Polymorphism and heterogeneity of mitochondrial and ribosomal DNA in *Pythium ultimum*

by

John Buchko

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Department of Microbiology
University of Manitoba
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POLYMORPHISM AND HETEROGENEITY OF MITOCHONDRIAL AND RIBOSOMAL DNA IN *Pythium ultimum*

BY

JOHN BUCHKO

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

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ABSTRACT

Restriction endonuclease analysis of mitochondrial DNA (mtDNA) was performed on 14 isolates of *Pythium ultimum*, two of *P. ultimum* var. *sporangiiferum*, six of *Pythium* sp. "group G", and one of *Pythium irregulare* with the purpose of studying the genetic variability among these isolates and to help assign names to asexual *Pythium* isolates. The degree of genetic variability is represented by the distance dendogram which resulted in the majority of isolates being placed into two major groupings. Thus, little genetic variability seems to be present in the mtDNA of *P. ultimum* although exceptions exist for two of the *P. ultimum* isolates, the two *P. ultimum* var. *sporangiiferum* isolates and all of the asexual *Pythium* isolates. The mtDNA data support the classification of at least three of the "group G" isolates as belonging to the species *P. ultimum*. The mtDNA restriction digestion presented here was used as supporting evidence by Huang *et al.* (1992) to classify one asexual isolate of *Pythium* (BR583) as *P. ultimum*.

The ribosomal DNA restriction endonuclease banding patterns of numerous *P. ultimum* isolates (including *P. ultimum* var. *sporangiiferum* and asexual "group G" *Pythiums*) were also studied. The digestion revealed the presence of two regions of heterogeneity occurring in the nontranscribed spacer (NTS). A region located about one kb downstream of the 3′ end of the large subunit ribosomal RNA (LSrRNA) gene

contains a segment present in multiple versions differing in size by as much as 0.9 kb. Each isolate shows a unique pattern of heterogeneity in this region. The other region of heterogeneity is located nearer to the centre of the NTS and was identified by digestion of the rDNA which resulted in the appearance of a ladder-like banding pattern. Each band of the ladder increased in size by an increment of approximately 380 bp. Therefore, the NTS in *P. ultimum* is dominated by an array of uniform subrepeats. The size of the repeat is identical in all isolates, but the number of subrepeats (6-12), and the relative abundance of a length variant is highly polymorphic.

In addition to the discovery of subrepeat heterogeneity in *P. ultimum*, the rDNA restriction endonuclease banding pattern was correlated with the distance dendogram generated using the mtDNA data. The rDNA data demonstrated that all of the *P. ultimum* var. *ultimum* isolates which belong to the two major grouping identified by mtDNA restriction analysis, also have very similar rDNA restriction banding patterns. In cases where mtDNA polymorphisms were observed, significant polymorphism was also observed in the rDNA restriction data. The data showed that many of the *Pythium* sp. "group G" isolates possessed subrepeat heterogeneity, with the exception of two isolates, which is consistent with differences seen in the mtDNA data. The rDNA data also demonstrated that *P. ultimum* var. *sporangiiferum* possesses

subrepeat heterogeneity. Thus, all three forms of *P. ultimum* (var. *ultimum*, var. *sporangiiferum*, and *Pythium* sp. "group G" have subrepeat heterogeneity of the same incremental nature (380 bp). Therefore, the NTS organization observed in *P. ultimum* can be used as a character in assigning isolates to *Pythium* species.

The polymerase chain reaction (PCR) was used to amplify the NTS of two isolates of *P. ultimum* which have subrepeat heterogeneity. The amplified region includes a small part of the LSrRNA gene, about half of the LSrRNA gene, and all of the NTS. PCR amplification of the heterogenous target DNA resulted in sets of fragments which accurately reflect the heterogeneity in the target DNA, although there was a preferential amplification of the smaller targets. PCR product sizes ranged from 4.6 to 5.8 kb.

In this thesis the first complete sequence of the NTS of an Oomycete is presented. The NTS of *P. ultimum* (BR471) was amplified using PCR and subsequently cloned into Bluescript M13ks+. The entire sequence of the NTS was deduced for the shortest length variant (pJB1A). The nucleotide sequence revealed the presence of two repeat families, A and B. There were four A repeats present in a tandem array, each repeat about 62 bp. In addition, there were 13 B repeats, each about 40 bp. There is some indication that the A and B repeats are related and could have evolved from a common sequence. The next to shortest

length variant clone (pJB2) was partially sequenced to reveal the existence of a third family of repeats, referred to as the C repeats. The PCR was then used to further characterize the next larger length variant (pJB3 and pJB3A). The sequence and PCR data identified the exact location and size of the subrepeat, which causes the major length heterogeneity observed previously in rDNA restriction endonuclease analysis. The sequence data reveals that the corrected subrepeat size (compared to the previously estimated size determined from the rDNA digestion data) is in fact 366 bp. In addition, primer extension was used to determine the putative transcription initiation site. The general organization of the NTS of *P. ultimum* is similar to what has been observed in plants and animals (Reeder 1984, Rogers *et al.* 1987) and unlike what is seen in other fungi. This observation supports the separation of the Oomycetes from the kingdom of Fungi (Cavalier-Smith 1989).

Using the DNA sequence of the NTS for *P. ultimum*, two oligonucleotide probes were designed. Their ability to hybridize to total DNA was tested against 26 other *Pythium* species, several other Oomycetes, one member of the Hyphochytriomycetes, and four Ascomycetes. The probe PS2 was found to be very effective in specifically hybridizing to *P. ultimum* isolates only, including *P. ultimum* asexual isolates ("group G") as well as the type culture of *P. ultimum* var. *sporangiiferum*.

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A	adenine
bp	base pairs
C	cytosine
CTAB	hexadecyltrimethyl ammonium bromide
cm	centimetre
DNA	deoxyribonucleic acid
dATP	2'-deoxyribonucleic 5'-triphosphate
EDTA	ethylenediamine-tetra-acetic acid
ETS	external transcribed spacer
Fig(s).	figure(s)
G	guanine
g	gram(s)
h	hour(s)
IGR	intergenic region
IPTG	isopropylthiogalactoside
ITS	internal transcribed spacer
kb	kilobase pairs
L	litre(s)
LSrRNA	large subunit ribosomal RNA
M	molar
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)

mM	millimolar
mol	molecular
ng	nanogram(s)
NTS	nontranscribed spacer
PCR	polymerase chain reaction
pol	polymerase
RFLP	restriction fragment length polymorphism
rDNA	ribosomal DNA
RNA	ribonucleic acid
rRNA	ribosomal RNA
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SSC	sodium saline citrate
SSrRNA	small subunit ribosomal RNA
T	thymine
μg	microgram(s)
μl	microlitre(s)
v/cm	volts/centimetre
vol	volume(s)
v/v	volume/volume
wt	weight
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Introduction

Pythium ultimum belongs to the class Oomycetes, a group of organisms which have been considered to be fungi on the basis of nutrition and morphology. More recent classification systems have placed the Oomycetes within the newly created kingdom Chromista (Cavalier-Smith 1989).

Pythium ultimum has been identified throughout the world as a major plant pathogen. It affects a wide variety of plant species, especially in the seedling stage, causing preemergence and postemergence damping off, root rot, and lower stem rot (Hendrix and Campbell 1973).

It is relatively easy to identify the genus *Pythium* among other fungi but there are few morphological features for species assignment within the genus. *P. ultimum* has been misidentified in the past (Drechsler 1927) and can be confused with a morphologically similar species such as *P. irregulare* or *P. paroecandrum*. The problem of species assignment within the genus is further compounded by the existence of many *Pythium* isolates which are unable to produce sexual structures, which are essential in the proper identification of species. The problem of identifying species is beginning to be addressed by using molecular methods (Martin 1990, Martin 1991, Lee *et al.* 1994, Levesque *et al.* 1994).

In the study presented here mitochondrial DNA (mtDNA)

and ribosomal DNA (rDNA) restriction banding patterns are compared among different *P. ultimum* isolates. Included in the study are some asexual "group G" *Pythium* isolates, as well as some *P. ultimum* var. *sporangiiferum* isolates. The goal is to assign the asexual isolates to a species, as well as to study the intraspecific variability among the group of organisms. The results of the study may also help to confirm the separation of Oomycetes from the true fungi. In addition, DNA sequence of the nontranscribed (NTS) spacer for *P. ultimum* was used to design a species-specific probe.

The ribosomal DNA (rDNA) genes are composed of tandemly repeating units each consisting of a large and small subunit ribosomal RNA genes (LSrRNA and SSrRNA respectively) plus a 5.8S component. Between each repeat unit is a region referred to as the nontranscribed spacer (NTS). It is known that the rRNA genes are highly conserved in sequence and structure, whereas the NTS can be highly variable in sequence, even between closely related species. In higher eukaryotes (plants and animals) the NTS has been shown to possess repeat elements which may or may not have homology with the promoter region (Bach *et al.* 1981, Rogers *et al.* 1987, Reeder 1984). There is often more than one family of repeats, whose numbers and sizes can vary between species. It is also believed that such repeat elements are responsible for the length heterogeneity which can occur in the NTS of the rRNA gene family (Dover 1982, Dover and

Flavell 1984). In addition, it has been shown that many of these repeat elements can enhance transcription and bind transcription factors involved in RNA polymerase I transcription (Reeder 1984, Sollner-Webb and Moughey 1991).

The goal of this project is to study the degree of genetic variability in *P. ultimum*. This will be achieved by doing restriction fragment length polymorphism (RFLP) studies of the mtDNA and rDNA of numerous *P. ultimum* isolates. These studies will also contribute molecular data which can be useful for species identification purposes. The next goal is to characterize the length heterogeneity present in the rDNA family, which most frequently occurs in the NTS region (Bach *et al.* 1981, Reeder 1984, Rogers *et al.* 1987). Once the region of heterogeneity has been identified, PCR and cloning techniques can be used to further characterize the nature of the heterogeneity. The ultimate goal is to describe the length heterogeneity at the nucleotide sequence level. The sequence generated from such a study can then be used to design a species-specific probe for *P. ultimum* based on the NTS.

LITERATURE REVIEW

I. *Pythium ultimum* and the Oomycetes

Pythium ultimum is a member of the class Oomycetes, a group of zoosporic fungi with typical fungal nutritional needs and morphology. A closer look at these organisms reveals four key distinguishing features which set them apart from true fungi (Alexopoulous and Mims 1979):

1. They often have biflagellate zoospores with one anteriorly directed tinsel flagellum and a posteriorly directed whiplash flagellum.
2. Their cell wall contains cellulose, while fungi have cell walls that consist mainly of chitin.
3. Their sexual reproduction is oogamous (ie. they have male and female reproductive structures).
4. Their somatic nuclei are diploid, and nuclei only become haploid in the gametangial structures where meiosis takes place before fertilization of the egg. Meiosis happens after fertilization in true fungi.

Therefore there are many morphological and cytological features which set Oomycetes apart from all the other fungi. Their phlyogenetic relationship to other major fungal groups, including the Chytridiomycetes, is probably very distant (reviewed in Barr 1983). McNabb and Klassen (1988) have shown that the mode of evolution of the mitochondrial DNA of Oomycetes is distinct from that of other fungi. In

addition, analysis of the 18S rRNA gene sequence from different species supports the separation of Oomycetes from the fungi (Gunderson *et al.* 1987; Förster *et al.* 1990). Cavalier-Smith (1989) has removed Oomycetes from the kingdom Fungi altogether and placed them in the newly created kingdom Chromista. The new kingdom has the Oomycetes grouping with the diatoms and brown algae (Cavalier-Smith 1989).

The Oomycetes (or "water molds") are largely aquatic organisms, but can also inhabit soils. It is the terrestrial Oomycetes which have the greatest social and economic impact on humans, since they are commonly parasitic on agriculturally important plants. For example, *Phytophtora infestans* caused the great potato famines in Ireland of 1845-47. The organism was therefore partially responsible for the massive influx of Irish immigration into the United States. It should be noted that many of these plant pathogens still possess the ability to produce zoospores, no doubt a remnant of their aquatic ancestry.

Trow isolated *Pythium ultimum* from a very rotten cress seedling that had been left lying prone on water-saturated soil. He went on to publish a thorough description of the organism (Trow 1901). It is likely that *P. ultimum* was first observed by de Bary (1881) but he chose to assign it to the species *Pythium debaryanum*, a species already described by Hesse (1874). De Bary's description more

closely resembles Trow's *P. ultimum* than Hesse's *P. debaryanum*. Drechsler (1927) stated that *P. ultimum* has often been misidentified as *P. debaryanum*. It now is apparent that Hesse did not describe a pure culture and Plaats-Niterink (1981) has chosen to exclude the species altogether. Hence with all the conflicting descriptions associated with *P. debaryanum*, Trow's name, *Pythium ultimum* has been given priority.

P. ultimum forms a cottony aerial mycelium when grown on cornmeal agar (Plaats-Niterink 1981). The hyphae are coenocytic and up to 11 μm wide with cellulose walls. Sporangia are mostly not formed and zoospores are very rarely produced. Hyphal swellings are formed which are globose, intercalary and sometimes terminal. The oogonia are mostly terminal, sometimes intercalary, globose, smooth-walled, and their diameters can vary a great deal (14-25 μm). There can be 1-3 antheridia per oogonium. Antheridia are sac-like, monoclinous, diclinous or sometimes hypogynous. Each oogonium will produce one oospore which is aplerotic, globose, 12-21 μm in diameter, with a wall often 2 μm or more thick.

Trow (1901) believed that the new species he had described stood at the end of a series since it alone had lost its ability to produce zoospores. He considered this to be an adaptation to its terrestrial existence. That is why he proposed the name *Pythium ultimum*. Of course today

there are many known examples of non-zoospore producing *Pythiums* (Dick 1990).

Drechsler (1946) demonstrated that oospores from 3-month old cultures of *P. ultimum* var. *ultimum* can produce zoospores when they are placed in water, affirming their aquatic ancestry. Drechsler (1960) later described an isolate resembling *P. ultimum* which readily produced zoospores. He believed it to be the same species and chose to call it *P. ultimum* var. *sporangiiferum*. Drechsler's new variety automatically established the species described by Trow (1901) as *P. ultimum* var. *ultimum*. In the following discussions "*P. ultimum*" will refer to *P. ultimum* var. *ultimum*.

In his original description of *Pythium ultimum*, Trow (1901) considered it to be non-parasitic. Today it is regarded as a major plant pathogen with worldwide distribution. It has been isolated throughout the United States (Miller *et al*. 1957, McLauglin 1946, Sprague 1942) and Canada (Vaartaja and Agnihotri 1969), Tahiti (Scott 1960), South America (Avarez-Garcia and Cortes-Monllor 1971), Iceland (Johnson 1971), Africa (Wager 1931, Ravise and Boccas 1969, Filani 1975), Europe (Koyeas 1977, Domsch et al. 1968, Plaats-Niterink 1975, Cejp 1961), Japan (Alicbusan *et al*. 1965) and Australia (Vaartaja and Bumbieris 1964).

P. ultimum affects a wide variety of plant species,

especially in the seedling stage, causing preemergence and postemergence damping off, root rot, and lower stem rot (for review of *Pythiums* as plant pathogens see Hendrix and Campbell 1973). Examples of plant hosts from which *P. ultimum* has been isolated include: tulips (Moore and Buddin 1937), sweet potato (Poole 1934), sugar beet (Gindrat 1976), coffee (Filani 1975), apple (Bielenin 1976), pumpkin and watermelon (Tompkins *et al.* 1939), cotton (Arndt 1943), peach (Miller *et al.* 1966), sugar cane (Stevenson and Rands 1938), citrus (Mahmood 1971) , soya beans (Laviolette and Athow 1971) and safflower (Klisiewicz 1968).

P. ultimum is one of the most prevalent of *Pythium* species found in the soil (Plaats-Niterink 1981). In soils where it is predominant, plants not immediately killed by the pathogen can go on to mature, but are likely to experience poor root development, stunting and reduced yields. Reduced plant vigor has been described as the only above-ground symptom which may be manifested in crops infected with *P. ultimum* (Yuen *et al.* 1991).

Miller *et al.* (1966) described the process of infection of peach roots by *P. ultimum*. The organism penetrates the roots within 5-8 hours after inoculation, usually between epidermal cell via an infection peg formed from an appressorium. It colonizes the cortex within 24 hours and moves on into the stelar region by 36 hours. At that point the cell collapses and separation occurs. Cells which lack

secondary thickening, such as feeder roots and root tips, are more prone to infection. Once feeder root necrosis takes place, the peach tree will be less able to absorb nutrients (Hendrix *et al*. 1966), which can lead to the death of the tree. More than one *Pythium* species is often involved in the infection of the feeder roots.

Pringsheim (1858) established the genus *Pythium* and placed it in the Family Saprolegniaceae. The genus became better defined as more new species were described over the next few decades. By the end of the century the relationship of *Pythiums* to other Oomycetes was better understood. Schroter (1897) believed that it was more appropriate to place the genus in a new family called *Pythiaceae*. The genus and its relative taxonomic position remain unchanged since that time.

It is relatively easy to identify the genus *Pythium* among other fungi but there are few morphological features for species assignment within the genus. Size and shape of sporangia, oospore and oogonia, oospores wall thickness, and the mode of antheridial attachment are some of the major characters used in assigning isolates to species. *P. ultimum* may become confused with closely related species such as *P. irregulare* or *P. paroecandrum*, where ranges of oogonial and oospore size and oospore wall thickness may overlap. The problem is further compounded by the inability of some isolates to produce sexual structures, essential in

the proper identification of species. Asexual isolates cannot be identified as *P. ultimum* and are assigned to groups designated "F", "T", "G", "P", or "HS" on the basis of the morphology of the sporangia or hyphal swellings (Plaats-Niterink 1981). Thus, there is a problem in assigning these asexual isolates to a species.

Problems in properly identifying species have been addressed by molecular methods. Species-specificity of mitochondrial DNA (mtDNA) restriction endonuclease banding patterns within the genus *Pythium* has been observed (Martin and Kistler 1990). Mitochondrial DNA (mtDNA) restriction analysis was used to identify a *Pythium* sp. "group G" isolate as *P. ultimum* (Huang *et al. 1992*). A number of isolates of "group HS" *Pythium* have been shown to be identical to *P. ultimum* on the basis of mtDNA restriction profiles (Martin 1990). DNA probes have been reported useful in the identification of other species (Lee *et al.* 1993, Martin 1991). Yuen *et al.* (1993) produced a species-specific monoclonal antibody reported to be effective in identifying *P. ultimum*. Using PCR, a species-specific DNA probe based on the internal transcribed spacer was developed by Levesque *et al.* (1994).

Mullis and Faloona (1987) devised the polymerase chain reaction (PCR), an *in vitro* method of amplifying a targetted segment of DNA with a DNA polymerase using a pair of oligonucleotide primers directed toward each other.

Consecutive rounds of template denaturation, primer annealing, and DNA polymerization are performed until the target sequence is amplified by a factor of about 10 million fold. Mullis and Faloona had to add fresh Klenow polymerase after each round of denaturation, but Saiki *et al.* (1988) improved upon this method greatly by using a thermostable DNA polymerase (Taq polymerase) instead.

PCR technology has many applications (reviewed in Erlich (ed.) 1989, and Innis *et al.* (eds.) 1990). It has been used for fungal phlyogenetic studies (White *et al.* 1990), as well as countless medical applications (also reviewed in Erlich 1989). Belkhiri *et al.* (1992) used PCR to identify the location of the 5S ribosomal RNA genes in different species of *Pythium* and in several Oomycetes.

II. Ribosomal RNA genes

A. Function

Ribosomal RNA (rRNA) along with numerous proteins make up the ribosome. It was once generally accepted that its protein component was largely responsible for the enzymatic reactions involved in protein synthesis, rRNA simply acted as a scaffold which supported and positioned the various proteins within the ribosome. This idea was reinforced with the discovery that mutations in the gene for ribosomal protein S12 could cause streptomycin resistance (Ozaki *et*

al. 1969) or dependence (Birge and Kurland 1969). As early as 1963 Santer (1963) proposed that rRNA had a direct role in ribosome function. Crick (1968) and Woese (1972) suggested that the major or sole component of the primordial ribosome may have been RNA. Woese (1980) has hypothesized that rRNA is responsible for the basic translational mechanism, putting less emphasis on the proteins in the ribosome.

There is mounting evidence supporting the idea that rRNA is an essential catalytic component of the ribosome. The discovery of self-splicing introns and catalytic RNA (Cech *et al.* 1981, Guerrier-Takada 1983, reviewed by Cech and Bass 1986) has given more credibility to this idea.

B. Ribosomal RNA gene organization

In eukaryotic cells, over half of the transcriptional capacity is dedicated to the production of rRNA. They synthesize a 35-47S precursor rRNA that is further processed into the 17-18S (Small Subunit rRNA or SSrRNA), 5.8S, and 25-28S (Large Subunit rRNA or LSrRNA) components of the ribosome. These ribosomal genes are exclusively transcribed by RNA polymerase I (reviewed by Gerbi 1985, Sollner-Webb and Tower 1986, Sollner-Webb and Mougey 1991).

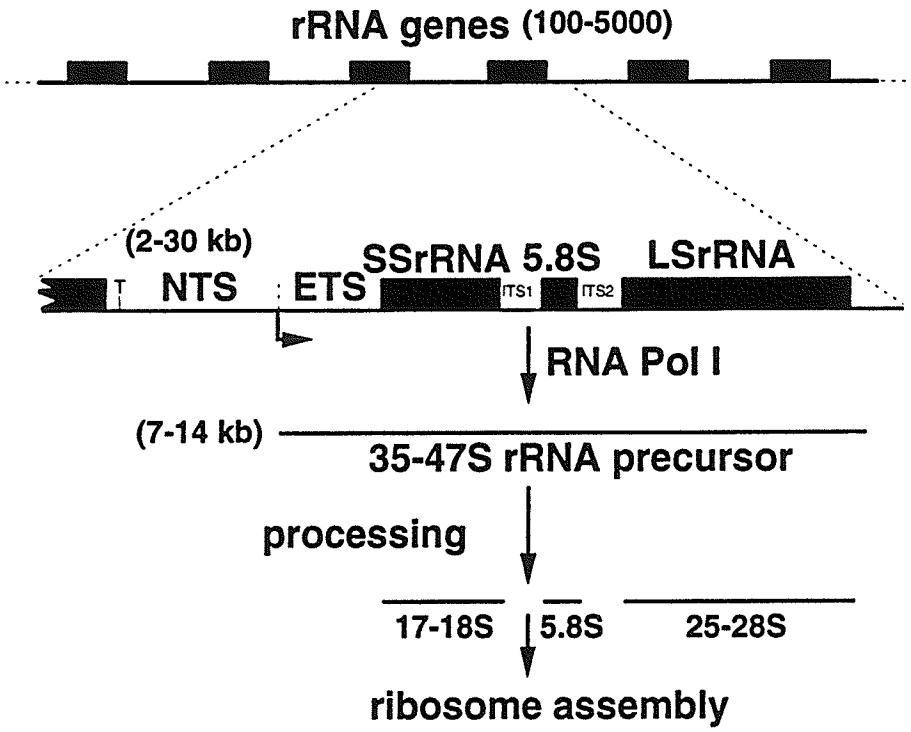
The cell requires more than a million new ribosomes per generation to meet its requirements for protein synthesis in daughter cells. Assuming the cell is actively transcribing

rRNA at the maximal rate, over 100 rRNA gene copies will be required to meet its demands. Thus it is not surprising that various eukaryotes have between 100 and 5000 rRNA gene copies per haploid genome (Long and Dawid 1980).

The rRNA gene (refer to fig. 1) copies are arranged in tandem, head to tail (Reeder 1974), located on one to several chromosomes (Busch and Smetana 1970, Lima-de-Faria 1976, 1980). The location of the rRNA genes is referred to as the nucleolus organizer region (NOR) (Ritosssa and Spiegelman 1965, Ritossa *et al*. 1966, Birnstiel *et al*. 1966) whose active transcription can be visualized in the area of the nucleus called the nucleolus. Each rRNA operon is separated by a non-transcribed spacer (NTS, also referred to as intergenic spacer or IGS, or intergenic region or IGR), a region that appears to be devoid of transcription (Miller and Beatty 1969, Miller and Bakken 1972, Federoff 1979). Transcription begins in the external transcribed spacer (ETS) traversing the SSrRNA, 5.8S rRNA and LSrRNA genes (in that order) creating one precursor rRNA molecule (also called "primary transcript") of 35-47S (6-15 kb). The three rRNA genes are separated by two internal transcribed spacers which are later removed to create the mature rRNA species.

The basic organization of the rRNA genes was understood even before the age of recombinant DNA technology (Reeder 1974, Miller 1981). The task was made easier because rRNA genes are so actively transcribed, and because amphibian DNA

Fig. 1. Organization, transcription and processing of the rRNA genes. A schematic representation showing from top to bottom: the tandemly organized rDNA repeat units, the structure of a single rDNA repeat unit, its transcription by RNA polymerase I (RNA pol I) resulting in the production of an rRNA precursor molecule, processing of the precursor molecule into the LSrRNA, SSrRNA and 5.8S rRNA components. The range of sizes and numbers are given in brackets. The dashed line separates the NTS (nontranscribed spacer) and the ETS (external transcribed spacer). "T" refers to the termination site. The arrow shows the transcription initiation site.


rRNA genes (100-5000)
(2-30 kb)
NTS
ETS
SSrRNA 5.8S
ITS1
ITS2
LSrRNA
RNA Pol I
(7-14 kb)
35-47S rRNA precursor
processing
17-18S
5.8S
25-28S
ribosome assembly

is very GC-rich and highly amplified in oocytes (Brown and Dawid 1968, Gall 1968). The first rRNA gene to be isolated was from *Xenopus laevis* (Birnstiel *et al.* 1968) and the first eukaryotic gene to be cloned was the rRNA genes from *X. laevis* (Morrow *et al.* 1974). The first genes whose transcriptional pattern was visualized by electron microscopy were the amphibian rRNA genes (Miller and Beatty 1969, Miller and Bakken 1972) and rRNA was the first eukaryotic transcript whose basic processing pattern was discerned (Perry 1976).

Not all organisms have their rRNA genes organized as in Fig. 1. *Tetrahymena pyriformis* has just one copy of the rRNA operon integrated into its chromosome (Yao and Gall 1977). In *Tetrahymena* the rRNA copy number is increased by generating several hundred, mostly linear, extrachromosomal molecules that contain two copies of the rRNA gene cluster in a palindromic arrangement (Karrer and Gall 1976, Engberg *et al.* 1976). Each half of the linear molecule contains one rRNA transcription unit oriented in opposite directions (the 5S gene is absent). *Dictyostelium discoideum* (Cockburn *et al.* 1978) and *Physarum polycephalum* (Vogt and Braun 1976, 1977, Molgaard *et al.* 1976) also have the rDNA repeat units on palindromic extrachromosomes, the former having the 5S gene present in each half of the palindrome. *Xenopus* amplifies about 3000 fold more rDNA on circular and linear extrachromosomes during oogenesis (Brown and Dawid 1968,

Gall 1968), each molecule containing several rDNA transcription units (Hourcade *et al.* 1973, Rochaix *et al.* 1974). Extrachromosomal rDNA transcription units have been identified in *Drosophila* (Graziani *et al.* 1977) and *Saccharomyces* (Meyerink *et al.* 1979, Clark-Walker and Azad 1980, Larionov *et al. 1980*), as well. Childs *et al.* (1981) discovered the presence of single rDNA repeat units dispersed throughout the genome of *Saccharomyces cerevisiae* and *Drosophila melanogaster*. Such transcription units outside of the tandem arrays are referred to as "orphans".

In eukaryotes the 5.8S rRNA is separated from the SSrRNA and LSrRNA genes by the internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2) respectively. These are "pseudo-introns" in the sense that they are excised during processing of the primary transcript, but no ligation reaction occurs afterwards. The 5.8S rRNA can associate with the LSrRNA molecule by hydrogen bonding, therefore no covalent ligation is necessary (Pace *et al.* 1977, Nazar and Sitz 1980).

Introns have been discovered in some of the LSrDNA of *Drosophila melanogaster* (Glover and Hogness 1977, White and Hogness 1977, Wellauer and Dawid 1977, Pellegrini *et al.* 1977), although such intron-containing rDNA units appear to be transcriptionally silent (Levis and Penman 1978, Long and Dawid 1979, Jolly and Thomas 1980, Kidd and Glover 1981). One type of intron found in *D. melanogaster* is flanked by

short direct repeats (Roiha *et al.* 1981), very reminiscent of transposable elements. Many, but not all *Tetrahymena* species and strains have been shown to possess an intron in all their LSrDNA copies (Wild and Gall 1979, Din and Engberg 1979). Unlike the situation in *Drosophila*, the intron containing rDNA repeat units are transcriptionally active. The intron is excised from the transcript as a linear molecule (Carin *et al.* 1980, Zaug and Cech 1980, 1982), cyclized (Grabowski *et al.* 1981, Zaug *et al.* 1983) and the cut transcript is ligated (Zaug *et al.* 1983). Kruger *et al.* (1982) observed that this reaction can occur *in vitro* in the absence of any protein factors (self-catalytic). Ribosomal DNA introns of presumably transcriptionally active genes have been observed in mitochondria (Bos *et al.* 1978, Hahn *et al.* 1979) and chloroplasts (Rochaix and Malnoe 1978). All the examples of rDNA introns listed above occur in the LSrDNA. The occurrence of introns in the SSU rDNA seems rarer, but cases of self-splicing introns have been reported for fungi (Sogin and Edman 1989), lichens (Wilcox *et al.* 1992), and green algae (Davila-Aponte *et al.* 1991, Wilcox *et al.* 1992).

5S rRNA

In eukaryotes the 5S rRNA genes are transcribed by RNA polymerase III, unlike the other rRNA genes which are transcribed by RNA polymerase I (reviewed by Gerbi 1985).

It is not surprising then to find that the 5S gene is often found unlinked to the rDNA repeat unit. In fungi it can be found dispersed throughout the genome (Selker *et al.* 1981, Mao *et al.* 1982, Bartnik 1986) or present in the NTS of the rDNA repeat (Kramer *et al.* 1978, Cassidy and Pukkila 1987, Cihlar and Sypherd 1980, Rozek and Timberlake 1979). In plants and animals the 5S gene is commonly organized in tandem arrays (Brown and Sugimoto 1973, Hemleben and Grierson 1978, Gerlach and Dyer 1980, and Benes and Cave 1985), separated from the RNA polymerase I-transcribed arrays.

Belkhiri *et al.* (1992) surveyed for the genomic locations of the 5S genes in different *Pythiums*, plus other Oomycetes. The 5S rRNA gene was found in the rDNA repeat unit for some species and outside of the repeat unit for others. The orientation of the NTS-located 5S genes was usually inverted (relative to the direction of the rRNA transcription unit), with the exception of *Phytophthora cryptogea* and *Achlya klebsiana*. Their data agree with the study done by Howlett *et al.* (1992). Belkhiri *et al.* (1992) presented evidence showing that the 5S gene is located in tandem arrays outside of the rDNA NTS for *Pythium ultimum*, and that there was some indication of dispersed genes as well. This kind of organization is typical of higher eukaryotes.

Evolution of the rRNA operon

In typical eubacteria there are several rRNA transcription units, each containing the 16S, 23S and 5S rRNA genes (in that order). They are transcribed as a single precursor rRNA molecule (Kiss *et al.* 1977, Morgan 1982). The 5.8S is absent in bacteria, but a closer look at the 5′ end of the 23S rRNA reveals a region which shares a good deal of sequence and/or structural similarity with the 5.8S rRNA (Nazar 1980, Jacq 1981, Clark and Gerbi 1982). Several archaebacteria have only a single copy of the operon in the genome (Hofman *et al.* 1979, Tu and Zillig 1982), but in the case of *Thermoplasma acidophilum* there are three rRNA genes which may have separate promoters (Tu and Zillig 1982). Clark and Gerbi (1982) proposed that the original rRNA operon consisted of a single transcription unit which included the 5S gene. The transcription unit was eventually duplicated producing several operons over time (Gerbi 1985). As new species evolved the 5S gene may have become separated, allowing it to develop its own promoter. Clark (1987) later offered an alternative hypothesis whereby the ancestral 5S gene was unlinked and over time became linked with the other rRNA genes in some fungi and protozoa.

The primary and secondary structure of rRNA is well conserved across the various kingdoms. This lends support to the belief that the rRNA genes originated from a common ancestral gene (Gerbi 1985). The conservation of structure

may indicate the functional importance of rRNA as it relates to the ribosome and protein synthesis (Wool 1979, Noller 1991). Comparison of these sequences has been done in molecular phylogeny studies to help ascertain the evolutionary relationships of different organisms (Gray *et al.* 1984).

Brown *et al.* (1972) discovered that when they compared the sequence of the rDNA repeat unit between *Xenopus laevis* and *Xenopus borealis* that the rRNA genes were remarkably conserved, whereas the NTS showed a good deal of variation. The manner in which these tandemly organized repeat units have evolved, leading to intraspecific homogeneity along with interspecific variation, has been termed "concerted evolution" (reviewed by: Dover 1982, Dover *et al.* 1982, Arnheim 1983, Dover and Flavell 1984).

Dover (1982) proposed that "molecular drive" was responsible for the homogenization of sequence within tandemly repeated families (Dover and Flavell 1984). The mechanisms responsible for molecular drive involve unequal crossing over, gene conversion and transposition (mechanisms reviewed by Gerbi 1985).

Nontranscribed spacer

Each rRNA transcription unit is separated from the next by a non-transcribed spacer (reviewed by Reeder 1984, Sollner-Webb and Mougey 1991). The length of the NTS can

vary from 2 kb (eg. *Saccharomyces cerevisiae*) to 30 kb (eg. mouse)(Long and Dawid 1980). Length heterogeneity is not only observed between species but within the rDNA tandem array of an individual. Wellauer *et al.* (1974, 1976) described the presence of variable length spacers for *Xenopus* within an individual. Length heterogeneity within an individual has also been observed in Chinese hamster (Stambrook 1978), mouse and man (Arnheim and Southern), *Drosophila* (Glover and Hogness 1977, Wellauer and Dawid 1977), *Vicia faba* (Rogers *et al.* 1986), *Arabidopsis thaliana* (Gruendler *et al.* 1991), wheat (Appels and Dvorak 1982) and barley (Saighai-Maroof 1984). Intraspecific length heterogeneity of the rDNA spacer has commonly been observed within populations as well (Yakura *et al.* 1984, Appels and Dvorak 1982, Ellis *et al.* 1984, Pruit and Meyerowitz 1986, Rogers and Bendich 1987). These length variants are usually observed as differences in spacer lengths of 100 bp or more.

The major length heterogeneity described above is common in "higher eukaryotes" (plants and animals). It is usually due to variable numbers of a repetitive element(s) (also referred to as subrepeats) present in the rDNA NTS (Reeder 1984, Rogers *et al.* 1986, Rogers and Bendich 1987). The size of subrepeats can vary from 35 bp (*Xenopus*) to 617 bp (shrimp) in different species (Reeder 1984, Koller *et al.* 1987). The number of size classes within a species and/or individual can vary greatly (Rogers and Bendich 1987). The

most dramatic length heterogeneity observed is probably that in *Vicia faba*, in which 20 size classes have been identified (Rogers *et al.* 1986). More than one class of subrepeats may be present in the rDNA spacer of any one repeat unit (Reeder 1984, Gruendler *et al.* 1991). It is believed that unequal crossing over during meiosis and mitosis is the major mechanism responsible for length heterogeneity of the spacer region (Smith 1973, 1976, Dover 1982, Dover and Flavell 1984).

The presence of length heterogeneity appears to be rare in the true fungi. The ascomycete *Yarrowia lipolytica* has been reported to have variably-sized spacers, but this heterogeneity does not appear to be due to variations in the number of subrepeats (van Heerikhuizen *et al.* 1985). This pattern is therefore different from that commonly seen in plants and animals. Zerucha (Thesis, 1992) observed what appears to be length heterogeneity due to varying numbers of a 100 bp motif in the basidiomycetous fungus *Puccinia graminis*. Sometimes length heterogeneity is not obvious from restriction analysis. Minor length variations in yeast, due to a variable number of a direct repeat of 4-16 nucleotides, were identified after sequence analysis of the NTS (Skryabin *et al.* 1984). A recent report (Morton *et al.* 1995) of short subrepeats in the Hyphomycetes *Verticullum alboatrum* and *V. dahliae* indicate that they are complex in organization and may not have arisen by simple reiteration

of the whole units.

The occurrence of major length heterogeneity seems to be more common in oomycetes than in other fungi. Martin (1990a) discovered a region of heterogeneity in the NTS near the 3' end of the LSrRNA gene in *P. paroecandrum*, *P. spinosum*, *P. sylvaticum*, and *P. irregulare*. The results strongly suggest the presence of subrepeat heterogeneity. Belkhiri *et al.* (1992) discovered that upon amplifying the region between the 5S and LSrRNA genes, more than one PCR product was often produced for many *Pythium* and other Oomycete isolates. This suggests that some sort of length heterogeneity, possibly due to subrepeat reiterations, is causing the multiple amplification products to occur. The above evidence supports the belief that Oomycetes are phylogenetically distinct from other fungi (Barr 1983, Cavalier-Smith 1989), which often lack this kind of length heterogeneity.

Subrepeats in the NTS will not always be perfect copies of each other, and the degree of homogeneity can vary between species (Barker *et al.* 1988, Toloczyki and Feix 1986, Moss *et al.* 1980). Also, there are examples of spacers which have subrepeats, but no length heterogeneity (Roger and Bendich 1987, Sharp *et al.* 1986).

Other kinds of heterogeneities can also exist. Micro-heterogeneities in the form of sequence changes and/or base modification of the NTS have been observed in onions,

pumpkins, peas, sea urchins and humans (Maggini and Carmona 1981, Siegel and Kolacz 1983, Jorgensen *et al*. 1987, Simmen *et al*. 1985, Sylvester *et al*. 1986). Nucleotide insertions or deletions in the rDNA spacer within the tandem array have also been reported (Siegel and Kolacz 1983, May and Appels 1987).

Function of the NTS

Electron micrographs of the region between the LSrRNA and SSrRNA genes show a region of non-transcription (Miller and Beatty 1969, Miller and Bakken 1972), hence the term "non-transcribed spacer". In fact, transcripts within the NTS have been observed on rare occasions (Scheer *et al*. 1973, 1977, Franke *et al*. 1976). At the very least it is a region where transcription initiates and where it terminates. Enhancer elements, present as subrepeats and promoter duplications, have been identified in the NTS (Reeder 1984, Sollner-Webb and Moughey 1991), and the mechanisms of transcription by RNA polymerase I and the transcription factors involved are being elucidated (Sollner-Webb and Moughey 1991). In addition, origins of replication have been discovered in the NTS of yeast (Szostak and Wu 1979), *Lytechinus* (Botchan and Dayton 1982) and *Drosophila* (McKnight and Miller 1975). It would therefore seem that the NTS is a hotbed of activity and not just a region where no transcription appears to take place.

That is why some have chosen to refer to the rDNA spacer region as the intergenic spacer (IGS) or intergenic region (IGR).

The relative location of the transcription start site can vary a great deal between species. Some examples of ETS sizes are: *Xenopus laevis* approx. 710 bp (Sollner-Webb and Reeder 1979, Bosely *et al.* 1979); Maize, approx. 820 bp (McMullen *et al.* 1986); wheat, approx. 1130 bp (Barker *et al.* 1988); *Drosophila melanogaster*, approx. 860 bp (Simeone *et al.* 1985); *Saccharomyces cerevisiae*, approx. 700 bp (Klemenz, R., and E.P. Geiduschek 1980); *Tetrahymena thermophila*, approx. 500 bp (Engberg *et al.* 1980); Mouse, approx. 4.0 kb (Mishima *et al.* 1980); and man, approx. 4.0 kb (Miesfeld and Arnheim 1982).

The sequence at the site of initiation shows very little homology between species (Gerbi 1985, Gerstner *et al.* 1988, Cordesse *et al.* 1993). In plants a very weak homology at the region immediately around the transcription start of TATAT(G/A)GGGG (the (G/A) representing the initiation site) has been identified. The first nucleotide transcribed is usually a purine (more often an A than a G).

Similarly when the sequences of the promoter are compared between species, there is virtually no sequence homology seen, except in very closely related species (Sollner-Webb and Moughey 1991). This is consistent with studies which showed that transcription factors were highly

species-specific (Grummt 1982). Although there is little sequence homology, the general spatial organization of the promoter appears to be relatively conserved in eukaryotes (Sollner-Webb and Moughey 1991). A region of 35 residues upstream of the initiation site seems critical for transcription; it is referred to as the "core promoter domain" (Sollner-Webb and Tower 1986). Another region up to 150 residues upstream of the start site enhances transcription initiation. It is referred to as the "upstream promoter domain" (Sollner-Webb and Tower 1986, Miller et al. 1985). The spacing of the two domains has been shown to be critical in yeast (Choe *et al.* 1992).

One may have assumed that with the functional constraints placed upon the RNA polymerase I promoter, conservation of sequence across species would be observed, but it is not. This high rate of evolution is probably due to the fact that RNA polymerase I only has to recognize one type of promoter (Sollner-Webb and Moughey 1991). Since the rRNA genes are organized in tandem arrays, any subtle changes in the promoter sequence can be amplified through the family by the mechanisms responsible for molecular drive (unequal crossing over, transposition and gene conversion)(Dover 1982, Dover and Flavell 1984). Any changes in the promoter sequence may have to be followed by compensatory changes in the transcription factors. Such co-evolution would be much more difficult for the other two

classes of RNA polymerase since they must recognize the promoters from many different genes.

The site of termination in the rRNA genes is not easily defined in eukaryotes. Although dyad symmetry followed by a stretch of T's is common at the end of the LSrRNA gene (Gerbi 1985), this is not likely to be the true termination site. Tautz and Dover (1986) showed that there is no fixed point of termination in the rDNA of *Drosophila melanogaster*. Three termination sites have been identified in *Xenopus laevis*, with one terminator proximal to the initiation site (Labhart and Reeder 1986). In mouse there is a promoter distal (Grummt *et al.* 1986a) and promoter proximal terminator (Grummt *et al.* 1986b). Both regions are capable of binding protein factors believed to be responsible for termination. Promoter-proximal terminators seem to be quite common and may be necessary to prevent disruption of the initiation complex (Henderson *et al.* 1989). In addition, evidence suggests they have enhancer-like properties (McStay and Reeder 1990). Safrany *et al.* (1989) identified four possible termination sites in humans. Lang *et al.* (1994) have proposed a model for termination involving a pause element mediated by a protein factor (Reb1p) and a 5' flanking release element.

Sequence organization on the NTS

The best studied and understood NTS in higher

eukaryotes is that for *Xenopus laevis* (reviewed by Reeder 1984, Sollner-Webb and Moughey 1991). The complete sequence of the NTS in *Xenopus* has been known since the early 1980's with many researchers contributing data (Bosely *et al.* 1979, Sollner-Webb and Reeder 1979, Moss and Birnstiel 1979, Moss *et al.* 1980, Maden *et al.* 1982).

A large part of the *Xenopus* rDNA spacer is made up of repetitive sequence (reviewed by Reeder 1984)(refer to Fig. 2). There are 2-8 promoter duplications present at approximately 1 kb intervals starting from the gene promoter. The spacer promoters (approx. 150 bp) have about 90% homology with the gene promoter. The restriction site *Bam*H1 is present in each duplicated promoter, defining the so-called "Bam islands" of the NTS. Separating each Bam island is a region containing 6-12 60/81 bp repeats. The 81 bp repeat is essentially the same as the 60 bp repeat, except it has an additional 21 bases. Each of the 60/81 bp elements shares homology with a 42 bp stretch found in the gene promoter. Two other classes of 35 and 100 bp repeats are present nearer to the 3' end of the LSrDNA. These two classes show little similarity with any of the other repeats or promoter sequences. A major cause for the observed length heterogeneity appears to be the variable numbers of "super-repeats" (defined as one spacer promoter plus 6-12 60/81 bp elements).

The organization of the rDNA of *Drosophila melanogaster*

is similar to that in *Xenopus* (refer to Fig. 2). A good deal of its NTS is composed of three classes of repeats that are 95, 330 and 240 bp long (Coen and Dover 1982, Simeone *et al.* 1985). It appears that the 330 bp repeat element is composed of the 240 bp and 95 bp repeats. The 330 and 240 bp elements each contain a 42 bp stretch which is homologous to the promoter region (Simeone *et al.* 1982, 1985, Coen and Dover 1982). The observed variability in spacer length of 3.3 to 5.4 kb is due mostly to the variable numbers of the 240 bp element (Long and Dawid 1979).

Mouse and humans have comparatively large rDNA spacer regions of over 20 kb each (Pikaard *et al.* 1990, Sylvester *et al.* 1986, La Volpe *et al.* 1985). Mouse has numerous 140 bp repeat elements flanked by the promoter and a spacer promoter (Pickaard *et al.* 1990)(refer to Fig. 2). The 140 bp repeats do not have any sequence homology with the promoter. The spacer in mouse is not entirely made up of repeats. In fact, a majority of the mouse spacer is devoid of any 140 bp motifs (Pickaard *et al.* 1990). La Volpe *et al.* (1985) has identified a 700 bp repeat in the rDNA spacer in humans which can vary in number from 1-3. Length heterogeneity is known to exist in the NTS of both of these organisms (Arnheim and Southern 1977).

Even in the so-called "primitive eukaryote", *Trypanosoma cruzi*, a repeat element has been observed just upstream of the promoter (Dietrich *et al.* 1993). The

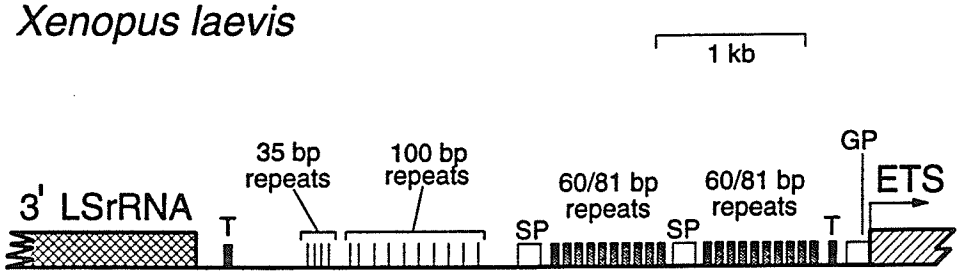
subrepeat was shown to have some similarity with its promoter region as well as with the promoter of *T. brucei*.

Four repeat families are present in the NTS of wheat (refer to Fig. 2)(Barker *et al.* 1988). A majority of the spacer is made up of numerous tandemly arranged 135/136 "A repeats". The A repeat family, interestingly, is flanked by diverged copies of the repetitive element. Three other repeat families also exist, "B", "C", and "D", of approximately 150, 172, and 30 base pairs respectively. There is no apparent promoter duplication present. The major cause of the observed length heterogeneity is the variable number of A repeats (Appels and Dvorak 1982, Flavell *et al.* 1990). The C-repeats actually are positioned downstream of the transcription start site, a situation commonly observed in other plants (Zentgraf *et al.* 1990, Appels *et al.* 1986, Delcasso-Tremousaygue *et al.* 1988).

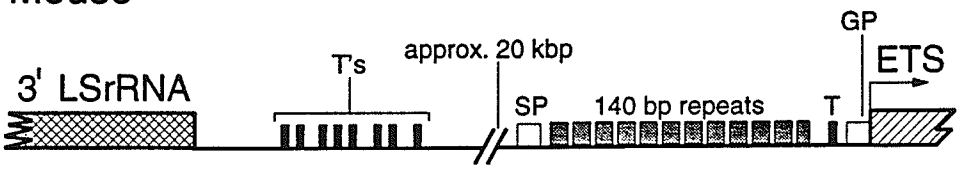
The general organization of intergenic spacer is relatively conserved across plants, although there is little sequence homology except for closely related species. Repeat elements are common in many plants, as well as the spacer length heterogeneity often associated with them. Promoter duplication can be present in some spacers such as those in *Arabidopsis thaliana* (Gruendler *et al.* 1991). *Cucumis sativus* is unusual in that all of its repeats are located downstream of the transcription start site (Zentgraf *et al.* 1990).

Fig. 2. General organization of the NTS of *Xenopus (Reeder 1984)*, mouse (Pickaard 1990), wheat (Barker *et al.* 1988) and Drosophila (Coen and Dover 1982, Simeone *et al.* 1985). T. termination site; SP, spacer promoter; GP, gene promoter; ETS, external transcribed spacer; 3′ LSrRNA, 5′ end of large subunit ribosomal RNA; 5′ SSrRNA, 5′ end of small subunit ribosomal RNA. The solid arrow represents the transcription initiation site for the gene promoter. The shaded arrow represents the transcription initiation site for the spacer promoter.

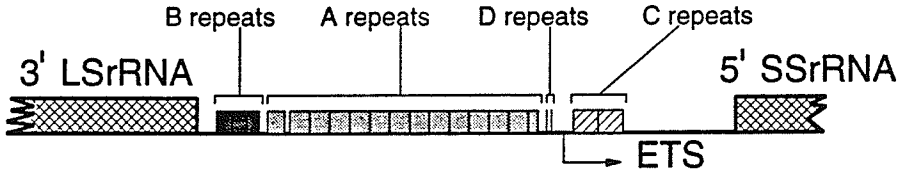
Xenopus laevis



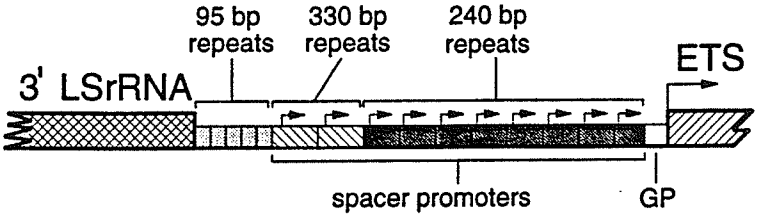
Mouse



Wheat



Drosophila melanogaster



Enhancers of the rRNA genes

Since the early 1980's the possible function of rDNA spacer promoters and subrepeats have been elucidated (Reeder 1984, Sollner-Webb and Moughey 1991). Although the reasons were unknown at the time, the observation of nucleolar dominance of *X. laevis* rRNA genes over *X. borealis* in interspecies hybrids of the two (Cassidy and Blackler 1974, Honjo and Reeder 1973) can be attributed to rDNA spacer organization.

The complete sequence of the rDNA spacer for *X. laevis* and *X. borealis* became known in the early 1980's (Moss *et al.* 1980, Bach *et al.* 1981). Analysis of the sequence data revealed the presence of promoter duplications and the promoter-homologous 42 bp repeat elements (part of the 60/81 bp motif in *X. laevis*) in both species. It was observed that the *X. laevis* spacer had considerably more spacer promoters and 42 bp motifs than did *X. borealis*. Oocyte competition assays were performed, in which rRNA genes with variable length spacers were coinjected into oocyte nuclei. It was observed that gene constructs with longer spacers (ie. more repeat elements) were transcriptionally dominant over constructs with shorter spacers (Moss 1983, Reeder *et al.* 1983, Labhart and Reeder 1984). Reeder and Roan (1984) were able to mimic nucleolar dominance by microinjecting rRNA gene plasmids from *X. laevis* and *X. borealis* into oocytes of either species. Reeder *et al.* (1983) proposed

that nucleolar dominance was due to increased numbers of 60/81 bp elements in the rDNA spacer. The evidence indicates that the repeats found in the NTS have enhancer-like qualities, similar to those seen by RNA polymerase II transcribed genes.

Similarly, Martini and Flavell (1985) have observed differential activity of the rDNA loci in the same nucleus of wheat. Nucleolar dominance of loci correlated with the length of the spacer. The larger rDNA spacers, which contain a higher number of repeat elements, were more transcriptionally active (Flavell *et al.* 1990). Evidence of enhancer-like function has also been demonstrated for repeats in mouse (Pikaard *et al.* 1990) and rat (Ghosh *et al.* 1993).

Pape *et al.*. (1989) were able to demonstrate, using both *in vivo* and *in vitro* methods, the manner in which the 60/81 bp repeats are able to enhance transcription. They showed that the repeat would enhance transcription in any orientation and up to about 1 kb from the gene promoter. If the enhancers were positioned any farther than 2 kb from the promoter they actually acted as silencers, presumably by sequestering transcription factors. The repeats would not increase the level of transcription when situated downstream of the promoter. Using *in vitro* methods, Pape *et al.* (1989) also demonstrated that the 60/81 bp motifs would enhance transcription in *cis* and compete with (or silence)

transcription in *trans*.

Beside repeat elements, spacer promoters are often present in the NTS. In *Drosophila* a large portion of the spacer is made up of spacer promoters which appear to stimulate transcription of the downstream gene promoter in an orientation-dependent manner (Giovanna and Di Nocera 1988). Moss (1983) proposed that spacer promoters may load the RNA polymerase I molecules and deliver them to the gene promoter. It is also possible that transcription from upstream promoters may aid in creating an open chromatin structure, making the rRNA gene promoters more accessible to transcription factors (Giovanna and Di Nocera 1988). Both mechanisms may be playing a role in transcription activation.

Spacer promoters are present in plants and vertebrate animals as well, but not to the extent observed in Drosophila. Although De Winter and Moss (1986) demonstrated that the spacer promoter stimulates transcription of the downstream gene promoter in *Xenopus*, the mechanism of enhancement remains unclear. Conversely, there are numerous examples where spacer promoters, or repeat elements with promoter homology, are not present. The NTS of maize, wheat, tomato and potato lack promoter duplications (McMullen *et al.* 1986, Barker *et al.* 1988, Perry and Palukaitis 1990, Borisjuk and Hemleben 1993). The mouse 140 bp repeats lack gene promoter homology, yet they have been

shown to enhance transcription (Pikaard *et al.* 1990).

A region in yeast has been discovered which greatly enhances transcription (Johnson and Warner 1989). It is of a nonrepetitive nature and seems to play a role in termination of RNA polymerase I transcription.

Transcription factors of RNA polymerase I

At least three transcription factors (not including RNA polymerase I) are involved in RNA polymerase I transcription (Sollner-Webb and Moughey 1991). There is some confusion in the literature due to naming of analogous factors isolated from various organisms with different names.

The best characterized of the transcription factors is "upstream-binding factor" (UBF). The name is misleading since it is now known that the protein interacts with both the upstream and core promoter domains (Bell *et al.* 1988). Bell *et al.* (1988) showed that UBF, along with other protein factors, can activate transcription by RNA polymerase I. UBF appears to be relatively sequence independent as demonstrated by its ability to bind to heterologous rDNA templates (Bell *et al.* 1989). This is not surprising since UBF shares homology with the non-specific DNA binding protein HMG-1 (high mobility group protein 1) (Jantzen *et al.* 1990).

The second major transcription factor is SL1 (Bell *et al.* 1988). SL1 is probably analogous to other identified

transcription factors which include SL-1, D, TF1D, T1F-1B, and T1F (Sollner-Webb and Moughey 1991). It is SL1, possibly in combination with UBF, that interacts species-specifically with an rRNA gene promoter (Bell *et al.* 1988). It appears that SL1 interacts with the upstream half of the core promoter domain and the upstream promoter domain. Various models have been presented (Bell *et al.* 1988, Bell *et al.* 1989, Smith *et al.* 1990, Schnapp and Grummt 1991, Kwon and Green 1994). The structure of SL1 has been characterized by Comai *et al.* (1992) as a multisubunit complex. They discovered a subunit common to all three classes of RNA polymerase, referred to as TATA-binding factor (TBF).

An "activating component" which is closely associated with RNA polymerase I appears to be required for initiation and elongation at residue +1 (Paule *et al.* 1984, Tower and Sollner-Webb 1987, Schnapp *et al.* 1994). The activating component has numerous nomenclatures which include: TF1C, C, T1F-1A and T1F-1C (Sollner-Webb and Moughey 1991, Schnapp *et al.* 1994).

Interaction of transcription factors with rDNA enhancers

It has been hypothesized that the 60/81 bp repeats in *Xenopus* act as enhancers by binding transcription factors, increasing the level of initiation complexes on the adjacent promoter (Reeder 1984). Evidence supporting this idea was

presented by Dunaway (1989) and Pickaard *et al.* (1989). They demonstrated that the 60/81 bp rDNA repeats in *Xenopus* are bound by the transcription factor UBF. In addition, the ability of the enhancer to compete in *trans* can be eliminated by the addition of purified UBF (Pickaard *et al.* 1989). Bound UBF spans two enhancer sequence elements as a dimer (Putman and Pickaard 1992), and it binds in a cooperative manner. A UBF dimer bends and wraps DNA in a right-handed direction in a manner analogous to a nucleosome (Putman *et al.* 1994, Bazzet-Jones *et al.* 1994). Bazzet-Jones *et al.* (1994) has referred to the complex consisting of the looping of a pair of *Xenopus* rDNA enhancers by a UBF dimer as an "enhancesome".

It is believed that the enhancesomes may inhibit histone-mediated gene repression (Putman *et al.* 1994), therefore stimulating transcription. At the promoter, UBF may encourage interaction between the upstream promoter domain and core promoter domain by bending or looping the DNA. Alternatively, it may present the transcription factor SL1 with an ideal binding site and lead to the subsequent formation of the initiation complex (Bazzet-Jones *et al.* 1994).

Mutagenesis of the enhancer sequence in *Xenopus* removes the enhancing ability of UBF, but UBF can still bind to the altered sequence. This suggests that other protein factors are involved in enhancement, possibly SL1, which complexes

with UBF in some manner (Pikaard 1994).

Transcription factors in other organisms analogous to *Xenopus* UBF have been shown to act in the same manner. The binding of UBF or UBF-like proteins to rDNA spacer repeat elements has been observed in mouse (Pickaard *et al.* 1990), rat (Ghosh *et al.* 1993), maize (Schmitz *et al.* 1989), cucumber (Zentgraf and Hemleben 1993), and wheat (Jackson and Flavell 1992). The ability of the enhancers to bind transcription factors is therefore conserved in many species.

No consensus sequence has been proposed as a binding site for UBF or other RNA polymerase 1 transcription factors, therefore it is difficult to understand how rRNA gene promoters and enhancers may have evolved. In *Xenopus*, at least, it is possible that enhancers originated from the promoter domains (Bosely *et al.* 1979). It has been theorized that UBF, which is sequence tolerant, recognizes structured nucleic acids (Copenhauer *et al.* 1994). Consistent with this belief is the observation that little or no sequence homology exists between mouse and frog enhancers. Yet, if a mouse enhancer is joined to a *Xenopus* promoter in a *Xenopus* cell it will enhance transcription (Pickaard *et al.* 1990). In addition, enhancers from both species can bind UBF from either species and compete with promoters in *trans*. These observations indicate that some kind of structural organization of the enhancers has been

conserved across species (Pikaard 1994, Pikaard *et al.* 1990). Even *Arabidopsis* spacer sequences can enhance rRNA transcription of a *Xenopus* ribosomal RNA gene promoter (Doelling *et al.* 1993). This evidence suggests that plant and vertebrate spacer elements perform the same function.

The ribosomal DNA spacer therefore appear to exhibit a general conservation of structure in higher eukaryotes (Gerbi 1985, Sollner-Webb and Moughey 1991). Length heterogeneity is very common in plants and animals and has been observed in *Pythiums* and other Oomycetes (Belkhiri *et al.* 1992, Martin 1990a). In most cases, major length heterogeneity is due to variation in subrepeat numbers (Reeder 1984, Roger *et al.* 1987). Additional evidence clearly demonstrates that the repeat elements are acting as enhancers by binding protein factor(s) which activate transcription of the adjacent rRNA gene promoter (Reeder 1984, Sollner-Webb and Moughey 1991).

MATERIALS AND METHODS

Cultures and strains utilized.

All cultures used in Sections 1 and 2 as a source of mtDNA and total genomic DNA are listed in Table 1. All *Pythium ultimum* and asexual *Pythium* isolates used in these two sections were acquired from the Biosystematics Research Centre, Ottawa, Canada.

The *E. coli* strain NM522 was used in all cloning protocols.

Culturing method

All cultures listed in Table 1 were grown on PYG media (3.0 g glucose, 1.0 g yeast extract, 1.0 g peptone, made up to 1.0 L with water). Stock cultures on agar slants were used to inoculate a petri-plate filled with 20 ml PYG medium. After 2-5 days growth, when the plate was filled with a mat of mycelia, the mat was broken up into smaller mycelial pieces using a blender. The blended mycelia was then aliquoted into 8 Roux bottles containing 50 ml PYG medium. They were allowed to grow until a mycelial mat covered the entire surface of the medium. Once growth was complete, 2 mats of mycelial growth was added to shaker flasks containing 400 ml of PYG. The cultures were then allowed to grow under vigorous agitation for about 3-5 days.

All incubation were done at room temperature. The mycelium was then harvested from the shaker by vacumn filtration through a Whatman no. 1 filter (Whatman Laboratory Products, Clinton, N.J.), followed by several washes with water.

Table 1. A list of *Pythium ultimum* isolates used for restriction endonuclease analysis of mitochondrial and ribosomal DNA.

Isolates[a]	Host/Source[b]	Origin
P. ultimum var. *ultimum* BR128	carrot	Ontario
P. ultimum var. *ultimum* BR144	tobacco	Ontario
P. ultimum var. *ultimum* BR319	papaya	California
P. ultimum var. *ultimum* BR406	alfalfa	Quebec
P. ultimum var. *ultimum* BR425	cucumber	Alberta
P. ultimum var. *ultimum* BR447	rapeseed	Alberta
P. ultimum var. *ultimum* BR471	soil	U.S.A.
P. ultimum var. *ultimum* BR511	geranium	Alberta
P. ultimum var. *ultimum* BR634	-	-
P. ultimum var. *ultimum* BR639	pea	Alberta

P. ultimum var. *ultimum* BR640	cucumber	Alberta
P. ultimum var. *ultimum* BR418	alfalfa	Quebec
P. ultimum var. *ultimum* BR443	soil	Saskatchewan
P. ultimum var. *ultimum* BR638	pea	Alberta
P. ultimum var. *ultimum* BR600	bean	B.C.
P. ultimum var. *ultimum* BR628	-	-
P. ultimum var. *sporangiiferum* BR650	C. alba	Maryland
P. ultimum var. *sporangiiferum* BR651	soil	Spain
Pythium sp. (group G) BR583	safflower	Alberta
Pythium sp. (group G) BR612	geranium	Ontario
Pythium sp. (group G) BR656	safflower	Alberta
Pythium sp. (group G) BR657	safflower	Alberta
Pythium sp. (group G) BR658	safflower	Alberta
Pythium sp. (group G) BR613[c]	geranium	Ontario
Pythium sp. (group G) BR659[c]	safflower	Alberta

P. irregulare BR174	spinach	Ontario

[a]All isolates were obtained from the Biosystematics Research Centre, Ottawa, Canada.

[b]The source or the host from which the organism was isolated is given.

[c]The species assignment of the isolate is unknown. was then harvested from the shaker by vacumn filtration through a Whatman no. 1 filter (Whatman Laboratory Products, Clinton, N.J.), followed by several washes with water.

DNA isolation

DNA was isolated from samples in Table 1 as previously described (Klassen *et al.* 1987). 8 to 10 g (wet weight) of mycelium were used for each DNA extraction. The mycelium was extracted by grinding in a pre-cooled mortar with pestle in the presence of liquid nitrogen, until a fine powder resulted. The powder was then suspended in 50 ml ice-cold lysis buffer (150 mM NaCl, 50 mM EDTA, 10 mM Tris-HCl, pH 7.4) and proteinase K (Sigma, St. Louis, Mo.) was added to a final concentration of 20 μg/ml. SDS (10%) was added to produce a final concentration of 2% and this mixture was incubated for at least 30 min at 65°C with occasional stirring. The solution was transferred to a 150 ml Corex centrifuge tube and extracted with an equal vol of phenol, followed by centrifugation at 5,000 rpm. The DNA was precipitated in a 250 ml centrifuge tube by adding 0.08 vol of 5M LiCl and 2.25 vol of 95% ethanol to the aqueous phase and letting it stand at -20°C for at least 1 h. This was followed by centrifugation at 7,000 rpm for 1 h at 4°C. The resulting DNA pellet was resuspended in 4 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). The DNA containing solution was transferred to a 15 ml corex centrifuge tube and extracted with phenol, and centrifuged at 10,000 rpm for 10 min at room temperature. The aqueous phase was re-extracted with an equal vol of phenol until no debris was evident at the interphase. Then, a phenol/chloroform/isoamyl (25:24:1,

v/v) extraction was done, followed by a final chloroform/isoamyl (24:1, v/v) extraction. The DNA was precipitated with LiCl and ethanol (as described above). After centrifugation and drying of the pellet, the DNA was resuspended in 300-500 μl TE.

The DNA extracted by the protocol described above was then further purified into mitochondrial and genomic DNA fractions by CsCl-bisbenzimide density gradient centrifugation (Garber and Yoder 1983). The top band collected by this method corresponded to the mtDNA fraction, while the lower band(s) contained the rDNA (along with the rest of the genome) fraction (Klassen *et al.* 1987).

A DNA "mini" preparation protocol was used to isolate total genomic DNA from all the APCC (Aquatic Phycomycete Culture Collection, University of Reading, U.K.) isolates listed in Table 5 (Section 5). Cultures were grown on PYG medium in petri plates (15 cm diameter). Mycelium harvested from 2 to 4 petri plates (0.5 to 4.0 g wet weight) was used for each DNA preparation. The method used was based on a protocol previously described (Hausner *et al.* 1992a) with one minor modification (samples were not freeze-dried before DNA extraction).

Plasmid DNA isolation was performed using the Magic Miniprep Purification System (Promega Co., Madison, WI)

following the instructions given by the manufacturer. A 10 ml culture of *E. coli* NM522 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) was used for plasmid purification. Plasmid DNA was resuspended in TE (pH 8.0) or water.

Single stranded DNA was isolated from the phagemid Bluescript M13 (Stratagene, La Jolla, CA) by infecting phagemid bearing *E. coli* NM522 with approximately 1.5 X 10^9 helper phage R408 particles (Promega). The infected culture was grown overnight in LB-amp broth (2.5 ml) at 37°C. Once growth was complete the culture was transferred to two microcentrifuge tubes and the cells were spun down (12,000 rpm at room temperature). The supernatant was separated from the cells and transferred to microcentrifuge tubes. The phage was precipitated by adding 0.25 vol of 1.5M NaCl/20% polyethylene glycol (mol wt 8000) to the supernatant, and allowed to sit at room temperature for 15 min. The phage was pelleted by centrifugation at 12,000 rpm for 15 min at room temperature. The resulting supernatant was removed and each phage pellet was resuspended in 100 μl TE buffer (pH 8.0). The resuspended phage was then extracted once with an equal vol of phenol, then once with an equal vol of phenol/chloroform (1:1) and finally with an equal vol of chloroform. Each extraction was followed by a

2 min centrifugation at 12,000 rpm (room temperature). After the last chloroform extraction, the ssDNA was precipitated by addition of 0.5 vol of 7.5M ammonium acetate, followed by addition of 2.25 vol of 95% ethanol. It was allowed to sit at -20°C for at least 20 min before centrifugation at 12,000 rpm (4°C). The ssDNA was then resuspended in 10-20 μl water.

Restriction endonuclease reactions and electrophoresis

Endonuclease digestion were performed using enzymes obtained from Pharmacia Canada Ltd. (Dorval, Que) and BRL (Bethesda Research Laboratories Inc., Gaithersburg, MD) as recommended by the manufacturers. Approximately 1 μg of DNA was used for each digestion. Loading buffer was added to the DNA restriction reactions after the recommended incubation period (6% v/v glycerol, 2mM EDTA, 0.05% w/v bromophenol blue). TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 7.6) or TAE (40 mM Tris-acetate, 1 mM EDTA, pH 7.6) running buffer was used for electrophoresis. The samples were run on 0.8 to 1.0% agarose (BRL) gels. Voltage used varied from 2 V/cm to 10 V/cm, depending on what size fragments needed to be resolved (the smaller the fragments the higher the voltage used). The DNA size marker 1 Kb Ladder (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 transilluminator (Fotodyne Incorporated, Mississauga, Ont.). Photographs were taken using a Polaroid camera and Polaroid 667 film.

Nick Translation

The recombinant plasmid, pMF2, which has an insert containing the gene region of the rDNA repeat of *Neurospora crassa* was labelled with [α-^{32}P]dATP (Dupont, New Research Products, Boston, Ma.) as previously described (Rigby *et al.* 1977, Maniatis *et al.* 1982).

Southern blotting and hybridization

DNA which was digested with restriction endonuclease(s) and run on agarose gels was transferred onto Hybond-N nylon membranes (Amersham International, Oakville, Ont.) according to the manufacturer's instructions. The blots were prehybridized at 55°C for 2 h in 1 M NaCl (Fisher Scientific, Nepean, Ont.) and 1% SDS (Fisher Scientific) with gentle shaking. The probe (nick translated) was boiled for 10 min and added to the prehybridization solution. The blot and probe were incubated at 55°C with gentle agitation for 12-14 h. Once hybridization was complete, the membranes were washed twice with 2X sodium saline citrate (SSC; 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at room temperature for 5 min each, then three times in 2X SSC and 1% SDS at 55°C for 30 min each. All solutions were gently agitated

during washes. After the final wash, the probe-hybridized blot was exposed to X-ray film (X-OMAT RP or X-OMAT AR)(Kodak, New Haven, CT) ranging from one to five days before developing.

5' end-labelled oligonucleotide probes

Oligonucleotide were labelled at the 5' end with [γ-^{32}P]ATP (Dupont) using T4 polynucleotide kinase (BRL) as described by Sambrook *et al.* (1989). Oligonucleotides PS1c and PS2 (refer to Table 2) were labelled in this manner.

Dot blotting and hybridization

Approximately 1 μg of total genomic DNA (sample listed in Table 5) was dot blotted onto Hybond-N+ (Amersham) using a dot blot manifold (BRL) as outlined by Ausubel *et al.* (1987). The dot blot was placed in prehybridization solution (1M NaCl, 1% SDS) for two h at 50°C before the end-labelled PS2 probe was added to the solution. After 18 h the probe was removed and the blot was washed twice with 4X SSC at room temperature. Three more washes with 1% SDS and 2X SSC were performed at 50°C (two washes for 30 min and then one wash for 60 min). The PS2-hybridized dot blot was then exposed to X-ray film (X-OMAT RP, Kodak) for a period of 18 h before developing.

The probe PS1c was hybridized in the same manner as described above, except that a hybridization temperature of

45°C was used. X-ray film exposure was for the same duration of time as PS2-probed blot.

Probes were removed from nylon membrane by bringing a 0.1% SDS solution to a boil. The hot solution was then poured onto the membrane and allowed to cool to room temperature. The procedure was repeated until no radioactivity was detected on the blot.

Synthesis of oligonucleotides

All oligonucleotides used for PCR, sequencing or probing are listed in Table 2. Oligonucleotides were synthesized with the PCR-MATE (391 DNA synthesizer, Applied Biosystems, Foster city, CA) by Jack Switala (Dept. of Microbiology, University of Manitoba). They were removed from the column by passing 0.5 ml of NH_4OH at least 10 times through the column and then left to incubate for a period of 15 min at room temperature. The solute was collected and two more extractions with NH_4OH were performed in the same manner. The solute from the three extractions was pooled together and incubated overnight at 55°C. The oligonucleotide was then dried in a SpeedVac (Savant SC110 Speedvac, Farmingdale, NY) to remove the ammonium hydroxide. The dried oligonucleotide was resuspended in ddH_2O to a final concentration of 40 pmol/μl.

Table 2. Oligonucleotides used for PCR amplification, DNA sequencing, or oligonucleotide probes.

Primer	Location	Sequence (5′ to 3′)
Fo	Bluescript[e]	GTAAAACGACGGCCAGT
G	1866-1887[a]	CCAAGAATTTCACCTCTGAC
H	2863-2880[b]	CTTCGATGTCGGCTCTTC
P	701-720[a]	GGCTCCCTCTCCGGAATC
P2	80-98[a]	ATACTTAGACATGCATGGC
P3	3101-3118[c]	CGCTTTGGCTATCATTCG
P3c	3101-3118[c]	CGAATGATAGCCAAAGCG
P4	2924-2942[c]	GAAGTTGCCCTCTGCACGC
P4c	2924-2942[c]	GCGTGCAGAGGGCAACTTC
P5	2699-2717[c]	GTTTCGCATTACGCATGAC
P5c	2699-2717[c]	GTCATGCGTAATGCGAAAC
P5.5	2556-2573[c]	GCATGCAGCACGTTTCAG
P6	2404-2422[c]	CATTAGTACCGACTCGTTC
P6c	2404-2422[c]	GAACGAGTCGGTACTAATG
P7	2132-2148[c]	CACACGTAATATAGACG
P7c	2132-2148[c]	CGTCTATATTACGTGTG
P7.1	2052-2072[c]	CAGCACAAATGGACATATCTC
Q	3110-3128[b]	ACGCCTCTAAGTCAGAATC
Q1.5	227-244[c]	GAGTAGTCTTGTACTACG
Q2	367-383[c]	AGCCCGCACCGCCTAGG
Re	Bluescript[e]	AACAGCTATGACCATG
PS1	1322-1340[c]	GCGTCACTAGCACGCTGC

PS1c	1322-1340[c]	GCAGCGTGCTAGTGACGC
PS2	780-800[c]	ATACTGTTAATATAGAGAAAT
XP1	1655-1673[c]	CTGTTGACAGTTGGTAGCG
XP1c	1655-1673[c]	CGCTACCAACTGTCAACAG
XP1.1c	155-175[d]	CTCCTGTGAACGCATCATGGT
XP1.2c	1759-1777[c]	GCGGATGTGCACCAGCGAG
XP2	1935-1953[c]	CCAGATGTCGTCAAATTGC
XP2c	1935-1953[c]	GCAATTTGACGACATCTGG
XP2.1	391-410[d]	GAGCGACCACGACCTGCTCA
XP2.1c	391-410[d]	TGAGCAGGTCGTGGTCGCTC

[a]Based on the SSrRNA sequence of *S. cerevisiae* (Rubstov et al. 1980).

[b]Based on the LSrRNA sequence of *S. cerevisiae* (Gutell and Fox 1988).

[c]Based on the sequence of pJB1A (Fig. 4.6).

[d]Based on the sequence of pJB2 (Fig. 4.16).

[e]Flanks the multiple cloning sites in Bluescript M13.

PCR amplification

Specific DNA fragments were amplified by the polymerase chain reaction technique (Saiki *et al.* 1988). Amplification were performed in a 100 or 50 μl reaction volume using the following components: 1X Taq DNA polymerase reaction buffer (Promega), 200 μM each dNTP (Pharmacia), 1.5 mM $MgCl_2$ (omit if 10X reaction buffer contains MgCl), 40 pmol of each primer (for 100 μl reaction volume), 50 to 100 ng template DNA, and 1.0 to 2.5 units Taq polymerase (Promega). The reaction mixture was covered with a layer of mineral oil (Fisher Scientific). All amplifications were performed using a Perkin Elmer-Cetus DNA thermal cycler (Norwalk, Conn.). The following general cycle was performed: denaturation was usually performed at 93°C for 1 min, primer annealing was done at 50-55°C for 1 min, polymerization temperature ranged from 70-72°C for 1-8 min. The cycle was repeated 17-35 times followed by a final extension of 10 min at 72°C after the final cycle. The number of cycles, temperature used, and times of incubations varied in order to optimize amplification of the desired DNA fragment.

Isolation of DNA fragments from agarose gels

The "freeze-squeeze" technique was used to extract DNA fragments from agarose gels (Tautz and Renz 1983, Hausner *et al.* 1992b). Bands were cut out of ethidium bromide-stained gels and frozen at -20°C. The gel plug was placed between

two pieces of parafilm (American National Can, Greenwich, Conn.) and pressure was applied using finger pressure causing the agarose plug to thaw. The resulting liquid was collected and placed in a 1.5 ml microcentrifuge tube and made up to 1.0 M NaCl and 1% hexadecyltrimethyl ammonium bromide (CTAB, Sigma). The mixture was incubated at 55°C for 10 min, followed by two chloroform/isoamyl alcohol (25:1, v/v) extractions. The solution was then precipitated by the addition of 2.25 vol of 95% ethanol.

Ligation of DNA fragments into Bluescript phagmid

Three forms of Bluescript M13 (Statagene) were utilized in cloning protocols: M13ks+, M13ks-, and M13sk-.

Ligations were performed using T4 DNA ligase (Pharmacia) as recommended by the manufacturer (Maniatis *et al.* 1982). The ligation reaction mixture of cohesive ends consisted of: 20-30 μg/ml Bluescript vector DNA, approximately 30 μg/ml insert DNA, 1X One-Phor-All buffer (Pharmacia), 1 mM ATP, 5 units T4 DNA ligase. The reaction mixture was incubated overnight at 15°C. The reaction was stopped by heating to 65°C for 10 minutes. The same procedure was followed for the ligation of blunt-ended fragment, except that 10-15 units of T4 DNA ligase were used instead.

Preparation of competent *E. coli* NM522 cells

E. coli NM522 was grown overnight on LB media at 37°C. The next day 0.2 ml of the culture was subcultured into 10 ml of LB containing 100 mM $MgCl_2$ and allowed to grow for 2.5 h. The cells were then placed on ice for 20 min before being spun down. After the cells were pelleted, they were resuspended in 3 ml of trituration buffer (100 mM $CaCl_2$, 70 mM $MgCl_2$, 40 mM sodium acetate, pH 5.5) and placed on ice for 15 minutes. The cells were spun down once again and resuspended in 0.5 ml trituration buffer. The cells were considered competent at that point.

Transformations

Approximately 1/2 of the ligation reaction was mixed with 0.1 ml of competent NM522 cells. The mixture was incubated on ice for at least 15 min. The cells were then heat shocked at 42°C for two min, followed by an incubation at room temperature for 10 min. 1.0 ml of pre-warmed (37°C) LB broth (0.1 M $MgCl_2$) was added and the mixture was allowed to incubate at 37°C for 45 min. From 5% to 100% of the mixture was then spread-plated onto LB-amp containing 32 μg/ml X-gal (5-bromo-4chloro-3-indoyl-β-D-galactopyranoside) (Sigma) and 6.4 μg/ml IPTG (isopropyl-β--D-galactopyranoside)(Sigma).

PCR of individual spacer length variants

The NTS of *P. ultimum* BR471 was amplified as previously described (Buchko and Klassen 1990, and Section 3) using primers G and H (Table 2) and DNA template which was extracted from isolate BR471 (Table 1). The resulting PCR amplification products were run on 0.8% agarose gels (Fig. 4.1, lane 1). The three smallest bands were cut out and isolated using the "freeze-squeeze" method. 5% of each recovered band was then used as template for amplification by the primer-pair P and Q (Fig. 4.1, lanes 2,3 and 4). The re-amplification products were precipitated by addition of 0.5 vol 7.5 M ammonium acetate and 2.25 vol of 95% ethanol. The precipitated DNA was resuspended in TE (pH 8.0) to a final concentration of approximately 0.5 μg/μl. The isolated PCR-amplified length variants were then cloned into Bluescript M13 (Stratagene).

Cloning of pJB1A and pJB1:

Phosphorylation and blunt-ending of PCR amplified DNA

Phosphorylation and blunt-ending of a PCR amplified product was done as previously described (Denney and Weissman 1990). The PCR product representing the shortest spacer length variant, referred to as Band 1 (Fig. 4.1, lane 2), was phosphorylated (primers used in amplification lack the 5′ phosphate group) in order to help facilitate the ligation of a PCR fragment. The enzyme T4 polynucleotide kinase (Pharmacia) was used as recommended by the

manufacturer. The reaction mixture was made up of 1X One-Phor-All Buffer (Pharmacia), 50 pmoles ATP (Pharmacia), approximately 2.5 μg DNA from Band 1 and 10 units T4 kinase (Pharmacia) in a total volume of 25 μl. The mixture was allowed to incubate at 37°C for 30 min. The reaction was inactivated by heating it to 65°C for 15 min. The following was then added to the above reaction mixture: 2.5 μl 1X One Phor-All Buffer (Pharmacia), 2.0 μl of 0.1M Dithiothreitol (Sigma), 2.0 μl of 2.5 mM of each dNTP, and 7 units Klenow DNA polymerase (Pharmacia). Water was added to a final volume of 50 μl. The new reaction mixture was incubated at 14°C for one h and then heat-inactivated at 65°C for 15 min. The resulting phosphorylated/blunt-ended product was then ethanol precipitated using 7.5M ammonium acetate (as previously described in this section). After precipitation of the DNA, it was resuspended in 10 μl TE (pH 8.0). 7.0 μl of the solution was then ligated into *Eco*RV digested Bluescript M13ks+ (Stratagene). The ligated product was then transformed into competent NM522 cells and plated onto LB-amp (with X-gal and IPTG, Sigma) and incubated overnight at 37°C. White colonies were screened for the Band 1 insert as described in a following section.

Cloning of pJB2, pJB3 and pJB3A:

The next two larger PCR-amplified length variants (Bands 2 and 3 of Fig. 4.1, lanes 3 and 4) were digested

with *Xba*I (refer to Fig. 3) and ligated into *Xba*I/*Eco*RV digested Bluescript M13ks+. When the ligation reaction was complete it was heat inactivated at 65°C for 15 min. The above reaction mixture was then treated with Klenow (Pharmacia)(1X One-Phor-All buffer, 1 mM ATP, 3 mM DTT, 7 units Klenow) at 37°C for 3 min, then 0.1 mM of each dNTP was added and the mixture was incubated for a further 37°C for 5 min. 5 units of T4 DNA ligase (Pharmacia) was then added to the mixture and incubated at room temperature for 1 h. Once ligation was complete, all of the mixture was transformed into NM522 and plated onto LB-amp (with X-gal and IPTG) and incubated overnight at 37°C. White colonies were screened for the possession of Bands 2 or 3.

Screening for positive NTS clones

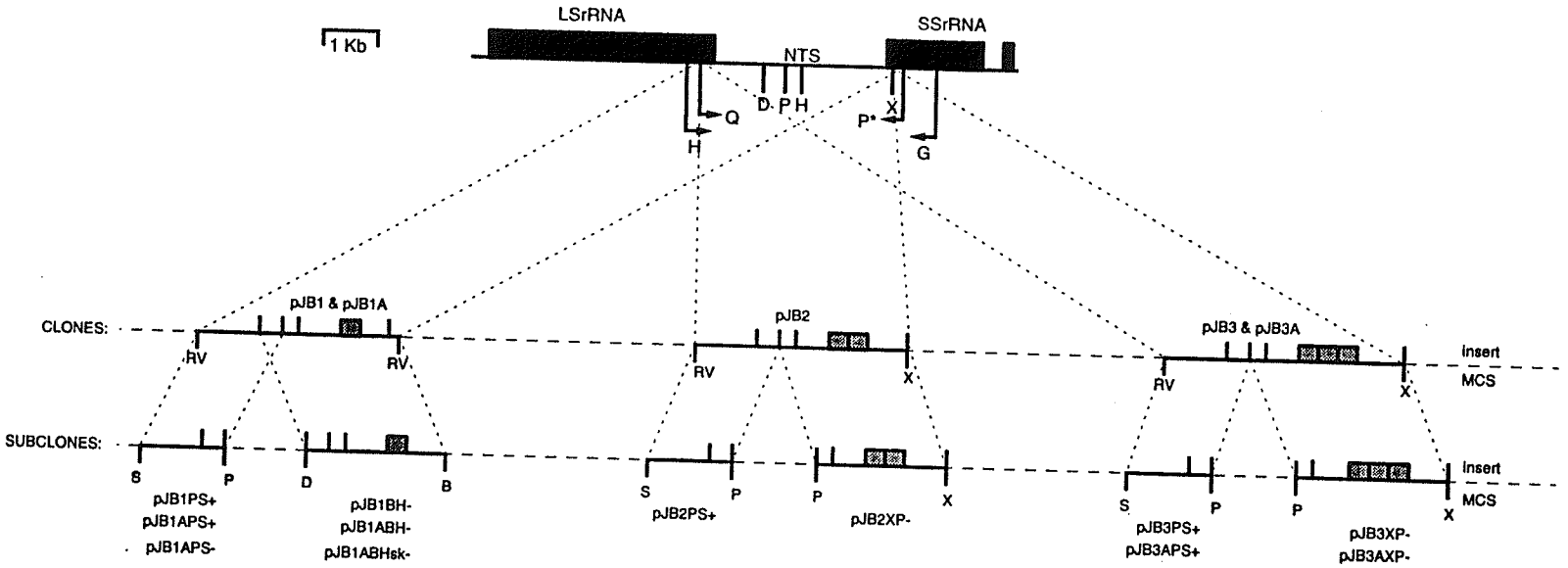
Plasmid DNA was isolated from white colonies using the Magic Miniprep DNA purification system (Promega). The shortest NTS length variant (Band 1, Fig. 4.1) was screened by digestion of plasmid DNA with *Eco*RI and electrophoresis on a 0.8% agarose gel. If the shortest length variant was successfully ligated into M13ks+ then a band of approximately 6.5 kb was expected. Positive clones could then be further confirmed by digestions with *Pst*I, *Hinc*II, *Hind*III and *Xba*I which are all present in the NTS (except *Xba*I which is near the 5′ end of SSrRNA gene) of *Pythium ultimum*. The clones pJB1 and pJB1A were identified in this

manner. The next two larger spacer length variant (Bands 2 and 3, Fig. 4.1) were screened by digestion with *Hin*dIII. If PCR amplified spacer DNA was successfully ligated into M13ks+ then two bands would be produced of approximately 1.4 kb (for both size variants) and 5.6 (Band 2, Fig. 4.1) or 6.0 kb (Band 3, Fig. 4.1). The positive clones were then further confirmed by digestion with *Pst*I, *Hinc*II, and *Xba*I. The three clones, pJB2 (Band 2, Fig. 4.1), pJB3 and pJB3A (Band 3, Fig. 4.1) were identified by this method.

Subcloning protocol

All of the clones were subcloned in a similar manner. They were digested with the appropriate restriction enzymes and run on a 0.8% agarose gel. DNA fragments were isolated from the gels using the "freeze squeeze" method and ligated into various Bluescript M13 vectors (M13ks+, M13ks-, and M13sk-) which were previously digested with restriction endonucleases that produced cohesive ends. A list of all the clones and subclones made is given in Fig. 3.

Fig. 3. A diagram of all the clones and subclones created containing the NTS of *P. ultimum* BR471. The region cloned and subcloned is traced by the dotted lines. Restriction sites below the dashed line are present in the MCS (multiple cloning site), site shown above the dashed line are present in the insert. Restriction sites are represented by the following symbols: D, *Hin*dIII; P, *Pst*I; H, *Hin*cII; S, *Sal*I; X, *Xba*I ; RV, *Eco*RV (cloning of the insert into the *Eco*RV site of the vector MCS destroyed the restriction site). Primer sites P* (P primer), Q, G, and H (see table 1) are indicated. Subclone names ending in: "-" are inserted into Bluescript M13ks-, those with "+" are inserted into Bluescript M13ks+, and those with "sk-" are inserted into Bluescript M13sk-. Shaded blocks represent the putative 385 bp subrepeats (Klassen and Buchko 1990).


1 Kb
LSrRNA
SSrRNA
NTS
D P H
Q
H
P*
X
G
CLONES:
pJB1 & pJB1A
RV
RV
pJB2
RV
X
pJB3 & pJB3A
RV
X
Insert
MCS
SUBCLONES:
S
P
pJB1PS+
pJB1APS+
pJB1APS-
D
B
pJB1BH-
pJB1ABH-
pJB1ABHsk-
S
P
pJB2PS+
P
X
pJB2XP-
S
P
pJB3PS+
pJB3APS+
P
X
pJB3XP-
pJB3AXP-
Insert
MCS

Construction of deletion clones for pJB1APS+/-

The Erase-a-Base system (Promega) was used to construct deletion clones of pJB1APS+ and pJB1APS- following instructions given by the manufacturer, with minor modifications. In order to ensure that the plasmid DNA used was not nicked, it was treated with T4 DNA ligase for 2 h at room temperature. Approximately 5 μg of plasmid DNA was used to generate each set of deletion clones. The subclone pJB1APS+ was digested with *Sac*I (Pharmacia), producing a 3′ overhang, which is resistant to exonuclease III digestion. A 5′ protruding end, which is sensitive to exonuclease III digestion, was created by digestion with *Bam*HI. This meant that deletions would be started near the middle of the NTS and directed toward the SSrRNA gene portion of the subclone pJB1APS (see Fig. 3). Exonuclease III digestion was performed at 35°C and samples (up to 10) were collected at 30 sec intervals. Deletion clones in the opposite direction were done by digesting pJB1APS- with *Kpn*I (resistant to exonuclease III) and *Cla*I (exonuclease III sensitive). All the restriction sites used flank the insert and are found in the multiple cloning site portion of Bluescript M13.

Screening of deletion clones

PCR amplification was used to screen for different sized deletion clones. Cells from colonies were picked and placed in 1.5 ml microcentrifuge tubes. 50 μl of water was

added to each sample followed by vortexing for 10-30 sec. The samples were then left on ice while the PCR reaction mixtures were prepared. The samples were then spun down briefly to pellet the cells and 2 μl from each supernatant was used as a template source for each PCR reaction. PCR reactions were carried out in 50 μl vol as previously described in this section. The primer-pair Re and Fo (refer to Table 2), which flank the multiple cloning site of Bluescript M13, were used for amplification. A control amplification using undeleted pJB1APS+ was used to compare the amplification products of colonies being screened. The cycle used was: denature at 93°C for 30 sec, anneal at 50°C for 1 min, and extend at 72°C for 2 min. 20% of the reaction mixture was then run on a 0.8% agarose gel to determine the size of the deletion clones. Appropriately sized deletion clones were then selected and used for sequencing purposes later.

DNA sequencing

Sequencing was performed using single-stranded and double-stranded template based on the Sanger dideoxy method (Sanger *et al.* 1977). Sequencing of single stranded DNA (ssDNA) was performed as recommended by the manufacturer using the Sequenase version 2.0 DNA sequencing kit (USB Biochemical, Cleveland, Ohio) and [α^{35}S]dATP (Dupont). Minor modifications of this protocol were used upon

sequencing double-stranded DNA (dsDNA) template. The annealing reaction was composed of 5% formamide (BRL), 3-5 μg of dsDNA plasmid (as opposed to 1 μg for ssDNA) and 5-15 pmol of primer (compared to 1 pmol for ssDNA) made up to 10 μl with water. The annealing reaction was placed in a boiling water bath for 2-5 min and then transferred into a -70°C ethanol bath for about 30 sec. Once the sample was frozen the annealing reaction was placed on ice and allowed to thaw. After this step the protocol was the same as given by the manufacturer. Sequencing samples were electrophoresed in a 0.8% polyacrylamide gel for 2-6 h at a temperature of approximately 50°C. The gels were fixed in a 10% ethanol and 10% acetic acid solution for 20 min and then blotted onto 3MM paper (Whatman). Sequencing gels were dried on gel dryer (Savant) and exposed to Kodak X-Omat AR film for 1-5 days at room temperature.

Isolation of total cellular RNA from *P. ultimum* BR471

A method described by Auubel *et al.* (1987) was used to isolate total RNA from mycelia of *P. ultimum*. A culture of *P. ultimum* BR471 was grown and harvested as previously described in this section (culturing methods). Approximately 23 g (wet weight) of mycelia was homogenized using liquid nitrogen with mortar and pestle. Once cells were ground to a fine powder, 230 ml of denaturing solution (4M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0,

0.1 M 2-β mercaptonethanol, 0.5% sarkosyl) was added and the homogenized cells were vortexed for 1 min. One-tenth vol of 3M sodium acetate (pH 5.2) was then added to the solution. An equal vol of phenol was added, followed by addition of one-tenth vol of chloroform. The mixture was incubated on ice for 15 min. The solution was centrifuged at 10,000X g for 20 min at 4°C. The aqueous phase was collected and the RNA was precipitated by addition of an equal vol of isopropanol, followed by incubation at -20°C for 20 min. After the RNA was spun down by centrifugation it was resuspended in 1 ml denaturing solution followed by addition of 1 vol of isopropanol. Further incubation at -20°C for 20 min followed before centrifugation was done to pellet the RNA. The pellet was allowed to dry and then it was resuspended in 0.5 ml DEPC (diethylpyrocarbonate, Sigma) treated sterile deionized water. CsCl (Sigma) and DEPC-treated water was then added to a final concentration of 0.1 g CsCl/ml solution in a final volume of 4 ml. The CsCl/RNA mixture was then placed in a 16 X 79 mm polyallomer centrifuge tube, overlaid with mineral oil to fill the tube, and centrifuged at 50,000 rpm in an 80 Ti rotor (Beckman) for 16 h at 20°C. After centrifugation, the supernatant was aspirated off and the pellet at the bottom (RNA) was resuspended in 0.5 ml DEPC-treated water. The solution was then precipitated once with ammonium acetate and ethanol, and finally resuspended 0.1 ml DEPC-treated water

(concentration approximately 13.3 $\mu g/\mu l$). This RNA was used for primer-extension, described in the next section.

Primer extension

A protocol previously described (Drolet and Lau 1992) with some modifications was used. Approximately 16 pmol of primer P6 (see Table 2) was end-labelled with [γ^{32}P]ATP (Dupont) using T4 polynucleotide kinase (BRL). The enzyme was inactivated by boiling, followed by ethanol precipitation. After the labelled primer was spun down and dried, it was resuspended in 5 $\mu g/\mu l$ RNA (BR471 total RNA), 20 mM Tris (pH 8.3), 200 mM NaCl, O.1 mM EDTA. The mixture was heated for 3 min at 100°C and then placed on ice for 45 min. The primer-annealed RNA mixture was then ethanol precipitated and resuspended in 50 units RNA guard (Pharmacia), 1X AMV reaction buffer (Promega), 0.1 $\mu g/\mu l$ Actinomycin D (Sigma), 1 mM each DNTP, 20 units AMV reverse transcriptase (Promega), in a total volume of 20 μl. The above mixture was then incubated for 1 hour at 45°C. The reaction was stopped by the addition of 13.5 μl of Sequenase stop buffer (USB) and 1 μl was run on a 0.8% acrylamide gel for about 2.0 hour. A sequencing reaction using pJB1ABH- plasmid DNA (see Fig. 3) and primer P6 was run alongside the primer extension product. The gel was fixed, dried and exposed to X-ray film (Kodak X-Omat AR) overnight.

RESULTS AND DISCUSSION

This research project began as a study of intraspecific variation in mitochondrial and ribosomal DNA sequences in *P. ultimum* with the aim of developing tools for isolate identification and taxonomy. Parts 1 and 2 present data from this phase of the project. During the investigation of rDNA polymorphism, subrepeat heterogeneity similar to that found in plants was discovered in all isolates of *P. ultimum*, and because of its novelty and importance, the characterization of this phenomenon superceded earlier aims to some extent. Parts 3 and 4, which account for the major portion of the project, report the results of this undertaking. In the end, sequencing of the NTS of *P. ultimum* BR471 led to the development of oligonucleotide probes with potential usefulness in the identification of both varieties of *P. ultimum*. These results are found in part 5.

1. Mitochondrial DNA RFLP Analysis

Chromosomal DNA was purified from all the isolates listed in Table 1 including 16 isolates of *P. ultimum*, two of *P. ultimum* var. *sporangiiferum*, six of *Pythium* sp. "group G", and one of *Pythium irregulare*. The isolates had been collected from various geographical locations and hosts, including many isolates from five provinces in Canada, two from the U.S.A and one from Spain. Total DNA from each

isolate was fractionated by CsCl-bisbenzimide gradient centrifugation. The top band, representing the mitochondrial DNA fraction, was collected and used for mtDNA RFLP analysis. The bottom band was also collected and used for rDNA RFLP analysis (presented in section 2).

In this study restriction endonuclease analysis of mtDNA was performed on 14 isolates of *Pythium ultimum*, five of *Pythium* sp. "group G" (possessing globose vegetative bodies), two of *P. ultimum* var. *sporangiiferum*, and one of *Pythium irregulare* (refer to Fig. 1.1) with the purpose of studying the level of genetic variability among these isolates, and to help assign names to asexual *Pythium* isolates. Some insights into the relationship of *P. ultimum* var. *ultimum* , asexual *Pythium* sp. "group G", and *P. ultimum* var. *sporangiiferum* were also expected from mtDNA RFLP analysis.

In total, 22 isolates were digested with *Hind*III and *Hpa*II (refer to Fig. 1.1). A binomial matrix for each restriction enzyme was constructed in which the restriction endonuclease banding patterns were compared (Tables 3 and 4). The presence or absence of a band was represented by a 1 or a 0, respectively. The two binomial matrix data sets were combined and used for analysis by programs available on PHYLIP, (Version 3.57, Felsenstein 1995). PHYLIP is a component of BIRCH (Biological Research Computer Hierarchy) which is part of the SUN Unix system at the University of

Fig. 1.1. Mitochondrial DNA restriction fragment length polymorphisms (RFLP) analysis. Samples in A and B were digested with *Hind*II. Samples in C and D were digested with *Hpa*II. DNA samples in A and C are from the following isolates: 1-BR443, 2-BR471, 3-BR628, 4-BR639, 5-BR650, 6-BR651, 7-BR657, 8-BR638, 9-BR583, 10-BR659, 11-BR174. DNA samples in B and D are from the following isolates: 1-BR443, 2-BR319, 3-BR425, 4-BR471, 5-BR144, 6-BR447, 7-BR511, 8-BR128, 9-BR406, 10-BR418, 11-BR612, 12-BR600, 13-BR583, 14-BR613, 15-BR418, 16-BR406.

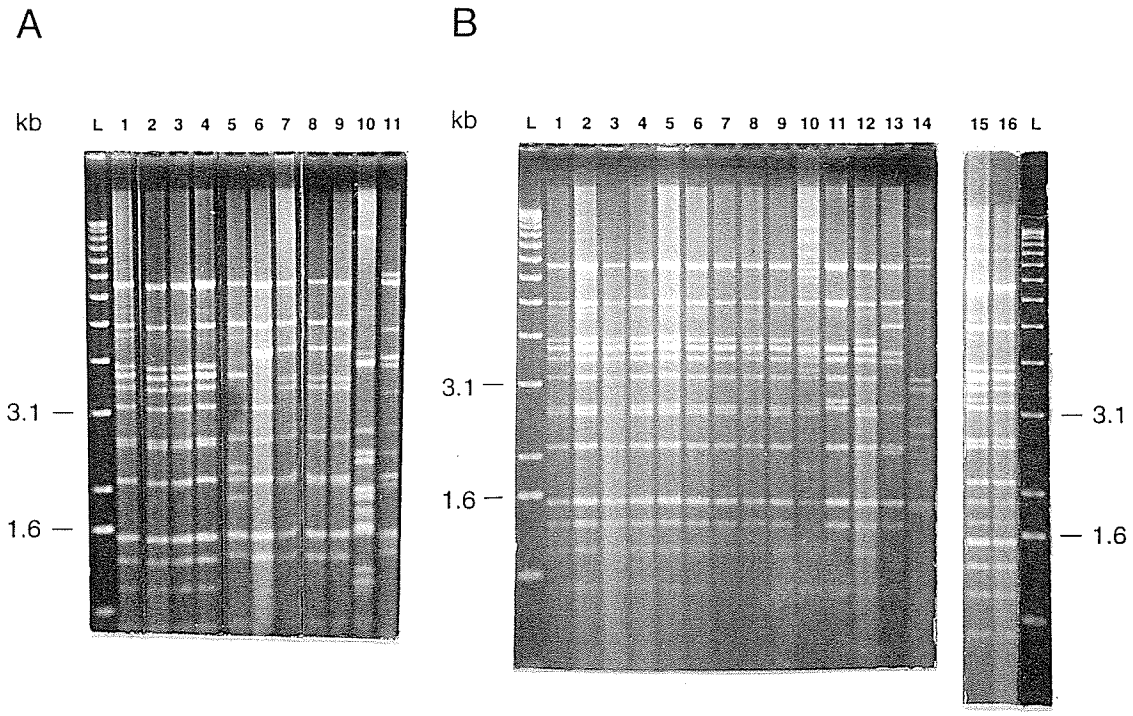

A
kb
L 1 2 3 4 5 6 7 8 9 10 11
3.1
1.6
B
kb
L 1 2 3 4 5 6 7 8 9 10 11 12 13 14
15 16 L
3.1
1.6
3.1
1.6
C
kb
L 1 2 3 4 5 6 7 8 9 10 11
3.1
1.6
D
kb
L 1 2 3 4 5 6 7 8 9 10 11 12 13 14
3.1
1.6

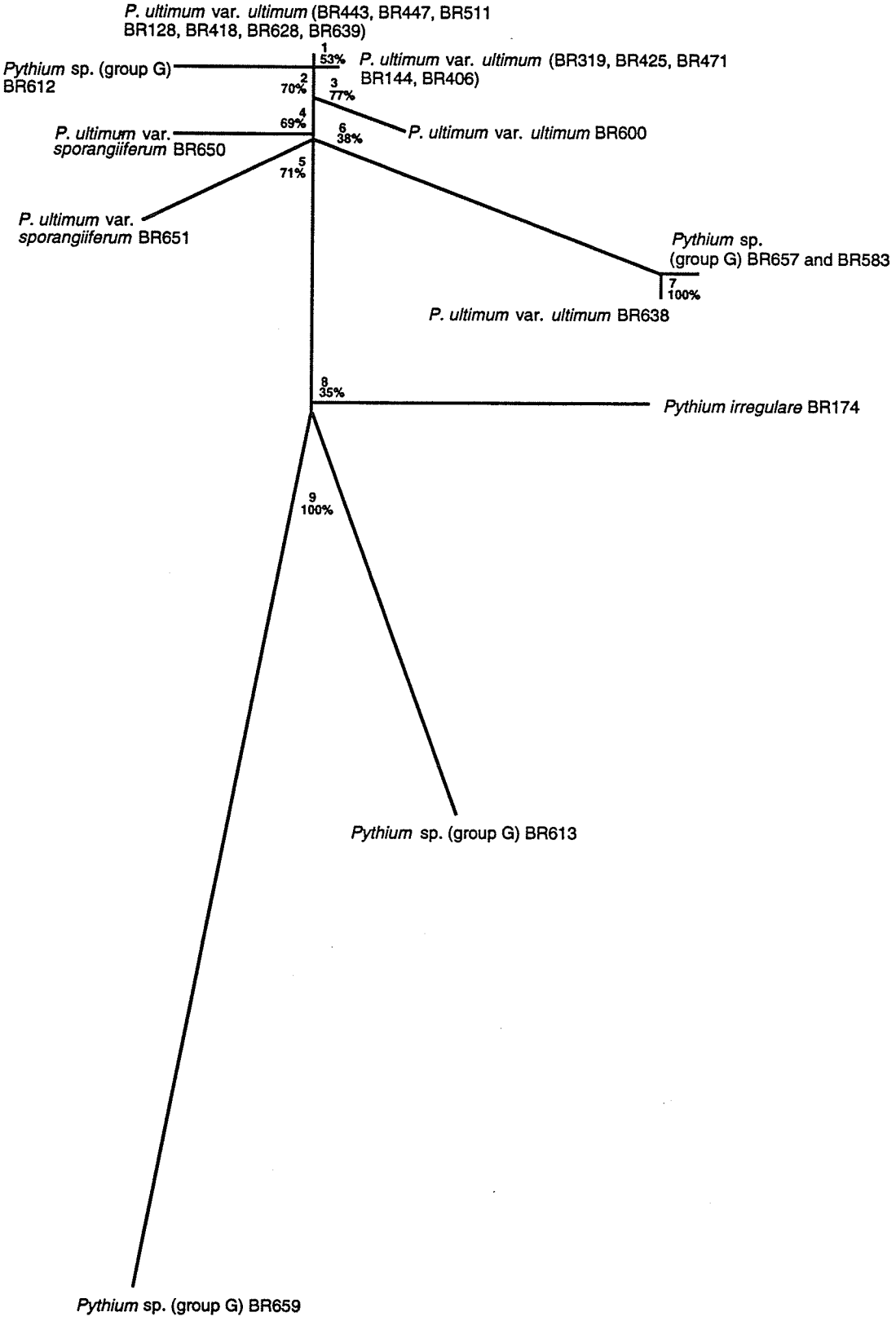
Table 3. A binomial matrix of the *Hpa*II restriction fragments observed in figures 1.1C and 1.1D. Fragment present=1, fragment absent=0.

Isolates	Fragment Size (kb)	16.0	15.5	13.5	12.5	12.0	11.0	10.5	10.0	9.50	8.30	8.00	7.50	7.20	6.80	6.60	6.10	5.60	5.40	5.30	5.00	4.50	4.30	4.20	4.10	3.90	3.80	3.85	3.30	3.20	3.10	3.00	2.80	2.40	2.35	3.30	2.20	2.10	1.80	1.60	1.65	1.20	1.00
	443	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
	447	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
	511	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
	128	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
	418	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
	628	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
	639	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
	319	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
	425	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
	471	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
	144	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
	406	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
	612	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1
	600	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1
	650	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1
	651	0	1	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
	657	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0
	583	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0
	638	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0
	174	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0
	613	0	0	0	0	0	1	0	0	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0
	659	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0

Table 4. A binomial matrix of the *Hind*III restriction fragments observed in figures 1.1A and 1.1B. Fragment present=1, fragment absent=0.

Isolates	10.5	9.50	9.00	7.10	6.60	5.10	4.80	4.30	4.25	4.20	4.10	4.00	3.80	3.70	3.60	3.40	3.30	3.10	2.90	2.70	2.50	2.40	2.30	2.25	2.20	2.15	2.10	2.05	2.00	1.90	1.80	1.70	1.65	1.50	1.45	1.40	1.30	1.20
443	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1
447	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1
511	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1
128	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1
418	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1
628	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1
639	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1
319	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1
425	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1
471	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1
144	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1
406	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1
612	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	1	0	1	0	1	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1
600	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1
650	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	1	0	0	1	0	0	0	1	0	1	0	0	0	1	0	0	1	0	1	0	0
651	0	0	0	0	1	1	0	0	1	1	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0
657	0	0	0	0	1	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	0
583	0	0	0	0	1	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	0
638	0	0	0	0	1	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	0
174	0	0	0	1	1	0	1	0	0	0	1	0	1	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0	1	0	0	0	0	1	0	1	0	0
613	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	1	0	1	0	0	1	1	1	0	0
659	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	1	1	1	1	0	0	0	1	1

Fig. 1.2. An unrooted Fitch-Margoliash network illustrating groupings between isolates of *Pythium ultimum*, asexual *Pythium* sp. and *Pythium irregulare*. The network was generated by the FITCH program (User Tree option, user-defined tree was produced by the BOOT program) and was based on the mtDNA digestion profiles shown in fig. 1.1 and the binomial matrices given in tables 3 and 4. The numbers along the branches represent the nodes. The percentages below the node numbers represent the number of times out of 100 replicates that a given branch appeared among the 100 bootstrap replicates. The confidence limits were determined by the BOOT program (Wagner option).


P. ultimum var. ultimum (BR443, BR447, BR511 BR128, BR418, BR628, BR639)
1
53%
Pythium sp. (group G) BR612
2
70%
3
77%
P. ultimum var. ultimum (BR319, BR425, BR471 BR144, BR406)
4
69%
6
38%
P. ultimum var. sporangiiferum BR650
P. ultimum var. ultimum BR600
5
71%
P. ultimum var. sporangiiferum BR651
Pythium sp. (group G) BR657 and BR583
7
100%
P. ultimum var. ultimum BR638
8
35%
Pythium irregulare BR174
9
100%
Pythium sp. (group G) BR613
Pythium sp. (group G) BR659

Manitoba (Fristensky 1991). A distance matrix was calculated for 11 of the RFLP profiles (11 of 22 profiles were used because identities were removed) by using RESTDIST (restriction fragments option). The FITCH program (User Tree option) was used to carry out Fitch and Margoliash's method for estimating phylogenies from the distance matrix (Fig. 1.2). The user-defined tree was generated using the data in Tables 3 and 4 by the program BOOT (Wagner option, 100 bootstrap replicates). The resulting distance dendogram (determined by Fitch) with confidence limits (determined by BOOT) based on the mtDNA RFLP data is shown in Fig. 1.2.

The resulting dendogram based on *Hin*dIII and *Hpa*II digestions of mtDNA divides the majority of the *P. ultimum* var. *ultimum* isolates into two closely related groupings which diverge at node 1 (Fig. 1.2). The first grouping, which have identical restriction banding patterns, includes isolates BR443, BR447, BR511, BR128, BR418, BR628 and BR639. The second major grouping, also possessing identical restriction banding patterns, includes isolates BR319, BR425, BR471, BR144, BR406. The second grouping has two additional restriction bands created by the enzyme *Hpa*II, all the other bands are shared between both of these groups (refer to Tables 3 and 4, and Fig. 1.1). Among these two groups various hosts and geographic locations are represented, thus there do not appear to be any links associating host and/or location with banding pattern.

There is some RFLP variability observed in other *Pythium ultimum* isolates. BR600 (node 3) and BR638 (node 7) are placed in a position on the dendogram which has them separated from the two major groups previously discussed. BR638 is unusual; its mtDNA banding pattern places it closer to the two "G" *Pythium* BR583 and BR657 at node 7 and it is positioned the furthest away from the two largest groupings of *P. ultimum* isolates on the dendogram at node 1. In fact, BR638 has an identical *Hind*III profile with BR657 and BR583, but is different at three positions for the enzyme *Hpa*II (refer to Table 3 and 4, and Fig. 1.1). The BR638 *Hpa*II mtDNA profile was used as supporting evidence for classifying BR583 (a "group G" *Pythium* sp.) with the species *P. ultimum* (Huang *et al.* 1992).

The two *P. ultimum* var. *sporangiiferum* isolates, BR650 and BR651, are placed at nodes 4 and 5 respectively. Although their banding patterns are not identical, they are placed closest to one another. They are positioned between the *P. ultimum* var. *ultimum* group at node 1 and *Pythium* sp. "group G" isolates (BR583 and BR657) at node 7.

It is not surprising that *P. irregulare* BR174 is placed very distant from the majority of the isolates. The two other *Pythium* sp. "group G" isolates, BR613 and BR659 at node 9, are placed even more distant than *P. irregulare*, at node 8, from all the other isolates. In addition, both BR613 and BR659 had noticeably slower growth rates and

mycelial growth was not as lush as a typical *P. ultimum*. Huang *et al.* (1992) stated that BR659 grew in a much narrower range of temperature and grew significantly more slowly at its optimum when compared to BR583. The differences in cultural characteristics and mtDNA banding patterns strongly suggest that BR613 and BR659 should not be assigned to the species *P. ultimum*.

The group G isolate BR612 is placed at node 2. It is positioned on the dendogram nearer to the major grouping of *P. ultimum* var. *ultimum* isolates at node 1 than to the two other "group G" isolates (BR657 and BR583). In addition, its relative position has it placed more closely to the majority of the *P. ultimum* isolates than to the two other "group G" *Pythiums* (BR657 and BR583). Its position on the dendogram becomes more understandable upon examination of the rDNA RFLP data, which will be discussed later in the next section.

In a previous study minimal intraspecific variation of mitochondrial DNA restriction banding patterns has been observed in different *Pythium ultimum* isolates (Martin and Kistler 1990). In another study selected asexual *Pythium* isolates belonging to "group HS" (possessing hyphal swelling) were shown to have very similar or identical mtDNA restriction banding patterns to that of *P. ultimum (Martin 1990)*. Martin proposed the re-classification of these asexual isolates as *P. ultimum* based on their observations.

Francis and St. Clair (1993) demonstrated that an HS isolate can outcross with *P. ultimum* and form viable progeny, which is evidence for conspecificity. Here, it was observed the two "group G" *Pythiums* (BR657 and BR 583) had the same *Hin*dIII restriction banding patterns as were observed for several "HS" isolates by Martin and Kistler (1990). In addition, Huang *et al.* (1992) have used the mtDNA RFLP data presented here along with morphological observations as evidence supporting the reclassification of group G *Pythium* BR583 as *P. ultimum*.

2. Ribosomal DNA RFLP analysis

DNA used for rDNA RFLP analysis was extracted from sixteen *P. ultimum* var. *ultimum*, two *P. ultimum* var. *sporangiiferum*, and seven *Pythium* sp. "group G" isolates (Table 1) that were collected from various geographical locations and host sources. The bottom band fraction of a CsCl bisbenzimide centrifugation gradient was used as the source of DNA.

A restriction site map of the rDNA repeat unit of *P. ultimum* BR471 was constructed by means of numerous single and double enzyme digestions (Fig. 2.5). It became apparent that fragments overlapping the NTS actually consisted of families of fragments, forming regular ladders of bands on gels (Figs. 2.1-2.4). Ladders of bands, such as those generated in this investigation, are produced either by partial digestion of arrays of tandem repeats or by the presence of length heterogeneity within the arrays. Other enzymes (*Hin*dIII, *Bgl*II, *Sst*I) also produced the observed ladder-like pattern (data not shown). For this to be due to partial digestion, all of these sites would have to be present exactly once in each of the postulated subrepeats, which is highly improbable. There is also no evidence of partial digestion for sites in the gene regions. It therefore must be concluded that the ladders are due to the existence of heterogenous arrays within each isolate of *Pythium*, very similar to those found in *Vicia faba* (Rogers

et al. 1986).

Two sets of DNA digestions are presented (Figs. 2.1-2.4) to show the approximate location of heterogenous regions (these are more precisely located in sequence data presented in section 4), to characterize the heterogeneous regions to some extent, and to compare band profiles of all the isolates in order to correlate rDNA RFLPs with those obtained from mtDNA.

Digestion of DNA from all the isolates in Table 1 (except *P. irregulare* and *Pythium* sp. "group G" BR659) with *Pst*I and detection of fragments by hybridization to pMF2, a recombinant plasmid containing the ribosomal genes of *N. crassa* (Free *et al.* 1979) are shown in figures 2.1 and 2.2. Results for BR443, BR406. BR471 and BR583 appear in both figures as a way of checking the reproducibility of the bands. Reproduction of major bands and most minor ones is excellent, extending even to the relative intensities of bands in the ladders of heterogeneity. Fragments I and II are not heterogeneous or polymorphic, as expected from their location in the LSrRNA gene (Fig. 2.5A). Fragment III is highly heterogeneous in all isolates except BR613. This result indicates that the main region of heterogeneity is in the large fragment downstream of the *Pst*I site near the centre of the NTS. The unnamed fragment upstream of the *Pst*I site in the NTS is not expected to hybridize to the probe because it contains little of the LSrRNA gene and it

is unlikely that much homology with the *N. crassa* probe exists in this region, which is known to be nonconserved.

The upward displacement of fragment III ladders in the two *P. ultimum* var. *sporangiiferum* isolates (BR650 and BR651) suggests that the *Pst*I site in the NTS is missing in these isolates, which has the effect of adding about 1.5 kb to each of the fragments in the ladder. This possibility is supported by evidence in the figures which will be discussed next. Isolate BR613 also seems to be missing the *Pst*I site in the NTS and it also does not have the ladder of bands which would indicate absence of the type of heterogeneity seen in the other isolates. (The hybridization signal at 1.6 kb is due to spillover of standard DNA from the adjacent lane not shown in the figure.)

Simultaneous digestion with *Hinc*II and *Eco*RV (Figs. 2.3-2.4) confirms the observations above and reveals a second region of heterogeneity in the upstream region of the NTS. The autoradiograms may be interpreted by referring to figures 2.5B and 2.5C. The gene regions, represented by fragments I, II, and III in both figures, are highly conserved except in an isolate (BR659) that was added to the isolate set for this experiment.

Fragment V in figure 2.3 and fragment VI in figure 2.4 show the same ladder-like pattern of heterogeneity as that seen in figures 2.1 and 2.2, with the exception of isolates BR613 and BR659. The location of the heterogeneous region,

downstream of the *Hinc*II site in the NTS, is consistent with the findings obtained after *Pst*I digestion. Band intensities in figures 2.1 and 2.2 match with those in figures 2.3 and 2.4, and band intervals are surprisingly uniform in all the isolates. The subrepeat interval size was estimated by plotting fragment size against the integers for fragment VI of isolate BR406 in figure Fig. 2.6. The linearity of the relationship between fragment size and integers confirms the size uniformity of the subrepeats and allows for an accurate estimate of subrepeat size at 380 bp. The ladders of bands for BR656, BR657, BR658, and BR583 (Fig. 2.4) are offset with respect to the others presumably due to an insertion outside of the subrepeat array, but increment size is the same as the others. The upward displacement of the ladder of bands for the two *P. ultimum* var. *sporangiiferum* isolates (BR650 and BR651) by about 1 kb is likely due to the absence of the *Hinc*II site in the NTS. (As discussed below, the *P. ultimum* var. *sporangiiferum* profiles are interpreted to mean that the central *Hinc*II site in figure 2.5C is absent and that the other *Hinc*II site, indicated by H* is always present.)

A second region of heterogeneity in the NTS can be inferred from the band patterns for fragment IV in figure 2.3 and fragment V in figure 2.4 by the map in figure 2.6C. A large group of isolates (BR443, BR418, BR319, BR128, BR447, BR406, BR144, BR471, BR612, BR425, BR600, BR511,

BR628, BR639, BR640, BR634) have families of hybridizing bands ranging from 2.5 to 3.0 kb. Many differences between isolates occur. This is interpreted to mean that the *Hinc*II band overlapping the NTS at its upstream end contains a region of length heterogeneity and that this region is polymorphic. The heterogeneity appears complex in organization, not like that in the downstream portion of the NTS. Another group of isolates (BR650, BR651, BR656, BR657, BR658, BR583, BR638) have prominent fragments at 1.7 kb with absent or less prominent fragments at 2.5-3.0 kb (Fig. 2.4). This is interpreted to mean that there is another *Hinc*II site in the NTS upstream of the region of heterogeneity which is present in most of the rDNA repeats, but not in all (Fig. 2.5C). In the case of *P. ultimum* var. *sporangiiferum* (BR650 and BR651) this *Hinc*II site appears to be always present because no bands appear in the 2.5-3.0 kb region. In the others, faint bands (except for BR638 in which the bands are intense) appear in this region, indicating that some rDNA repeats do not have the upstream *Hinc*II site in the NTS. Three isolates (BR656, BR657, BR658) also show a ladder of fainter bands just above the 1.7 kb band. This may mean that the variable *Hinc*II site is inside the region of heterogeneity and that fragment IV (Figs. 2.4, 2.5C) for these isolates can also form a ladder of bands. The band patterns for isolates BR613 and BR659 can not be interpreted fully using the *P. ultimum* maps, but neither of them shows

Fig. 2.1. Autoradiogram of *P. ultimum* var. *ultimum* and *Pythium* sp. "group G" (listed in Table 1; numbers represent culture numbers with the prefix "BR" omitted) chromosomal DNA digested with *Pst*I and hybridized to pMF2. Roman numerals refer to DNA fragments shown in Fig. 2.5A. The migration of the BRL 1 kb ladder DNA fragments are indicated.

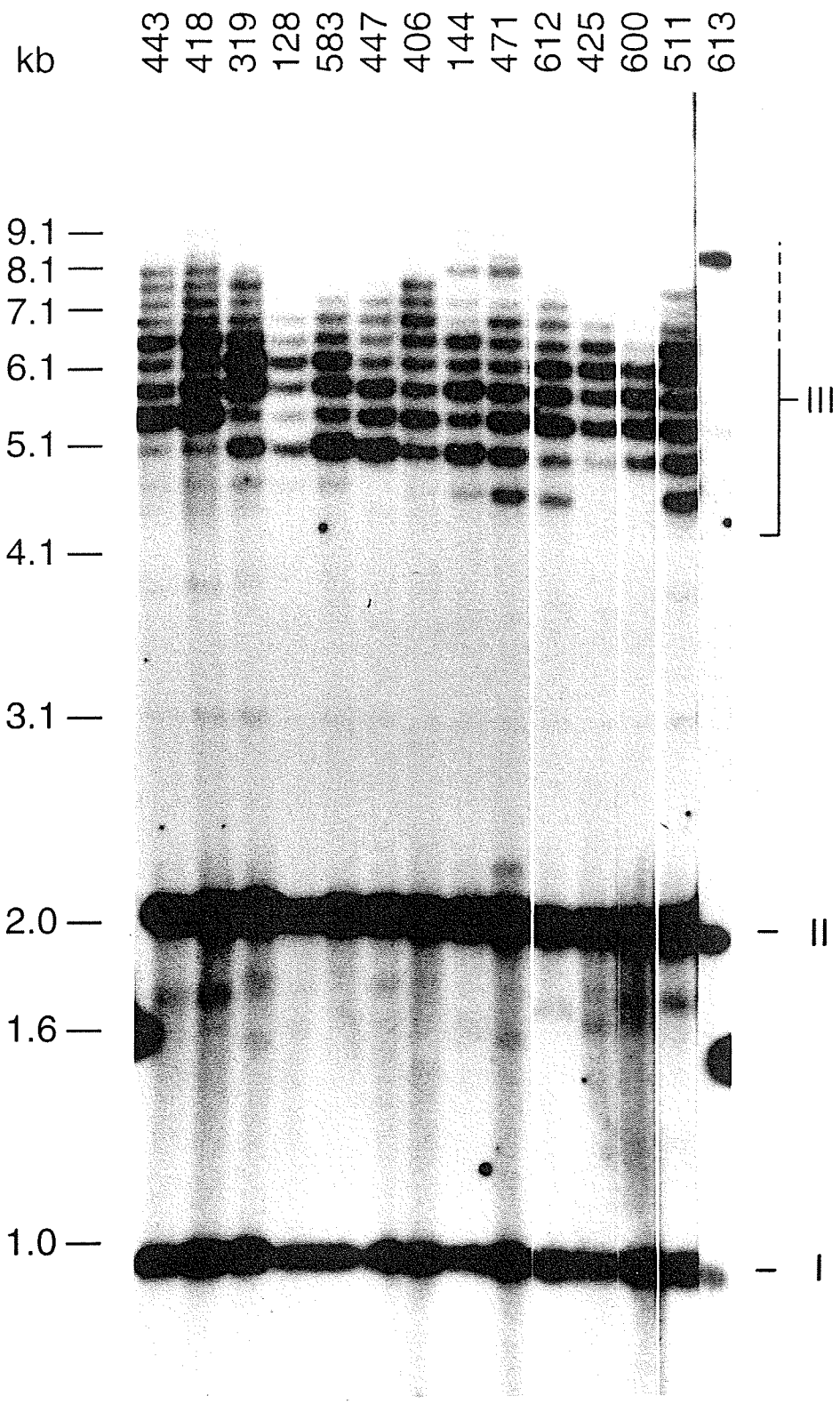

kb
443
418
319
128
583
447
406
144
471
612
425
600
511
613
9.1
8.1
7.1
6.1
5.1
4.1
3.1
2.0
1.6
1.0
III
II
I

Fig. 2.2. Autoradiogram of *P. ultimum* var. *ultimum*, *P. ultimum* var. *sporangiiferum*, and *Pythium* sp. "group G" (listed in Table 1; numbers represent culture numbers with the prefix "BR" omitted) chromosomal DNA digested *with PstI* and hybridized to pMF2. Roman numerals refer to DNA fragments shown in Fig. 2.5A. The migration of the BRL 1 kb ladder DNA fragments are indicated.

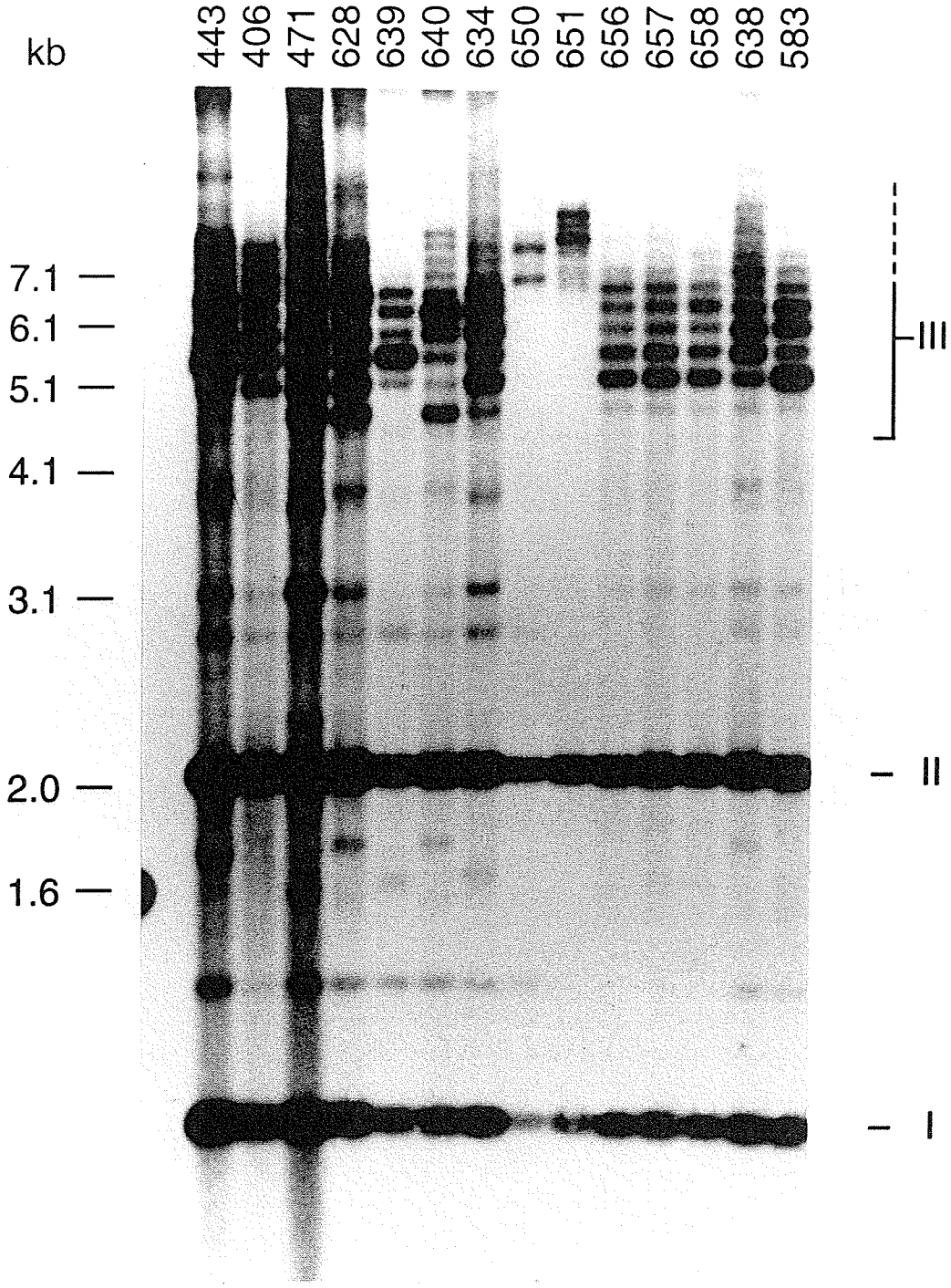

kb
443
406
471
628
639
640
634
650
651
656
657
658
638
583
7.1
6.1
5.1
4.1
3.1
2.0
1.6
III
II
I

Fig. 2.3. Autoradiogram of *P. ultimum* var. *ultimum* and *Pythium* sp. "group G" (listed in Table 1; numbers represent culture numbers with the prefix "BR" omitted) chromosomal DNA digested simultaneously with *Hinc*II and *Eco*RV and hybridized to pMF2. Roman numerals refer to DNA fragments shown in Fig. 2.5B. The migration of the BRL 1 kb ladder DNA fragments is indicated.

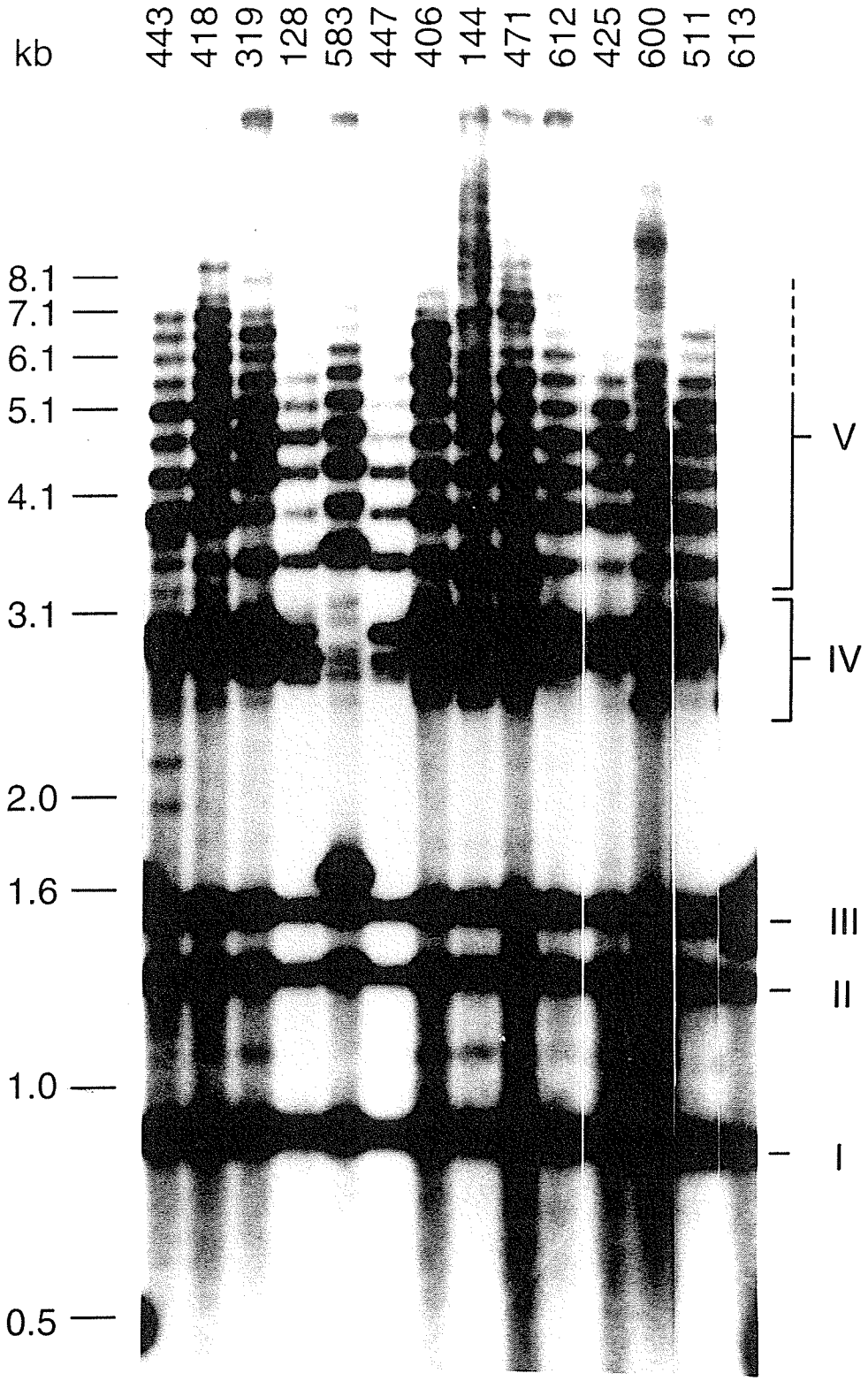

kb
443
418
319
128
583
447
406
144
471
612
425
600
511
613
8.1
7.1
6.1
5.1
4.1
3.1
2.0
1.6
1.0
0.5
V
IV
III
II
I

Fig. 2.4. Autoradiogram of *P. ultimum* var. *ultimum*, *P. ultimum* var. *sporangiiferum*, and *Pythium* sp. "group G" (listed in Table 1; numbers represent culture numbers with the "BR" prefix omitted) chromosomal DNA digested simultaneously with *Hinc*II and *Eco*RV and hybridized to pMF2. Roman numerals refer to DNA fragments shown in Fig. 2.5C. The migration of the BRL 1 kb ladder DNA fragments are indicated.

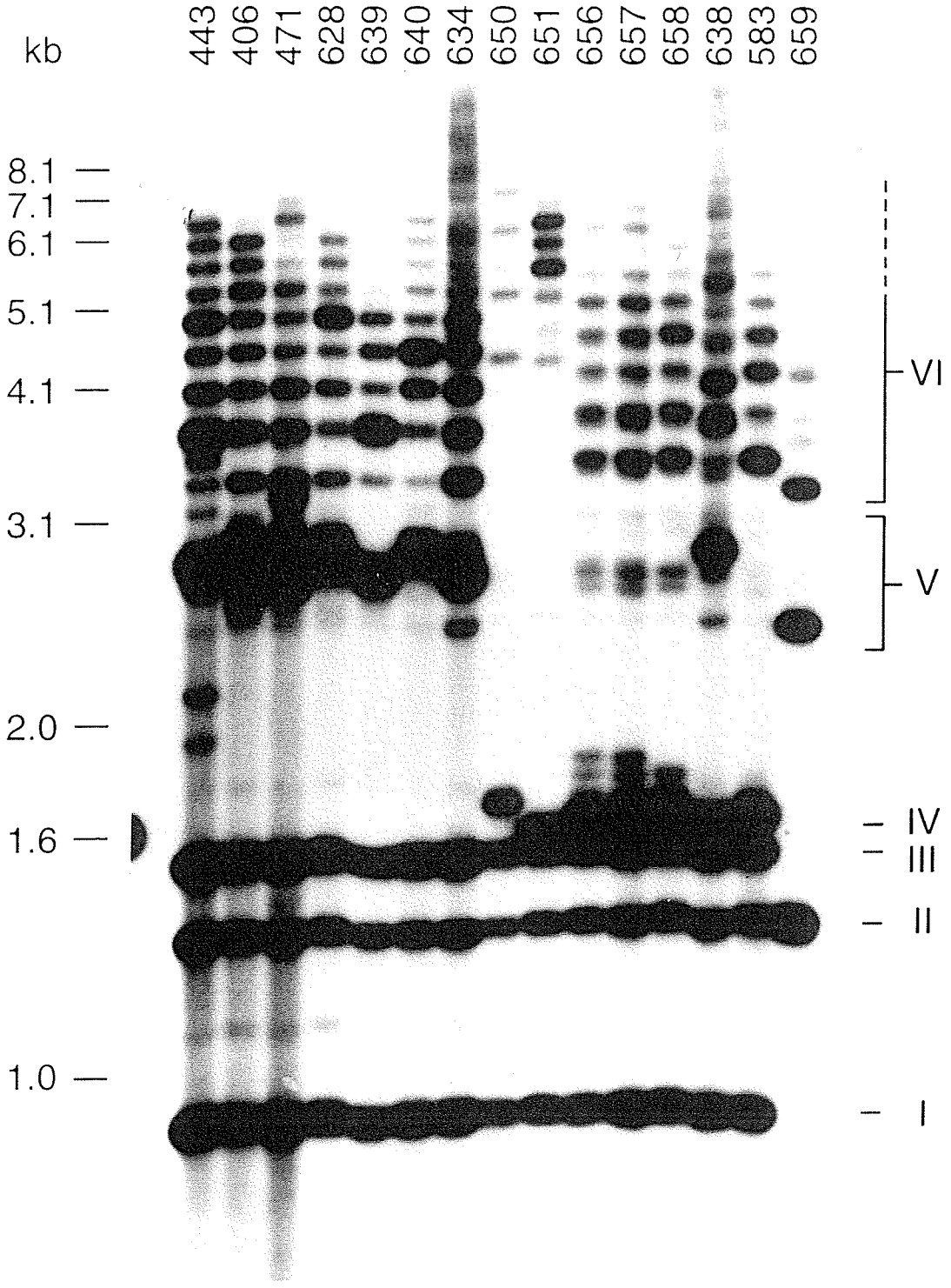

kb
443
406
471
628
639
640
634
650
651
656
657
658
638
583
659
8.1
7.1
6.1
5.1
4.1
3.1
2.0
1.6
1.0
VI
V
IV
III
II
I

Fig. 2.5. Endonuclease restriction map of the rDNA repeat unit in *P. ultimum* isolates. The roman numerals correspond to bands shown in Figs. 2.1-2.4. Letter "b" indicates the region of complex length heterogeneity downstream of the LSrRNA gene. Letter "a" indicates the region of 380 bp subrepeat length heterogeneity located upstream of the SSrRNA gene. A) Restriction map corresponding to the autoradiograms in figs. 2.1 and 2.2. The letter P symbolizes the *Pst*I site. The *Pst*I site in the middle of the NTS is absent for BR650 and BR651. B) Restriction map corresponding to the autoradiogram in Fig. 2.3. Symbols for restriction sites are as follows: H-*Hinc*II, RV-*Eco*RV. C) Restriction map corresponding to the autoradiogram in Fig. 2.4. Symbols for the restriction sites are the same as above except that the *Hinc*II site symbolized by H* is observed in BR650, BR651, BR656, BR657, BR658, BR638, BR583 only. The *Hinc*II site in the middle of the NTS is absent in BR650 and BR651.

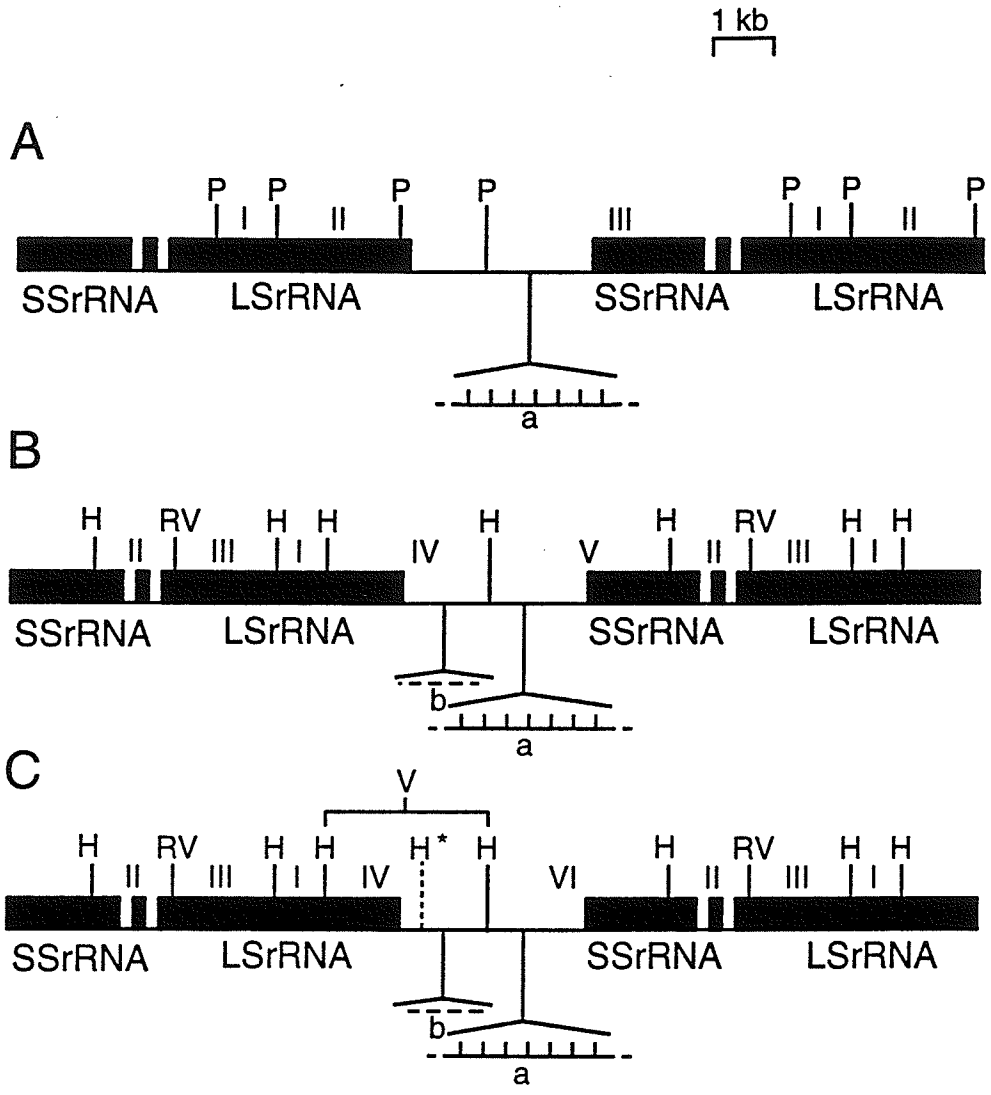
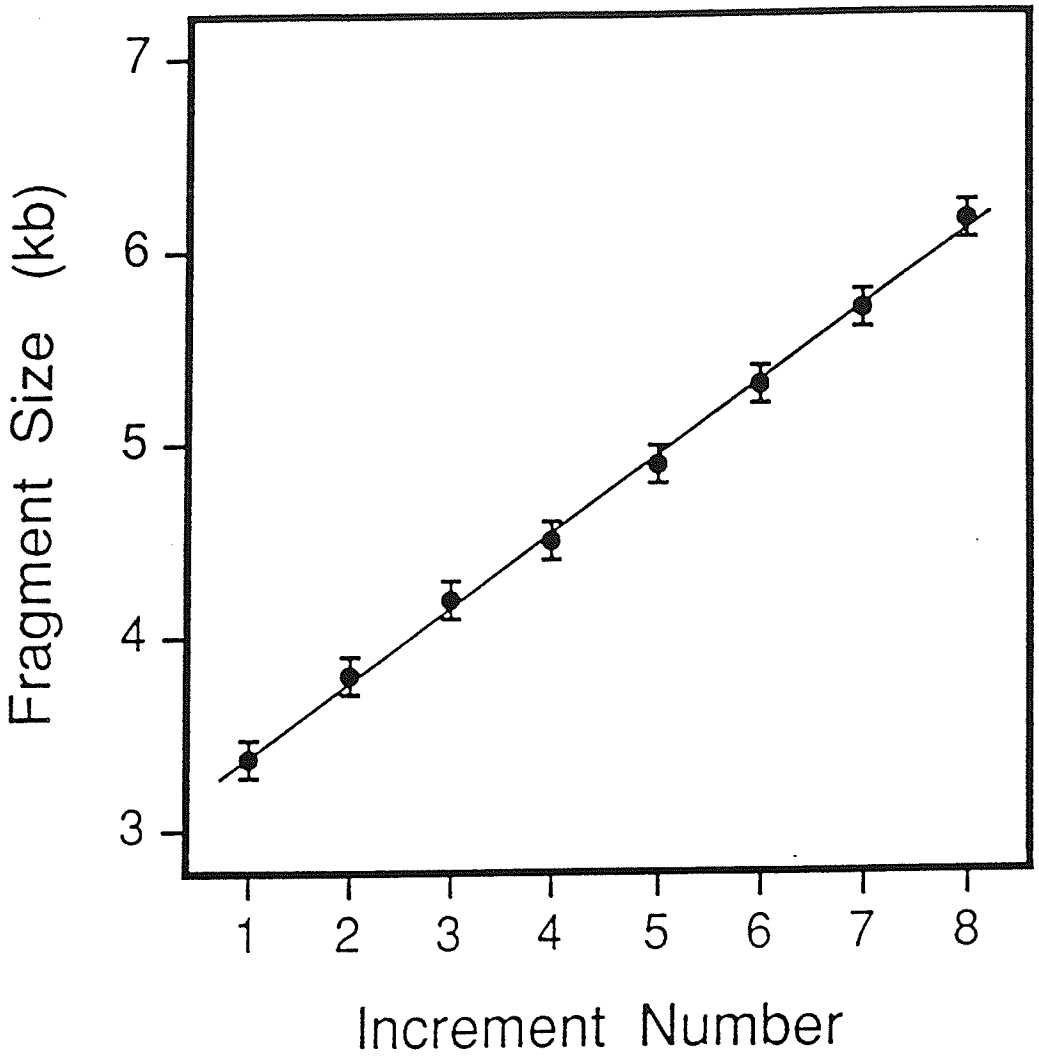

1 kb
A
P
I
P
II
P
P
III
P
I
P
II
P
SSrRNA
LSrRNA
SSrRNA
LSrRNA
a
B
H
II
RV
III
H
I
H
IV
H
V
H
II
RV
III
H
I
H
SSrRNA
LSrRNA
SSrRNA
LSrRNA
b
a
C
V
H
II
RV
III
H
I
H
IV
H*
H
VI
H
II
RV
III
H
I
H
SSrRNA
LSrRNA
SSrRNA
LSrRNA
b
a

Fig. 2.6. The incremental nature of the heterogeneity of fragment V (see Figs. 2.1 and 2.5B) in *P. ultimum* BR406. The sizes of the eight visible versions of fragments V are plotted against integers. Error bars are estimates of the accuracy of measurement of band mobilities on the gel.


Fragment Size (kb)
7
6
5
4
3
1
2
3
4
5
6
7
8
Increment Number

any sign of length heterogeneity.

The region containing the complex heterogeneity described above may correspond to regions of heterogeneity reported for *P. paroecandrum., P. spinosum, P. sylvaticum,* and *P. irregulare* (Martin 1990). In these cases, insertion-deletions were found in multiples of approximately 60 bp or 90 bp, strongly suggesting subrepeat organization of the region. Similarly, Belkhiri *et al.* (1992) demonstrated length heterogeneity within the NTS upon amplifying the region between the 5S rRNA and the LSrRNA gene. Thus, length heterogeneity of the NTS may be common within the genus *Pythium*, although the degree of heterogeneity in *P. ultimum* may be exceptional.

The organization of the NTS observed among the *P. ultimum* isolates is very similar to what was observed by Rogers *et al. (1986)* for the species *Vicia faba*. Digestion of *V. faba* rDNA with certain restriction enzymes resulted in the production of ladders of bands, caused by 325 bp subrepeats, much like that reported in this study for *P. ultimum*. It has been demonstrated that subrepeats which are present in the NTS of other organisms have the ability to enhance transcription of the rRNA genes by RNA polymerase I (Sollner-Webb and Moughey 1991). The evidence of major subrepeat heterogeneity in *P. ultimum*, and length heterogeneity in Oomycetes, supports the belief that oomycetes are phylogenetically distinct from other fungi

(Barr 1983, Cavalier-Smith 1989), which often lack this kind of length heterogeneity.

The presence of subrepeat heterogeneity was found in all the *P. ultimum* var. *ultimum* isolates, both *P. ultimum* var. *sporangiiferum* isolates and five of seven "group G" *Pythium* sp. isolates. The size of the subrepeat is conserved in all the isolates which possess them. The isolates differed in the number of size variants they had and the relative abundance of each variant appears to be unique for each isolate. In addition, the other region of length heterogeneity (Band IV in Fig. 2.3 and Band V in Fig. 2.4) is unique for each isolate. Thus, the generation of size heterogeneity at both locations of the NTS will be useful for the differentiation of very closely related strains of *P. ultimum*.

The fact that the 380 base pair subrepeat is present in all the isolates, except for BR613 and BR659, supports the classification of the asexual isolates (BR612, BR656, BR657, BR658 and BR583) as belonging to the species *P. ultimum*. All the other isolates can be more easily identified based on morphological characters since they are capable of producing sexual structures. The rDNA RFLP data support the species assignment of these isolates since they all share the character of subrepeat heterogeneity. Conversely, the lack of the subrepeat heterogeneity in the two asexual isolates BR613 and BR659 suggest that they should not be

assigned to the species *P. ultimum*.

Some parallels can be drawn between the dendogram presented in Figure 1.2 and the rDNA RFLP analysis done in this section. Most of the *P. ultimum* var. *ultimum* were clustered very close to each other on the dendogram (BR443, BR447, BR511, BR128, BR418, BR628, BR639, BR319, BR425, BR471 BR144 and BR406) into two major groupings. It is not surprising that the organization of the NTS for all of these isolates are very similar (refer to Figs. 2.1-2.5).

Two "group G" *Pythium* sp. (BR657 and BR583) are clustered together in the dendogram. The organization of the NTS supports the clustering the two isolates on the dendogram. The ladder-like banding patterns for both of these isolates run slightly out of register with the other *P. ultimum* isolates, but are in register with each other. In addition, the two isolates both possess an added *Hinc*II site in the NTS. These characteristics set them apart from all the other *P. ultimum* isolates. Huang *et al.* (1992) has proposed that BR583 should be assigned to the species *P. ultimum*. The NTS structure of BR583 is very similar to that of the isolates BR656, BR657 and BR658, and supports the assignment of these three isolates to the species *P. ultimum*, as well.

BR638 (*P. ultimum* var. *ultimum*) is placed very close to the asexual isolates BR583 and BR657 on the dendogram (Fig.

1.2). Interestingly, its NTS has characteristics which are common to both *P. ultimum* and the "group G" *Pythium* sp. The bands corresponding to the subrepeat length heterogeneity are in register with all the other *P. ultimum* var. *ultimum* isolates, but out of register with the asexual isolates (BR656, BR657, BR658, and BR583). In addition, it has the added *Hinc*II restriction site present in its NTS producing Band IV (Fig. 2.4). It is therefore a very unusual isolate and may represent some kind of a evolutionary intermediate between *P. ultimum* var. *ultimum* and its "group G" asexual form.

The other "group G" *Pythium* sp. isolate BR612 is positioned closer to the majority of the *P. ultimum* isolates than to the other asexual isolates (BR657 and BR583) on the dendogram. The organization of the NTS for BR612 more closely resembles the sexual structure-producing isolates. BR612 may represent a *P. ultimum* var. *ultimum* isolate which has more recently lost its ability to produce reproductive structures.

The correlation between the dendogram results and the rDNA data is not as easily seen for *P. ultimum* var. *sporangiiferum*. The two isolates are positioned somewhere in the middle of the dendogram. Although they are placed on separate nodes, they are positioned closer to each other than to any other isolate. Their NTS organization is somewhat similar, but significant polymorphisms are observed

between the two (Fig 2.4).

The two other "group G" *Pythium* sp. isolates BR613 and BR659 are positioned more distantly from any *P. ultimum* isolate than *P. irregulare* BR174 on the dendogram. The mtDNA data alone suggests that they belong to a species other than *P. ultimum*. Similarly, the rDNA RFLP analysis demonstrates that each of the two isolates has a very distinctive restriction banding pattern from all the other isolates looked at. Specifically, neither isolate appeared to have subrepeat heterogeneity of the NTS. It is a characteristic which is common to all the other isolates looked at in this study. Thus, the rDNA RFLP analysis agrees with the mtDNA RFLP analysis for BR613 and BR659.

It can be stated with some confidence that the asexual isolates BR656, BR657, BR658, BR612, and BR583 are a form of the species *P. ultimum*. This conclusion is based on the mtDNA data, morphological data provided by Huang *et al.* (1992) and the rDNA restriction banding pattern analysis (eg. subrepeat heterogeneity).

3. PCR amplification of the NTS of *Pythium ultimum*

The non-transcribed spacer of the rDNA repeat unit in *P. ultimum* is extremely heterogeneous due to the presence of an array of tandem subrepeats and other forms of variability (Klassen and Buchko 1990). This type of NTS organization is commonly seen in plants and animals where such subrepeats have been implicated in transcriptional control (Flavell 1990, Reeder 1984, Sollner-Webb and Moughey 1991). Although there are examples of similar types of length heterogeneity in true fungi (van Heerikhuizen *et al.* 1985, Zerucha 1992, Morton *et al.* 1995) it is much less common than it is in higher eukaryotes. If the presence of this type of sequence organization is typical of oomycetes, it would set them apart from the other fungi (Barr 1983, Cavalier-Smith 1989). Therefore there is a need for a protocol which can easily identify this trait without going through gene cloning procedures. It is possible to use PCR technology (Mullis and Faloona 1987, Saiki *et al.* 1988) to amplify the NTS, providing clues to the organization of that region.

A large number of SSrRNA and LSrRNA genes have been sequenced for numerous organisms (Dams *et al.* 1988, Gutell and Fox 1988). These genes are highly conserved in sequence, even between distantly related species. It is therefore possible to find conserved regions in different species (and kingdoms). The sequence data in the literature was used to design primers that could be used to amplify the

non-transcribed spacer of *P. ultimum* using PCR. With this goal in mind, primers G and H were synthesized (refer to Table 2). Primer G is based on a conserved sequence located about 900 bp downstream of the 5′ end of the SSrRNA gene and it is directed upstream toward the NTS (Rubstov *et al.* 1980). Primer H is based on a sequence located about 500 bp upstream of the 3′ end of the LSrRNA gene, and it is directed downstream towards the NTS (refer to Fig. 3.1)(Gutell and Fox 1988). Using the polymerase chain reaction technique, the two primers could therefore be used to amplify the region between the SSrRNA and LSrRNA gene (NTS) to yield products containing the NTS as well as enough gene sequence for hybridization to gene probes such as pMF2.

The restriction map of the rDNA repeat unit for BR471 is given in Fig. 3.1. The heterogeneous region in the 3′ half of the NTS would appear in products of HG amplification (above the map) and also in the largest *Pst*I fragment (below the map). The HG products are expected to be 4.6 kb and larger and the *Pst*I fragments about the same.

In Figure 3.2 DNA from the two *P. ultimum* isolates, BR471 and BR425, was digested with *Pst*1 followed by hybridization to pMF2 (rDNA probe from *N. crassa*, Free *et al.* 1979). The ladder of bands between 4.7 and 8.6 kb for BR471 is due to the presence of length heterogeneity caused by variable numbers of a 380 bp element (Klassen and Buchko 1990). In isolate BR425, the ladder of bands range from

Fig. 3.1. Amplified region of the rRNA repeat unit in *P. ultimum BR471*. One complete repeat is shown with gene locations and the nontranscribed spacer region. Endonuclease sites are symbolized as follows: P-*Pst*I, H-*Hinc*II, D-*Hin*dIII. Arrow symbols indicate the annealing site for the H and G primers (listed in table 2). Horizontal lines under the map represent *Pst*I fragments with incremental subrepeat arrays. Horizontal lines above the map represent the shortest three members of the set of PCR products produced by the HG amplification.

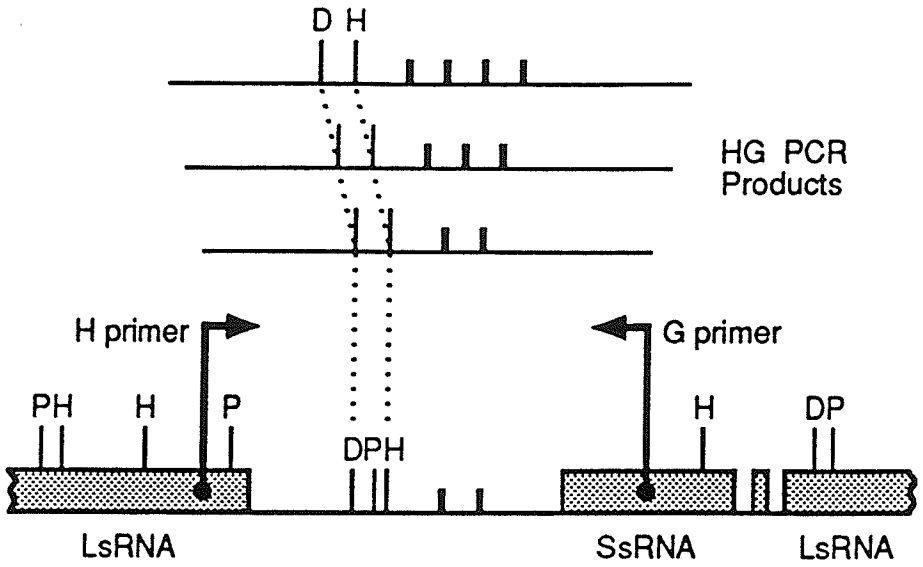

D H
HG PCR
Products
H primer
G primer
PH
H
P
DPH
H
DP
LsRNA
SsRNA
LsRNA

Fig. 3.2. *Pst*I fragments produced by digestion of ribosomal DNA of *P. ultimum* BR471 and BR425 visualized by hybridization to pMF2. Band sizes are based on BRL 1 kb ladder which was used as a DNA size standard.

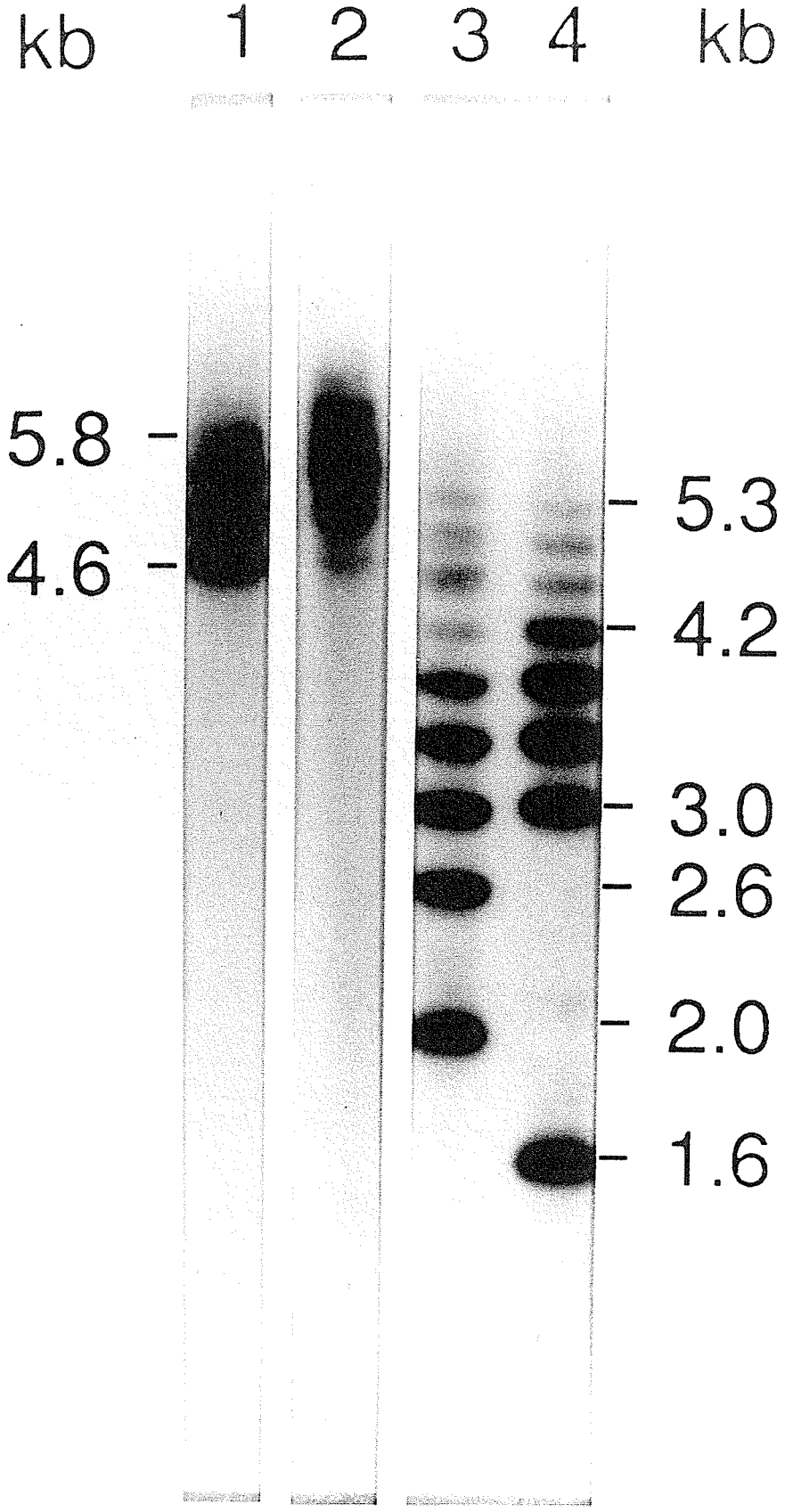

kb
471
425
8.6
7.0
5.1
4.7
2.0
1.7
1.6
1.0

about 4.7 kb to 7.0 kb. This observed difference is caused by differences in frequencies of certain length variants between the two isolates. The bands at 2.0 and 1.0 kb represent fragments that are near the 3' end of the LSrRNA gene and near the middle of the LSrRNA gene, respectively. The 1.6 (BR471) and 1.7 (BR425) kb bands represent the region from the 3' end of the LSrRNA gene to the middle of the NTS for the two isolates respectively. The 3' end of the LSrRNA gene is highly variable in sequence between species. It is for this reason that pMF2 (a probe containing rDNA repeat unit from the ascomycete *N. crassa*) hybridizes weakly to the 1.6 and 1.7 kb DNA fragments from the two oomycetous isolates (BR471 and BR425). The observed difference in length seen between these two fragments must be due to an insertion or deletion of a sequence element in this region.

Amplification of DNA from *P. ultimum* BR471, using the HG primer pair, resulted in an incremental set of fragments ranging form 4.6 to 5.8 kb with increments of about 0.4 kb (Fig. 3.3, lane 1). The pattern and size of bands is very similar to the ladder of bands produced by digestion of total DNA with *Pst*I, as predicted (see Fig. 3.2). Since both primer annealing locations are situated in the rRNA gene region, the resulting PCR product can be detected using the pMF2 probe. Hybridization to pMF2 was done to help visualize the faintest bands, but PCR products yield was

good enough so that ethidium bromide-stained bands were clearly observed in the gel when 20% of the PCR reaction volume was run (there is an example of an ethidium bromide-stained gel given in Fig. 4.1 of the next section). In order to confirm that the PCR product represented the NTS region, amplified DNA was digested with *Hin*dIII and *Hinc*II, enzymes with sites in the NTS (see Fig. 3.1). Both enzymes produced fragments predicted by the map (Fig. 3.3, lanes 3 and 4). *Hinc*II digestion produced a 2.0 kb fragment which represents the 5′ end of all the PCR products. It also produced a series of bands ranging from 2.6 to 5.3 kb, representing the 3′ heterogenous ends (Fig. 3.3, lane 3). The faint restriction bands at the top of the profile are obscured by a small amount of the unrestricted PCR product remaining due to incomplete digestion. The top three fragments appear to be full length PCR products like those seen in lane 1. The *Hin*dIII digestion of the PCR products produced a 1.6 kb band, representing the 5′ ends of the PCR products and a series of bands ranging from 3.0 to 5.3 kb, representing the 3′ ends (Fig. 3.3, lane 4). Unlike *Hinc*II, *Hin*dIII digested the DNA fragments fully. *Hin*dIII digestion of the PCR fragments consisted of four abundant bands in the lower size range and three less abundant bands in the upper range. This pattern is very reminiscent of what is observed for the *Pst*I digestion of DNA from BR471 (see Fig 3.2, lane 1), although the smallest bands seem to be preferentially

Fig. 3.3. Polymerase chain reaction products produced by amplification of ribosomal DNA of *P. ultimum* BR471 and BR425 using primers H and G. Lane 1, PCR product from BR471 target DNA; 20% of the reaction mixture was loaded onto a 0.8% agarose gel. Lane 2, PCR products from BR425 target DNA; 80% of the reaction mixture was applied to an 0.8% agarose gel. PCR cycle used: denature at 93°C for 30 sec, anneal at 55°C for 1 min, ramp up to 70° in 30 sec, and extend at 70°C for 8 min. For BR471 template DNA, the cycle was repeated 17 times with a final extension step of 10 min at 70°C. For BR425 template DNA, the same procedure was followed except that 18 cycles of PCR were carried out. Lane 3, PCR products from BR471 digested with *Hinc*II. Lane 4, PCR products from BR471 digested with *Hind*III. All bands were visualized by hybridization to pMF2.


kb
1
2
3
4
kb
5.8
4.6
5.3
4.2
3.0
2.6
2.0
1.6

amplified. Both sets of restrictions have four strong lower bands and three or more weaker bands above them. It appears that PCR is capable of amplifying a set of fragments and roughly preserving relative abundance except that there is a bias towards the shorter fragments.

Amplification using DNA template from BR425 yielded a set of fragments seen in Fig. 3.3, lane 2. The ladder of bands varied in size from 4.6 to 6.6 kb. No band corresponding to the 4.6 kb PCR band is visible in the genomic DNA ladder (Fig. 3.2, land 2). It appears that PCR has amplified a template that is so low in abundance that it is undetectable by the hybridization probe before amplification. The five bands visible above the 4.6 kb band correspond to the shortest five fragments of the ladder seen in genomic DNA (5.1 to 6.2 kb). The longest fragment of genomic DNA, producing a faint band in Fig. 3.2 (lane 2), was not amplified by PCR. Again PCR amplifies most of the fragments of the set, but shows a decided bias towards the smaller members.

The PCR bands produced by BR425 are all slightly larger than the ladder of fragments produce by BR471. This is due to a difference in length in the region flanking the LSrRNA genes between the two isolates. Fig. 3.2 shows that the regions downstream of the LSrRNA gene up to the *Pst*I site, in the middle of the NTS, are different in size. Genomic DNA from BR471 and BR425 produced 1.6 and 1.7 kb fragments

respectively upon digestion with *Pst*I (Fig. 3.2). This size difference accounts for the slight difference observed in the HG amplification products for the two isolates. It should be noted that the region immediately downstream of the LSrRNA gene is subject to length heterogeneity (see Fig. 2.2 and 2.3), although one size variant is usually predominant. This reinforces the previous observation whereby the PCR products seem to reflect the relative abundance of a certain template.

In this section the amplification of extremely long DNA targets as well as the simultaneous co-amplification of families of DNA targets are described. In order to successfully generate a coherent ladder of bands it was critical that the number of PCR cycles was kept to a minimum (17 or 18 cycles in this case), an observation which agrees with the study by Jeffreys *et al.* (1988). Even one additional cycle beyond the optimum has been observed to result in significant degeneration of the product pattern, presumably due to out-of-register annealing (Jeffreys *et al.* 1988).

The faithfulness of the PCR amplification in reproducing the pattern of heterogeneity in ribosomal DNA makes feasible the use of this method for the detection of heterogeneity from very small samples of crude DNA in large numbers of species. The potential for cloning specific PCR products directly without resorting to constructing genomic

libraries, is thus feasible. This is the technique which is used to clone and study the NTS for the *P. ultimum* isolate BR471, work presented in the next section.

4. Cloning, sequencing, and characterization of the ribosomal RNA spacer region of *Pythium ultimum*

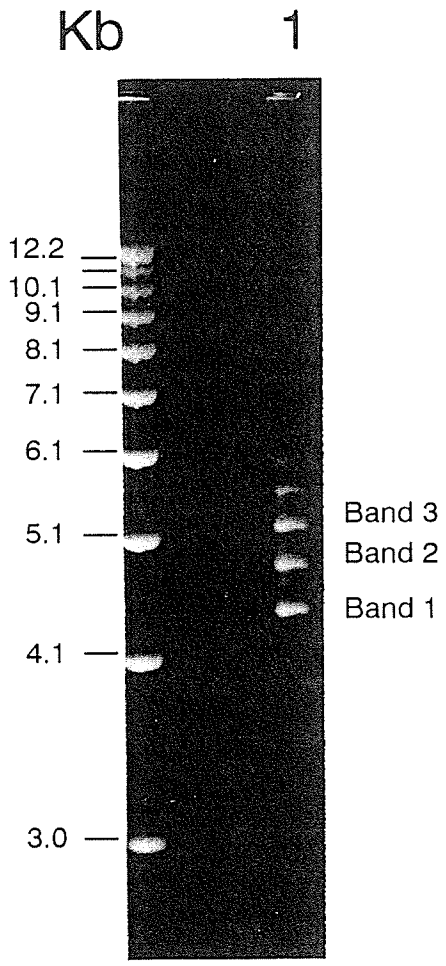
Restriction endonuclease analysis of the rDNA repeat unit of numerous *P. ultimum* isolates has revealed a high degree of length heterogeneity in the NTS region. The variable length spacers are due mainly to the presence of a repetitive element of approximately 380 bp, which can differ in numbers among the rRNA gene family (Klassen and Buchko 1990). Another region of length heterogeneity, located downstream of the LSrRNA gene, has also been identified. The heterogeneity is more complex and it does not seem to be of the same incremental nature as the region containing the 380 bp subrepeat. In addition, one or two size variants usually predominate in this region (refer to Figs. 2.1 and 2.2). The region between the 3′ end of LSrRNA gene and the 5′ end of the SSrRNA gene has been successfully amplified using PCR (Buchko and Klassen 1990). Both of these previous studies have given insights into the organization of the rDNA spacer region for *P. ultimum*.

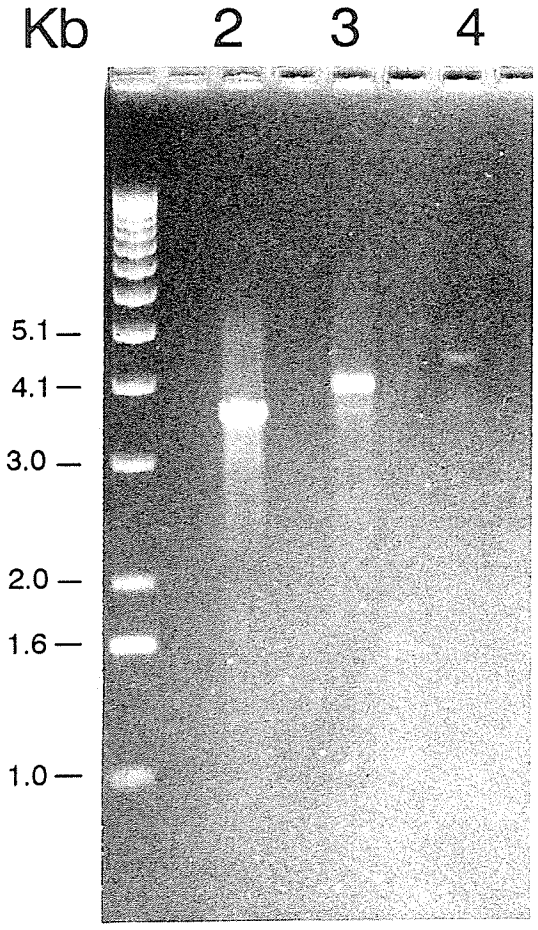
Successful amplification of the NTS region has made it possible to study the organization of the rDNA spacer at the sequence level without subcloning from a genomic library. After cloning the PCR products, the location and sequence of the 385 bp subrepeat can be obtained by sequencing at least two spacer length variants (the shortest and the next larger one). The nature of the length heterogeneity present

immediately downstream of the LSrRNA gene may also be ascertained.

With these goals in mind, one *P. ultimum* var. *ultimum* isolate was selected for the purpose of amplifying the rDNA spacer region. DNA from isolate BR471 was used as template, along with the primer pair HG for PCR amplification (as outlined in section 3, Fig. 3.1). The resulting PCR fragments are shown in Fig. 4.1 (lane 1). At least four bands are visible on the ethidium bromide-stained agarose gel when 20% of the total reaction mixture is loaded onto the gel. The bands, as expected, differ by an increment of approximately 0.4 kb. Bands 1, 2, and 3 (Fig. 4.1, lane 1) were cut out and extracted from the gel. Each isolated band was then used as template for amplification with the primer pair P and Q (refer to Table 2). If the HG PCR fragments represent a true amplification of the region of interest, and not an artefact, then primer annealing sites P and Q should be present on the HG PCR products (refer to Fig. 4.2 for the relative location of P and Q). The PCR amplification products of each isolated band (from the HG amplification) using the PQ primer pair is given in Fig. 4.1. The PCR fragments ranging from 3.8 to 4.6 kb in lanes 2, 3, and 4 (Fig. 4.1) differ by an increment of about 0.4 kb, as predicted. Fragments in lanes 2, 3, and 4 represent Bands 1, 2, and 3 respectively (Fig 4.1, lane 1) whereby each band is approximately 0.8 kb shorter than the length of

Fig. 4.1. Amplification of individual length variants for *P. ultimum* BR471. Lane 1, PCR products of BR471 using primer pair H and G (described in previous section). Lane 2, PCR product using Band 1 as template and primers P and Q. Lane 2, PCR product using Band 2 as template and primers P and Q. Lane 3, PCR products using Band 3 as template and primer P and Q. PCR cycle utilized in lanes 2-4: denature at 93°C for 30 sec, anneal at 50°C for 1 min, ramp up to 70°C in 30 sec, and extend at 70°C for 5 min. The cycle was repeated 30 times, with a final extension at 70°C for 10 min. 25% of the PCR reaction mixture was loaded onto a 0.8% agarose gel for each sample and visualized by ethidium bromide staining. 1.0 kb ladder (BRL) size fragment are shown.


Kb
1
12.2
10.1
9.1
8.1
7.1
6.1
5.1
4.1
3.0
Band 3
Band 2
Band 1


Kb
2
3
4
5.1
4.1
3.0
2.0
1.6
1.0

their respective templates (HG products), as expected. The results, therefore indicate that individual length variants of the rDNA intergenic region have been successfully amplified.

DNA fragments represented in lanes 2, 3, and 4 (Fig. 4.1) were then cloned into the Bluescript M13 vectors (procedure outlined in Materials and Methods section). Five clones were constructed representing three length variants (Fig. 4.2). The clones pJB1 and pJB1A have the same size inserts and are the shortest clones. It would be expected that they should contain the minimum number of 380 bp subrepeats, or none at all. If it is assumed that pJB1 and pJB1A each have one subrepeat, then pJB2 would have two subrepeats, while pJB3 and PJB3A would have three subrepeats (refer to Fig. 4.2).

In order to confirm that the clones are in fact representative of the rDNA spacer for *P. ultimum*, the clones were digested with the enzymes *Pst*I, *Sal*I and *Xba*I simultaneously. The restriction sites for *Pst*I and *Xba*I are present in the NTS clones (see Fig. 4.2), the *Sal*I site is present in the multiple cloning site of Bluescript M13. The result of the digestion is shown in Fig. 4.3. All of the clones produce the 3.0 kb fragment, which is vector DNA. They also all have a 1.50 kb fragment which represents the region between the *Pst*I site in the NTS and the Q site in the LSrRNA gene (refer to Fig. 4.2). Although a complex

Fig. 4.2. A list of all the NTS clones derived from *P. ultimum* BR471 DNA. The large and small subunit rRNA genes are displayed (LSrRNA and SSrRNA respectively). The fine dashed line indicates the portion of the rDNA repeat unit which was cloned. Restriction sites are represented by the following letters: H-*Hin*cII, P-*Pst*I, D-*Hin*dIII, X-*Xba*I. The primer annealing sites, P (represented by P*), Q, H and G (refer to table 2) are represented by arrows. The area in which subrepeat heterogeneity occurs is indicated below the map of the rDNA repeat unit. The putative number of subrepeats in each clone is represented by the vertical dashed line and subrepeat symbol above each clone.

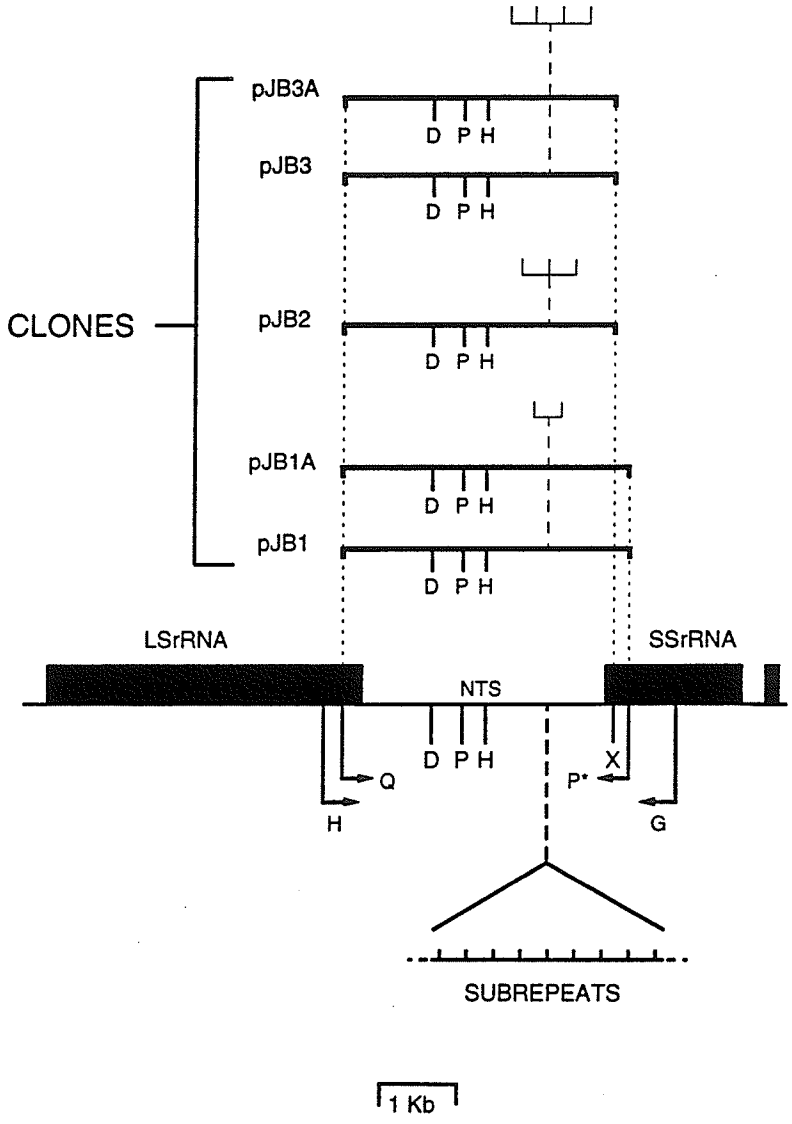
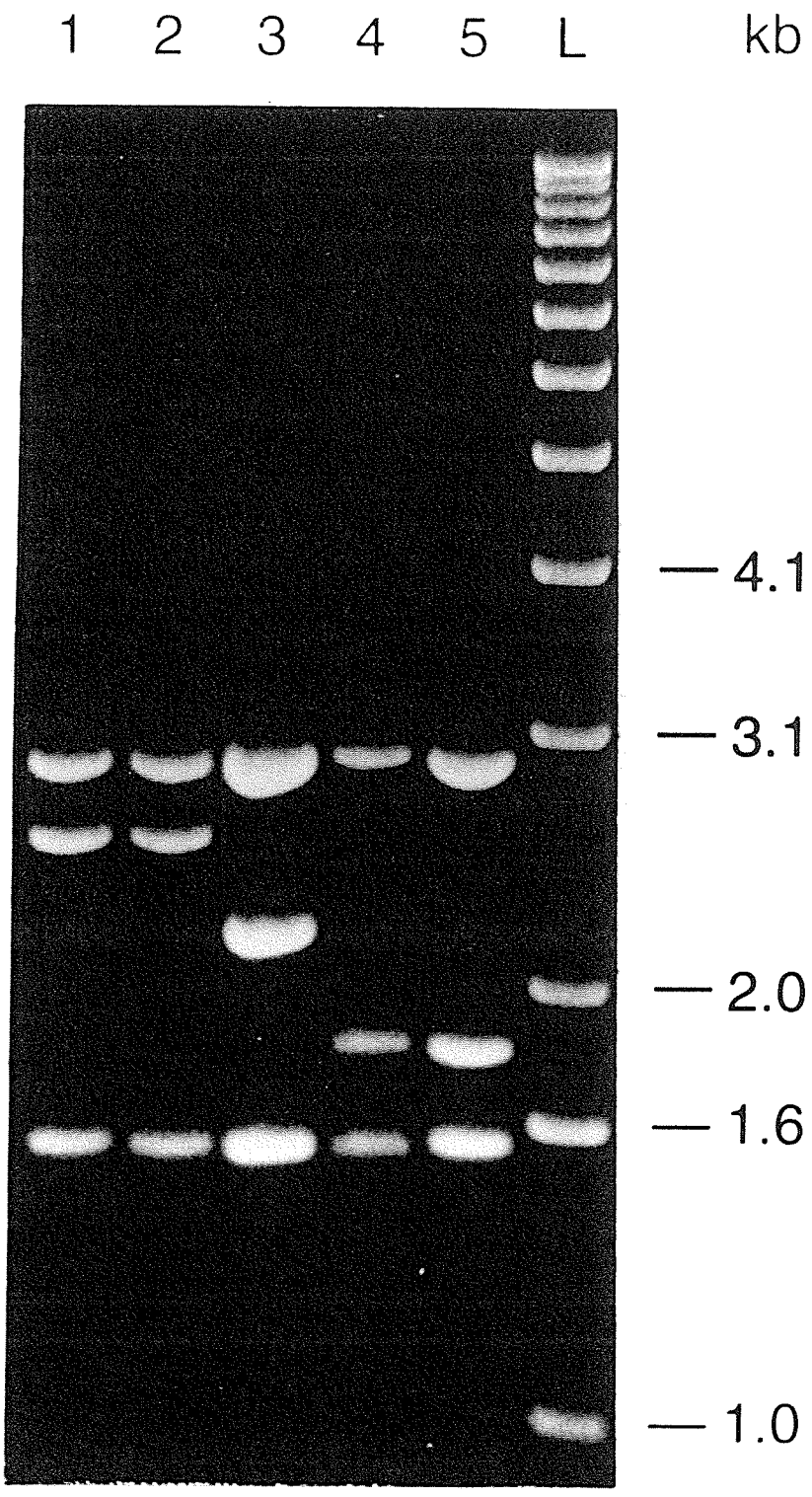

CLONES
pJB3A
pJB3
pJB2
pJB1A
pJB1
D P H
LSrRNA
SSrRNA
NTS
Q
H
P*
X
G
SUBREPEATS
1 Kb

Fig. 4.3. Restriction endonuclease analysis of the five NTS clones. Each clone was digested with *Pst*I, *Sal*I, and *Xba*I simultaneously. Lane 1, pJB3A. Lane 2, pJB3. Lane 3, pJB2. Lane 4, pJB1A. Lane 5, pJB1. Lane L, 1.0 kb ladder (BRL).


1
2
3
4
5
L
kb
4.1
3.1
2.0
1.6
1.0

form of heterogeneity is known to exist in this region, just one length variant has been cloned. It is likely that this length variant is the most abundant one in the rRNA gene family for *P. ultimum* BR471. Previous observations upon amplifying the NTS demonstrated that the relative abundance of a PCR fragment usually reflected its relative abundance in the genomic DNA (Buchko and Klassen 1990). The clones pJB3A and pJB3 (lanes 1 and 2), pJB2 (lane 2), pJB1A and pJB1 (lanes 4 and 5) each have another band which is approximately 2.6, 2.2 and 1.8 kb respectively (Fig. 4.3). These fragments represent the region from the *Pst*I site to the *Xba*I site, the region of major subrepeat heterogeneity (see Fig. 4.2). These bands differ by increments of about 0.4 kb for each length variant among the clones. The 0.4 kb size is consistent with the PCR and rDNA data (sections 2 and 3), which indicate about the same size for the subrepeat. The digestion of the clones with *Pst*I, *Xba*I and *Sal*I (Fig. 4.3), therefore agrees with the restriction map given in Fig. 4.2. Thus, it appears that three length variant (5 clones) of the ribosomal DNA spacer of *P. ultimum* BR471 has been successfully inserted into the vector Bluescript M13.

Digestion of DNA from different *P. ultimum* isolates with *Bgl*II had previously given ambiguous results which were difficult to explain. Some light was shed upon this problem when the clones pJB1 and pJB1A were restricted with *Bgl*II

(Fig. 4.4). PJB1 yields a single band (6.5 kb) including both vector and insert. PJB1A has two bands (4.7 kb and 1.8 kb), indicating the presence of a second *Bgl*II site in the NTS, 1.8 kb downstream of the first one. This may reflect true heterogeneity in BR471, but it is also possible that the additional site seen in pJB1A is a PCR artefact, due to Taq DNA polymerase error. In order to confirm that this was not a PCR artefact, the rDNA RFLP data was looked at again (Fig. 4.4, Lanes 471, 600, 511).

Total genomic DNA from isolates BR471, BR600 and BR511 was digested with *Bgl*II, followed by hybridization to pMF2 (rDNA probe). A physical map of the rDNA repeat unit for *P. ultimum* is shown in Fig. 4.5. The map predicts three major bands, I, II, and III. The autoradiogram in Fig. 4.4 shows that DNA digested with *Bgl*II from all three isolates (BR471, B600, and BR511) produce bands I (0.9 kb) and II (3.9 kb), as predicted from the map. Isolate BR471, which is the source of the clones, produces a ladder of bands above 4.5 kb representing the area of subrepeat heterogeneity. It also produces a series of bands, ranging from approximately 3.0 to 3.6 kb which corresponds to the other more complex heterogeneity present immediately downstream of the LSrRNA gene (see Figs. 2.3, 2.4 and 2.5). These bands would only appear if another *Bgl*II site is present in the spacer, downstream of the region of heterogeneity (refer to Fig. 4.5). If this other *Bgl*II site was present in all the rDNA

Fig. 4.4. Variable *Bgl*II site in *P. ultimum*. Lanes 471, 600, and 511 represents *P. ultimum* isolates (refer to Table 1; number represents culture numbers with the "BR" prefix omitted) which were digested with *Bgl*II and hybridized with pMF2. Roman numerals and the asterisk correspond to fragments predicted by the restriction map shown in fig. 4.5. Lane L, 1.0 kb ladder. Lanes pJB1 and pJB1A contain plasmid DNA of the NTS clones (with the same name) that were digested with *Bgl*II.

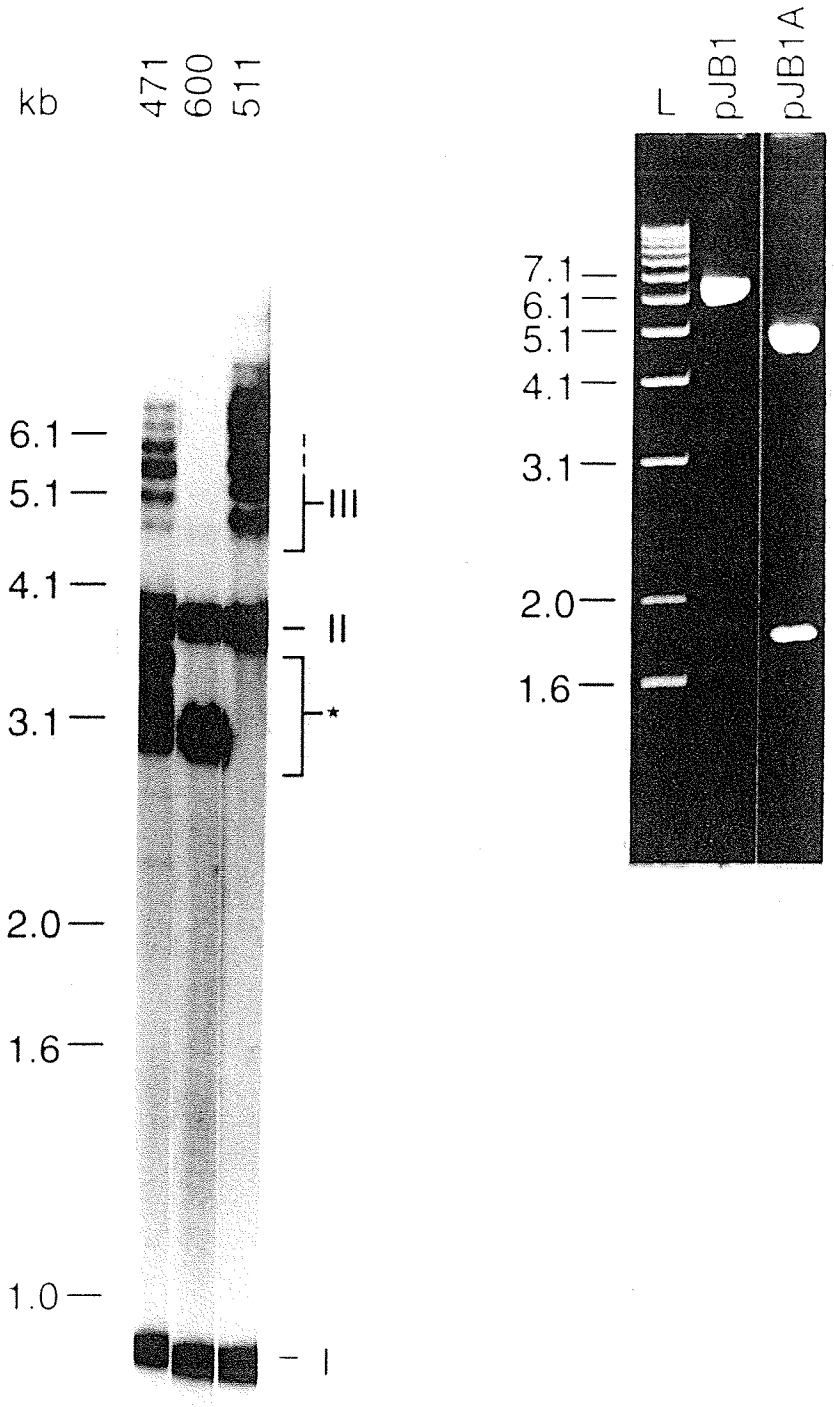
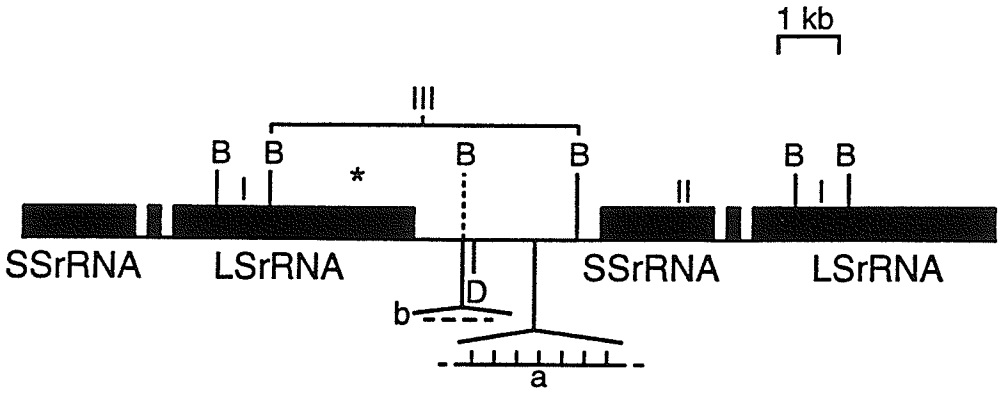

kb
471
600
511
6.1
5.1
4.1
3.1
2.0
1.6
1.0
III
II
*
I
L
pJB1
pJB1A
7.1
6.1
5.1
4.1
3.1
2.0
1.6

Fig. 4.5. Heterogeneous *Bgl*II sites in *P. ultimum*. A restriction map of the rDNA repeat unit for *P. ultimum* is shown. Restriction sites are represented by the letters: B-*Bgl*II, D-*Hin*dIII. Roman numeral and asterisk refer to fragments labelled in fig. 4.4. The vertical dashed line indicates the position of a variable *Bgl*II site. The asterisk represent the fragment generated by the additional *Bgl*II site.


1 kb
III
B
B
*
B
B
II
B
B
SSrRNA
LSrRNA
SSrRNA
LSrRNA
D
b
a

repeat units then the upper ladder of bands should disappear, because it would no longer be able to hybridize to the rDNA probe (pMF2). Since both regions of heterogeneity are appearing, this can only be explained if there is a *Bgl*II site upstream of the *Hind*III site in a certain number of rDNA repeats (Fig. 4.5)(the exact location of the other *Bgl*II site has been confirmed by the sequence data given in Fig. 4.6). At least two other kinds of NTS organization seem to exist in *P. ultimum*. In BR600 the ladder (Band III, Figs. 4.4 and 4.5), due to subrepeat heterogeneity, is absent. Three bands centred around the 3.1 kb marker are present, representing the region of length heterogeneity just downstream of the LSrRNA gene (not the 380 bp subrepeat heterogeneity). This can only be explained if every spacer within the rRNA gene family had an additional *Bgl*II site present in the NTS. Thus, for BR600, no *Bgl*II heterogeneity appears. Yet another kind of NTS organization is observed in isolate BR511. When DNA from BR511 is digested with *Bgl*II it lacks the bands at around 3.1 kb, which the other two isolates possess (Fig. 4.4). This means that it has only one *Bgl*II site in the NTS in all of its rDNA repeats, and that if it has the two regions of heterogeneity seen in BR471, both contribute to the ladder of bands above 4.5 kb. Thus, for BR600, the upstream *Bgl*II site is always present in the NTS, for BR511 it is never present, and for BR471 it may of may not be present.

The observed heterogeneity of the *Bgl*II site in the NTS of *P. ultimum* BR471 agrees with the difference seen in the spacer clones, pJB1 and pJB1A (Fig. 4.4). Thus, it strongly suggests that the additional *Bgl*II site in pJB1A is not due to PCR error. The differences between the three isolates of *P. ultimum* may reflect a high rate of evolution in the NTS. It is known that the NTS shows a high degree of variability between species, even closely related species (Rogers and Bendich 1987, Tautz *et al*. 1987). Thus, it is a region which appears to be evolving at a fast rate.

After it was confirmed that the clones contained the intergenic region of the rDNA repeat unit, the next step was to devise a strategy for sequencing the smallest length variant clone, pJB1A. Two subclones were constructed, pJB1ABH and pJB1APS (refer to Materials and Methods section). The subclone pJB1APS represents the region from the *Pst*I site up to the Q primer site (see Fig. 4.2). Two sets of deletion clones (in opposing directions) for pJB1APS were made using "Erase A Base" (Promega Corp., Madison, Wis.). The subclone pJB1ABH was sequenced by synthesizing new primers as needed and "walking" along the insert until it was completely sequenced. Both single and double stranded sequencing methods were employed. After the sequencing was complete for pJB1A, ASSEMGEL (PCGENE; IntelliGenetics Inc., Mountain View, Cal.) was used to link up all the sequences. A more detailed account of the

sequencing strategy employed is given in the Materials and Methods section.

The sequence, which includes the region between the Q primer annealing site and the *Xba*I restriction site is given for pJB1A in Fig. 4.6. The nucleotide sequence presented includes all of the NTS and ETS, the 3′ end of the LSrRNA gene, and the 5′ end of the SSrRNA. The 3′ end of the LSrRNA gene (nt. pos. 250, Fig 4.6) was inferred by alignment with the sequences given for *Phytophthora megasperma* (Van der Auwera 1994) and *Saccharomyces cerevisiae* (Gutell and Fox 1988)(see Fig. 4.7). As expected, it was much easier to align the LSrDNA portion of pJB1A with the LSrDNA sequence of the oomycete *Phytophthora megasperma*, than with *S. cerevisiae*. A question mark placed in the alignment of Fig. 4.7A indicates another potential 3′ end. The 3′ end of the LSrRNA gene in *P. megasperma* was determined solely by comparison with other LSrDNA sequences. In contrast, the 3′ end of *S. cerevisiae* was determined experimentally. That is why a question mark is placed over the C residue (nt. pos. 282, see Figs. 4.6 and 4.7), representing an alternative 3′ end. The 5′ end of the SSrRNA (nt. pos. 3244) gene was determined by alignment with the sequence given for *Achlya bisexualis* (Dams *et al.* 1988)(Fig. 4.7). The location of key restriction sites such as *Pst*I, *Hind*III, *Hinc*II, and *Xba*I are shown in Fig. 4.6. The restriction sites are found in the positions as

Fig. 4.6. Complete nucleotide sequence of the NTS for *P. ultimum BR471 (clone pJB1A)*. All primer annealing sites are indicated below the sequence, arrows giving the orientation of the primer sites. Two primer names and bidirectional arrows indicate two primers which are complementary to each other. Dashed lines and arrows in boldface below the sequence indicate A and B repeat arrays (positions 448-1186). The possible 3′ end of the LSrRNA gene is indicated by a vertical line below the sequence at position 280 (aligned with *S. cerevisiae)*; the ? indicate another possible termination site at position 282 (aligned with *Phytophthora megasperma*). The positions of the following restrictions sites are given: *Bgl*II, *Hin*dIII, *Pst*I, *Hinc*II, *Sac*I, and *Xba*I. The sequence in boldface is repeated twice (not perfectly) in pJB2. The transcription initiation site (TIS) (position 2319) and the beginning of the SSrRNA gene is shown (position 3244).

```
1    CCTCTAAGTC AGAATCCATG CTGGAATAGA CGATAATCAC CTTTCCTGAT
     |-------Q------>|

51   GTACCGCGAA TAGCGATAGA TGTCTTTTGG GCATCCAACA TCATAAAATT

101  GCAACGCACT CGCATTGCCT GACAAATGTT GGTAGTGGAG AGTATGCTGG

151  ATTGTAATTT CAAATATTGG GAAAGATAAA TCCTTTGTAG ACGACTTAAA

201  TACAGAACGG GGTGTTGTAA GCACGAGAGT AGTCTTGTAC TACGATCTGC
                               |-------Q1.5------>|

251  TGAGATTTAG CCCTTGTTCT ATTGATTTGT TCATTTCTGA ACATATCTCC
                     <--LSrRNA---|--?-NTS--->

301  CCCCCTACCA TATACGTCTA CTCCCCCCTA TATGAGGATT TCTATACTAT

351  CATACTATCA TATACTAGCC CGCACCGCCT AGGCGCCTGC TGGCAAAAGG
                    |-------Q2------->|

401  TTCCTTACCT TCGCGCGCGT GTCGAGTCCA TTTTTATTTT AGCGCTGGTA
                                                        |--

451  AATACGGCAA GTGTTTTGTT GTATTGGGCT TTCGGTTCCT TTTCAGTTTG
     ----------------------A1 repeat-------------------------
```

```
501  CTGAAAAAGC ACCAAGTCAT TTTGCTGTTA TAGTTGGCTC GGCCTTTGCG
     ->|-----------------A2 repeat-------------------------

551  TTCCTTTTCG GCAACGAAAA AGCACCAAGT CATTTCGCTA TTATAACTGG
     --------------->|---A3 repeat-------------------------

601  CTCGGCTTTC GGTTCCTTTT CGGCAACGAA AAAGCACCAA GTCATTTCGC
     ---------------------------->|----------------------

651  TATTATAACT GGCTCGGCTT TCGGCTCTTT CAGTTTGCTG AAAATGCGCC
     --------------------A4 repeat------------>|-----------

701  AAGTCATTTT TCCTGTATTA ACAGTATGGG AATCGGCGAG CATTTGCCTG
     -------B1 repeat-------------->|-------B2 repeat------

751  TATTAGCAGT ATGGGAAAAT CTCCGAGTCA TTTCTCTATA TTAACAGTAT
     -------------->|---------------B3 repeat--------------

801  GGGAAATCCT TCGACCATTC TCCTATATTA ACAGTATGGG AAAATCTGCC
     ->|------------------B4 repeat----------->|-----------

851  GAGCATTTCT CTATATTAAC AGTATGAGAA AATCTCCGAG TCATTTCTCT
     ----------B5 repeat--------->|------B6 repeat---------

901  ATATTAACAG TATGGGAAAA TCTGCCGAGC ATTTGCCTAT ATTAACAGTA
     --------------->|--------------B7 repeat--------------

951  TAGGAAAACC ATCCAACCAA TTTCTCTATA TTAACAGTAT CGGAAAATCA
     -->|------------------B8 repeat-------------->|-------
```

1001 CTGACCAATT TCTCTATATT AACAGTATCG GAAAATCACT GACCAATTTC
---------**B9 repeat**-------------> |-----**B10 repeat**-----

1051 TCTATATTAA CAGTATCGGA AAACCAGCCA ACCAATTTCT CTATATTAAC
------------------>|-----------**B11 repeat**------------

|*Bgl*II
1101 ACTATGGAGA AACCATCCAA CCAAGATCTC TATATTAACT GTATAGGAAA
-------->|---------------**B12 repeat**--------------->|---

1151 ATCTCACAAG TCGTTCTTCT ATATTAACAC GATCGAGAAA GCTGGCTATT
---------------**B13 repeat**------------>

1201 GTAGCACTAT AAAGAAAAAA TGAAAAAGTG ACGCGAATAT GCACACGTTC

|*Hind*III
1251 CAGCATGTCC ATATACATGC ACACATGGAA GCTTTCCACC CAAATATGCA

1301 AAACCAATAC GCACACGCAC ATGCAGCGTG CTAGTGACGC ATATCCATTT
|<----PS1/PS1c---->|

1351 CCATGTGCAT GCGACTAATA TGCGTCGCAT AGTACAATAC AGTACAGTAC

1401 ATACAGCGCA AATGCAAAAT GCCAAATGCA GACCGCGAGA AGCACGACAA

1451 GTACCTCTCA TTTATTATCT CCCCCACAAT GTCAAATAAC AAATGCTACC

1501 ATTTTTGACG GGGACCCCTT TATTGTAATA CTCGGGACGC AGGCTACTGC

|*PstI*
1551 AGCTATAACA ACAAACAGAT CCTGCATCAA TTGTTGTATA TATGTTCACA

1601 GTGTATTTAT TTTCACAATC CGCAAACCTC TCTATTATTA TTCCCTATTA

|*HincII*
1651 TTTACGCTAC CAACTGTCAA CAGATATGAC AGGTACCTCT TTAATTCTAT
|<-----XP1/XP1c---->|

|*SacI*
1701 TACATGGGGA CAAAAACCTT CTTGAATCTG TATACCGAGC TCAAGACAAA

1751 AAACGTTTGC GGATGTGCAC CAGCGAGTAC **AATGGCAATG CGAGTCCCGA**
|------XP1.2c------>|

1801 **GATTTGAGTG GTATAGGGGT TATTCATCAC GGGGAACACG CTCGTGCACT**

1851 **CGCATCAAGT CTCCTGTGAA CGCATCATGG TCAAACACGC GTTTTCGTGC**
|---------XP1.1c------>

1901 **TAAAATGGCC ATATTTGCGT TTGTGGAGGT TTTTGCAATT TGACGACATC**
|<--XP2/XP2c-----

1951 **TGGACGCACA CTAGCCCACC AGATGTTTTT GCAATCGTAT TGTCCTACTT**
->|

2001 **GCGTAGATAT GTTTAGACCC CAGCAGCGTG TCTACATGTT TTCT**ACAGCT

```
2051 CGAGATATGT CCATTTGTGC TGAAAAATGT GCACTTTTGT GTCGTTTTGT
      |<--------P7.1--------|

2101 TGCTATTTCT TTCATGTGTT TGCGTAAATT ACGTATATAT TACGTGTGAA
                                          |<----P7/P7c---->|

2151 TATTGTGTTA TAGAGGTTCC CTTTATATGT GATTATAATT TCCCTGTGTA

2201 TATGTCGAGG ATATGTCTGT AATAGAGGTT TTTGCAGCAA ATGGTGCCTA

2251 TGTTTTTTTG TTTGGTTTGG AATTTTGTGA ATATGGCGTA TTTGTCGTCG

                           |TIS------>
2301 TTCGGATTCC CCATATATGT GGAGTGTGTA AGCATGGTTG CGAGGAAGTG

2351 AAAAATGGAA ATGATAAAGC CGTCGTGAAA AGTGGCCTAA ATGAGTAATT

2401 TTTGAACGAG TCGGTACTAA TGTAATAAGC ATTTCGCATT GAACGTTTTG
       |<------P6/P6c----->|

2451 TTCAGGCAAA TATGGCATTT TGACTGTGTT TTCGCAGGGA TGATTCTGTC

2501 ATTTCTAAAG CATGGCAAAA GGCAAAAGTT AGTGTGCAGT GTTGTTTTGG

2551 AGAATCTGAA ACGTGCTGCA TGCGCCCTGT TTATTGTGAT TGTGTTGTTT
         |<------P5.5-------|
```

2601 TGCGTTGCAT AACGATACAG TATCAATGAC GCAAAATGGT GTTGCGCTGG

2651 GTATGTTTGC TCCTCTTTAT CCGAGACTGT GGTTGTGCGT GTGGCGTTGT
|<

2701 CATGCGTAAT GCGAAACACT TGGGTTTGTG AGTGCAGCGG CTTGCTACAA
-----P5/P5c----->|

2751 CAACACTCCT GGAGTTTAGG GATAACTTGG TTGTTGGCAT AATGTGTGGG

2801 CGATGATTGG CGTTGTCAGT CGATGTCTGT GCTGTCTTGC TGGCGGTCGG

|*Bgl*II
2851 GTTGCGCCTT GGATTTCTCG GGAAACTATT AATTCAATGA TGGTAGATCT

2901 GAATTGAATT GTGTGGTATT GCTGCGTGCA GAGGGCAACT TCTGTGTGTG
|<------P4/P4c----->|

2951 GCGGTGCTAT TTTTGATATG TGTTTTCTTT TCTGTTTGTT TCTGATTTTC

3001 TCTTGATGTA AAAGTCTAGT GAAAGGAAGG AATAAATAGG GAGAGAACAA

3051 GTTTCAATCT AGTTTTTGTC TTTCGATAAG AGAGTACGAA AGCGACTGTG

3101 CGAATGATAG CCAAAGCGTG CTGTATGTGT GTGTGTGTGT GGTTGTTCTG
|<-----P3/P3c---->|

```
3151 AGTTTTTCAG TTCTGACTGT ATGCATGCAT TTGGCGTGCG GGTGTTTGAA

                                                        |SSrRNA->
3201 TTTGTTGGGT CGTTTTTTGT GTATTTTTTG TATTGAAGAT ATTAACCTGG

3251 TTGATCCTGC CAGTAGTCAT ACGCTTGTCT CAAAGATTAA GCCATGCATG
                                                  |<----P2--

3301 TCTAAGTATA AACAATTTTG TACTGTGAAA CTGCGAACGG CTCATTATAT
     --------|

3351 CAGTTATAGT CTACTCGATA GTACCTTACT ACTTGGATAA CCGTAGTAAT

      |XbaI
3401 TCTAGA
```

Fig. 4.7. Alignment of the 3′ end of the LSrDNA and 5′ end of the SSrDNA with other species. Asterisks indicate nucleotide positions which are conserved. A. Alignment of *P. ultimum* BR471 nucleotide sequence of pJB1A (PULS) with 3′ end sequence of LSrRNA genes of *P. megasperma* (PMLS) (Van der Auwera *et al. 1994*) and *S. cerevisiae* (SCLS) (Dams *et al. 1988*). The arrow and the question mark symbol indicate the putative ends of the large subunit rRNA gene in *P. ultimum*. B. Alignment of the *P. ultimum* BR471 nucleotide sequence of pJB1A (PUSS) with the 5′ end of the SSrRNA gene of *Achlya bisexualis* (ABSS) (Gutell *et al.* 1988). The arrow indicates the putative start site of the small subunit rRNA gene in *P. ultimum*.

A. LSrDNA alignment


```
PULS  T--ACAGAACGGGGTGTTGTAAGCACGAGAGTAGTCTTGT-ACTACGATCT
PMLS  T--ACAGAACGGGGTGTTGTAAGCATGAGAGTAGTCTTGT-ACTACGATCT
SCLS  TGTAC--AACGGGGTATTGTAAGCGGTAGAGTAGCCTTGTTGTTACGATCT
      *  **  ******** ********   ******* *****   ********

                              <-LSrRNA-| ?
PULS  GCTGAGATTTAGCCCTTGTTCTATTGATTTGTTCATTTCTGAACATATCTCC
PMLS  GCTGAGATTTAGCCCTTGTTCTATTGATTTGTTC
SCLS  GCTGAGATTAAGCCTTTGTTGT-CTGATTTGT
      ********* **** ***** *  ********
```

B. SSrDNA alignment

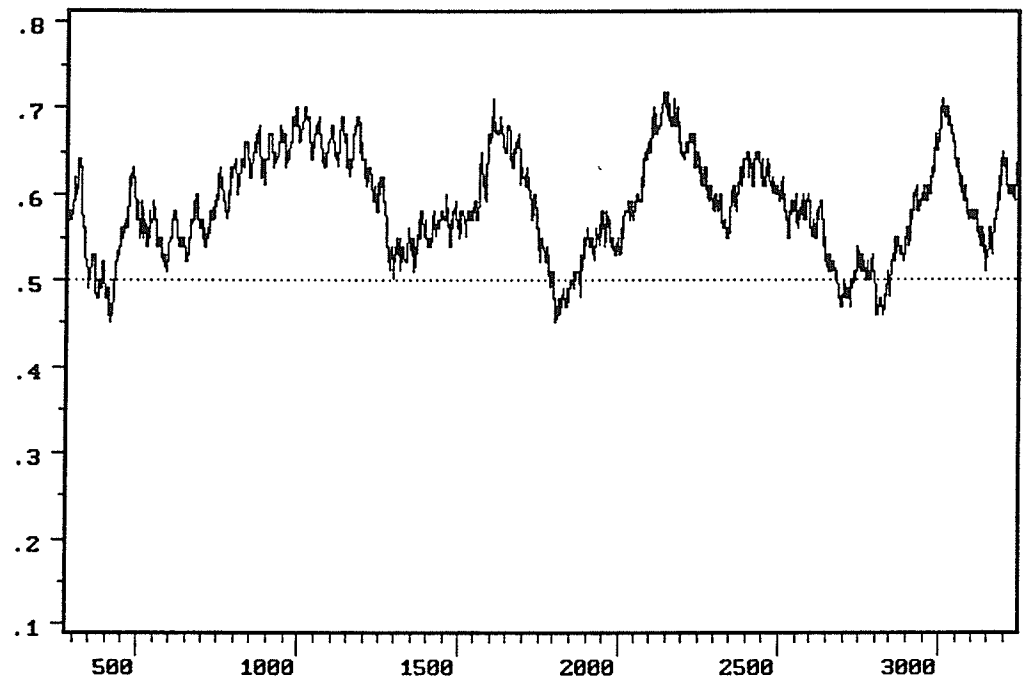
```
                                 |----SSrRNA---->
ABSS                             AACCTGGTTGATCCTGCCAGTAGTCATAC
PUSS       ATTTTTTGTATTGAAGATATTAACCTGGTTGATCCTGCCAGTAGTCATAC
                                 ******************************

ABSS       GCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAACAATTTTGTA
PUSS       GCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAACAATTTTGTA
           **************************************************

ABSS        CTGTGAAACTGCGAATGGCTC
PUSS        CTGTGAAACTGCGAACGGCTC
            *************** *****
```

predicted from the physical maps displayed in Fig. 2.5 and 4.2. Thus, the sequence data agree with the restriction map data.

An analysis of the sequence of pJB1A reveals that the rDNA spacer of *P. ultimum* is rich in A and T residues (see Figs. 4.6 and 4.8). Of the 2963 bases representing the intergenic region, the number of G, C, A and T residues are 739, 669, 915 and 1108, respectively. Thus, the spacer is relatively A and T rich, whereby the two residue make up approximately 59% of the spacer region. The A+T residues are not distributed evenly throughout the IGR. Using the program PBASE (PCGENE), a plot of AT/ATGC shown in Fig. 4.8 reveals that the proportion of A+T residues in the IGR is not uniform. In comparison, the rDNA IGR of *Arabidopsis thaliana* has a proportion that is uniform along the IGR at about 50% G+C and A+T residues (Gruendler *et al.* 1991).

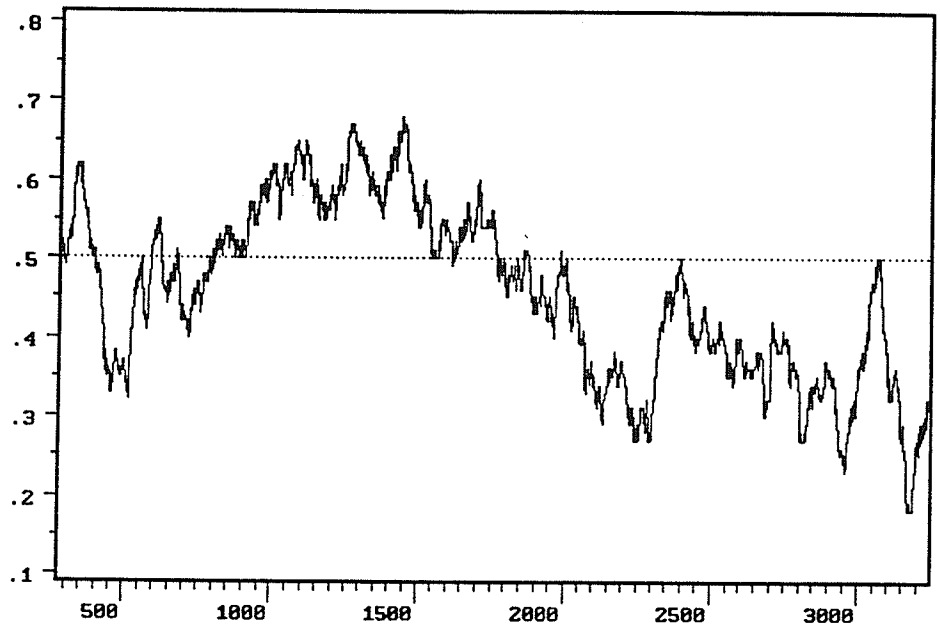
An unusual pattern of base composition is observed when the proportion of A+C to ACTG residues is plotted for the rDNA spacer (Fig. 4.9). The 5′ end of the spacer is predominantly A+C nucleotides on one strand and G+T residues on the other DNA strand. The opposite situation occurs at the 3′ end of the spacer where it is mostly made up of G+T residues. This asymmetric base composition is similar to what was observed for the 5S rRNA IGR of *P. ultimum* and *P. spinosum* (Belkhiri 1994). Similarly, Belkhiri observed that the 5′ end of the spacer was predominantly A+C, while the 3′

Fig. 4.8. Base composition of NTS of *P. ultimum*. Plot of AT/ATGC bases was computed using an interval of 100 bases (PBASE program, PCGENE, Intelligenetics, Inc.). Base position and frequency are indicated at the X and Y axes respectively.



Plot of AT/ATGC in sequence PJB1A.
From base 281 to base 3243 computed using an interval of 100 bases.

Fig. 4.9. Base composition asymmetry in the NTS of *P. ultimum*. Plot of the AC/ATGC bases was computed using an interval of 100 bases (PBASE program, PCGENE, Intellegenetics, Inc.). Base position and frequency are indicated at the X and Y axes respectively.



Plot of AC/AIGC in sequence PJB1A.
From base 281 to base 3243 computed using an interval of 100 bases.

end was largely G+T. Like the 5S rRNA genes, the rRNA genes (LSrRNA, SSrRNA and 5.8S) are also arranged in tandem arrays in *P. ultimum*. The strand asymmetry may be a function of the way the array have been assembled and this characteristic may be unique to *Pythiums*. More rRNA spacers and other spacer of tandemly arranged genes from other *Pythium* species will have to be investigated to test this hypothesis.

There is also some evidence for the presence of simple sequence just upstream of the beginning of the SSrRNA gene. A stretch of eight tandemly organized TG repeats are present from positions 3126 to 3141, and there are several TG dinucleotides found immediately flanking the array (refer to Fig. 4.6). Belkhiri (1994) reported the presence of 22 tandemly organized TG repeats in the IGR of the 5S rRNA genes of *P. spinosum*. He also observed a region rich in TG dinucleotides in the same region for *P. ultimum*, but it was not repeated in as regular a manner as in *P. spinosum*.

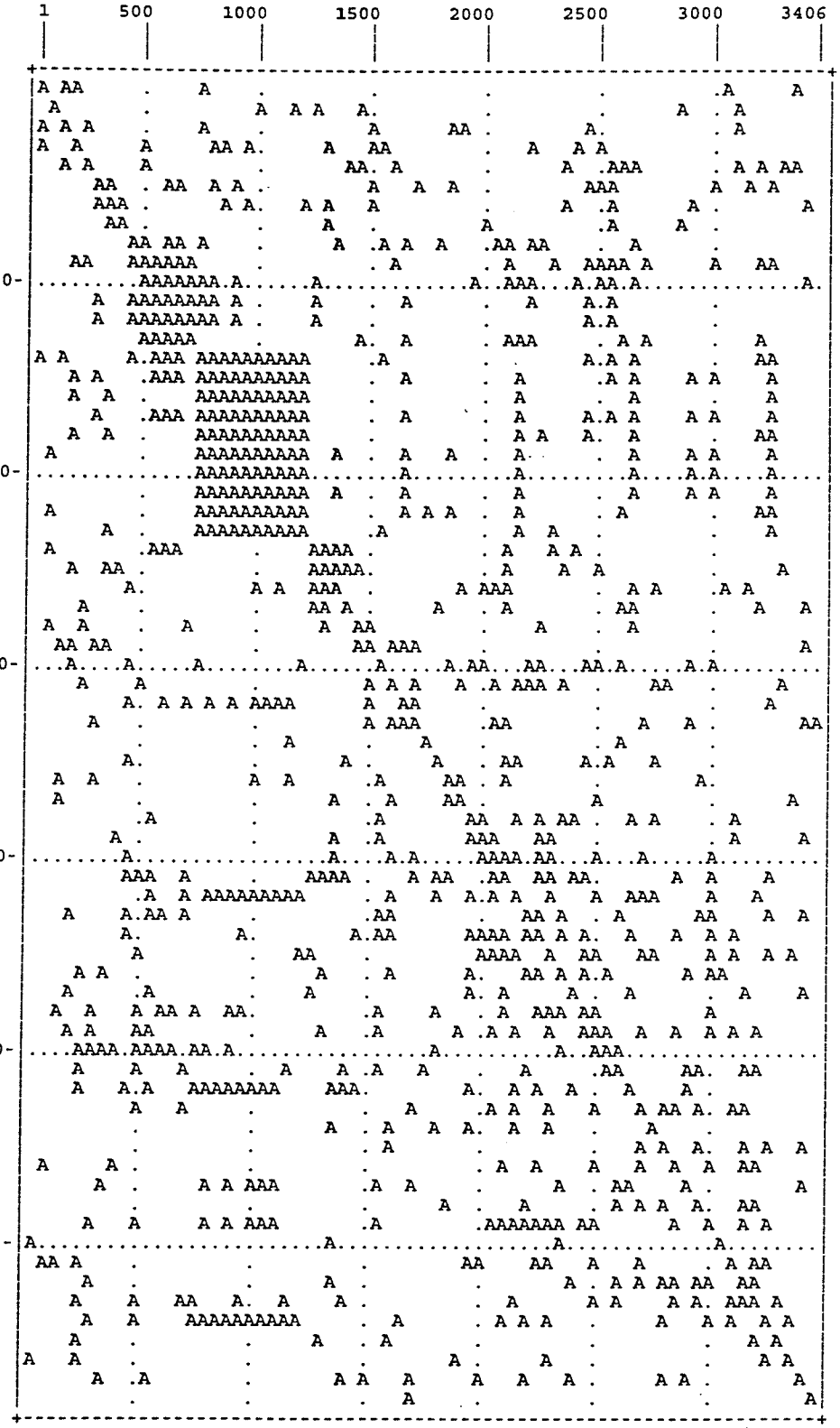
The TG dinucleotide repeat has been found associated with an intron splicing site for the human cardiac muscle actin gene, containing considerably more TG repeats (25) than found in the rDNA IGR of *P. ultimum* (Hamada and Kakunaga 1982). As well, the TG dinucleotide has been implicated in the formation of Z-DNA (Hamada and Kakunaga, 1982, Hamada *et al.* 1982). Since the simple sequence of TG dinucleotides has the potential to form Z-DNA, such a

conformational change may aid it in being recognized by specific protein factors. In the same manner, the TG dinucleotide repeat unit observed in the rDNA IGR of *P. ultimum* may have a functional purpose, although it also possible that it represents the random evolution of simple sequence.

P. ultimum has length heterogeneity in the NTS of the rDNA repeat unit, much like that observed in plants and animals. The cause of this length heterogeneity, in plants and animals, is known to be due to varying numbers of repetitive elements in the spacer (Reeder 1984). NMATPUS (in PCGENE) was used to compare the sequence of pJB1A with itself, generating a nucleic acid matrix comparison using Pustell's method (Pustell and Kafatos 1982, Pustell and Kafatos 1984). If tandemly arranged repeats are present in the sequence being compared with itself, then a block or a series of diagonal letters will be generated. Each letter is representative of the percent similarity between the sequences being compared, within a chosen window size.

The results of the sequence self-comparison of pJB1A is given in Fig. 4.10. The analysis reveals two regions which appear to contain repetitive elements, one between nucleotide positions 450-700 and the other between 700-1200. These two blocks of repeats are found in the region of complex heterogeneity which exists immediately downstream of the LSrRNA gene (refer to Section 2). Notably, the

Fig. 4.10. Identification of two blocks of repetitive arrays. NMATPUS (PCGENE, IntelliGenetics Inc.) was used to compare the sequence of pJB1A with itself. A range of 3 bases and scale of one was used. X and Y axis compression factor is 50X. The letter A indicates 100% similarity.


1
500
1000
1500
2000
2500
3000
3406
500-
1000-
1500-
2000-
2500-
3000-

comparison matrix did not identify any repeats in the area of the spacer which is known to possess the 380 bp subrepeat (Klassen and Buchko 1990). The failure to identify the subrepeat in the clone means that pJB1A represents a spacer with only one subrepeat, or no subrepeat at all. If one subrepeat is present, then it is not likely to be a promoter duplication like that observed in *Drosophila* (Coen and Dover 1982, Simeone *et al.* 1985) since there are no duplications of any sequence upstream of the SSrRNA gene where the promoter is likely to be situated.

The first block of repeats between nucleotide positions 450-700 contains 4 direct repeats, one truncated version followed by three longer ones. An alignment of the 4 repeats, called "A repeats", is shown in Fig. 4.11 and precise positioning in the sequence is given in Fig. 4.6. The length of the first repeat, A1, is 55 bp, while the remaining repeats, A2, A3, and A4, are 63, 62, and 62 bp respectively. None of the repeats are perfect copies of each other.

Another family of repeats was identified by the sequence comparison matrix between nucleotide positions 700-1200. A closer analysis of that region reveals the presence of 13 repeats of about 40 bp. The repeats in this region are referred to as the "B repeats", and an alignment of the B repeat family is given in Fig. 4.12. The repeat sequences were compared to a consensus sequence, which consisted of

Fig. 4.11. Alignment of the A repeats. Number of nucleotides (Nts.) and their nucleotide position (Nt. Pos.)(refer to Fig. 4.6) are given. The number of mismatches with a consensus sequence is shown. Dots represent identities with the consensus sequence.

		Nts.	Nt. Pos.	mismatches
A1 REPEAT	.T...TA.GG....T-G...T.---..G..-T...G.-----.....A.TTT...	55	502	25
A2 REPEAT	.....T...G.....GT.....C...GC.....-.-	63	565	9
A3 REPEAT	.....-.-	62	622	2
A4 REPEAT	.....C..--....A.TTT...	62	689	7
consensus	GAAAAAGCACCAAGTCATTTCGCTATTATAACTGG-CTCGGC-TTTCGGTTCCTTTTCGGCAAGCT			

Fig. 4.12. Alignment of the B repeats. Number of nucleotides (Nts.) and their nucleotide position (Nt. Pos.)(refer to Fig. 4.6) are given. The number of mismatches with a consensus sequence is given. The asterisk represents a nucleotide which is conserved in all the repeats, while the vertical arrowhead indicates that the position is conserved in all but one or two positions. Dots indicate identities with the consensus sequence. The underlined sequence in boldface below the alignment represents a well-conserved core sequence. [a]The GAAA motif overlaps with the beginning each B repeat.

		Nts.	Nt. Pos	mismatches
consensus	GAAAATCTGCCGAGCAATTTCTC-TATATTAACAGTATGG-			
B1 REPEAT	......GC...A..TC....T..C.G.....-	40	729	8
B2 REPEAT	...--..G.-.....-....G-.C.G.....G.....-	35	764	10
B3 REPEAT	.....-.....TC.....-.....-	38	802	3
B4 REPEAT	....TC..T-...C.-...-...C.....-	37	839	8
B5 REPEAT	.....-.....-.....A-	38	877	1
B6 REPEAT	.....-.....TC.....-.....-	38	915	3
B7 REPEAT	.....-....GC.-.....A.-	38	953	3
B8 REPEAT	.....C.AT..A.C.....-.....C.-	39	992	4
B9 REPEAT	.....A-.T..C.....-.....C.-	38	1030	5
B10 REPEAT	.....A-.T..C.....-.....C.-	38	1068	5
B11 REPEAT	.....C.A...A.C.....-.....C.....A	40	1108	6
B12 REPEAT	....-C.AT..A.C...GA....-.....T....A.-	38	1146	9
B13 REPEAT	.....CA.A..TCG..CT..-.....CG..C.A	40	1186	12
	^ ^ * ^^^^ ^* *^***^*^^^** ^^			

ATTT-TC-TATATTAACAGTAT-G-[GAAA][a]

the most frequent base at each position of the alignment. The number of mismatches with the consensus sequence is greatest in the flanking repeats (B1, B2, B12, and B13), and the core repeats (B3-B11) seemed to be more homogeneous in nucleotide content. Two pairs of repeats, B3 and B6, and B9 and B10, have identical nucleotide sequences. This observation is similar to what is seen in the rDNA spacer of wheat (Barker *et al.* 1988) in which the flanking repeats in a repeat family are more diverged from the consensus than are the central repeats.

The sequence motif ATTT-TC-TATATTAACAGTAT-GGAAA (the "GAAA" motif is actually part of the 5′ end of the next tandemly positioned repeat element) is highly conserved in all of the repeats. Thirteen of the 25 residues are found in all of the repeats, the remainder are found in all but one or two of the B repeats (refer to Fig. 4.12). The conservation of the 25 bp sequence motif suggests that it may have functional importance. These repeats may be enhancers, much like the ones that have been characterized in *Xenopus*, mouse, *Drosophila* and wheat (Reeder 1984, Sollner-Webb and Moughey 1991, Pikaard *et al.* 1990, Grimaldi and Di Nocera 1988, Jackson and Flavell 1992) where repetitive elements in the NTS have be linked with enhancement of transcription. The same may apply for the A repeats. Alternatively though, the possibility exists that these repetitive motifs may not have any function

whatsoever, but may simply represent a random amplification of a sequence motif in the NTS.

There is a tendency for tandemly organized sequences to become homogenized over time, a process referred to as concerted evolution (Dover 1982). The 5′ ends of the B repeats seem to be more variable than the 3′ ends. There are considerable differences in sequence between the B repeats, although two pairs of repeats are identical. There does not seem to be as much homogenization of sequence as is observed in the family A repeats in wheat (Barker *et al.* 1988). It is believed that mechanisms such as unequal crossing over, and to a lesser extent gene conversion, are responsible for homogenizing tandem arrays. Recurrent unequal exchange (and possibly gene conversion) may lead to the stochastic fixation of one or another variant member throughout the array. In the case of *P. ultimum* BR471, there appears to be a high degree of flux in sequence within the B repeat array. It is therefore possible that the B repeats may still be in the process of homogenization, and that over time one variant member in the array will become predominant.

Comparison of the consensus sequence of the A repeats with that of the B repeats reveals a possible relationship between the two repeat families (Fig. 4.13). When the B repeat consensus is aligned with the 40 bp at the 5′ end of the A repeat consensus, mismatches occur at 14 positions.

Fig. 4.13. Identification of conserved motifs found in both A and B repeats. An alignment of the A with the B repeats is given, which includes the consensus sequence for each repeat family. The boxed areas represents regions of significant homology. The nucleotides in boldface are identical with the motif sequences given at the top of the alignment. The number of matches of each repeat with the motifs are shown (maximum = 27).

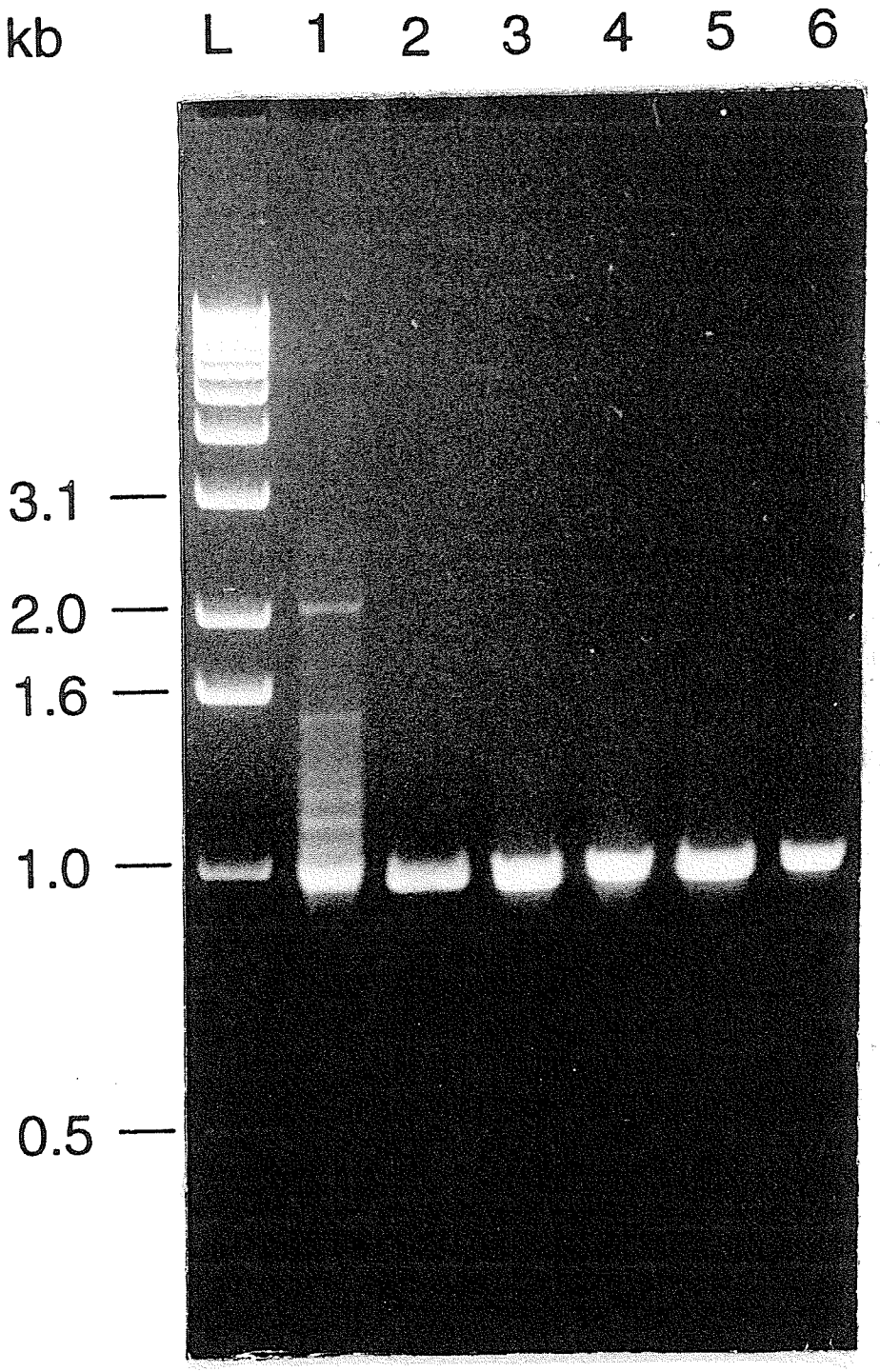
motifs	GAAAA		CCAAGTCATTTC		TATT		AAC		TGG		matches
A1 REPEAT	**GTAAA**	TACG	**GCAAGT**-GTTTT	G-	--TT		**A**-T		**TGG**	GC------TTTCGGTTCCTTTTCAGTTTGCT	18
A2 REPEAT	**GAAAA**	AGCA	**CCAAGTCATTTT**	GC	**TGTT**	AT	**AGT**		**TGG**	-CTCGGCCTTTGCGTTCCTTTTCGGCAA-C-	23
A3 REPEAT	**GAAAA**	AGCA	**CCAAGTCATTTC**	GC	**TATT**	AT	**AAC**		**TGG**	-CTCGGC-TTTCGGTTCCTTTTCGGCAA-C-	27
A4 REPEAT	**GAAAA**	AGCA	**CCAAGTCATTTC**	GC	**TATT**	AT	**AAC**		**TGG**	-CTCGGC-TTTCGGCTC--TTTCAGTTTGCT	27
A consensus	**GAAAA**	AGCA	**CCAAGTCATTTC**	GC	**TATT**	AT	**AAC**		**TGG**	-CTCGGC-TTTCGGTTCCTTTTCGGCAAGCT	27
B consensus	**GAAAA**	TCTG	**CCGAGCAATTTC**	TC-TA	**TATT**		**AAC**	AGTA	**TGG**	-	24
B1 REPEAT	**GAAAA**	TGCG	**CCAAGTCATTTT**	TCCTG	**TATT**		**AAC**	AGTA	**TGG**	-	26
B2 REPEAT	**GAA--**	TCGG	**-CGAGC-ATTTG**	-CCTG	**TATT**		**AGC**	AGTA	**TGG**	-	19
B3 REPEAT	**GAAAA**	TCT-	**CCGAGTCATTTC**	TC-TA	**TATT**		**AAC**	AGTA	**TGG**	-	26
B4 REPEAT	**GAAAT**	CCTT	**-CGACC-ATT-C**	TCCTA	**TATT**		**AAC**	AGTA	**TGG**	-	20
B5 REPEAT	**GAAAA**	TCTG	**CCGAGC-ATTTC**	TC-TA	**TATT**		**AAC**	AGTA	**TGA**	-	23
B6 REPEAT	**GAAAA**	TCT-	**CCGAGTCATTTC**	TC-TA	**TATT**		**AAC**	AGTA	**TGG**	-	26
B7 REPEAT	**GAAAA**	TCTG	**CCGAGC-ATTTG**	CC-TA	**TATT**		**AAC**	AGTA	**TAG**	-	23
B8 REPEAT	**GAAAA**	CCAT	**CCAACCAATTTC**	TC-TA	**TATT**		**AAC**	AGTA	**TCG**	-	23
B9 REPEAT	**GAAAA**	TCA-	**CTGACCAATTTC**	TC-TA	**TATT**		**AAC**	AGTA	**TCG**	-	21
B10 REPEAT	**GAAAA**	TCA-	**CTGACCAATTTC**	TC-TA	**TATT**		**AAC**	AGTA	**TCG**	-	21
B11 REPEAT	**GAAAA**	CCAG	**CCAACCAATTTC**	TC-TA	**TATT**		**AAC**	ACTA	**TGG**	A	22
B12 REPEAT	**GAAA-**	CCAT	**CCAACCAAGATC**	TC-TA	**TATT**		**AAC**	TGTA	**TAG**	-	18
B13 REPEAT	**GAAAA**	TCTC	**ACAAGTCGTTCT**	TC-TA	**TATT**		**AAC**	ACGA	**TCG**	A	21

This is higher than the degree of mismatch between the peripheral B repeats (B1, B2, B12, B14), and the B sequence consensus (8-12), but not excessively so. The homology between the B repeats and the A repeats can still be taken as highly significant, especially since motifs such as GAAAA, CCAAGTCATTC, TATT, AAC and TGG are conserved (refer to Fig. 4.13). A similar relationship between two families of subrepeats was observed in wheat (Barker *et al.* 1988).

The existence of A and B repeats in the region just downstream of the LSrRNA gene raises the question of whether variations in the number of subrepeats in these arrays is responsible for the length heterogeneity observed in this region (see Figs. 2.1, 2.2, and 2.7). Length heterogeneity is commonly associated with tandemly organized repetitive arrays (Reeder 1984, Roger *et al.* 1986) where unequal crossing over is believed to be responsible for generating heterogeneity.

PCR amplification was used to confirm and further identify the precise region where length heterogeneity is occurring. Primers flanking the A and B repeat region, Q2 and PS1 (refer to Fig. 4.6), and total genomic DNA extracted from *P. ultimum* BR471 were used for PCR amplification. The same amplification was also done using all the clones as templates. The results are shown in Fig. 4.14. The resulting amplification using genomic DNA template resulted in the production of multiple bands (Fig.

Fig. 4.14. Length heterogeneity occurring in the region of the A and B repeats. PCR amplification with Q2 and PS1 using the five different spacer clones and total genomic DNA as template, (refer to Table 1 and Fig. 4.6). PCR protocol used: denaturation at 93°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 2 min. 17 cycles were performed, followed by a final extension of 10 minutes at 72°C. 20% of the reaction mixture was run on a 0.8% agarose gel. Lane L, 1 kb ladder. Lanes 1-6 are the Q2/PS1 PCR products using the following templates: Lane 1, total genomic DNA of *P. ultimum* BR471; Lane 2, pJB1; Lane 3, pJB1A; Lane 4, pJB2; Lane 5, pJB3; Lane 6, pJB3A.


kb
L
1
2
3
4
5
6
3.1
2.0
1.6
1.0
0.5

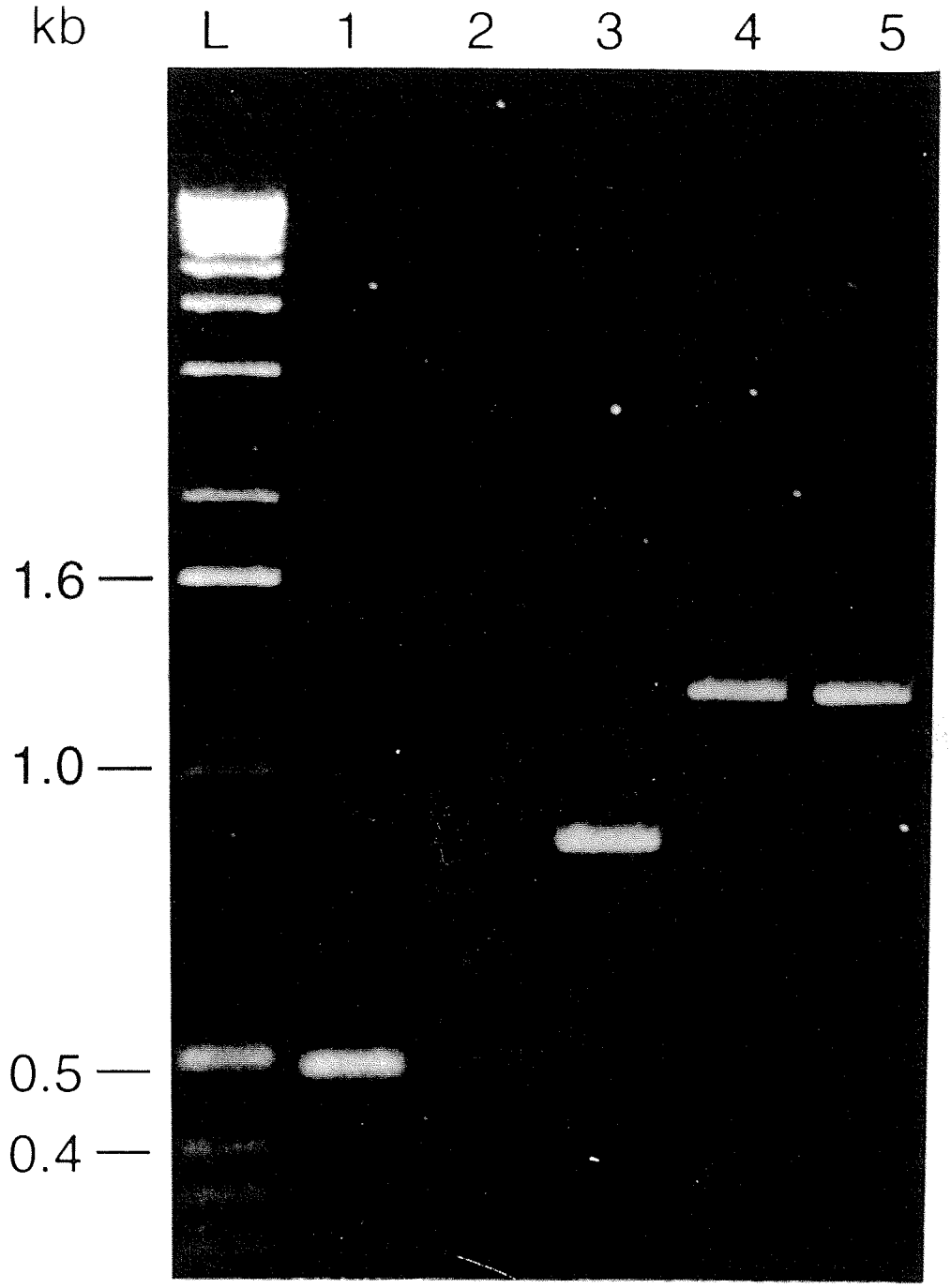
4.14, lane 1), whereas all of the clones gave a single band just under 1.0 kb in length as predicted by the sequence data for pJB1A. Multiple bands could be due to out of register annealing or heterogeneity. If out of register annealing had occurred, it would have resulted in multiple banding appearing in all of the amplifications. Since this did not happen, the multiple banding seen in lane 1 is likely not a PCR artefact, but representative of a region between the primers which is variable in length. The most intense band in the genomic amplification is the same size as that in all of the spacer clones. It is likely that the 1.0 kb size version is most abundant in the rDNA repeats and so it was preferentially cloned. Similar results were observed in the genomic digests where one or two size variants seem to predominate (the genomic digest in Fig. 5.4 is the best example illustrating this point for the isolate BR471). The relative yield of a certain band size after PCR was previously found to correlate well with relative genomic abundance of a size variant (Buchko and Klassen 1990, refer to Section 3).

The bands observed in Fig. 4.14 (lane 1) range in size from approximately 0.8 to 2.0 kb. The heterogeneity appears to be complex, and does not follow the stepwise pattern in length observed for the 385 bp subrepeat heterogeneity found nearer to the 3′ end of the NTS in *P. ultimum* (Klassen and Buchko 1990, Buchko and Klassen 1990). It is likely that

the multiple bands observed in lane 1 are due to length heterogeneity caused by variable numbers of A and/or B repeats. This is likely because tandemly arranged arrays are more prone to unequal exchange, which can shorten or lengthen such an array. The PCR amplification of the genomic DNA therefore is consistent with the rDNA digestion studies which show that this area is subject to length heterogeneity. Also, it pin-points the location of length heterogeneity to a region between the Q2 and PS1 primers.

The next task was to identify the location of the major 380 bp subrepeat length heterogeneity using PCR. The resulting PCR amplification using primers XP1c and P7 (location given in Fig. 4.6), along with the DNA from the 5 different spacer clones is shown in Fig. 4.15. Bands migrating at approximately 0.5 (lanes 1 and 2), 0.9 (lane 3) and 1.3 kb (lanes 4 and 5) were produced by the templates pJB1 and pJB1A, pJB2, plus pJB3 and pJB3A respectively. The size of the fragment in lane 2 (pJB1A used as template) is about the size as predicted by the nucleotide sequence (493 bp) (refer to Fig. 4.6). The size of the next larger PCR fragment is approximately 0.4 kb larger (lane 3) where the clone pJB2 was used as a template. Similarly, the fragments in lanes 3 and 4 (Fig. 4.15) are 0.4 kb larger than the fragment in lane 2. The length variations seen between the clones can therefore be accounted for by the region between the primers XP1c and P7 (see Fig. 4.6). The results of the

Fig. 4.15. Localization of the subrepeat to an area between primers XP1c and P7 (refer to Fig. 4.6). Primers XP1c and P7 along with phagemid DNA subclones were used for PCR amplification. The following protocol was used: denaturation at 93°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 3 min. 30 cycles were performed, followed by a final extension at 72°C for 10 min. 15% of the reaction mixture was run on a 0.8% agarose gel. Lane L, 1 kb ladder. Lanes 1-5 are the PCR product using the following templates: Lane 1, pJB1BH-; Lane 2, pJB1ABH-; Lane 3, pJB2XP-; Lane 4, pJB3XP-; Lane 5, pJB3AXP-.


kb
L
1
2
3
4
5
1.6
1.0
0.5
0.4

PCR amplifications agree with the genomic DNA digests (Section 2), the rDNA spacer amplification (Section 3), and the size variations seen in the five spacer clones (pJB1, pJB1A, pJB2, pJB3, and pJB3A). Since the primer pair XP1c (nt. pos. 1655 of pJB1A) and P7 (nt. pos. 2148 of pJB1A) result in a DNA fragment of approximately 0.5 kb and the subrepeat is believed to be about 380 bp, then the location of subrepeat heterogeneity has been located to within about 100 bp (assuming pJB1 and pJB1A have one subrepeat). If the NTS in *P. ultimum* is organized like that seen in other higher eukaryotes, then it is likely that a nucleotide sequence between the primers XP1c and P7 is repeated once in pJB2, and twice in pJB3 and pJB3A.

Once the location of the subrepeat had been identified using the primers which flank the subrepeat, the next larger clone was sequenced in order to elucidate the precise nucleotide content of the 380 bp subrepeat. Additional primers were synthesized as needed until the region spanning the two subrepeats was completely sequenced. The complete sequence of the region possessing the subrepeats is given in Fig. 4.16.

The region spanning from nucleotide position 75 to 704 contains two repeats that are well conserved (referred to as C1 and C2)(see Fig. 4.17). An alignment of the C1 and C2 repeats of pJB2, and the homologous sequence found in pJB1A, is given in Fig. 4.17. The two regions of homology (in

Fig. 4.16. Nucleotide sequence of the region spanning the subrepeat of the clone pJB2. Location of the primer annealing sites are shown by arrows indicating the orientation of primers. The bases in boldface represent sequence which is repeated with repeats being obviously similar (positions 75-344). The asterisks indicate a span of sequence which is not observed in pJB1A (position 345-440).

```
                                                        |Sac I
1     GGGACAAAAA CCTTCTTGAA TCTGTATACC GAGCTCAAGA CAAAAAACGT

51    TTGCGGATGT GCACCAGCGA GTACAATGGC AATGCGAGTC CCGAGATTTG
        |------XP1.2c------>|    |------C1 repeat----->

101   AGTGGTATAG GGGTTATTCA TCACGGGGAA CACGCTCGTG CACTCGTATC

151   AAGTCTCCTG TGAACGCATC ATGGTCAAAC GCGCGTTTTC GTGCTCCAAA
          |--------XP1.1c------->

201   AGTGGTACCA TGGTATCAAA TGTTGGAGGT TTTTGCGAGC TGGCGGGCTA

251   GGGGTGACAC GTAGACCACC AGATGTTTTT GCAATCGTAT TGTCCTACTT

                                                       ******
301   GCGTACATAT ATTTAGAGGC CAGCAGCGTG TCGAAATGTT TTCTGGATCA

      ********** ********** ********** ********** **********
351   AAAGTTAGGA CCATTTGCGT CAAAAGTTGG AGTGAAATGG TGAGCAGGTC
                                                  |<--XP2.1/

      ********** ********** ********** **********
401   GTGGTCGCTC GGACGTTGGC GCGGCCCACC GATTGTGCTA AATGGCAATG
      XP2.1c-->|                                  |---------

451   CGAGTCCCGA GATTTGAGTG GTATAGGGGT TATTCATCAC GGGGAACACG
      ---C2 repeat----->
```

501 **CTCGTGCACT CGTATCAAGT CTCCTGTGAA CGCATCATGG TCAAACACGC**
|--------XP1.1c------->

551 **GTTTTCGTGC TAAAATGGCC ATATTTGCGT TTGTGGAGGT TTTTGCAATT**
|<----

601 **TGACGACATC TGGACGCACA CTAGCCCACC AGATGTTTTT GCAATCGTAT**
-XP2/XP2c--->|

651 **TGTCCTACTT GCGTAGATAT GTTTAGACCC CAGCAGCGTG TCTACATGTT**

701 **TTCT**ACAGCT CGAGATATGT CCATTTGTGC TGAAAAATGT GCACTTTTGT
|<------P7.1----------|

751 GTCGTTTTGT TGCTATTTCT TT

Fig. 4.17. Alignment of C repeats. The two repeats found in pJB2 (C1 repeat, 2C1; C2 repeat, 2C2) are aligned with the homologous sequence found in pJB1A (1AC1). Asterisks indicate nucleotides which are conserved in all the C repeats. The number listed to the right of the sequence refers to the nucleotide position of the final base in each line (refer to Figs. 4.6 and 4.16). The size (bp) of each repeat is also given for each C repeat, at the bottom of the figure.

```
1AC1    AATGGCAATGCGAGTCCCGAGATTTGAGTGGTATAGGGGTTATTCATCAC  1830
2C2     AATGGCAATGCGAGTCCCGAGATTTGAGTGGTATAGGGGTTATTCATCAC  490
2C1     AATGGCAATGCGAGTCCCGAGATTTGAGTGGTATAGGGGTTATTCATCAC  124
        **************************************************

1AC1    GGGGAACACGCTCGTGCACTCGCATCAAGTCTCCTGTGAACGCATCATGG  1880
2C2     GGGGAACACGCTCGTGCACTCGTATCAAGTCTCCTGTGAACGCATCATGG  540
2C1     GGGGAACACGCTCGTGCACTCGTATCAAGTCTCCTGTGAACGCATCATGG  174
        ********************** ***************************

1AC1    TCAAACACGCGTTTTCGTGCT--AAAA-TGG--CCATATTTGCGTTTGT-  1924
2C2     TCAAACACGCGTTTTCGTGCT--AAAA-TGG--CCATATTTGCGTTTGT-  584
2C1     TCAAACGCGCGTTTTCGTGCTCCAAAAGTGGTACCATGGTATCAAATGTT  224
        ****** **************  **** ***  ****  *  *   ***

1AC1    GGAGGTTTTTGCAATTTGACGA-CATCTGGACGCACACTAGCCCACCAGA  1973
2C2     GGAGGTTTTTGCAATTTGACGA-CATCTGGACGCACACTAGCCCACCAGA  633
2C1     GGAGGTTTTTGCGAGCTGGCGGGCTAGGGGTGACACG-TAGACCACCAGA  273
        ************ *  ** **  *    **   ***  *** ********

1AC1    TGTTTTTGCAATCGTATTGTCCTACTTGCGTAGATATGTTTAGACCCCAG  2023
2C2     TGTTTTTGCAATCGTATTGTCCTACTTGCGTAGATATGTTTAGACCCCAG  683
2C2     TGTTTTTGCAATCGTATTGTCCTACTTGCGTACATATATTTAGAGGCCAG  323
        ********************************* **** ******  ****

1AC1    CAGCGTGTCTACATGTTTTCT  2044
2C2     CAGCGTGTCTACATGTTTTCT  704
2C1     CAGCGTGTCGAAATGTTTTCT  344
        ********* * *********

1AC1    Size:264
2C2     Size:264
2C1     Size:270
```

pJB2) flank a region which contains sequence which is unique to the clone pJB2 (indicated by asterisks in Fig. 4.16) and is not found at all in the shorter clone pJB1A. The repeats C1 and C2 are 270 and 264 bp respectively (nucleotides in boldface in Fig. 4.16). This at first seemed odd, since the expected size of the subrepeat was expected to be around 380 bp. This leads to the model shown in figure 4.19. The schematic figures for pJB1,pJB1A and pJB2 are based on the sequences presented earlier. It appears that at the core of each subrepeat is a 270 bp repeat sequence containing the downstream primer site XP1.1c. The 96 bp region between the two repeats sequences in pJB2 is not found in pJB1 and pJB1A. It bears the primer annealing sites for XP2.1 and XP2.1c. To arrive at the estimated subrepeat size of 385 bp, it is reasonable to suppose that when the 270 bp unit is repeated, the 96 bp is always placed between the repeating units so that subrepeat length becomes 366 bp (270 + 96). This is close to the estimate; the difference may be due to inherent limitations in accurately resolving fragment sizes on agarose gels.

No sequence has yet been determined for the pJB3 and pJB3A clones, but amplifications with primers used to sequence pJB1, pJB1A and pJB2 clones lead to the hypothesis shown in figure 4.19.But if you add on the region of unique sequence, which is 96 bp to the size of C1, the total size then becomes 366 bp.

Figure 4.18 shows the result of a PCR amplification when the primer pair XP2.1 and XP2.1c, and plasmid DNA of pJB3XP- (lane 1) and pJB3AXP- (lane 2) are used. A single DNA fragment migrating between the 0.34 and 0.40 kb marker was generated by the PCR reaction. Because these primers anneal to the 96 bp region in pJB2, it appears that a similar region is present twice in pJB3 and pJB3A, otherwise no such PCR fragment would be generated. The fact that the PCR product is approximately the same size as the 366 bp subrepeat in pJB2 agrees with the model which is presented in Fig. 4.19.

Further PCR evidence confirms the organization of the subrepeats in pJB3 and pJB3A (Fig. 4.18, lanes 3-6). The primer pairs XP2.1c-P7 and XP1.1c and P7 were used along with template DNA of pJB3 (or pJB3XP-) or pJB3A (or pJB3AXP-). The primer pair XP2.1c-P7 produces two products of approximately 0.4 and 0.8 kb (Fig. 4.18, lanes 3 and 4). This confirms the idea that there are only two XP2.1/XP2.1c sites in the clone and also confirms the prediction from figure 4.19 that they are about 0.4 kb apart. The size of the smallest band (0.4) indicates that the P7 primer site in pJB3 and pJB3A is in the same relationship with the XP2.1c site as it is in pJB2, and that there is no XP2.1c site downstream of the C3 repeat. These results support, but do not prove, that the pJB3 and pJB3A array terminates in the same way as the pJB2 array, without the 96 bp inter-repeat

Fig. 4.18. Organization of the subrepeats in pJB3 and pJB3A. PCR was used to amplify template from pJB3A (or pJB3AXP-) and pJB3 (or pJB3XP-) along with primers flanking or present in the subrepeat (refer to fig. 4.19). PCR protocol used: denaturation at 93°C for 1 min (lanes 1-6), annealing at 55°C (lanes 1 and 2) or 50°C (lanes 3-6), extension at 72°C for 1 min (lanes 1-4) or 2 min (lanes 5 and 6). 25 cycles were performed for all PCR reactions with a final extension at 72°C for 10 min. Lanes 1-6 contain PCR products using different templates and primer combinations. Lane 1, 10% of PCR reaction using pJB3XP- and primers XP2.1 and XP2.1c. Lane 2, 10% of PCR reaction using pJB3AXP- and primers XP2.1 and XP2.1c. Lane 3, 12% of PCR reaction using pB3XP- and primers XP2.1c and P7. Lane 4, 12% of PCR reaction using pJB3AXP- and primers XP2.1c and P7. Lane 5, 20% of PCR reaction using pJB3A and primers XP1.1c and P7. Lane 6, 20% of PCR reaction using pJB3 and primers XP1.1c and P7. Lane L, 1 kb ladder (BRL).

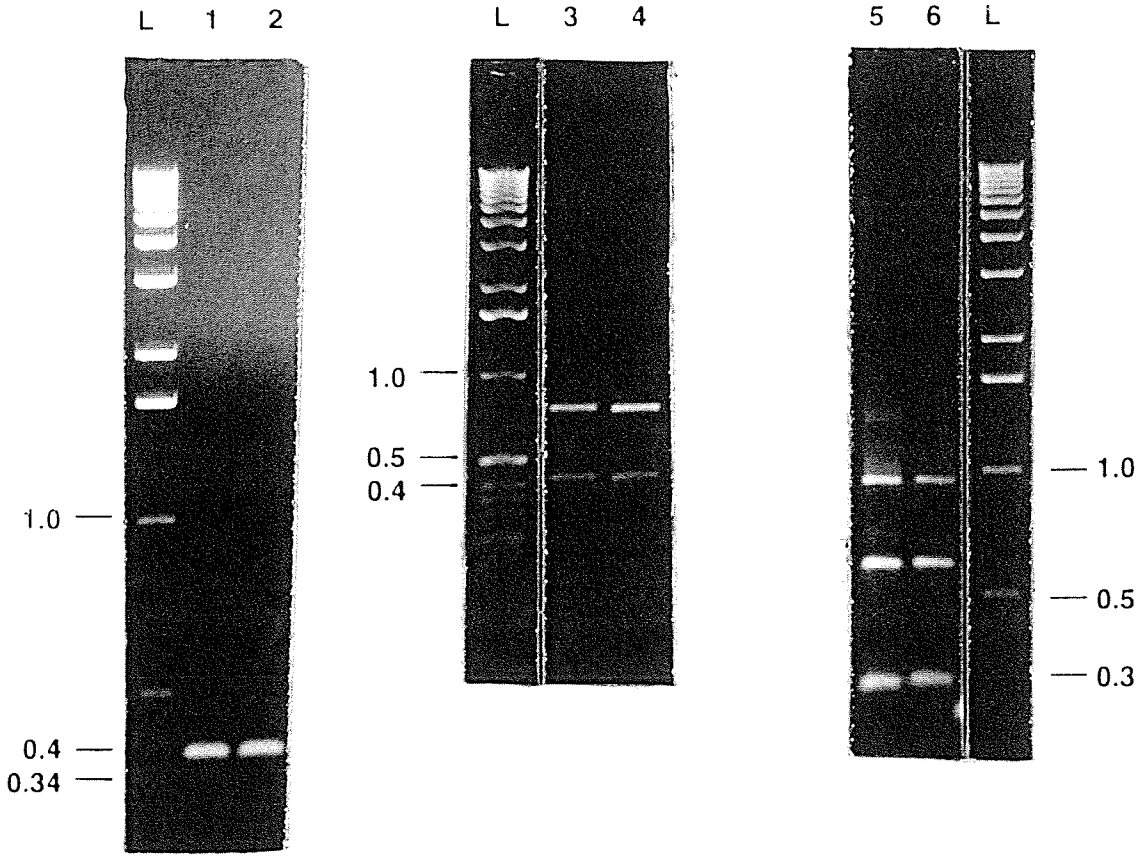
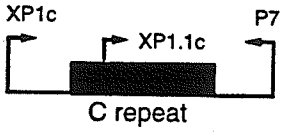
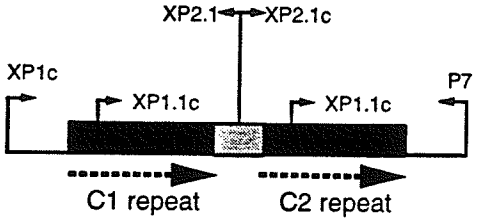

L
1
2
1.0
0.4
0.34
L
3
4
1.0
0.5
0.4
5
6
L
1.0
0.5
0.3

Fig. 4.19. Model of the organization of the C repeat arrays for three length variants (pJB1, pJB1A, pJB2, pJB3 and pJB3A). The filled in block represents a portion of the C repeat highly homologous in all length variants (264-270 bp). The shaded blocked area represents the 96 bp portion which was identified in pJB2 but absent in pJB1A. The primer annealing locations are indicated by arrows. The dashed arrow indicated the head to tail arrangement of the C repeats.

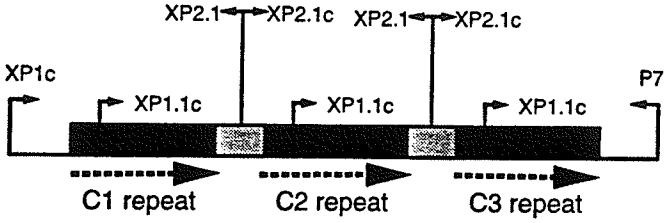
pJB1 & pJB1A



pJB2



pJB3 & pJB3A



region. The organization is further confirmed by the amplification of the NTS clones with the primer pair XP1.1c-P7 (Fig. 4.19, lanes 5 and 6). The XP1.1c annealing site is in both repeat regions in pJB2 (see Fig. 4.16), therefore if it is present in all subrepeats in pJB3 and pJB3A, amplification with XP1.1c and P7 should produce three PCR fragments with templates pJB3 and pJB3A. Three bands are, in fact produced (see Fig. 4.19, lanes 5 and 6). They differ by about 0.4 kb increments, as predicted by the model. Such products can only be produced if the subrepeats are organized in a manner as outlined in Fig. 4.19.

As part of the analysis of the complete sequence of the NTS of *P. ultimum*, it was of interest to locate the transcription initiation site (TIS). It is known that the promoter of the rRNA genes is not well conserved across species, although there is some similarity seen at the transcription initiation site (Sollner-Webb and Moughey 1991, Gerbi 1985, Gerstner *et al.* 1988, Cordesse *et al.* 1993). Using the information given in the literature a putative transcription initiation site was identified somewhere between nucleotide positions 2311-2330 (refer to sequence of pJB1A in Fig. 4.6). Total RNA was extracted from a culture of *P. ultimum* BR471 and used as template for primer extension.

The results of the primer extension experiment is shown in Figure 4.20. A major fragment is produced which

corresponds to the G residue at nucleotide position 2319 (refer to Fig. 4.6). Although there are some smaller fragments below the largest band, they may be due to the RNA polymerase falling off before reaching the end of the transcript. There are no fragments seen above the band which appears at residue number 2319.

If the start site is at nucleotide position 2319, then that would make the ETS 924 bp long. This is comparable to the sizes seen in some plants where the ETS is known to range from 800 and 1000 bp in maize and wheat, respectively (McMullen *et al.* 1986, Barker *et al.* 1988). In *S. cerevisiae* the ETS is approximately 700 bp. Thus, the position of the putative start site in *P. ultimum* is plausible when compared with other organisms with similar length spacers. It is neither too close nor too far away from the start of the SSrRNA gene.

The TIS of *P. ultimum* is compared with the start site from other organisms in Fig. 4.21. The boxed residues in Fig. 4.21 represent the regions which appear to be most conserved between different species. The start site for *P. ultimum* appears to be more similar to plants than to fungi (the TIS shown for *S. cerevisiae* is identical to the initiation sites in *S. rosei* (Verbeet *et al.* 1983), *S. carlsbergenis* (Veldman 1982), *Kluyveromyces lactis* (Verbeet *et al.* 1984), and *Hansenula wingei* (Verbeet *et al.* 1984)). The observation that the start site of *P. ultimum* is more

Fig. 4.20. Location of the putative transcription initiation site (TIS) of the rRNA genes in *P. ultimum*. The primer extension product in lane P is run alongside the sequencing reaction products of pJB1A in lanes C, T, A, and G. The nucleotide sequence of the coding strand is given. The arrowheads give the location of the TIS.

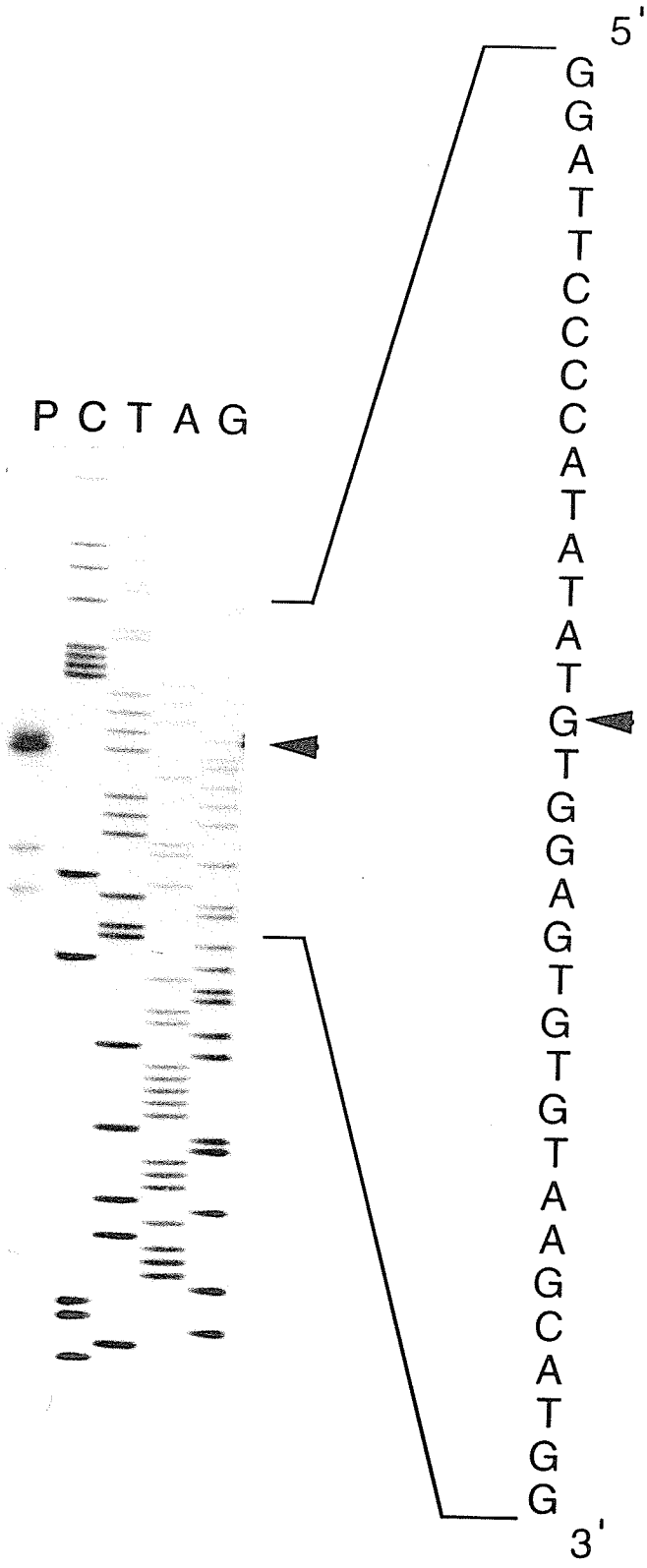
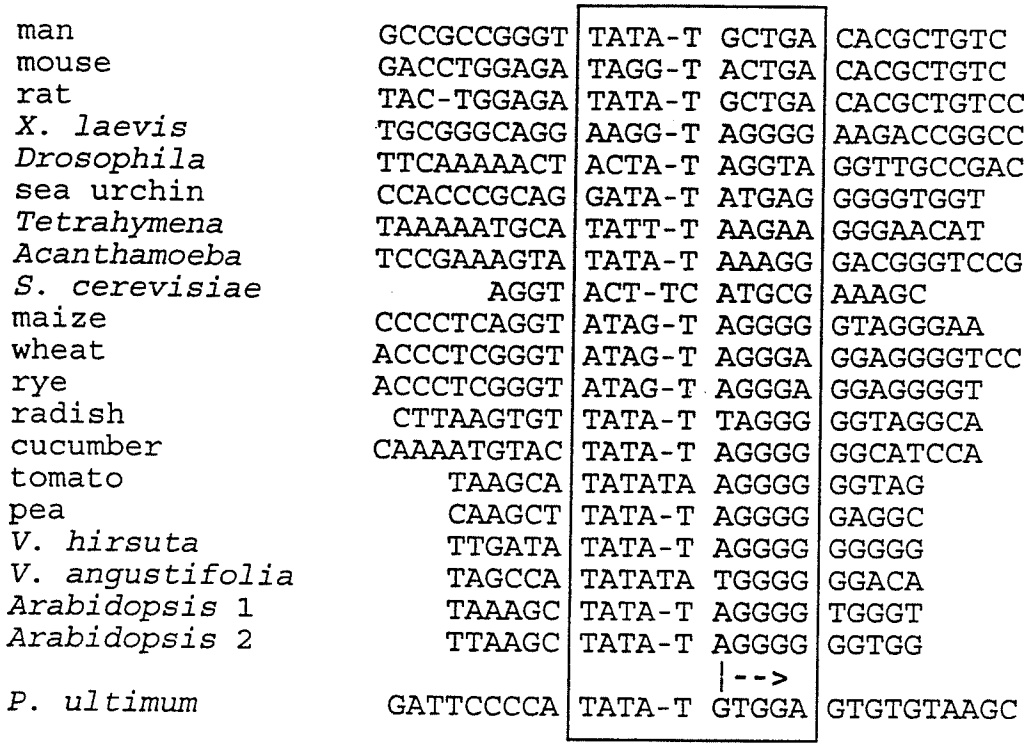

P C T A G
5'
G G A T T C C C C A T A T A T G G T G G A G T G T G T A A G C A T G G
3'

Fig. 4.21. Comparison of the TIS of *P. ultimum* with other organisms. Alignment of the TIS given by Gerstner *et al.* (1988) and Cordesse *et al.* (1993) were used to align the start site of *P. ultimum* BR471 with other species. The TIS of the following species are given: man (Haltiner *et al.* 1986), mouse (Skinner *et al.*1984), rat (Rothblum *et al.* 1982), *Xenopus* (Sollner-Webb *et al.* 1983), *Drosophila* (Kohorn and Rae 1983), sea urchin (Ho and Stafford 1985), *Tetrahymena* (Saigi *et al.* 1982), *Acanthamoeba* (Kownin *et al.* 1985), maize (McMullen *et al.* 1986, Toloczyki and Feix 1986), wheat (Lassner 1987), radish (Delcasso-Tremousaygue *et al.* 1988), cucumber (Ganal and Hemleben 1986), rye (Appels *et al.* 1986), tomato (Schmidt-Puchta *et al.* 1989), pea (Kato *et al.* 1990), *Arabidopsis* (Gruendler *et al.* 1989), *Vicia hirsuta* (Yakura and Mishkawa unpublished, EMBL accession number X62122), *Vicia angustifolia* (Ueki *et al.* 1992). The vertical line and arrow indicates the transcription initiation site and direction of transcription.


man GCCGCCGGGT TATA-T GCTGA CACGCTGTC
mouse GACCTGGAGA TAGG-T ACTGA CACGCTGTC
rat TAC-TGGAGA TATA-T GCTGA CACGCTGTCC
X. laevis TGCGGGCAGG AAGG-T AGGGG AAGACCGGCC
Drosophila TTCAAAAACT ACTA-T AGGTA GGTTGCCGAC
sea urchin CCACCCGCAG GATA-T ATGAG GGGGTGGT
Tetrahymena TAAAAATGCA TATT-T AAGAA GGGAACAT
Acanthamoeba TCCGAAAGTA TATA-T AAAGG GACGGGTCCG
S. cerevisiae AGGT ACT-TC ATGCG AAAGC
maize CCCCTCAGGT ATAG-T AGGGG GTAGGGAA
wheat ACCCTCGGGT ATAG-T AGGGA GGAGGGGTCC
rye ACCCTCGGGT ATAG-T AGGGA GGAGGGGT
radish CTTAAGTGT TATA-T TAGGG GGTAGGCA
cucumber CAAAATGTAC TATA-T AGGGG GGCATCCA
tomato TAAGCA TATATA AGGGG GGTAG
pea CAAGCT TATA-T AGGGG GAGGC
V. hirsuta TTGATA TATA-T AGGGG GGGGG
V. angustifolia TAGCCA TATATA TGGGG GGACA
Arabidopsis 1 TAAAGC TATA-T AGGGG TGGGT
Arabidopsis 2 TTAAGC TATA-T AGGGG GGTGG
|-->
P. ultimum GATTCCCCA TATA-T GTGGA GTGTGTAAGC

like that in man and rat, then to *S. cerevisiae*, certainly lends support to the belief that this organism is distinct from fungi. Thus far, the TIS has not been identified in any other Oomycete. It would be interesting to see whether the start site is conserved in other *Pythiums* and Oomycetes. If their organizations are similar, yet differ greatly from other fungi, then it would lend support to the belief that oomycetes are distinct from other fungi and are more closely related to higher eukaryotes (Barr 1983).

It is known that the promoter of the rRNA genes is about 150 bp long (Sollner-Webb and Moughey 1991). Using this rule as a guideline, the nucleotide content between bases 2151 and 2319 was examined (see Fig. 4.6). The region is made up of 24 A, 75 T, 20 C and 40 G residues. Thus, the region corresponding to the promoter is made up of a predominance of A and T residues (63%). Zentgraf *et al.* (1990) noticed that the same phenomenon occurred in dicotyledonous plants. Perhaps this kind of base composition helps facilitate initiation of transcription by RNA polymerase I by lowering the energy needed to melt the duplex DNA.

The primer extension data, along with the sequence comparison with other species, strongly suggests that the start site identified here is authentic. Once the TIS is characterized in other *Pythiums* and Oomycetes, these observation may be confirmed.

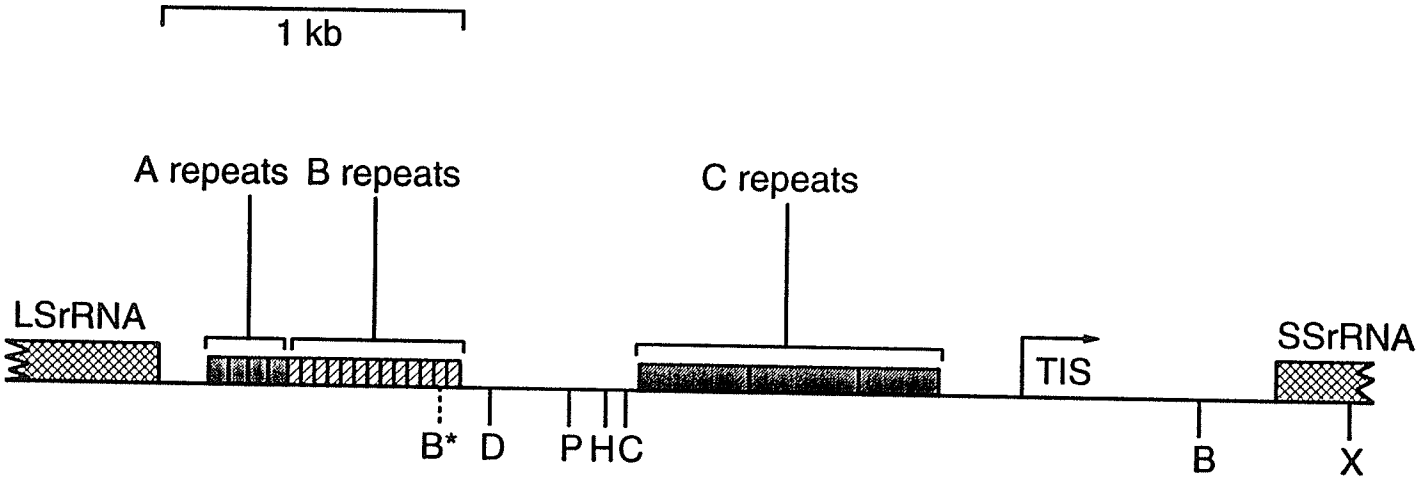
It is common for there to be a partial or more complete duplications of the promoter in the NTS. This kind of organization is known to exist in *Drosophila*, *Xenopus* and *Arabidopsis* (Coen and Dover 1982, Simeone *et al.* 1985, Reeder 1984, Gruendler *et al.* 1991). It is common for the promoter duplication to be found in subrepetitive elements found in the rDNA spacer. With the identification of the TIS in *P. ultimum*, a search of the NTS sequence was performed to see if there were any obvious promoter duplications in clones pJB1A and pJB2. No obvious duplications were identified in either of the clones pJB1A of pJB2. The only duplication that could be found was a stretch of 12 bp found in the C repeat of pJB1A (nt. pos. 1926-1937) and at the promoter region (nt. pos. 2225-2236). The same duplication is found in pJB2, although the C1 repeat has a 11 bp copy instead of 12 bp (nt. pos. 226-236), while the C2 repeat of pJB2 contains a 12 bp promoter duplication (nt. pos. 586-597). It is difficult to make any judgement on whether this duplication is significant. If the subrepeats in *P. ultimum* act as enhancers as in other organisms, then it may be possible that this 12 bp promoter duplication acts in a similar manner. It is possible that the 12 bp element may play a role of some sort in binding factor(s) relevant to transcription by RNA polymerase I.

Taking into account all of the rDNA RFLP data (Section 2), PCR data (Section 3 and 4), and sequence data (Section

4) a model of the rDNA spacer organization can be proposed (refer to Fig. 4.22). The rDNA RFLP data identified two regions of length heterogeneity. There is a region of complex heterogeneity which occurs immediately downstream of the LSrRNA gene. This region is composed of the A and B repeats. PCR amplification using primers flanking this region have confirmed that this area is prone to length heterogeneity, although one length variant predominates. The A and B repeats are made up of approximately 62 and 40 bp elements, respectively. In addition, at least one heterogeneous restriction site (*Bgl*II) has been identified between the spacer clones pJB1 and pJB1A and confirmed by digestion of genomic DNA with *Bgl*II. It appears that C repeats and their 96 bp spacer are responsible for the observed 366 bp (initially identified as 380 bp) length variants causing the dramatic ladders of bands which were seen in the genomic DNA digests. The PCR data and sequence data of pJB1A and pJB2 suggest that the clones pJB3 and pJB3A have two full length 366 bp subrepeats and one truncated 264 bp subrepeat. It is believed that there can be up to six C repeats in the rRNA spacer of *P. ultimum* and that this number varies within the gene family (Klassen and Buchko 1990).

Unfortunately, the sequence data for pJB1A and pJB2 cannot be compared with any other closely related *Pythium* or other Oomycete. The sequence presented here is the first

Fig. 4.22. A model of the NTS for *P. ultimum* based on the sequence, rDNA RFLP and PCR data. The large subunit rRNA (LSrRNA) and small subunit rRNA (SSrRNA) gene positions are given. The relative location of the A, B and C repeats are shown. The TIS is indicated by an arrow. The following restriction enzymes are represented by the letters: B-*Bgl*II, D-*Hin*dIII, P-*Pst*I, H-*Hin*cII,C-*Sac*I, B*-heterogeneous *Bgl*II site.


1 kb
A repeats
B repeats
C repeats
LSrRNA
SSrRNA
TIS
B*
D
P
H
C
B
X

complete sequence representing the rDNA spacer of an oomycete. Belkhiri has been able to show significant sequence similarity of a small portion of the ETS among numerous *Pythiums* with filamentous zoosporangia (Belkhiri 1994). There is no homology seen between *P. ultimum* and any of the *Pythiums* with filamentous zoosporangia.

5. Species-specific Oligonucleotide Probe for *Pythium ultimum* Based on the Intergenic Region of the Ribosomal RNA Gene.

It is relatively easy to identify the genus *Pythium* among other fungi but there are few morphological features for species assignment within the genus. It becomes more difficult if the isolate does not produce sexual structures, which are essential for the proper identification of a species. The development of species-specific probes can greatly enhance the ability to identify species. Martin (1991) demonstrated species-specificity of DNA probes derived from mtDNA for two *Pythium* species. A DNA probe for the identification of *P. irregulare* was presented by Matthew *et al.* (1995). Yuen *et al.* (1993) were able to demonstrate species-specificity of a monoclonal antibody for *P. ultimum*, although it showed minor cross hybridization with a few other species. Levesque *et al.* (1994) reported the use of a PCR amplified fragment of the internal transcribed spacer as a species-specific probe for *P. ultimum*. The goal of this project was to develop an oligonucleotide probe, based on the NTS, which would only hybridize to total DNA extracted from *P. ultimum* var. *ultimum*, *P. ultimum* var. *sporangiiferum*, and asexual forms of *P. ultimum* (group G).

It is known that the nucleotide sequences coding for the rRNA genes are highly conserved throughout plant, animal and fungal evolution, whereas the rDNA intergenic region

shows a great deal of length and sequence variation between species (Rogers and Bendich 1987, Tautz *et al.* 1987, Sollner-Webb and Moughey 1991). This makes the NTS ideal for the development of species-specific oligonucleotide probes. The other goal of this project is to test this hypothesis.

The sequence of pJB1A (see Fig. 4.6) was used to design two oligonucleotide probes, PS2 and PS1c. PS2 was derived from the family of 40 bp B repeats which were identified upon sequencing the spacer clone pJB1A (refer to Fig. 5.1). The probe recognizes a conserved region in each repeat, six of which share perfect identity to the probe, the other seven repeats having two to five mismatches. Ten of the 21 nucleotide positions in the probe are absolutely conserved in all the repeats.

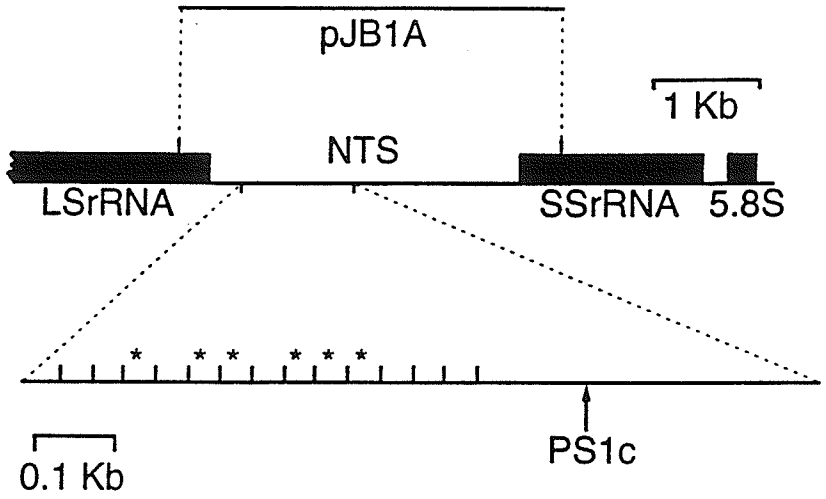
PS1c is based on unique sequence found about 150 bp downstream of the B repeats (Fig. 5.1).

DNA samples (listed in Table 5). extracted from several *P. ultimum* isolates, as well as 26 other *Pythium* species, five Oomycetes, one member of Hyphochytriomycetes, and four Ascomycetes were used for dot blot analysis (Fig 5.2). The ability of the two oligonucleotide probes (PS2 and PS1c) to hybridize to the various isolates was tested.

Figure 5.2A represents a positive control for the DNA samples used in the dot blot analysis. The clone pMF2 (a recombinant plasmid containing the gene region of the rDNA

Fig. 5.1. Map and partial nucleotide sequence of the rDNA repeat unit nontranscribed spacer (NTS) in *P. ultimum* BR471. A. Map of the rDNA repeat unit showing the locations of the NTS and the rRNA genes. Horizontal line above the rDNA map represents the NTS clone (pJB1A). The horizontal line below the rDNA map is an enlarged view of a portion of the NTS showing the positions of the B repeats. Asterisks identify repeats which have perfect homology to the PS2 primer. The arrow indicates PS1c primer binding site. B. Partial nucleotide sequence of pJB1A (refer to fig. 4.6) showing the alignment of the B repeats. The nucleotide stretches in bold print are perfect complements of the PS2 primer. The nucleotides under the asterisks indicate the region of PS2 binding. The number of nucleotide mismatches with PS2 within each repeat are given. The bold and underlined nucleotide sequence at the bottom of the figure corresponds to the region recognized by PS1c.

A



B

		Nt.Pos.	Mismatches
	**********		
REPEAT 1	GAAAATGCGCCAAGTCATTTTTCCTGTATTAACAGTATGG-	729	3
REPEAT 2	GAA--TCGG-CGAGC-ATTTG-CCTGTATTAGCAGTATGG-	764	5
REPEAT 3	GAAAATCT-CCGAGT**CATTTCTC-TATATTAACAGTAT**GG-	802	0
REPEAT 4	GAAATCCTT-CGACC-ATT-CTCCTATATTAACAGTATGG-	839	2
REPEAT 5	GAAAATCTGCCGAGC-**ATTTCTC-TATATTAACAGTAT**GA-	877	0
REPEAT 6	GAAAATCT-CCGAGT**CATTTCTC-TATATTAACAGTAT**GG-	915	0
REPEAT 7	GAAAATCTGCCGAGC-ATTTGCC-TATATTAACAGTATAG-	953	2
REPEAT 8	GAAAACCATCCAACC**AATTTCTC-TATATTAACAGTAT**CG-	992	0
REPEAT 9	GAAAATCA-CTGACC**AATTTCTC-TATATTAACAGTAT**CG-	1030	0
REPEAT 10	GAAAATCA-CTGACC**AATTTCTC-TATATTAACAGTAT**CG-	1068	0
REPEAT 11	GAAAACCAGCCAACCAATTTCTC-TATATTAACACTATGGA	1108	1
REPEAT 12	GAAA-CCATCCAACCAAGATCTC-TATATTAACTGTATAG-	1146	3
REPEAT 13	GAAAATCTCACAAGTCGTTCTTC-TATATTAACACGATCGA	1186	5
	GAAAGCTGGCTATTGTAGCACTATAAAGAAAAAATGAAAA	1226	
	AGTGACGCGAATATGCACACGTTCCAGCATGTCCATATAC	1266	
	ATGCACACATGGAGCTTTCCACCCAAATATGCAAAACCAA	1306	
	TACGCACACGCACAT**GCAGCGCTGCTAGTGACGC**	1340	

Table 5. DNA Samples Used for Dot Blot Hybridization

No.[a]	DNA SAMPLES[b]
1a	pJB1A
1b	*Pythium ultimum* var. *ultimum* BR471
1c	*Pythium* sp. (group G) BR612
1d	*Pythium ultimum* var. *ultimum* BR406
1e	*Pythium* sp. (group G) BR583
1f	*Pythium* sp. (group G) BR657
1g	*P. ultimum* var. *sporangiiferum* 4333c[c]
1h	*P. ultimum* var. *sporangiiferum* BR650
1i	Bluescript KS M13+
1j	pJB1A
2a	*Pythium dissimile* 4204d
2b	*Pythium graminicola* 4205b
2c	*Pythium arrhenomanes* 4201d
2d	*Pythium vanterpoolii* 4213a
2e	*Pythium myriotylum* 4216a
2f	*Pythium aphanidermatum* 4104b
2g	*Pythium torulosum* 4212a
2h	*Pythium vexans* 321561
2i	*Pythium volutum* 4214a
2j	*Pythium spinosum* 4012b
3a	*Pythium mamillatum* 4311c
3b	*Pythium echinulatum* 4306a
3c	*Pythium pachycaule* 4117a

3d *Pythium nagaii* 4321c

3e *Pythium australe* 2

3f *Pythium oligandrum* 4410f

3g *Pythium violae* MA2160

3h *Pythium hydnosporum* 434a

3i *Pythium rostratum* 4329J

3j *Pythium salpingophorum/conidiophorum* 4331e

4a *Pythium irregulare* 672

4b *Pythium tardicrescens* 4215a

4c *Pythium parvum* 4009b

4d *Pythium hypogynum* 4326b

4e *Pythium periplocum* SS78622

4f *Pythium ascophallon* 4004a

4g *Pythium periplocum* 4461a

4h *Beauveria brongniartii* CBS/128.53

4i *Gelasinospora tetrasperma* ATCC/11345

4j *Ophiostoma ulmi* WIN(M)/780

5a pJB1A

5b *Neosartorya fischeri* CBS/525.65

5c *Rhizidiomyces apophysatus* BR296

5d *Aplanopsis terrestris* 3102b

5e *Apodachlya brachynema* 501a

5f *Phytophthora cryptogea* BR522

5g *Pachymetra chaunorhiza*

5h *Verrucalvus flavofaciens*

5i pJB1A

---

[a]The number and letter refers to the row and column, respectively, as they are indicated on fig. 5.2.

[b]"BR" before the isolation number code indicates that the DNA was extracted from isolates obtained from the Biosystematics Research Centre, Ottawa, Canada. Samples 4h and 5b, 4i, and 4j were obtained from the CBS[d], ATCC[e] and and WIN(M)[f] culture collections respectively. DNA for all the other samples were extracted from isolates obtained from the APCC[g].

[c]Its species assignment remains in question.

[d]Central Bureau voor Shimmelcultures, Baarn, the Netherlands.

[e]American Type Culture Collection, Rockville, Maryland, U.S.A.

[f]The culture collection of Dr. Reid, University of Manitoba, Department of Botany, Winnipeg, Canada.

[g]Aquatic Phycomycete Culture Collection, University of Reading, U.K.

repeat unit for *Neurospora crassa*) hybridizes to all the samples, confirming DNA integrity and the presence of rDNA. Sample 1i (Bluescript KS M13+) gives a hybridization signal because pMF2 and Bluescript DNA both contain nucleotide sequence from pBR322 (Free *et al.* 1979). Some of the variation in hybridization intensity can be attributed to different degrees of similarity between DNA samples and pMF2.

The dotblot shown in Figure 5.2A was washed free of pMF2 and reprobed with PS2 to test its species-specificity (Fig. 5.2B). PS2 successfully hybridized to *P. ultimum* DNA samples only (except for sample no. 1g), as well as to the clone containing the rDNA NTS (pJB1A)(at the corners of the blot). The hybridization signal for the *P. ultimum* samples is of approximate equal intensity with the exception of 1g (Fig. 5.2B). There was no obvious hybridization signal observed for any of the other 26 *Pythium* isolates, nor for any of the Oomycete, Ascomycete or Hyphochytrid DNA samples. Bluescript KS M13+ DNA (Fig 5.2, sample 1i), which was present as a known source of DNA lacking the PS2 binding site, shows that the probe does not bind nonspecifically to DNA. Sample 1g did not give a hybridization signal, and for this reason along with a further explanation to be given later on in this section, it is believed this sample has been misidentified and is not representative of a true *P. ultimum*.

In Figure 5.2C the hybridization of PS1c to the same blot is shown. Again, PS1c binds to pJB1A DNA and to the same *P. ultimum* samples as the PS2 probe (like PS2, PS1c did not hybridize to sample 1g), while not hybridizing to any of the other *Pythium*, Oomycete, Ascomycete, Hyphochytrid, and Bluescript DNA samples. Unlike PS2, the intensity of the hybridization signal appears to vary among the *P. ultimum* isolates. BR471 (1b), BR612 (1c), and BR406 (1d) give the strongest signals, while for BR583 (1e) and BR657 (1f) the signal is much weaker and the signal for BR650 is extremely faint. Thus, PS1c has a narrower range of specificity for *Pythium* species, and may be useful for discrimination within the species.

The ability of PS2 to hybridize to a larger sampling of *P. ultimum* isolates was tested, including more *P. ultimum* var. *sporangiiferum*, and "group G" *Pythiums*. DNA from 14 *P. ultimum* isolates and one unidentified "group G" *Pythium* (BR659) was digested with *Hinc*II and *Eco*RV simultaneously and then probed with PS2 (Fig. 5.3, the same blot as the one shown in Fig. 2.4). All of the samples gave a positive hybridization signal except for BR659, an asexual isolate not believed to be a *P. ultimum*. Therefore PS2 hybridizes reliably to numerous assorted isolates of *P. ultimum* var. *ultimum*, *P. ultimum* var. *sporangiiferum* and asexual forms of *P. ultimum* ("group G").

For the strains *P. ultimum* var. *ultimum*, and the "group

Fig. 5.2. Dot blot analysis. Autoradiograph of DNA samples listed in table 5 hybridized to various probes. The same dot blot was used in all three hybridization experiments. A. pMF2 probe hybridization. B. PS2 probe hybridization. C. PS1c probe hybridization.

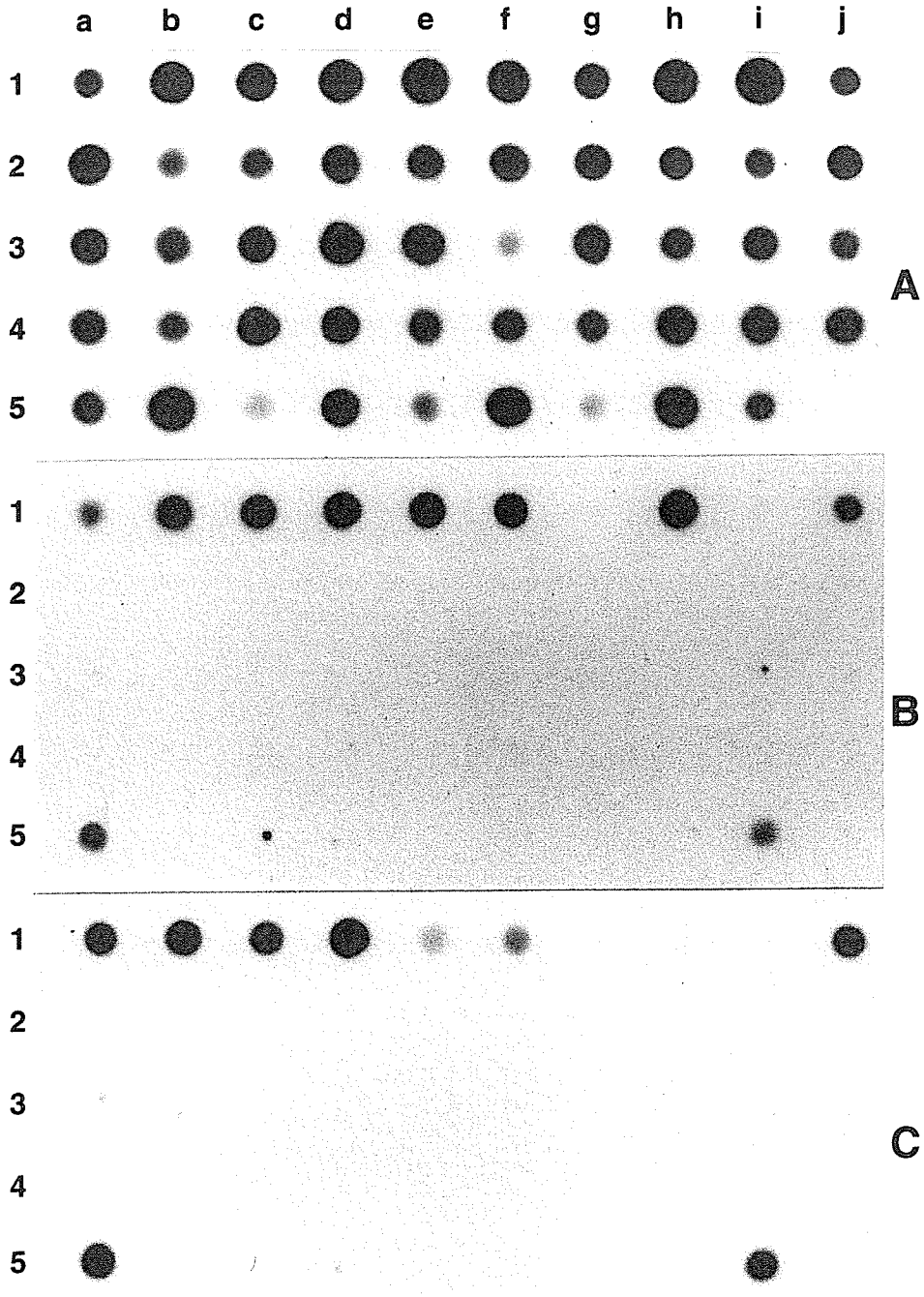
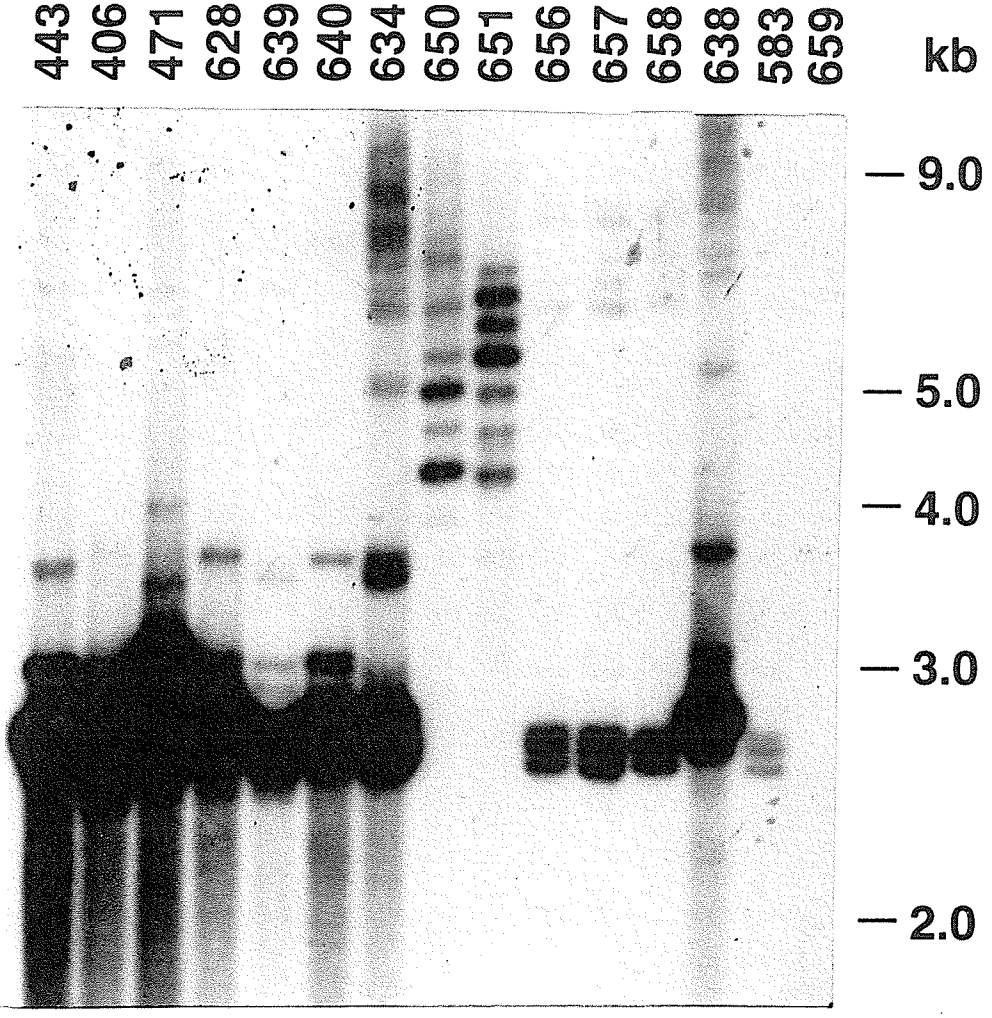

a
b
c
d
e
f
g
h
i
j
1
2
3
4
5
A
1
2
3
4
5
B
1
2
3
4
5
C

Fig. 5.3. Autoradiograph of *P. ultimum* DNA (listed in Table 1; numbers represent culture numbers with the "BR" prefix omitted) and one unidentified *Pythium* sp. "group G" DNA sample (659) digested simultaneously with *Hinc*II and *Eco*RV and hybridized to PS2. Kilobase (kb) size standard (based on the BRL 1 kb ladder) scale is shown.

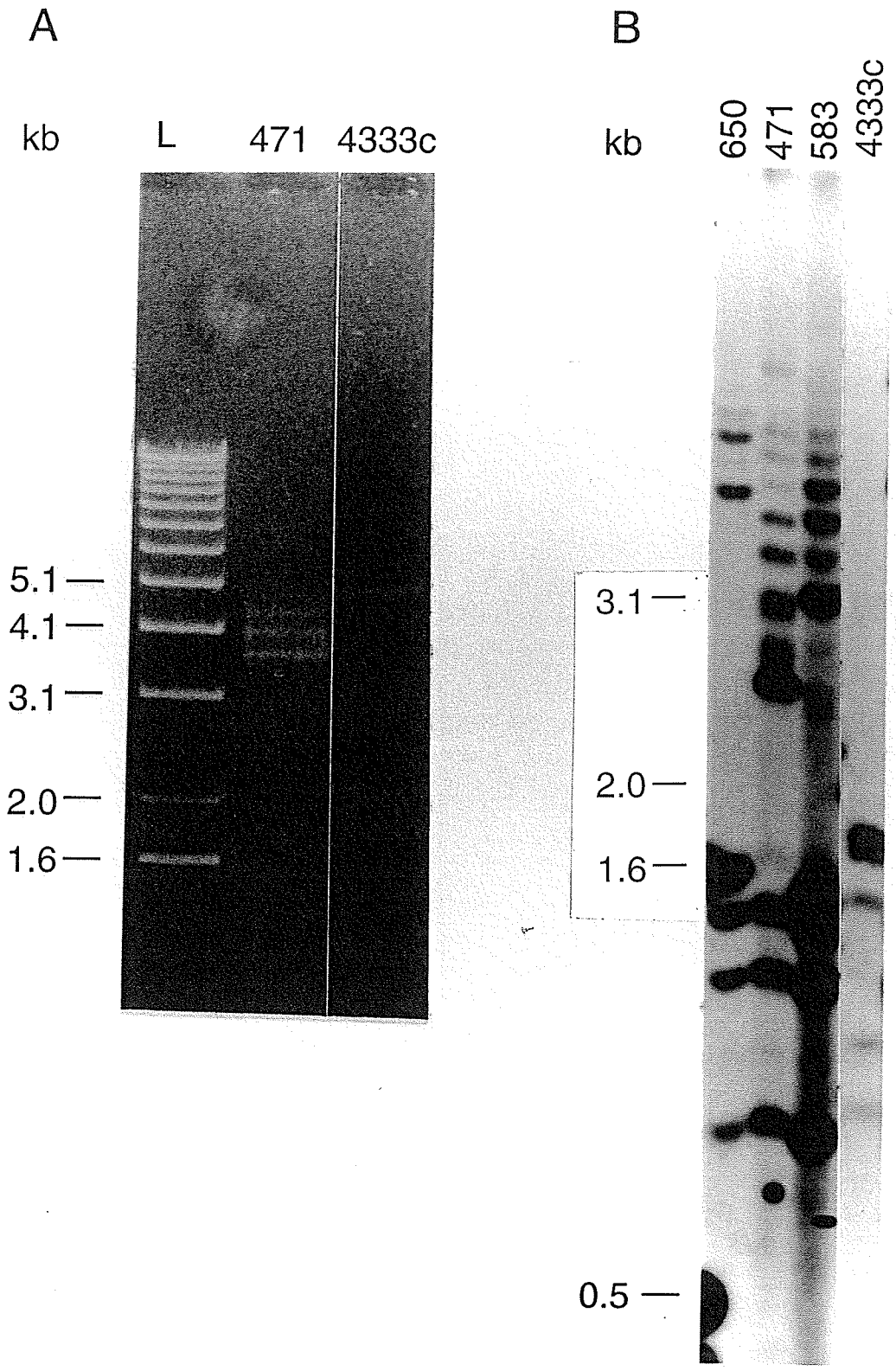

443
406
471
628
639
640
634
650
651
656
657
658
638
583
659
kb
9.0
5.0
4.0
3.0
2.0

G" isolates in Figure 5.3, most of the hybridization is to bands ranging from 2.5 to 3 kb. These bands represent the 5′ end of the NTS and do not reflect the more dramatic heterogeneity known to exist at the 3′ end (Klassen and Buchko 1990). The presence of the *Hinc*II site in the middle of the NTS (refer to map in Fig. 2.5) separates the two regions. This *Hinc*II is absent from *P. ultimum* var. *sporangiiferum* BR650 and BR651, so that the hybridizing bands, which range from 4 to 8 kb, represent the entire NTS. These multiple bands indicate that *P. ultimum* var. *sporangiiferum* has the same type of length heterogeneous NTS as the *P. ultimum* var. *ultimum* reported in Section 2 of this report (Figs. 2.1-2.4).

P. ultimum var. *sporangiiferum* 4333c was not recognized by either of the two probes (sample 1g in Fig. 5.1). In Section 2 of this report it has been shown that all of the *P. ultimum* strains which were investigated have dramatic length heterogeneity in the NTS of the rDNA repeat unit (Klassen and Buchko 1990) and it is shown here that the type culture (BR650), and one other isolate (BR651) of *P. ultimum* var. *sporangiiferum* also have length heterogeneity (Fig 5.3 and Figs. 2.2, 2.4 and 2.6). In order to help resolve this ambiguity relating to the isolate 4333c, further investigations were performed. In Figure 5.4 evidence is given which strongly suggests that 4333c does not belong to the species *P. ultimum* var. *sporangiiferum*. We used PCR to

check whether or not 4333c had length heterogeneity in the same manner as all of the previous *P. ultimum* isolates which were investigated (in Sections 1 and 2). Primers P (anneals to 5′ end of SSrDNA) and Q (anneals to the 3′ end of the LSrDNA) were used for amplification along with templates of BR471 (Fig. 5.4A) or 4333c (Fig. 5.4A)(relative locations of P and Q are given in Fig. 4.2 and Table 2). The resulting amplification is shown in Figure 5.4A. Clearly, template from BR471 produced a ladder of DNA fragment ranging from about 3.8 to 4.5 kb, while 4333c produced a single DNA fragment of approximately 5.0 kb. Thus, 4333c does not appear to have length heterogeneity, otherwise it would have given a series of bands like BR471 upon amplification with primers P and Q. To further confirm this result, digestion of total genomic DNA followed by probing with pMF2 (plasmid containing rDNA repeat unit) was performed for isolates BR650, BR471, BR583 and 4333c (Fig. 5.4B). Isolates BR650. BR471 and BR583 gave the RFLP patterns as observed previously (refer to Section 2). A ladder-like pattern of bands is observed for these three isolates, and bands corresponding to restriction sites in the rDNA regions are conserved (refer to restriction maps in Fig. 2.7). In comparison, 4333c lacks the ladder of DNA fragments corresponding to length heterogeneity. In addition, the restriction fragment banding pattern is considerably different from that of the other three isolates (Fig. 5.4B).

Fig. 5.4. Comparison of NTS organization of *P. ultimum* 4333c with other *P. ultimum* isolates (BR471, BR650, BR583) using PCR amplification and genomic DNA RFLP analysis. A. PCR amplification of the NTS using primer P and Q (refer to table 2) as described in section 3. Lane L, 1 kb ladder (BRL). Lane 471, PCR product using BR471 DNA and primers P and Q. Lane 4333c, PCR product using 4333c DNA and primers P and Q. B. Autoradiograph of various DNA sample digested simultaneously with *Hinc*II and *Eco*RV and hybridized with pMF2. Lane 650, BR650 DNA. Lane 471, BR471 DNA. Lane 583, BR583 DNA. Lane 4333c, 4333c DNA.


A
kb
L
471
4333c
5.1
4.1
3.1
2.0
1.6
B
kb
650
471
583
4333c
3.1
2.0
1.6
0.5

It can therefore be stated with some confidence, based on the evidence given in Figure 5.4, that 4333c does not belong to the species *P. ultimum*. That is why neither of the oligonucleotide probes hybridized to the DNA sample from isolate 4333c.

The oligonucleotide probe PS2 was designed to recognize the core sequence of the B-repeats. This probe recognized all strains of *P. ultimum*, including the type culture of *P. ultimum* var. *sporangiiferum* (BR650), and several isolates identified as "group G" (Figs. 5.2B, 5.3). This indicates that the B-repeat is conserved in *P. ultimum* var. *ultimum*, *P. ultimum* var. *sporangiiferum*, and in some asexual forms of *Pythium* ("group G"). The conservation of the repetitive element across the three forms suggests that it may have functional importance, possibly playing a role in transcriptional control of the rRNA genes (Reeder 1984, Sollner-Webb and Moughey 1991). In comparison, PS1c, which is downstream of the repeat array is less conserved across the three forms of *P. ultimum*, as demonstrated by the variable hybridization signals produced by the probe. Such intraspecific hypervariability within the rDNA spacer has been previously observed (Rogers *et al.* 1986, Rogers and Bendich 1987). The lack of conservation suggests that this sequence may not have functional constraints associated with it.

For *Pythium* "group G" isolates, recognition by the

probes is preliminary justification for assigning them to *P. ultimum*. Five "group G" isolates were included in this study and all but one (BR659) were recognized by probes PS2. The results support the classification of these *Pythiums* (BR656, BR657, BR658, BR583) as *P. ultimum*. It has been shown previously that isolate BR583 was believed to be a form of *P. ultimum* (Huang *et al.* 1992). In their study they also looked at isolates BR656, BR657, BR658 and BR659. They described BR656, BR657, BR658 as having similar vegetative characteristics and response to temperature whereas BR659 differed. The observations made by Huang *et al.* (1992) are consistent with the results given in Sections 1 and 2 of this reports. Mitochondrial and ribosomal DNA restriction fragment banding patterns for these isolates give strong support to the belief that the asexual isolates BR656, BR657, BR658 and BR583 belong to the species *P. ultimum*. The isolate BR659 consistently gave markedly different RFLP patterns when compared to the other "group G" *Pythiums*. Therefore the morphological and molecular data support the classification of BR656, BR657, BR658, and BR583 as *P. ultimum*.

The one other asexual isolate used in the dot-blot analysis was BR612. In Fig. 5.2C in hybridization intensity matched that of the var. *ultimum* more closely than the other two "group G" *Pythiums* (BR656 and BR583). This is not surprising since the NTS organization of BR612 more closely

resembles *P. ultimum* var. *ultimum* than that of the other asexual isolates looked at in this study (refer to Figs. 2.1 and 2.2). More specifically, BR612 lacks the additional *Hinc*II site which all the other "group G" *Pythiums* possess. In addition, mtDNA data place BR612 closer to var. *ultimum* than to the other asexual isolates (Section 1 of this report). Thus, the molecular data for BR612 also supports it placement with the species *Pythium ultimum*.

These studies have shown that rDNA NTS can be a source of diagnostic probes at the species level, and possibly also at the subspecific level. The hybridization signal is strong because the target sequence exists in hundreds of copies per genome, and if the probe recognizes subrepeats within the NTS, as is the case for PS2, the number of targets per genome is even greater.

Evidence has been given supporting PS2 and PS1c as species-specific. At the very least, a putative species-specific probe has been identified, and further studies will have to be done to confirm its specificity. If PS2 (a more promising probe than PS1c) proves to be species-specific, it may prove useful in identifying the presence of the fungi in infested soil and plants, especially in green houses. The PS2 probe may be useful in clarifying herbarium and culture collections, to identify asexual isolates which cannot be properly identified based on morphological characteristics alone.

CONCLUSIONS

This thesis demonstrates that there is some genetic variability among *Pythium ultimum* isolates, although the differences are not always dramatic. Mitochondrial DNA RFLP analysis demonstrates that the majority of *P. ultimum* isolates have very similar mtDNA organization, but notable differences are seen among some of the isolates. The asexual isolates (BR583, BR657) and the two *P. ultimum* var. *sporangiiferum* isolates demonstrated polymorphisms which set them apart from all the other isolates. The mtDNA data presented here was used as supporting evidence by Huang *et al.* (1992) to assign isolate BR583 to the species *P. ultimum*.

The rDNA RFLP data also demonstrate a significant level of genetic variability between numerous *P. ultimum* isolates most dramatically, the observed length heterogeneity producing the ladder-like series of bands. All of the isolates possessed subrepeat length heterogeneity except for two of the asexual isolates (BR613 and BR659). While the presence of subrepeat heterogeneity and the size of the major subrepeat are conserved in all *P. ultimum* isolates investigated, the number of size variants for each isolate and the relative abundance of each variant appear to be unique for each isolate. The observed difference seen between the *P. ultimum* isolates seems to correlate with the branching observed in the dendogram generated by the mtDNA

data. The rDNA data also confirms the assignment of the "group G", BR583, BR656, BR657, BR658 and BR612 to the species *P. ultimum*. The observed subrepeat length heterogeneity is similar to what is observed in plants and animals (Reeder 1984, Rogers *et al.* 1987).

A method for the detection of length heterogeneity in the rDNA in *P. ultimum* is given. The amplified fragments accurately reflect the rDNA digestions done previously. It was this method which ultimately led to the cloning of the amplified products, allowing for the complete characterization of the NTS in *P. ultimum*.

This work represents the first complete sequence of the NTS of an Oomycete. It reveals the presence of three families of repeats (A,B, and C). It can be hypothesized that the A and B repeat are responsible for the complex length heterogeneity observed downstream of LSrRNA gene and that the major length heterogeneity is due to a 366 bp repeat element. Such families of repeats can be hotspots for unequal crossing-over which lead to the observed length heterogeneities (Dover 1982). The NTS organization observed for *P. ultimum* is more like that observed in plants and animals and supports the separation of Oomycetes from the kingdom Fungi (Cavalier-Smith 1989). In other organisms such repeat elements have been shown to play an important role in transcription of the rRNA genes (Reeder 1984, Sollner-Webb and Moughey 1991).

The NTS of other Oomycetes and *Pythiums* will have to be sequenced to see whether or not the organization observed in *P. ultimum* is seen elsewhere. If any similarities are seen at all, it will provide insights into the evolution of the NTS in this group of organisms.

In the final portion of the thesis a species-specific probe for *P. ultimum* is presented. It is based on the B repeat nucleotide sequence found in the NTS of *P. ultimum*. The ability of the probe to hybridize specifically to numerous *P. ultimum* isolates (including some asexual isolates) is demonstrated. Such a probe would be useful for the rapid detection and preliminary identification of this species when isolated from infected plants, or in soil samples.

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