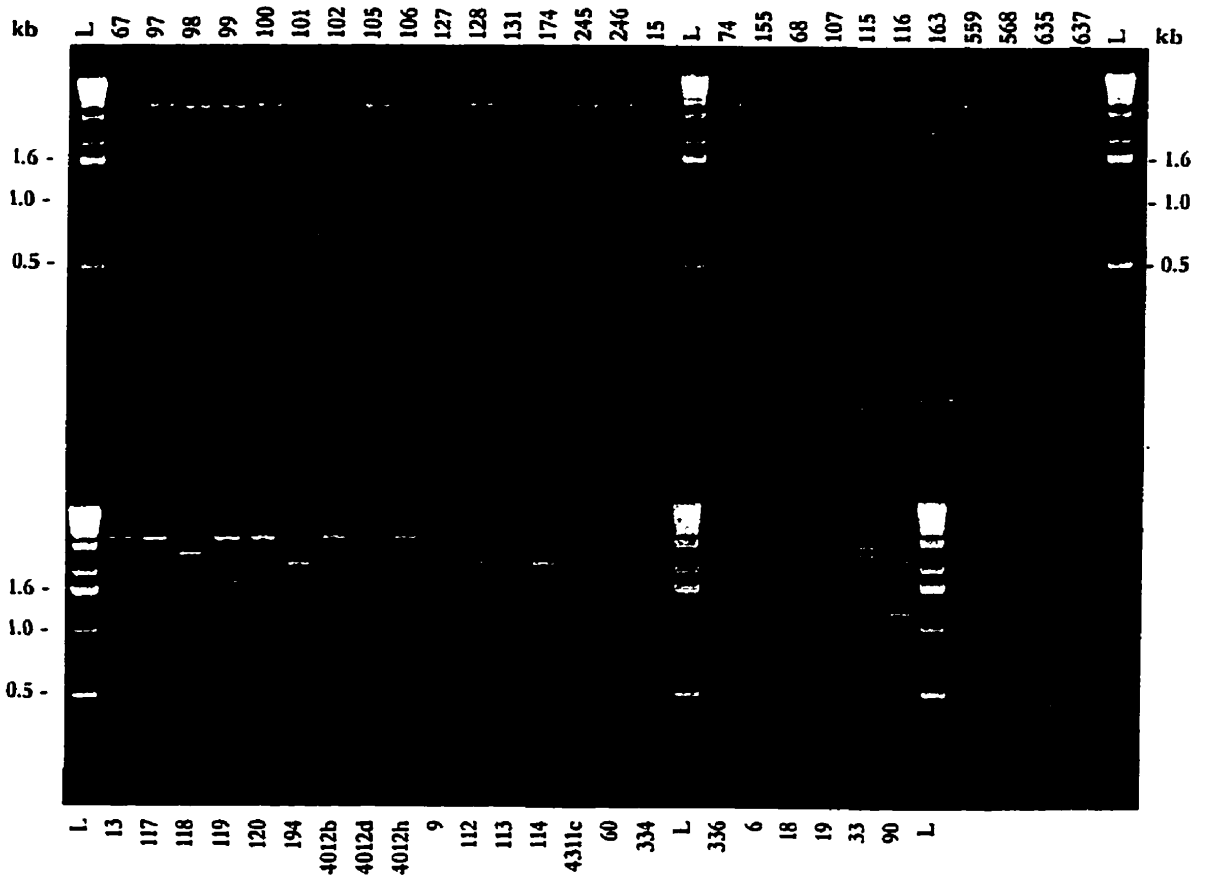
Figure 27. Restriction digestion of *Pythium* IGS spacers (Q1-P2 amplicons) with *Hae*III.

Numbers correspond to reference numbers of *Pythium* isolates provided in Table1: *P. irregulare* 67, 97, 98, 99, 100, 101, 105, 106, 127, 131, 174, 245, 246, *P. sylvaticum* 15, *P. cylindrosporum* 74, 155, *P. paroecandrum* 68, 107, 115, 116, 163, 559, 568, 635, 637, *P. spinosum* 13, 117, 118, 119, 120, 194, 4012b, 4012d, 4012h, *P. mamillatum* 9, 112, 113, 114, 4311c, *P. kunmingense* 60, *P. polymorphon* 334, *P. debaryanum* 336, *P. intermedium* 6, *P. ultimum* var. *ultimum* 18, *P. ultimum* var. *sporangiiferum* 19, *P. echinulatum* 33, *P. hydnosporum* 90. Lane L, 1 kb ladder.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4012b
4012d
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4311c
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L

Cluster analysis

A binomial matrix for each restriction enzyme was constructed in which the restriction endonuclease banding patterns were compared (data not shown). The presence or absence of a band was represented by a 1 or a 0, respectively. The seven binomial matrix data sets were combined and used to produce the distance matrices of the Kimura distance values by using "RESTDIST" (restriction fragments option) (PHYLIP 3.57) (Table 4). Phylogenetic relationships among isolates were evaluated by the Kitch method (Fig 28).

The phylogenetic analysis grouped 42 examined isolates representing eight *Pythium* species collected from diverse hosts and geographic origin, into 6 main clusters. The *P. mamillatum* cluster contains all five *P. mamillatum* isolates and one *P. spinosum* (194, adc 85.058) isolate. The *P. spinosum* cluster includes eight examined isolates of *P. spinosum* and the type culture for *P. kunmingense* (60, CBS 550.88). The type culture of *P. cylindrosporum* (74, CBS 218.94) shares identical RFLP fingerprints with *P. irregulare* (105, adc 94.02). Another examined isolate of *P. cylindrosporum* (155, CBS 219.94) seems to be closely related to the type culture of *P. paroecandrum* (68, CBS 157.64). Also, 11 of 15 examined *P. irregulare* isolates form one large cluster to which *P. polymorphon* (334, CBS 751.96) and two isolates of *P. paroecandrum*, 107 (CBS 219.94) and BR 559 can be assigned. Three other *P. irregulare* isolates, 174 (CBS 733.94), 224 (adc 94.10), 246 (adc 94.13), and one *P.*

parvocandrum, 116 (CBS 651.79), clustered together with the type culture for *P. sylvaticum* (15, CBS 452.67).

Table 4.
Restriction sites distance matrices for the IGS rgion of 42 isolates of eight *Pythium* species

		1	2	3	4	5	6	7	8
1	irr67	-	.	.	.	.	.	.	.
2	irr97	0.096	-	.	.	.	.	.	.
3	irr98	0.015	0.098	-	.	.	.	.	.
4	irr99	0.059	0.079	0.054	-	.	.	.	.
5	irr100	0.089	0.018	0.092	0.074	-	.	.	.
6	irr101	0.074	0.033	0.069	0.062	0.024	-	.	.
7	irr102	0.049	0.035	0.051	0.059	0.026	0.019	-	.
8	irr105	0.098	0.085	0.101	0.081	0.079	0.089	0.079	-
9	irr106	0.025	0.077	0.035	0.059	0.080	0.074	0.049	0.080
10	irr127	0.060	0.083	0.063	0.019	0.085	0.064	0.061	0.069
11	irr128	0.018	0.083	0.020	0.056	0.085	0.079	0.054	0.094
12	irr131	0.049	0.031	0.052	0.060	0.027	0.016	0.007	0.086
13	irr174	0.214	0.244	0.216	0.200	0.197	0.180	0.212	0.212
14	irr245	0.230	0.292	0.233	0.211	0.227	0.202	0.227	0.227
15	irr246	0.227	0.290	0.230	0.209	0.224	0.200	0.224	0.224
16	syl15	0.213	0.247	0.236	0.198	0.211	0.177	0.229	0.229
17	cyl74	0.101	0.087	0.104	0.084	0.074	0.083	0.081	0.002
18	cyl155	0.168	0.130	0.156	0.135	0.133	0.122	0.122	0.133
19	par68	0.156	0.133	0.146	0.127	0.135	0.114	0.114	0.124
20	par107	0.035	0.080	0.045	0.061	0.083	0.085	0.059	0.066
21	par115	0.353	0.260	0.312	0.266	0.263	0.275	0.263	0.293
22	par116	0.175	0.227	0.178	0.195	0.177	0.159	0.177	0.193
23	par163	0.156	0.133	0.146	0.127	0.135	0.114	0.114	0.124
24	par559	0.020	0.085	0.030	0.052	0.079	0.066	0.043	0.088
25	par635	0.199	0.178	0.166	0.221	0.165	0.177	0.181	0.165
26	par637	0.149	0.166	0.152	0.142	0.153	0.151	0.153	0.153
27	spn13	0.159	0.227	0.162	0.255	0.193	0.173	0.162	0.230
28	spn117	0.156	0.201	0.146	0.224	0.173	0.157	0.147	0.203
29	spn118	0.177	0.206	0.165	0.180	0.178	0.162	0.152	0.193
30	spn119	0.162	0.210	0.151	0.216	0.179	0.149	0.139	0.213
31	spn120	0.165	0.179	0.140	0.219	0.167	0.164	0.141	0.182
32	spn194	0.244	0.216	0.224	0.241	0.238	0.229	0.219	0.219
33	spnb	0.153	0.198	0.143	0.221	0.170	0.154	0.144	0.201
34	spnd	0.153	0.198	0.143	0.221	0.170	0.154	0.144	0.201
35	spnh	0.153	0.198	0.143	0.221	0.170	0.154	0.144	0.201
36	mam9	0.196	0.210	0.181	0.216	0.233	0.224	0.213	0.213
37	mam112	0.212	0.227	0.196	0.213	0.252	0.241	0.230	0.230
38	mam113	0.221	0.216	0.204	0.221	0.238	0.229	0.219	0.219
39	mam114	0.215	0.230	0.199	0.216	0.255	0.244	0.233	0.233
40	mamc	0.196	0.210	0.181	0.216	0.233	0.224	0.213	0.213
41	kun60	0.137	0.165	0.128	0.157	0.141	0.119	0.119	0.182
42	pol334	0.042	0.080	0.045	0.054	0.074	0.069	0.044	0.083

Table 4.
Restriction sites distance matrices for the IGS rgion of 42 isolates of eight *Pythium* species

		9	10	11	12	13	14	15	16
1	irr67	.	.	.	.	.	.	.	.
2	irr97	.	.	.	.	.	.	.	.
3	irr98	.	.	.	.	.	.	.	.
4	irr99	.	.	.	.	.	.	.	.
5	irr100	.	.	.	.	.	.	.	.
6	irr101	.	.	.	.	.	.	.	.
7	irr102	.	.	.	.	.	.	.	.
8	irr105	.	.	.	.	.	.	.	.
9	irr106	-	.	.	.	.	.	.	.
10	irr127	0.052	-	.	.	.	.	.	.
11	irr128	0.012	0.066	-	.	.	.	.	.
12	irr131	0.056	0.061	0.054	-	.	.	.	.
13	irr174	0.214	0.219	0.219	0.204	-	.	.	.
14	irr245	0.230	0.236	0.236	0.234	0.017	-	.	.
15	irr246	0.227	0.233	0.233	0.232	0.020	0.002	-	.
16	syl15	0.233	0.219	0.238	0.219	0.034	0.027	0.024	-
17	cyl74	0.083	0.071	0.097	0.089	0.200	0.211	0.209	0.214
18	cyl155	0.153	0.146	0.174	0.119	0.157	0.190	0.187	0.209
19	par68	0.143	0.136	0.162	0.112	0.148	0.178	0.175	0.195
20	par107	0.035	0.063	0.027	0.066	0.200	0.213	0.210	0.198
21	par115	0.308	0.250	0.280	0.272	0.406	0.381	0.378	0.384
22	par116	0.175	0.199	0.181	0.185	0.046	0.039	0.036	0.054
23	par163	0.143	0.136	0.162	0.112	0.148	0.178	0.175	0.195
24	par559	0.020	0.061	0.020	0.044	0.205	0.219	0.216	0.221
25	par635	0.199	0.228	0.186	0.190	0.227	0.272	0.269	0.249
26	par637	0.133	0.156	0.156	0.162	0.119	0.119	0.116	0.122
27	spn13	0.159	0.266	0.165	0.170	0.202	0.216	0.213	0.219
28	spn117	0.143	0.230	0.149	0.154	0.212	0.227	0.224	0.229
29	spn118	0.193	0.198	0.182	0.159	0.202	0.232	0.249	0.216
30	spn119	0.162	0.221	0.168	0.147	0.221	0.238	0.236	0.241
31	spn120	0.137	0.247	0.131	0.149	0.242	0.264	0.261	0.266
32	spn194	0.244	0.249	0.249	0.227	0.313	0.360	0.357	0.362
33	spnb	0.140	0.227	0.146	0.152	0.226	0.244	0.241	0.247
34	spnd	0.140	0.227	0.146	0.152	0.226	0.244	0.241	0.247
35	spnh	0.140	0.227	0.146	0.152	0.226	0.244	0.241	0.247
36	mam9	0.196	0.221	0.202	0.221	0.308	0.354	0.351	0.357
37	mam112	0.212	0.218	0.218	0.238	0.280	0.351	0.348	0.354
38	mam113	0.221	0.227	0.227	0.227	0.313	0.360	0.357	0.362
39	mam114	0.215	0.221	0.221	0.241	0.308	0.354	0.351	0.357
40	mamc	0.196	0.221	0.202	0.221	0.308	0.354	0.351	0.357
41	kun60	0.151	0.156	0.156	0.127	0.179	0.187	0.185	0.190
42	pol334	0.042	0.055	0.048	0.052	0.200	0.213	0.210	0.216

Table 4.
Restriction sites distance matrices for the IGS rgion of 42 isolates of eight *Pythium* species

		17	18	19	20	21	22	23	24
1	irr67	.	.	.	.	.	.	.	.
2	irr97	.	.	.	.	.	.	.	.
3	irr98	.	.	.	.	.	.	.	.
4	irr99	.	.	.	.	.	.	.	.
5	irr100	.	.	.	.	.	.	.	.
6	irr101	.	.	.	.	.	.	.	.
7	irr102	.	.	.	.	.	.	.	.
8	irr105	.	.	.	.	.	.	.	.
9	irr106	.	.	.	.	.	.	.	.
10	irr127	.	.	.	.	.	.	.	.
11	irr128	.	.	.	.	.	.	.	.
12	irr131	.	.	.	.	.	.	.	.
13	irr174	.	.	.	.	.	.	.	.
14	irr245	.	.	.	.	.	.	.	.
15	irr246	.	.	.	.	.	.	.	.
16	syl15	.	.	.	.	.	.	.	.
17	cyl74	-	.	.	.	.	.	.	.
18	cyl155	0.135	-	.	.	.	.	.	.
19	par68	0.127	0.002	-	.	.	.	.	.
20	par107	0.069	0.171	0.159	-	.	.	.	.
21	par115	0.296	0.234	0.237	0.247	-	.	.	.
22	par116	0.179	0.159	0.149	0.178	0.360	-	.	.
23	par163	0.127	0.002	0.000	0.159	0.237	0.149	-	.
24	par559	0.091	0.136	0.127	0.043	0.319	0.168	0.127	-
25	par635	0.168	0.148	0.151	0.202	0.236	0.186	0.151	0.209
26	par637	0.156	0.122	0.113	0.152	0.330	0.069	0.113	0.129
27	spn13	0.233	0.249	0.252	0.178	0.360	0.199	0.252	0.153
28	spn117	0.206	0.219	0.221	0.159	0.329	0.210	0.221	0.139
29	spn118	0.195	0.190	0.193	0.165	0.269	0.216	0.193	0.185
30	spn119	0.216	0.193	0.195	0.181	0.364	0.221	0.195	0.143
31	spn120	0.185	0.179	0.182	0.140	0.322	0.247	0.182	0.146
32	spn194	0.221	0.236	0.219	0.272	0.371	0.342	0.219	0.278
33	spnb	0.203	0.216	0.219	0.156	0.326	0.227	0.219	0.136
34	spnd	0.203	0.216	0.219	0.156	0.326	0.227	0.219	0.136
35	spnh	0.203	0.216	0.219	0.156	0.326	0.227	0.219	0.136
36	mam9	0.216	0.230	0.213	0.240	0.424	0.335	0.213	0.224
37	mam112	0.233	0.227	0.210	0.237	0.360	0.332	0.210	0.244
38	mam113	0.221	0.236	0.219	0.247	0.326	0.342	0.219	0.252
39	mam114	0.236	0.252	0.233	0.240	0.319	0.335	0.233	0.247
40	mamc	0.216	0.230	0.213	0.240	0.424	0.335	0.213	0.224
41	kun60	0.185	0.195	0.198	0.168	0.367	0.171	0.198	0.146
42	pol334	0.085	0.156	0.146	0.052	0.312	0.178	0.146	0.050

Table 4.
Restriction sites distance matrices for the IGS rgion of 42 isolates of eight *Pythium* species

		25	26	27	28	29	30	31	32
1	irr67	.	.	.	.	.	.	.	.
2	irr97	.	.	.	.	.	.	.	.
3	irr98	.	.	.	.	.	.	.	.
4	irr99	.	.	.	.	.	.	.	.
5	irr100	.	.	.	.	.	.	.	.
6	irr101	.	.	.	.	.	.	.	.
7	irr102	.	.	.	.	.	.	.	.
8	irr105	.	.	.	.	.	.	.	.
9	irr106	.	.	.	.	.	.	.	.
10	irr127	.	.	.	.	.	.	.	.
11	irr128	.	.	.	.	.	.	.	.
12	irr131	.	.	.	.	.	.	.	.
13	irr174	.	.	.	.	.	.	.	.
14	irr245	.	.	.	.	.	.	.	.
15	irr246	.	.	.	.	.	.	.	.
16	syl15	.	.	.	.	.	.	.	.
17	cyl74	.	.	.	.	.	.	.	.
18	cyl155	.	.	.	.	.	.	.	.
19	par68	.	.	.	.	.	.	.	.
20	par107	.	.	.	.	.	.	.	.
21	par115	.	.	.	.	.	.	.	.
22	par116	.	.	.	.	.	.	.	.
23	par163	.	.	.	.	.	.	.	.
24	par559	.	.	.	.	.	.	.	.
25	par635	-	.	.	.	.	.	.	.
26	par637	0.178	-	.	.	.	.	.	.
27	spn13	0.253	0.214	-	.	.	.	.	.
28	spn117	0.240	0.228	0.011	-	.	.	.	.
29	spn118	0.171	0.212	0.074	0.069	-	.	.	.
30	spn119	0.231	0.196	0.026	0.024	0.048	-	.	.
31	spn120	0.193	0.247	0.035	0.033	0.050	0.031	-	.
32	spn194	0.293	0.280	0.342	0.317	0.266	0.308	0.281	-
33	spnb	0.237	0.225	0.013	0.002	0.066	0.022	0.030	0.314
34	spnd	0.237	0.225	0.013	0.002	0.066	0.022	0.030	0.314
35	spnh	0.237	0.225	0.013	0.002	0.066	0.022	0.030	0.314
36	mam9	0.322	0.273	0.381	0.311	0.317	0.339	0.305	0.016
37	mam112	0.319	0.269	0.438	0.391	0.314	0.381	0.384	0.019
38	mam113	0.329	0.250	0.387	0.354	0.292	0.345	0.348	0.016
39	mam114	0.322	0.243	0.381	0.348	0.287	0.339	0.387	0.022
40	mamc	0.322	0.273	0.442	0.348	0.354	0.384	0.342	0.016
41	kun60	0.260	0.180	0.056	0.059	0.096	0.051	0.085	0.233
42	pol334	0.202	0.137	0.178	0.174	0.195	0.181	0.184	0.204

Table 4.
Restriction sites distance matrices for the IGS rgion of 42 isolates of eight *Pythium* species

		33	34	35	36	37	38	39	40
1	irr67	.	.	.	.	.	.	.	.
2	irr97	.	.	.	.	.	.	.	.
3	irr98	.	.	.	.	.	.	.	.
4	irr99	.	.	.	.	.	.	.	.
5	irr100	.	.	.	.	.	.	.	.
6	irr101	.	.	.	.	.	.	.	.
7	irr102	.	.	.	.	.	.	.	.
8	irr105	.	.	.	.	.	.	.	.
9	irr106	.	.	.	.	.	.	.	.
10	irr127	.	.	.	.	.	.	.	.
11	irr128	.	.	.	.	.	.	.	.
12	irr131	.	.	.	.	.	.	.	.
13	irr174	.	.	.	.	.	.	.	.
14	irr245	.	.	.	.	.	.	.	.
15	irr246	.	.	.	.	.	.	.	.
16	syl15	.	.	.	.	.	.	.	.
17	cyl74	.	.	.	.	.	.	.	.
18	cyl155	.	.	.	.	.	.	.	.
19	par68	.	.	.	.	.	.	.	.
20	par107	.	.	.	.	.	.	.	.
21	par115	.	.	.	.	.	.	.	.
22	par116	.	.	.	.	.	.	.	.
23	par163	.	.	.	.	.	.	.	.
24	par559	.	.	.	.	.	.	.	.
25	par635	.	.	.	.	.	.	.	.
26	par637	.	.	.	.	.	.	.	.
27	spn13	.	.	.	.	.	.	.	.
28	spn117	.	.	.	.	.	.	.	.
29	spn118	.	.	.	.	.	.	.	.
30	spn119	.	.	.	.	.	.	.	.
31	spn120	.	.	.	.	.	.	.	.
32	spn194	.	.	.	.	.	.	.	.
33	spnb	-	.	.	.	.	.	.	.
34	spnd	0.000	-	.	.	.	.	.	.
35	spnh	0.000	0.000	-	.	.	.	.	.
36	mam9	0.308	0.308	0.308	-	.	.	.	.
37	mam112	0.387	0.387	0.387	0.020	-	.	.	.
38	mam113	0.351	0.351	0.351	0.016	0.013	-	.	.
39	mam114	0.345	0.345	0.345	0.022	0.008	0.005	-	.
40	mamc	0.345	0.345	0.345	0.005	0.014	0.011	0.017	-
41	kun60	0.064	0.064	0.064	0.275	0.272	0.281	0.275	0.275
42	pol334	0.171	0.171	0.171	0.199	0.178	0.187	0.181	0.181

Table 4.
Restriction sites distance matrices for the IGS rgion of 42 isolates of eight *Pythium* species

		41	42
1	irr67	.	.
2	irr97	.	.
3	irr98	.	.
4	irr99	.	.
5	irr100	.	.
6	irr101	.	.
7	irr102	.	.
8	irr105	.	.
9	irr106	.	.
10	irr127	.	.
11	irr128	.	.
12	irr131	.	.
13	irr174	.	.
14	irr245	.	.
15	irr246	.	.
16	syl15	.	.
17	cyl74	.	.
18	cyl155	.	.
19	par68	.	.
20	par107	.	.
21	par115	.	.
22	par116	.	.
23	par163	.	.
24	par559	.	.
25	par635	.	.
26	par637	.	.
27	spn13	.	.
28	spn117	.	.
29	spn118	.	.
30	spn119	.	.
31	spn120	.	.
32	spn194	.	.
33	spnb	.	.
34	spnd	.	.
35	spnh	.	.
36	mam9	.	.
37	mam112	.	.
38	mam113	.	.
39	mam114	.	.
40	mamc	.	.
41	kun60	-	.
42	pol334	0.106	-

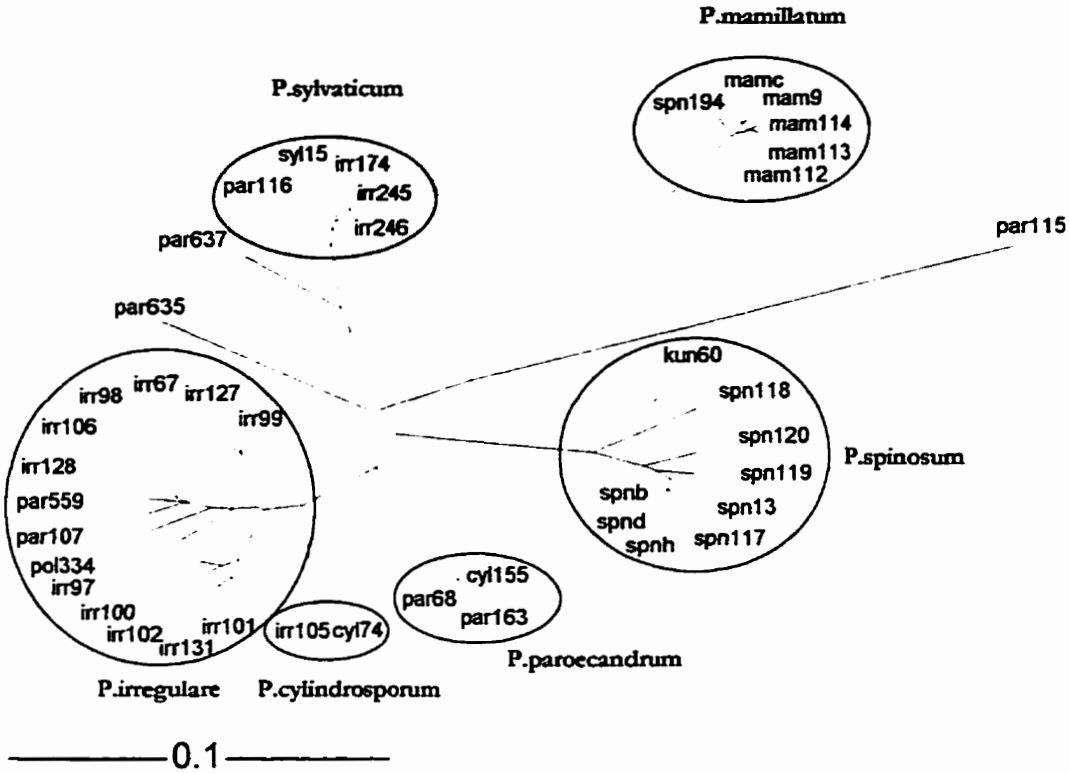


Figure 28. Unrooted distance tree showing relationships among isolates of eight *Pythium* species inferred by the Kitch method based on RLFP analysis of the intergenic spacer region.
Pythium species: irr, *P. irregulare*; pol, *P. polymorphon*; par, *P. paroecandrum*; cyl, *P. cylindrosporum*; spn, *P. spinosum*; kun, *P. kunmingense*; mam, *P. mamillatum*; syl, *P. sylvaticum*.

Species-specific fingerprints

The IGS region was amplified with primers Q and P2, digested with different 5 endonucleases, and probed with Q and P2 end labeled probes. The IGS was also amplified with Q1 and P2 primers, digested with *Rsa*I and *Hae*III, and hybridized to Q1 and P2 probes. Hybridization with Q and Q1 primers, that anneal at 280 bp and 40 bp, respectively from the 3' end of the large subunit rRNA gene, was done to determine the first restriction site for each endonuclease downstream of the lsrDNA. Similarly, probing with the P2 primer, that anneals at 98 bp from the 5' end of the small subunit rRNA gene, was performed to detect the restriction sites upstream of the ssrRNA.

Figures 29 and 30 show the autoradiogram of the amplified IGS region digested with *Hin*dII and hybridized with Q and P2 respectively. When probing with the Q primer, most isolates show multiple binding that indicates heterogeneity in a region located downstream of the 3' end of the large subunit rRNA gene. A different banding pattern characterizes each cluster of isolates. In most isolates one or two fragment lengths predominate. For example, isolates of the *P. irregulare* cluster have two main bands about 550 bp and 470 bp long. The isolates of the *P. sylvaticum* cluster share one common 3 kb band. No variation in banding pattern was observed among isolates of the *P. cylindrosporum* and *P. sylvaticum* clusters. The other isolates of *P. paroecandrum* (115, 568, 635 637), that were not grouped to any clusters, showed different banding patterns. The *P.*

spinosum cluster appears to have no conserved *Hin*dII sites in the region downstream of lsrRNA gene. The strong band about 750 bp long that is present in three isolates of *P. spinosum* (13, 119 and 120) represents an additional product of QP2 amplification. The banding pattern for isolates of the *P. mamillatum* cluster is almost uniform; they share one common 600 bp band.

Probing with the P2 primer resulted in one band that was common for isolates of each cluster. For example, isolates of the *P. irregulare* cluster have one band, approximately 1600 bp long, which is also present for two isolates of the *P. cylindrosporum* cluster. Isolates of the *P. sylraticum* cluster, the *P. paroecandrum* cluster and two more isolates of *P. paroecandrum*, 568 and 637, share one common band about 900 bp long. Two other isolates of *P. paroecandrum*, 115 and 635, show different banding patterns. *P. paroecandrum* 115 has a 400 bp band, whereas *P. paroecandrum* 635 has a 150 bp band. A band approximately 2500 bp long is characteristic of the *P. spinosum* group isolates. The isolates of the *P. mamillatum* cluster have a common 950 bp band.

Figure 29. Autoradiogram of *Pythium* IGS regions (Q-P2 amplicons) digested with *Hin*cII and hybridized to the Q primer.

Numbers correspond to reference numbers of *Pythium* isolates provided in Table1: *P. irregulare* 67, 97, 98, 99, 100, 101, 105, 106, 127, 131, 174, 245, 246, *P. sylvaticum* 15, *P. cylindrosporum* 74, 155, *P. paroecandrum* 68, 107, 115, 116, 163, 559, 568, 635, 637, *P. spinosum* 13, 117, 118, 119, 120, 194, 4012b, 4012d, 4012h, *P. mamillatum* 9, 112, 113, 114, 4311c, *P. kunmingense* 60, *P. polymorphon* 334, *P. debaryanum* 336, *P. intermedium* 6, *P. ultimum* var. *ultimum* 18, *P. ultimum* var. *sporangiiferum* 19, *P. acanthicum* 71, *P. hydnosporum* 90.

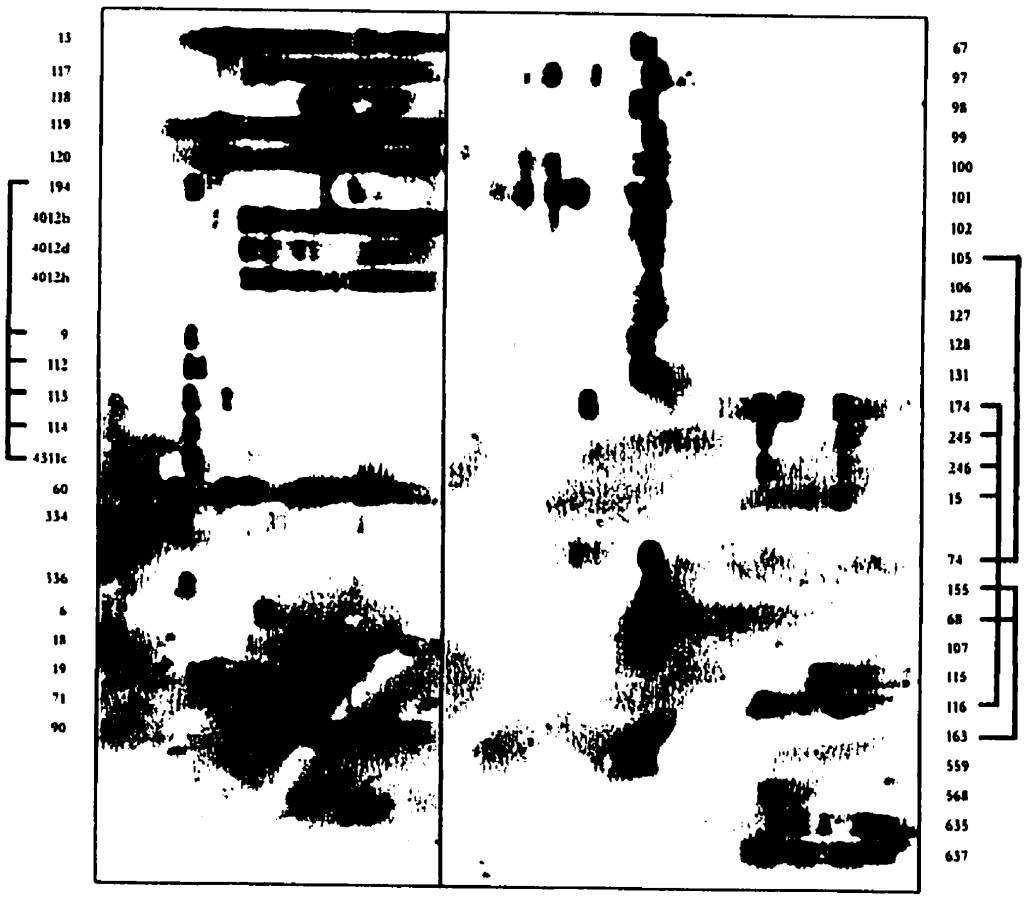
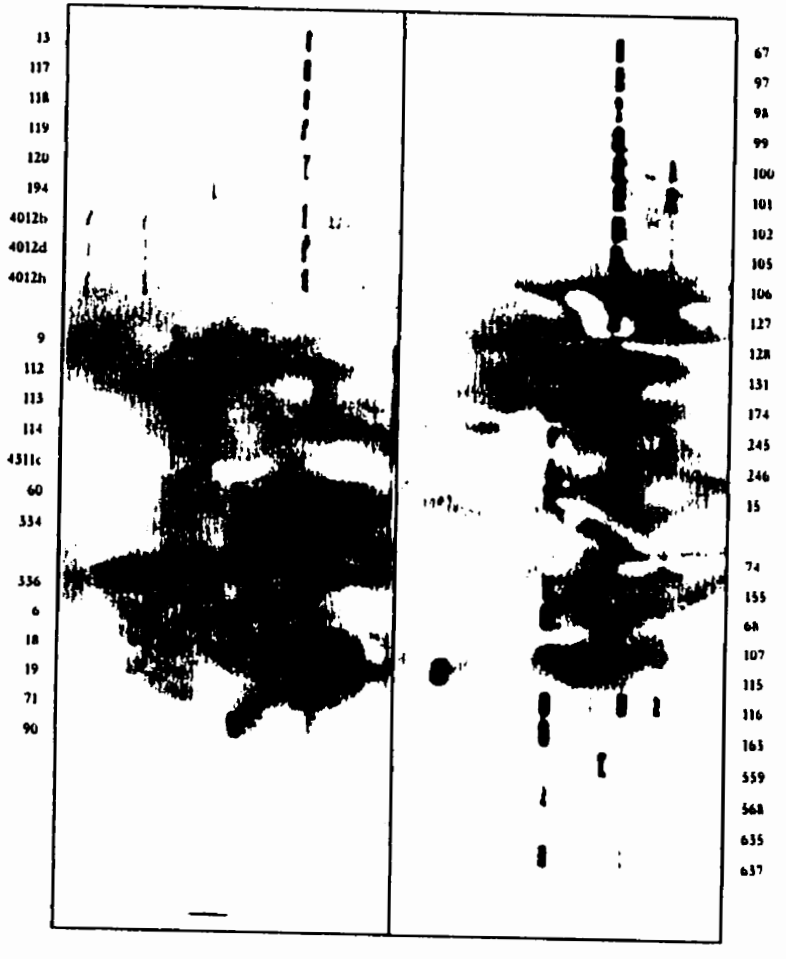

13
117
118
119
120
194
4012b
4012d
4012h
9
112
113
114
4311c
60
334
336
6
18
19
71
90
67
97
98
99
100
101
102
105
106
127
128
131
174
245
246
15
74
155
68
107
115
116
163
559
568
635
637

Figure 30. Autoradiogram of *Pythium* IGS regions (Q-P2 amplicons) digested with *Hin*dII and hybridized to the P2 primer.

Numbers correspond to reference numbers of *Pythium* isolates provided in Table1: *P. irregulare* 67, 97, 98, 99, 100, 101, 105, 106, 127, 131, 174, 245, 246, *P. sylvaticum* 15, *P. cylindrosporum* 74, 155, *P. paroecandrum* 68, 107, 115, 116, 163, 559, 568, 635, 637, *P. spinosum* 13, 117, 118, 119, 120, 194, 4012b, 4012d, 4012h, *P. mamillatum* 9, 112, 113, 114, 4311c, *P. kunmingense* 60, *P. polymorphon* 334, *P. debaryanum* 336, *P. intermedium* 6, *P. ultimum* var. *ultimum* 18, *P. ultimum* var. *sporangiiferum* 19, *P. acanthicum* 71, *P. hydnosporum* 90.


13
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336
6
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71
90
67
97
98
99
100
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74
155
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163
559
568
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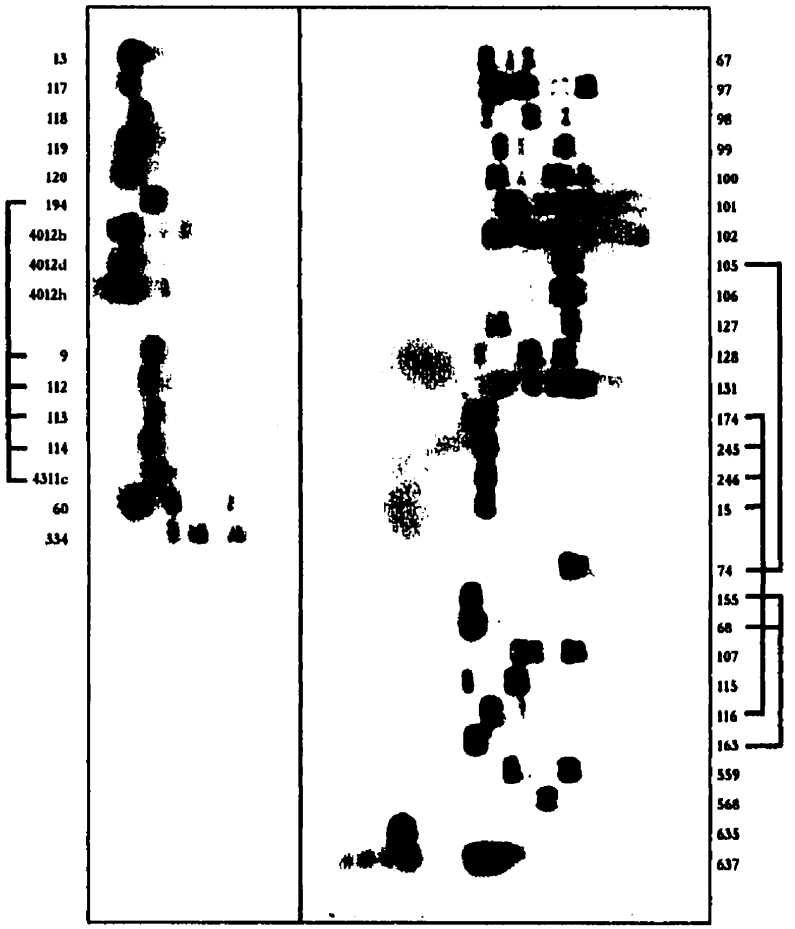
637

Fingerprints specific for isolates of the same cluster were also observed by digestion of amplified IGS spacer with *Taq*I and hybridization to the Q primer probe (Fig 31). The isolates of the *P. irregulare* cluster show multiple binding that indicates the presence of a region of heterogeneity. Almost all isolates of the *P. irregulare* group shows a unique pattern of heterogeneity in the region located 1.4 kb downstream of the 3' end of the lsrRNA gene. The isolates of the *P. sylvaticum* cluster have one main band, about 700 bp long. The 1.3 kb band is predominant for isolates of the *P. cylindrosporum* cluster. The three isolates grouped into the *P. paroecandrum* cluster have one 600 bp band. The other isolates of *P. paroecandrum* have unique banding patterns. The *P. spinosum* cluster has one predominant 550 bp fragment in common and the isolates of the *P. mamillatum* group share a 700 bp band in common.

When the *Taq*I digest of the IGS region was probed with the P2 primer, no major variation in banding patterns was observed among 42 examined isolates of the eight *Pythium* species (data not shown). Most isolates have one 220 bp band in common. The isolates of the *P. sylvaticum* and *P. mamillatum* clusters share a 250 bp band and the *P. spinosum* cluster has a 180 bp band.

Figure 31. Autoradiogram of *Pythium* IGS regions (Q-P2 amplicons) digested with *Taq*I and hybridized to the Q primer.

Numbers correspond to reference numbers of *Pythium* isolates provided in Table1: *P. irregulare* 67, 97, 98, 99, 100, 101, 105, 106, 127, 131, 174, 245, 246, *P. sylvaticum* 15, *P. cylindrosporum* 74, 155, *P. paroecandrum* 68, 107, 115, 116, 163, 559, 568, 635, 637, *P. spinosum* 13, 117, 118, 119, 120, 194, 4012b, 4012d, 4012h, *P. mamillatum* 9, 112, 113, 114, 4311c, *P. kunmingense* 60, *P. polymorphon* 334.


13
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4311c
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100
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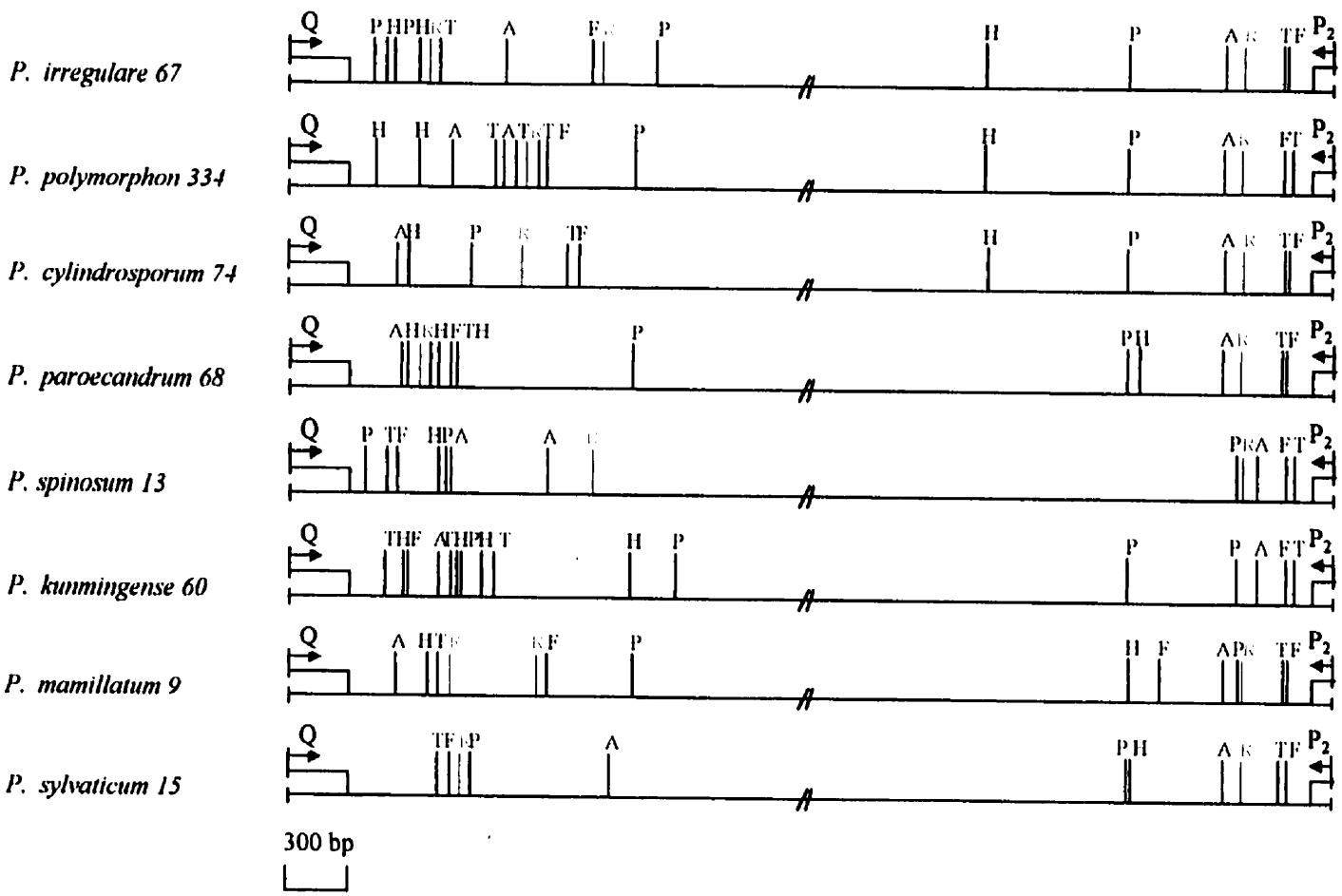
The physical map of the region downstream of the 3' end of the large subunit rRNA gene and the region upstream of the 5' end of the small subunit rRNA gene showing sites for seven endonucleases is given for eight *Pythium* species in Fig. 32. Many restriction sites in the region upstream of the 5' end of ssrDNA are conserved among the eight examined species. In contrast the region downstream of the 3' end of lsrDNA shows multiple restriction sites for most analyzed endonucleases (heterogeneity) and slightly different patterns of restriction sites for each species (inter-specific variation).

Figure 32. Restriction site maps of the region downstream of the 3' end of the large subunit rRNA gene and the region upstream of the 5' end of the small subunit rRNA gene.

Restriction endonucleases: A, *Aha*I; H, *Hind*III; F, *Hinf*I; P, *Hpa*II; R, *Rsa*I; and T, *Taq*I. *Rsa*I restriction site was not determined for *P. kunmingense*.


P. irregulare 67
P. polymorphon 334
P. cylindrosporum 74
P. paroecandrum 68
P. spinosum 13
P. kunmingense 60
P. mamillatum 9
P. sylvaticum 15
Q
P2
300 bp

Discussion

It is generally accepted that the various components of rDNA evolve at different rates (Jorgensen and Cluster 1988). In our studies of *Pythium* species, the spacer region between 5S rRNA genes showed length and sequence variation among species but also had intra-isolate and intra-specific length variations that precluded its use for identification of different isolates of the *P. irregulare* complex.

Contrary to what was found for the 5S spacer rDNA, the variability in restriction sites in the large IGS region allowed differentiation of isolates within the *P. irregulare* homology group.

PCR gave access to the large IGS region from *Pythium* species in pure form and in sufficient quantities for RFLP analysis. The species-specific banding patterns of restriction endonuclease-digested IGS amplicons suggest that this procedure provides a relatively reliable taxonomic tool for the identification of *Pythium* species. The pattern of variability detected between *Pythium* species indicates that the technique also can provide valuable information concerning *Pythium* systematics and phylogeny.

The IGS regions showed variations in restriction sites between species, but variation was relatively minimal within species. Based on the restriction endonuclease screened, many restriction sites were conserved in several isolates of different species, especially in the region upstream of the 5' end of the small ribosomal subunit. The region downstream from the 3' end of the large

ribosomal subunit seemed to be more variable between species and among the isolates of the same species. The restriction site polymorphism in this region could be caused by deletion and insertion events and point mutations. The region of heterogeneity located about one kilobase downstream of the 3' end of lsrRNA gene and near the centre of the IGS has been previously observed in *P. ultimum* (Klassen and Buchko 1990). This length heterogeneity in the IGS of *P. ultimum* was attributed to the presence of arrays of nearly identical subrepeats.

The clustering of isolates based on restriction sites in the IGS region of *Pythium* species is generally consistent with morphological differences. The digestion of the amplified IGS region with enzymes *Hin*dII, *Taq*I, *Hin*fI, *Hpa*II, *Alu*I, *Rsa*II and *Hae*III divided 42 examined isolates into 6 main clusters. The *P. irregulare* cluster includes 11 isolates of *P. irregulare* (inclusive of the neotype culture for this species, 67), two isolates of *P. paroecandrum* and one examined isolate of *P. polymorphon*. The clustering of *P. irregulare* with *P. polymorphon* is in agreement with our 5S spacer tree, which shows these two species to be very closely related. There is also no morphological evidence that can justify their separation into two different species (de Cock, personal communication). To further confirm the idea that *P. polymorphon* is identical with *P. irregulare*, the analysis of the authentic strain (CBS 263.30) of *P. polymorphon* (the type strain is not available) will be required.

The *P. cylindrosporum* cluster, which groups the type strain 74 (CBS 218.94) with the *P. irregulare* isolate 105 (adc 94.02), is closely related to the *P. irregulare*

cluster. *P. cylindrosporum* produces globose hyphal swellings, similar to the globose sporangia of *P. irregulare*, but zoospore production has not been observed in *P. cylindrosporum* (Paul 1992). The morphology of the type strain of *P. cylindrosporum* is very similar to that of *P. irregulare*, including the presence of a few digitate spines on some oogonia (de Cock, personal communication). Phylogenetic analysis of the 5S spacer sequences also confirms that these two species are very close related.

Eight of nine examined isolates of *P. spinosum* form one cluster to which the type strain of *P. kunmingense* (60, CBS 550. 88) can be assigned. This data is in accordance with strong similarity found between the 5S spacers of *P. spinosum* and *P. kunmingense*. The morphology of *P. kunmingense* is also very similar to *P. spinosum*. They both have globose, non-proliferating sporangia, plerotic oospores, and antheridia and oogonia of the same size (Van der Plaats-Niterink 1981). *P. kunmingense* oogonial projections are distinctly blunt and the longer ones are digitate as in *P. spinosum* (Yu 1973). It is interesting to note that these two species also share significant similarity in carbon source utilization. They were the only species out of 25 examined that could use none of 17 tested carbohydrates (Yu 1989).

The *P. mamillatum* cluster is almost uniform. It contains all five examined isolates of *P. mamillatum* and only one isolate of *P. spinosum* (194, adc 85.058). *P. mamillatum* shares some similarity with *P. spinosum* regarding sporangia and

position of oogonia (Middleton 1943). However, Middleton (1943) described the oogonial projections in *P. mamillatum* as conical and those in *P. spinosum* as digitate. This is in contrast with information from Arthur de Cock (personal communication) that *P. mamillatum* projections are also digitate as in *P. spinosum* but only slightly wider and of unequal length. This data indicates that some isolates of *P. spinosum* can be easily misidentified as *P. mamillatum* when classification is based only on morphological criteria. This may have been the case with *P. spinosum* 194 (adc 85.058).

The *P. sylvaticum* cluster grouped the type culture of *P. sylvaticum* (15, CBS 452.67) together with three isolates of *P. irregulare* (174, CBS 733.94; 224, adc 94.10 and 246, adc 94.13) and one isolate of *P. paroecandrum* (116, CBS 651. 79). Those isolates were also recognized by the 5S spacer species-specific *P. sylvaticum* probe. These data suggest that these isolates can be considered as members of *P. sylvaticum* and that their classification should be reconsidered.

P. paroecandrum isolates do not form a coherent cluster. Almost every isolate of this species can be assigned to a different species. There is much doubt about which isolate best represents *P. paroecandrum*. The representative strain (68, CBS 157.64) proposed by Van der Plaats-Niterink (1981) in her monograph of the genus *Pythium*, does not conform well to the original description, and is likely to be *P. irregulare* (D. Barr, personal communication). Middleton (1943) compared the asexual stage of *P. paroecandrum* with those of *P. debaryanum, P. irregulare, P.*

mamillatum and *P. ultimum* and noticed similarities between them. However, the sexual stage of *P. paroecandrum* was quite different from those of the species cited, with the exception of *P. ultimum*. According to Middleton (1943) *P. paroecandrum* and *P. ultimum* are similar and can be easily confused. Great genetic distances between two isolates of *P. paroecandrum* (115, CBS 203.79 and BR 635) and the other members of homology group suggest that these isolates cannot be assigned to *P. paroecandrum*. This hypothesis is also confirmed by the fact that *P. paroecandrum* 115 (CBS 203.79) did not hybridize with the *P. irregulare* 5S spacer group probes. If one of these isolates is considered to be a more credible member of *P. paroecandrum* than is the type culture, we may have to exclude *P. paroecandrum* from the *P. irregulare* homology group, find a neotype, and rename the *P. paroecandrum* (68) representative strain as *P. irregulare*.

In searching for molecular characters for systematic studies, the appropriate molecular markers should resolve relationships at the desired taxonomic level. The intergenic spacer between the large and small ribosomal subunit was shown to be variable between species, but relatively conserved within *Pythium* species. The present results indicate that the IGS region can be used as a taxonomic marker for the study of *Pythium* species by the detection of species-specific variation. The main advantage of this method is that the primers can be designed to specifically amplify fungal DNA from heterogeneous plant-fungal samples. This obviates the need for extraction of pure fungal cultures from

infected plant tissue. PCR can be performed on very small biological samples. This method could be employed to study *Pythium* populations in natural habitats. PCR-RFLP analysis should prove to be a powerful tool for plant pathogen detection and identification, and for basic studies of fungal systematics and phylogenetics.

A more definitive study of the taxonomic and phylogenetic relationships in *Pythium* species will require sequencing, aligning, and comparison of the IGS regions. Although our RFLP analysis provided a bounty of information for distinguishing and identifying *Pythium* species, IGS sequence comparisons can generate a large number of characters for phylogenetic inference and should provide a more accurate measurement of genetic distances between species.

Chapter 5

CONCLUSIONS

The major purpose of this study was to develop rapid, simple and reliable methods for the identification of *Pythium* species using molecular markers. Our results clearly showed that the spacer region between the 5S rRNA genes diverges rapidly after speciation and can, in many cases, be used as a species-specific hybridization probe for identification of *Pythium* species that have their 5S genes arranged into tandem arrays. Probe development was based on the amplification of the 5S-IGS region with primers complementary to the conserved ends of the 5S rRNA genes. This method could also allow for the quick generation of species-specific probes for many plants and animals demonstrating 5S gene tandem repeat arrangement and may lead to a more objective and quantitative criterion for species delimination.

Moreover, the evidence from this study provided an insight into phylogenetic relationships among *Pythium* species. Probing of genomic DNA with the 5S spacer probe was useful not only for identification of *Pythium* isolates to the species level but also allowed for detection of close relationships between species. The cross-hybridization signal observed between species that share similarity in morphology was an indication that these species are very closely

related. The IGS sequence comparison allowed me to draw a sharp discontinuity between the seven species that form the "*P. irregulare* 5S spacer homology group" and the other isolates that share some morphological characteristics but whose 5S spacers were significantly different. The common structure of the 5S spacer in the seven related species with its repeats within repeats indicates that unequal crossing over has occurred repeatedly to homogenize the 5S spacer repeats. This suggests that gene flow is occurring among the seven species and that perhaps they should be considered to be conspecific. Because 5S spacers in tandem arrays diverge rapidly after speciation but undergo homogenization before speciation, 5S spacer comparison should provide a readily exploitable tool for setting species boundaries in the genus *Pythium*.

Although the 5S gene spacer is a good source of a species-specific probe and can be a sensitive indicator of gene flow, its small size (approximately 500bp) and a large degree of intra-isolate heterogeneity in size and sequence precluded the use of the 5S spacer as a source of restriction site variation to yield adequate resolution of the relationships between different isolates of the *P. irregulare* complex. On the contrary, the large intergenic region between the large and the small rDNA gene subunit appeared to be variable between species but relatively conserved within species and allowed for differentiation of isolates within the *P. irregulare* homology group.

The clustering of isolates based on the RFLP of the IGS region is generally consistent with our grouping based on the 5S spacer tree. Two morphologically similar species, *P. irregulare* and *P. polymorphon* could not be differentiated from each other. These data suggest that they are not distinct species and need to be combined. RFLPs of the IGS region and phylogenetic analysis of the 5S spacer sequences also indicate that *P. spinosum* and *P. kunmingense* can possibly be considered as members of the same species. Some isolates of *P. paroecandrum* do not share 5S spacer sequence homology and RFLP fingerprints with the members of the *P. irregulare* homology group. Different isolates of *P. paroecandrum* can be inside or outside of the 5S spacer homology group. However, it must also be remembered that our 5S spacer group is the minimum *P. irregulare* group. It is possible that other 5S spacers exist within the genomes of members of the group which were not amplified by our PCR primers. Such elements, if they exist, might conceivably enlarge the membership of the group.

In searching for molecular characters for systematics studies, the appropriate markers should resolve relationships at the desired taxonomic level. Our results demonstrate that the IGS region can be used as a taxonomic marker for the study of *Pythium* species by the detection of species specific restriction site variations. RFLP analysis of the IGS region may allow for a more precise identification of many *Pythium* isolates whose taxonomic status is doubtful.

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IMAGE EVALUATION
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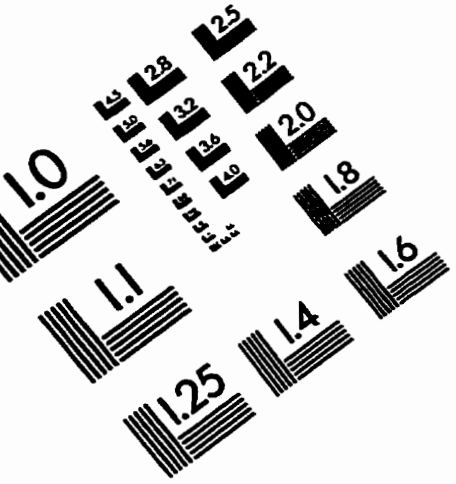

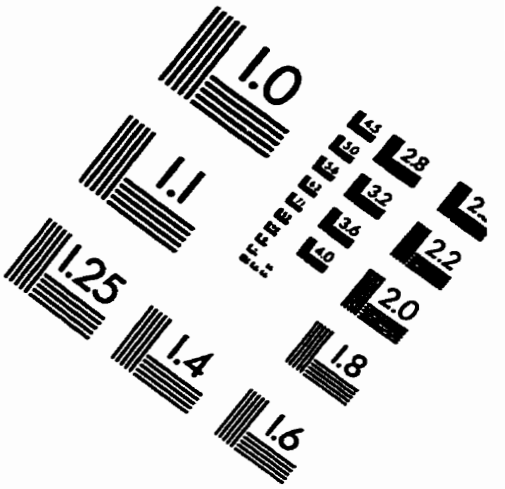

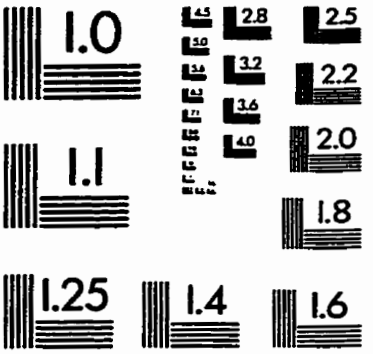

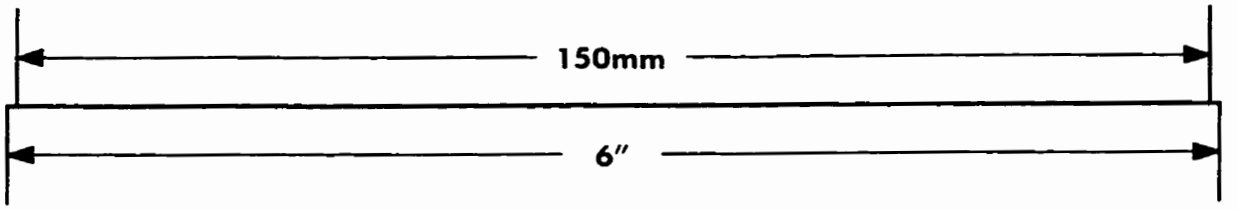

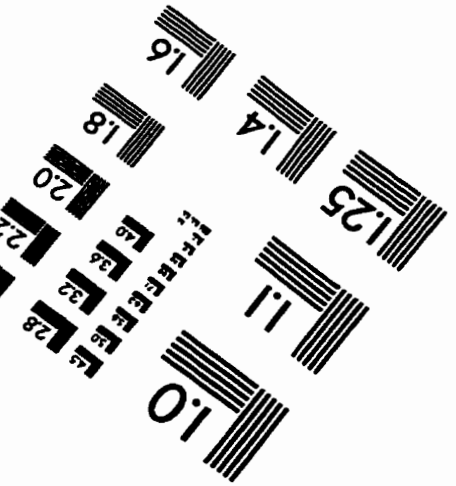

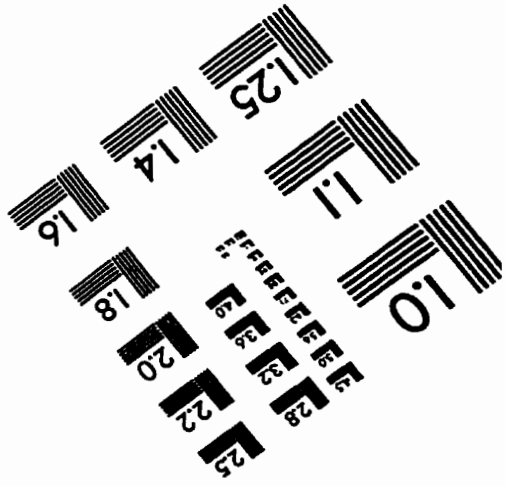

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Phone: 716/482-0300
Fax: 716/288-5989