Electrophorectic Karyotype of the Pathogenic Fungus *Penicillium marneffei*

By

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Penicillium marneffei

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12/12/02

Peter J. Kasvinsky, Dean of Graduate Studies — Date

ABSTRACT

An electrophoretic karyotype of *Penicillium marneffei*, an emerging AIDS-associated pathogen, was derived using contoured-clamped homogeneous electric field (CHEF) gel electrophoresis. Five chromosome-sized DNA fragments were clearly resolved in two different strains of *P. marneffei*. Chromosomal length polymorphisms were evident among the three largest molecules. Based upon these electrophoretic separation patterns, individual chromosomes were estimated to range in size from 2.2 to ≥ 6..1 Mbp with a total genome size of 19.6 to ≥ 20.6 Mbp. However, Southern blot analysis using a telomeric probe suggests that *P. marneffei* may possess at least six chromosomes. Further hybridization analysis also localized *P. marneffei* genes for chitin synthase, chitinase, malate synthase, isocitrate lyase, isocitrate dehydrogenase, actin, and 28S rDNA, to specific chromosomes. These analyses confirmed the existence of multiple chitin synthase and chitinase gene homologues in *P. marneffei*. The collective results of this study provide foundations for facilitating both the genetic characterization of *P. marneffei* and the molecular epidemilogical study of penicilliosis due to this fungus.

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Dad – Your loving memory and spirit often kept me from giving up during the tough times of my life. My success is as much yours as it is mine. We finally made it dad.

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CHAPTER I

INTRODUCTION

Species of the genus *Penicillium* are ubiquitous in nature (Pitt 1979). With well over 200 identified species, most are considered innocuous and few are known to cause infection in humans (Nelson, Kaufman et al. 1999). However, penicilliosis can be initiated by several species of *Penicillium*. Interestingly, most of the reported infections can be attributed to a single species of the *Penicillium* genus, *Penicillium marneffei*. A literature review throughout the past century has revealed that only 14 identified species of *Penicillium* (including *P. marneffei*) have ever acted as the documented pathogen of humans (Figure 1). Although 13 species other than *P. marneffei* accounted for only 25 cases of penicilliosis, over 5000 cases have been attributed to *P. marneffei* alone (Cooper 2002). In all probability, many more cases of penicilliosis due to *P. marneffei* have occurred. Since this organism

was not identified until the mid-20th century, many of the early cases were most likely attributed to similarly presenting pathogens, such as *Histoplasma capsulatum* or *Cryptococcus neoformans* (Cooper and McGinnis 1997; Nelson, Kaufman et al. 1999).

Penicillium marneffei was first isolated in 1956 from bamboo rats (*Rhizomys sinensis*) in central Vietnam (Capponi, Sureau et al. 1956). The first report of infection occurred three years later after a researcher was pricked by a contaminated needle (Segratain 1959). In 1973, the initial report of a naturally occurring infection came from the United States when a patient, afflicted by Hodgkin's disease, was diagnosed with penicilliosis due to *P. marneffei* two years following a visit to Vietnam (DiSalvo, Fickling et al. 1973). Since then, penicilliosis due to *Penicillium marneffei* has become an emerging infectious disease particularly among HIV-infected individuals residing in Southeast Asia (Cooper 1998; Cooper and Haycocks 2000). Without treatment, the disease is universally fatal. The disease has reached epidemic proportions with the apparent epicenter located in the northern part of Thailand (Figure 2). Since the early 1990's, the Maharaj Nakorn Chiang Mai University Hospital in the northern regional of this country has recorded more than 1600 culture-proven cases of penicilliosis due to *P. marneffei*. However, based upon clinical symptoms rather than culture-proven diagnoses, more than twice that number of patients have been treated empirically for infection by *P. marneffei* (Cooper 2002). Recent reports have

now expanded the endemic area to include India and Taiwan (Hung, Hsueh et al. 1998; Singh, Ranjana et al. 1999). Moreover, increased numbers of *P. marneffei* infections have been diagnosed outside the endemic area to include cases in North America (Cooper 1998; Cooper and Haycocks 2000), Australia, France, Germany, Italy, the Netherlands, Sweden, and Switzerland (Duong 1996). The vast majority of these cases have involved individuals who had lived or traveled in the endemic region and subsequently developed disease due to HIV-induced immune dysfunctions. However, penicilliosis due to *P. marneffei* has been reported in those groups traditionally not considered to be at risk for infection, including 39 cases without any known predisposing factors (Cooper and Haycocks 2000). Three recent cases of penicilliosis caused by *P. marneffei* have been reported in one African and two Mexican patients (Lo, Tintelnot et al. 2000; Fuentes 2001). Curiously, these infections occurred in non-autochthonous individuals who had never visited Southeast Asia nor had direct or indirect contact with the fungus, such as in a laboratory setting. These observations indicate that *P. marneffei* may not be strictly limited to its known geographic distribution and brings to question the reservoir of *P. marneffei.*

The actual distribution of *P. marneffei* itself is unknown. Although, *P. marneffei* is well documented as being harbored in several species of bamboo rats (Cooper 1998), it is not known if these rats actually act as a reservoir, or if they are merely another natural host. To date, the environmental niche of

this pathogen and its means of transmission have yet to be elucidated (Restrepo, Baumgardner et al. 2000). Attempts to isolate this organism from the environment or other animals have to date been unsuccessful (Cooper and Haycocks 2000; Restrepo, Baumgardner et al. 2000).

Among the penicillia, *P. marneffei* is unique in that it is the only dimorphic species in this taxon (Cooper and Haycocks 2000) (Figure 3). This fungus grows at 25°C as a mould bearing numerous conidia. When conidia or hyphal fragments are incubated at 37°C, *P. marneffei* undergoes phase transition to form thick-walled, single cells that appear to reproduce by fission (Figure 4). Closer examination of these cells shows that they are actually meristematic arthroconidia. Presumably, arthroconidial development is requisite for pathogenesis since the in vivo presentation of *P. marneffei* strongly resembles the 37°C-induced morphology (Cooper and McGinnis 1997). Until recently, the underlying molecular mechanisms regulating mould-to-arthrocondia phase transition have received little attention. In one study, investigators observed that an *Aspergillus*-like *abaA* sequence in *P. marneffei* directly regulates conidiogenesis while the absence of *abaA* function inhibits normal arthroconidiogenesis at 37°C (Borneman, Hynes et al. 2000). Other molecular-oriented studies have focused on various genes that could potentially serve as morphological regulators of dimorphism (Borneman, Hynes et al. 2001; Boyce, Hynes et al. 2001; Borneman, Hynes et al. 2002). However, none of these genes appear to regulate development of

the arthroconidial phase of *P. marneffei*. Still, previous studies have shown that arthroconidial formation correlates with increased expression of glyoxylate cycle genes (N. G. D. Haycocks, M. R. McGinnis, and C. R. Cooper, Jr., unpublished data). Obviously, more studies in this area are needed to understand the molecular basis of phase transition and pathogenesis in this fungus.

To facilitate the molecular studies of *P. marneffei*, it would be advantageous to know the basic genomic architecture of this fungus. To date, only two published studies have examined the karyotype of *P. marneffei*. One epidemiologically-oriented investigation utilized pulse-field gel electrophoresis (PFGE) to compare the restriction endonuclease-digested genomes of various clinical *P. marneffei* isolates (Trewatcharegon, Sirisinha et al. 2001). Another study identified three chromosomes in *P. marneffei*, but the location of any genes was not determined (Wu, Guo et al. 1996). A separate unpublished study reported finding four chromosomes in *P. marneffei*, with sizes significantly different from those found in the previously published report (Szaniszlo 1996).

This study was designed to specifically address the question of genomic architecture in *P. marneffei*. The specific goal and objectives are listed below.

Goal:

To more fully and accurately characterize the *P. marneffei* genome utilizing current molecular methodologies.

Objectives:

1. Determine the number of chromosomes and the genome size of two different strains of *P. marneffei.*
2. Document any chromosomal polymorphisms that exist between the two strains of *P. marneffei* studied.
3. Establish the chromosomal location of *P. marneffei* genes of interest and selected homologues.

Figure 1.

Reported cases of penicilliosis by all species of *Penicillium*.
Most species of *Penicillium* are unable to cause infection in humans. However, of the few that have, greater than 99% of the cases are attributed to *Penicillium marneffei* (Cooper 2000).

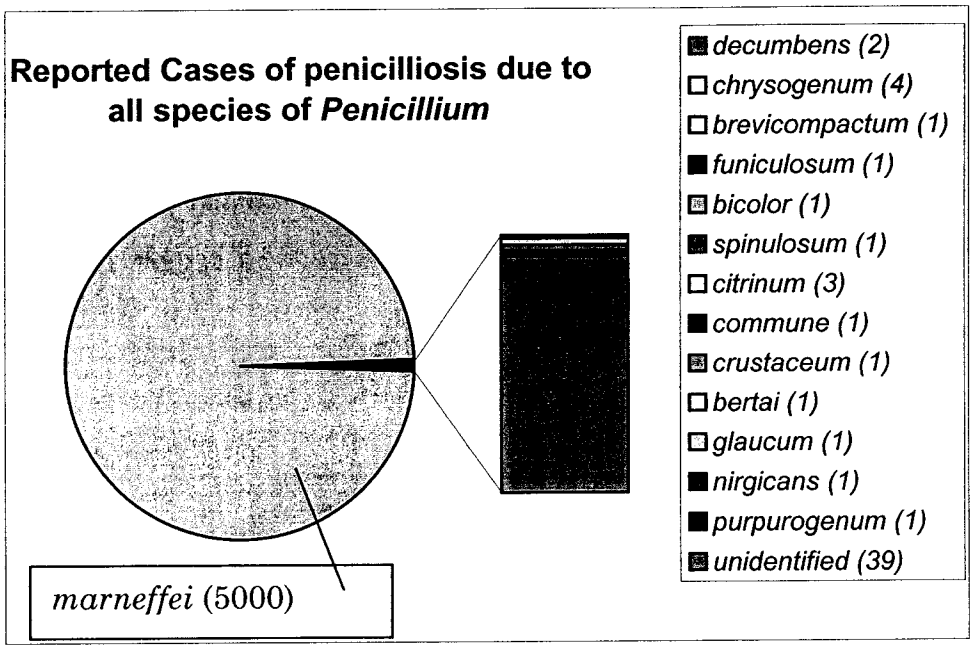
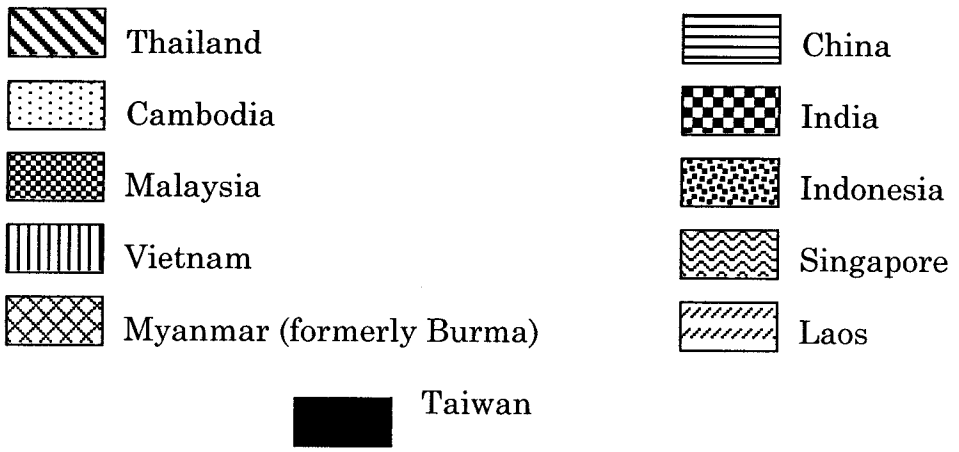

Reported Cases of penicilliosis due to all species of *Penicillium*
marneffei (5000)
decumbens (2)
chrysogenum (4)
brevicompactum (1)
funiculosum (1)
bicolor (1)
spinulosum (1)
citrinum (3)
commune (1)
crustaceum (1)
bertai (1)
glaucum (1)
nirgicans (1)
purpurogenum (1)
unidentified (39)

Figure 2.

Map of the identified endemic areas of penicilliosis due to *P. marneffei.*



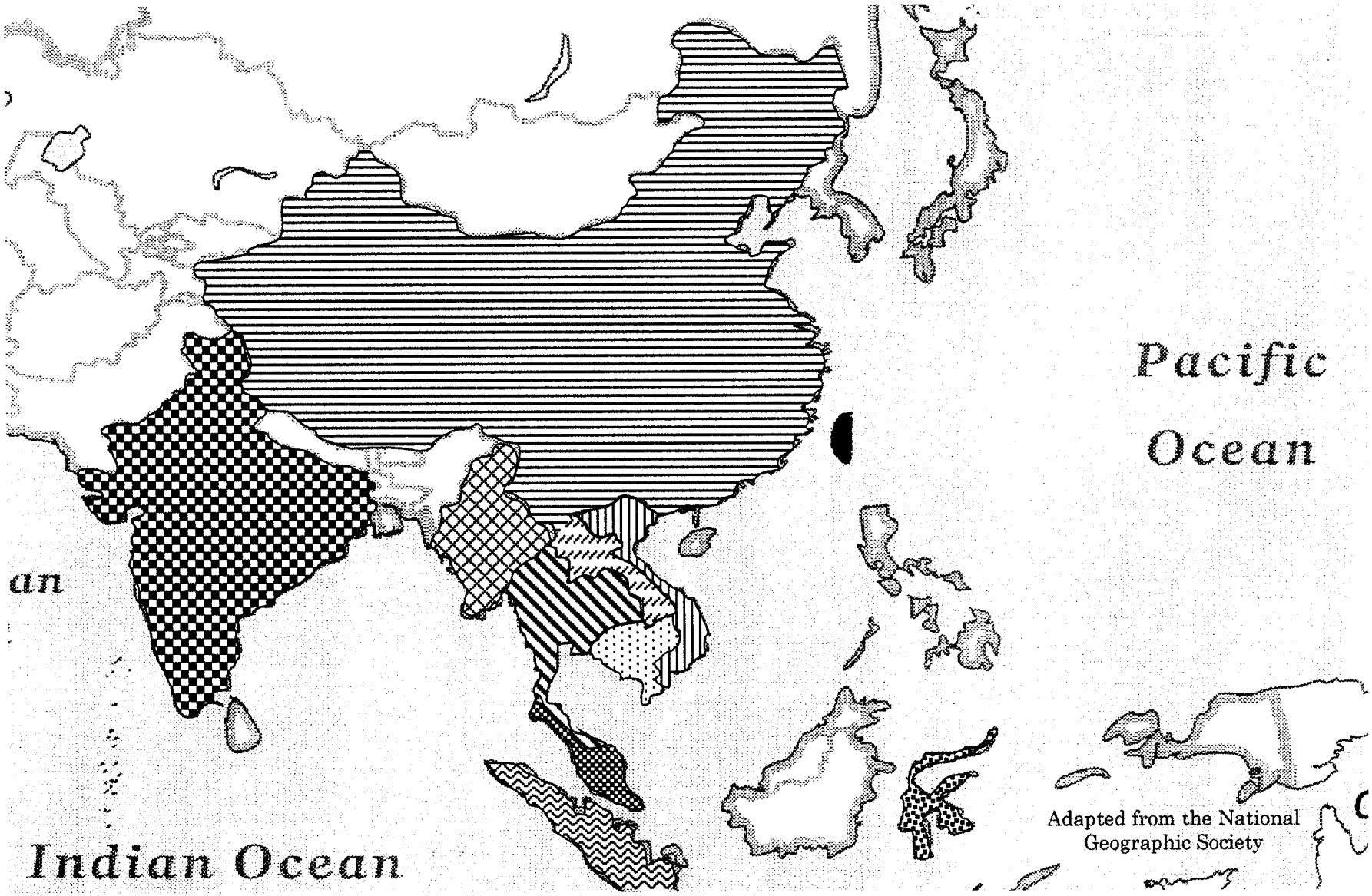

Pacific
Ocean
an
Indian Ocean
Adapted from the National
Geographic Society

Figure 3.

Colony morphology of *P. marneffei.*

A) Traditional "mold" form of *P. marneffei.* The haze surrounding this colony is pigment present only in the mycelial or "mold" form. This colony was incubated at 25°C.

B) Demonstrates the "yeast" or arthroconidial state of *P. marneffei.* The colony appears opaque with no pigment. This colony was incubated at 37°C.

Picture reproduced with permission of Dr. C.R. Cooper (Cooper and McGinnis 1997)

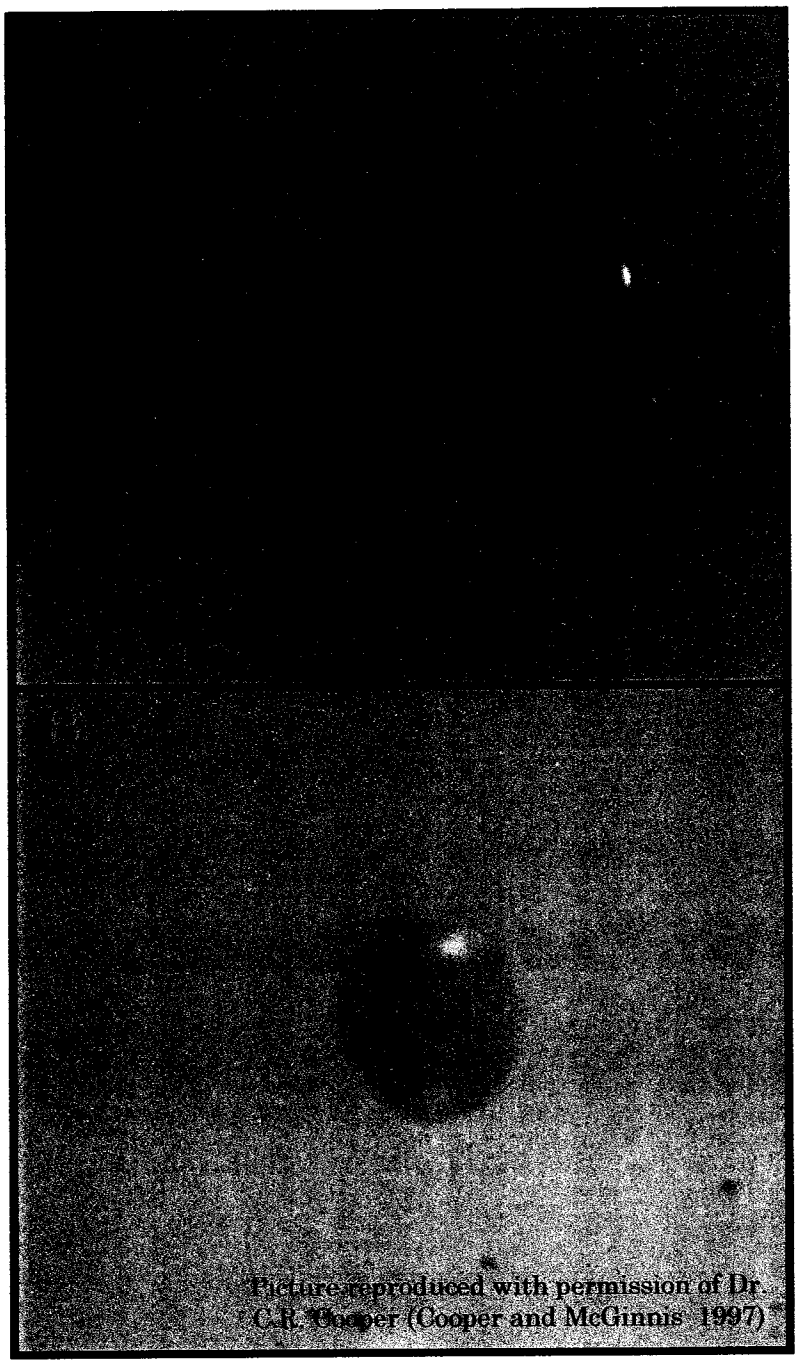


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Figure 4.

Dimorphism in *Penicillium marneffei.*
A) Typical penicillus structure bearing conidia of the mould phase at 25°C. B) Initial germination of conidia at 37°C results in the formation of short hyphae that eventually develop thickened septa (C) circumscribing individual arthroconidia. The arthroconidia subsequently are dispersed and continue to divide by fission at 37°C (D). If incubated at 25°C, instead, the mould form would develop (A).

The bar in each frame represents the following measurements:
A) 12 μm; B) 12 μm; C) 25 μm; D) 8 μm.

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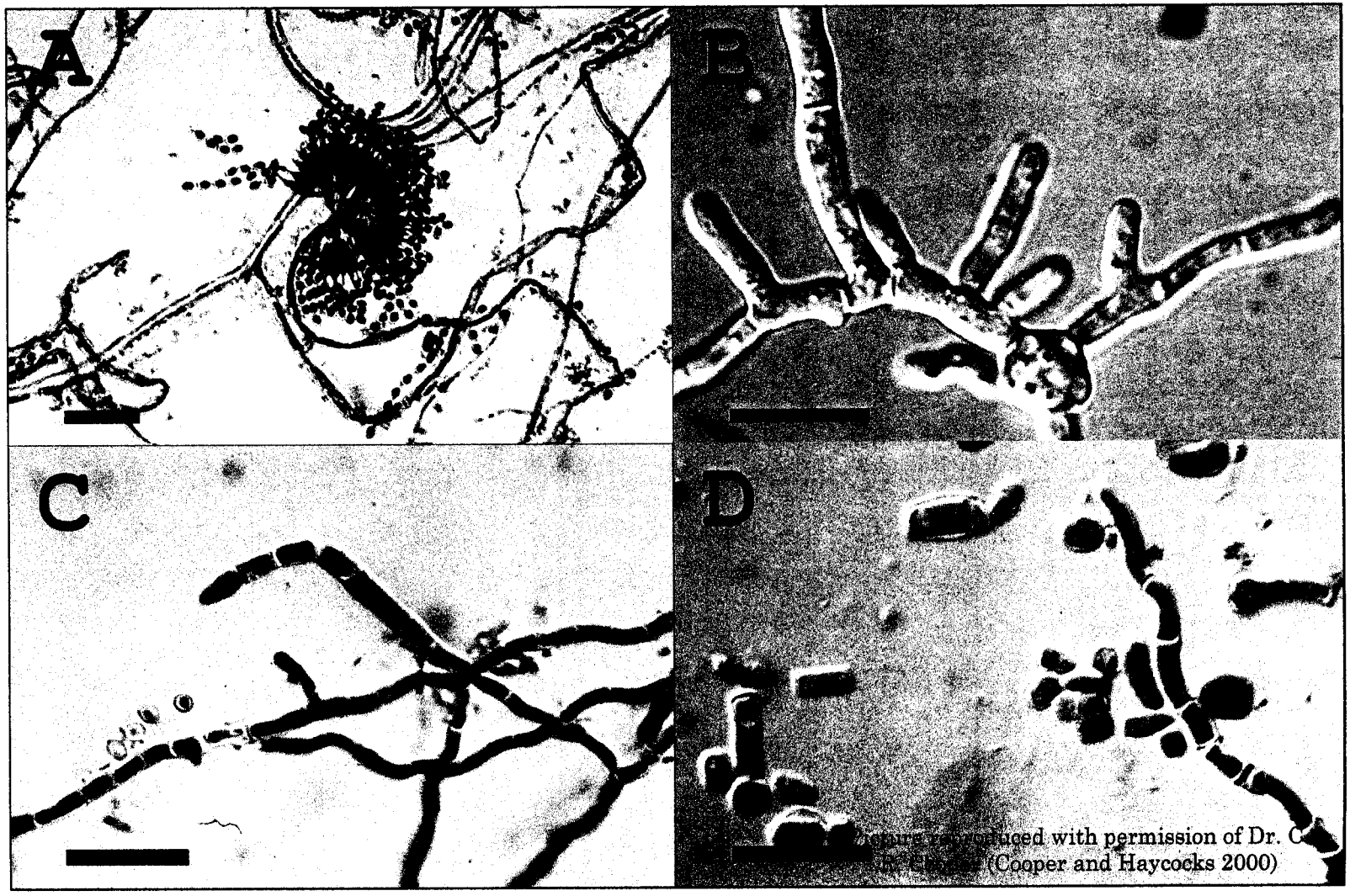


Picture reproduced with permission of Dr. C
[illegible] (Cooper and Haycocks 2000)

CHAPTER II

MATERIALS AND METHODS

FUNGAL STRAINS

Two strains of *P. marneffei* were used to derive an electrophoretic karyotype of this fungal species. Both strains, designated as Pm30 and PmF4, were isolated from HIV-positive individuals seen at the Maharaj Nakorn Chiang Mai University Hospital in Chiang Mai, Thailand. Strain Pm30, isolated from hemoculture in 1991, was among those isolates characterized in a previously published antifungal susceptibility study (Supparatpinyo, Nelson et al. 1993). Strain PmF4, a kind gift of Dr. Nongnuch Vanittanakom, was isolated in 1998 from a 36-year-old male patient who resided in the Mae Rim District of Chiang Mai, Thailand. Both strains were maintained on potato dextrose agar (Difco, Detroit, MI) slants.

PREPARATION OF AGAROSE-EMBEDDED CHROMOSOMES

To prepare intact chromosomes, conidia from strains Pm30 and PmF4 were used as inocula for broth cultures. The conidia from each strain were separately isolated following growth on potato dextrose agar slants for 7-10 days at 25°C. First, the surfaces of several slant cultures were washed with 7-10 ml of sterile 0.9% sodium chloride. The resulting suspension was passed through a sterile glass wool separation unit to remove hyphal fragments. The separation unit consisted of a 1 inch wedge of glass wool (Corning, Acton, MA; Catalogue number 3950-9989) placed between two screened caps (Bio-Rad, Hercules, CA; Catalogue number 170-3711) secured to each other by means of their screwed threads. The unit was wrapped in aluminum foil, then sterilized by autoclaving before use. Subsequently, the unit was secured to the end of a sterile 50-ml conical, screw-capped centrifuge. The *P. marneffei* cell suspension was transferred into the top screened cap tube, then enclosed with the cap from the centrifuge tube. Next, conidia were isolated by centrifugation of the completed separation unit in a swinging bucket rotor until a relative centrifugal force (RCF) of 100-150 x g was obtained. At the moment the proper RCF was attained, the centrifuge was switched off and the rotor was permitted to slow without braking until it stopped. The fluid that passed through the unit contained >99.9% conidia whereas the hyphae in the original suspension were retained by the glass

wool. The conidia, consisting mainly of individual cells, were counted in a haemocytometer, then used to inoculate Sabouraud glucose broth (Difco) at a final concentration of 1 x 10^7 conidia per ml. The conidial culture was incubated at 25°C under shaking conditions for 8-15 hours to produce germlings. The germlings were subsequently collected by centrifugation (RCF approximately 1250-2500 x g) for 10 minutes at room temperature. Agarose-embedded, intact chromosomes were prepared from these germlings using the Yeast Genomic DNA Plug Kit (Bio-Rad) according to the instructions provided by the manufacturer. All resulting agarose plugs were stored at 4°C until used. Each agarose plug was estimated to contain the chromosomal DNA from approximately 6 x 10^7 cells in a total plug volume of 100 μL.

PULSE-FIELD ELECTROPHORESIS

Chromosomes of the *P. marneffei* strains were separated by contoured-clamped homogeneous electric fields (CHEF)-gel electrophoresis (Chu, Vollrath et al. 1986; Birren and Lai 1993) using either a CHEF DRIII System (Bio-Rad) or a CHEF Mapper XA System (Bio-Rad). (Figure 5) The agarose (Agarose III) and buffers employed in these experiments were purchased from Amresco (Solon, OH). Separation of the agarose embedded chromosomes was accomplished by loading the well of a 13 cm x 14 cm agarose gel with the

plug containing *P. marneffei.* The plug was sealed within the well by covering the opening of the well with a sufficient quantity of the same agarose used to cast the gel. The loaded gel was next placed within the electrophoresis box of the CHEF Mapper or DRIII unit. Prior to beginning the run, the gel was allowed to equilibrate for 15 minutes with the chilled running buffer. Depending on the size range of chromosomes to be resolved, three separate sets of conditions were used to resolve the chromosomes (Table 1). Commerically purchased chromosome size standards of *Schizosaccharomyces pombe, Hansenula wingeii* and *Saccharomyces cerevisiae* (all Bio-Rad) were used in these experiments. Following electrophoresis, the chromosomes were stained by soaking the gel for 30 minutes at room temperature in gel running buffer containing ethidium bromide (0.5 μg/ml). Using the briefest exposures possible, stained chromosome images were captured either as photographs or as digital images. Photographs were made on type 667 film (Polaroid, Cambridge, MA) using an ultraviolet light transilluminator and subsequently digitally scanned. Alternatively, direct digitized images were derived using the Eagle Eye II Imaging System (Stratagene, La Jolla, CA). All digitized images were processed using Photoshop software (version 5.0; Adobe Systems, San Jose, CA).

LOCATION OF SELECTED CHROMOSOMAL LOCI

The specific chromosomal location of selected genes was determined by Southern blot analysis. Chromosomes resolved by CHEF gel electrophoresis were transferred to a charged nylon membrane (Zeta Probe GT, Bio-Rad) after staining and photographing by alkaline method (Birren and Lai 1993). To accomplish the transfer, the stained gel was UV irradiated with 60 mJoules of energy using a FisherBiotech UV XL 1000 crosslinker (Fisher Scientific). The gel was then soaked for 15 minutes in transfer solvent (0.4 N NaOH, 1.5 M NaCl) and then placed into a capillary transfer stack (Figure 6). Two liters of fresh transfer solvent was used to transfer the nicked DNA. After the transfer was completed (24 hours), the nylon membrane was removed and neutralized in 0.5 M TRIS (pH 7) for 5 minutes and then rinsed in 2 X SSC (Amresco).

The immobilized chromosomes were then probed with various *P. marneffei* gene fragments. All probes were amplified in a polymerase chain reaction (PCR) using gene-specific or –degenerate primers. Probes for isocitrate lyase (*PmICL1*), malate synthase (*PmMLS1*), isocitrate dehydrogenase (*PmICD1*), chitin synthase (*PmCHS1, PmCHS2, PmCHS3, and PmCHS4*), and chitinase (*PmCHT1*) were generously provided by other investigators (Table 2). A probe for 5.8S rDNA with associated internal transcribed spacer sequences (*PmITS*) was generated using primers ITS1 and

ITS4 in a PCR performed as previously described (White et al). Another probe for 23S rDNA was similarly created using primers generously provided by Dr. Gary Nunn (Applied Biosystems, Foster City, CA) (primer sequences are proprietary and not available for publication). The fragments were then inserted into a plasmid vector utilizing the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The isolated plasmid DNA was then sequenced using the CEQ Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, CA) to assure that the desired gene fragment had been isolated.

The plasmid constructs containing the gene fragments were then labeled, hybridized to target molecules, and the resulting chemiluminescent signals detected using the ECL DNA Labelling and Detection Kit (Amersham Pharmacia Biotech, Piscataway, NJ) according the to manufacturer's instructions (Figure 7). Both moderate and highly stringent hybridization conditions were used. Hyperfilm ECL media (Amersham) was used to capture the hybridization signals during a 30 minute exposure. The film was developed by hand using standard techniques with GBX developer and fixer (Eastman Kodak, Rochester, NY). To assign the chromosomal location of the hybridized signals, the hybridization signals on the film were compared to the karyotype images rendered by the initial ethidium bromide staining. Digital images were obtained by scanning these films and processing them using Photoshop software.

ESTIMATE OF CHROMOSOME SIZE

The sizes of particular *P. marneffei* chromosomes were estimated based upon their mobility relative to the known sizes of the *S. pombe* chromosomes and the largest of the *S. cerevisiae* chromosomes. The sizes were determined using the linear regression features of the Microsoft Excel (Figure 8). and the Eagle Eye II Imaging System software programs These methods utilize the known size of the commercial standards and the distance they traveled within the gel to estimate the sizes of the *P. marneffei* chromosomes.

TELOMERE DETERMINATIONS

The genome of *P. marneffei* strain Pm30 was analyzed by Southern blot analysis for telomere-like sequences. Genomic DNA was isolated utilizing the following method adapted from previously published studies (Cooper, Breslin et al. 1992; Vanittanakom, Cooper et al. 1996). Conidia was harvested from 7 to 10 day old slant cultures as previously described. The conidia were filtered and inoculated in broth to produce germlings as described. The cells were spun down and washed in 10 volumes of CPES buffer (20mM Sodium Citrate, 50mM Na_2HPO_4, 50mM EDTA, 0.9 M D-Sorbitol, pH to 5.6). They were next re-suspended in 10 volumes of fresh

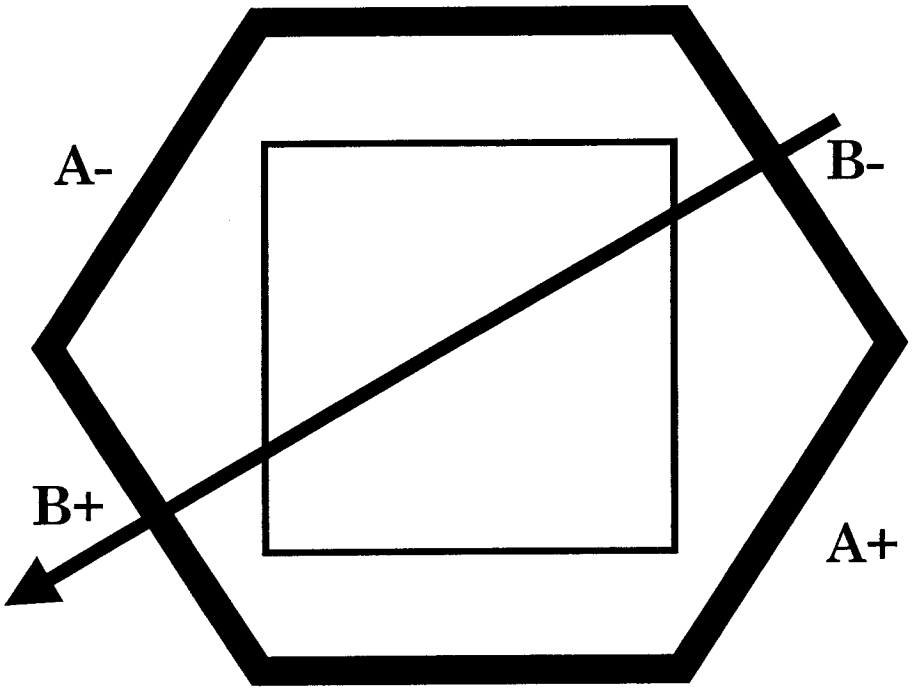
CPES buffer containing 0.1 M β-mercaptoethanol and 30 μL of Lyticase (Biorad) per mL of suspension. The suspension was allowed to incubate at 37°C for 4 hours before collecting the spheroplasts by centrifugation (2400 x g for 10 minutes). The collected spheroplasts were suspended in 10 mL of breaking buffer (2% Triton X100, 1% SDS, 1mM EDTA, 100 mM NaCl and 10 mM TRIS adjusted to pH 8.0) and vortexed briefly prior to incubation at 65°C for 1 hour. Following incubation, 2.75 mL of 5 M potassium acetate was added and the solution mixed by inversion followed by incubation on ice for 1 hour. The suspension was centrifuged (2400 x g for 10 minutes) and the supernatant removed to a clean tube. An equal volume of 2-propanol was added, mixed by inversion, and allowed to incubated at -20°C for 1 hour. The precipitate was next collected by centrifugation (2400 x g for 10 minutes) and re-suspended in sterile water. This suspension was extracted with phenol, phenol-chloroform, and chloroform, and precipitated with ethanol. The precipitate was collected by centrifuge and allowed to dry prior to suspension in TE buffer (10mM TRIS, 1mM EDTA, pH 8) and treatment with 50 μg/mL of RNase A (Amresco) to remove any RNA present.

Approximately 7 μg of isolated genomic DNA from strain Pm30 was digested in separate reactions with restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII *Pst*I, *Pvu*II, *Sal*I, and *Xho*I (all Promega, Madison, WI) used in accord with the manufacturer's instructions. The resulting digests and pertinent DNA size standards (Amresco) were mixed with loading dye

(Amresco), then loaded into the wells of a 1% agarose (Agarose IV; Amresco) gel prepared in 1X TAE buffer (Amresco). Subsequently, the samples were subjected to horizontal gel electrophoresis for 10 hours at 1 V/cm using 800 mL of the same buffer. Next, the resolved DNA fragments were transferred to a charged nylon membrane (Zeta Probe GT; Bio-Rad) using the alkaline conditions described previously and crossed linked with 60 mJoules UV radiation. The resulting blot was probed using an oligomer ($TTAGGG_6$) (IDT Technologies, Coralville, IA) specific for the telomeres of filamentous fungi (Levis, Giraud et al. 1997; Young, Itoh et al. 1998; Abdennadher and Mills 2000). The probe was first end labeled with fluorescein (Gene Images 3' Oligolabeling Module; Amersham, Piscataway, NJ), then hybridized to the blot using Ultrahyb buffer (Ambion, Austin, TX) in accord with the manufacturer's instructions. Using stringent wash conditions, hybridization signals were captured on Hyperfilm ECL media (Amersham) by employing a chemiluminescent detection method (Gene Images CDP-Star Detection Module; Amersham). Digital images were obtained by scanning and processing these films as previously described.

Firgure 5.

Clamped homogeneous electric fields gel electrophoresis.
This diagram represents the manner in which separation of large DNA molecules is achieved. By alternating the electric field between electrodes placed on different sides of the chamber, it causes large DNAs to reorient and move at different speeds through the pores of the gel. Initially, electrode bank A is switched on, and at a given time interval, bank A is turned off and bank B is switched on. This causes a zig zag pattern of migration that results in a net downward travel and separation of the DNAs.


A-
B-
B+
A+

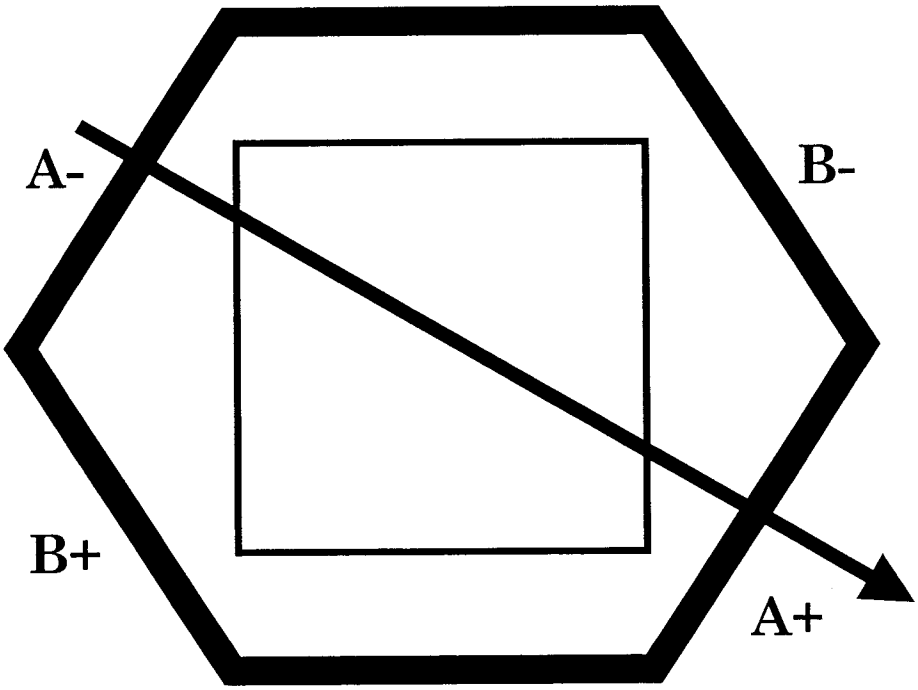
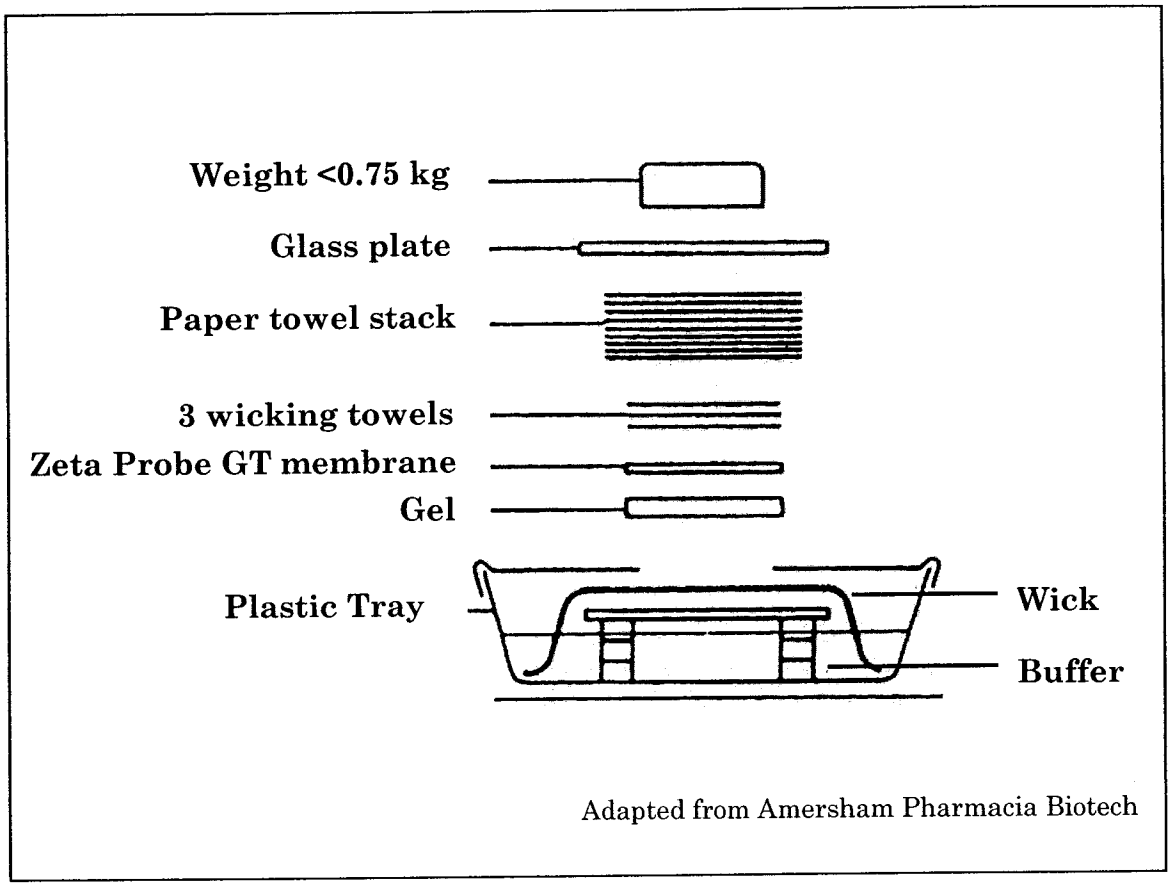

A-
B-
B+
A+

Figure 6.

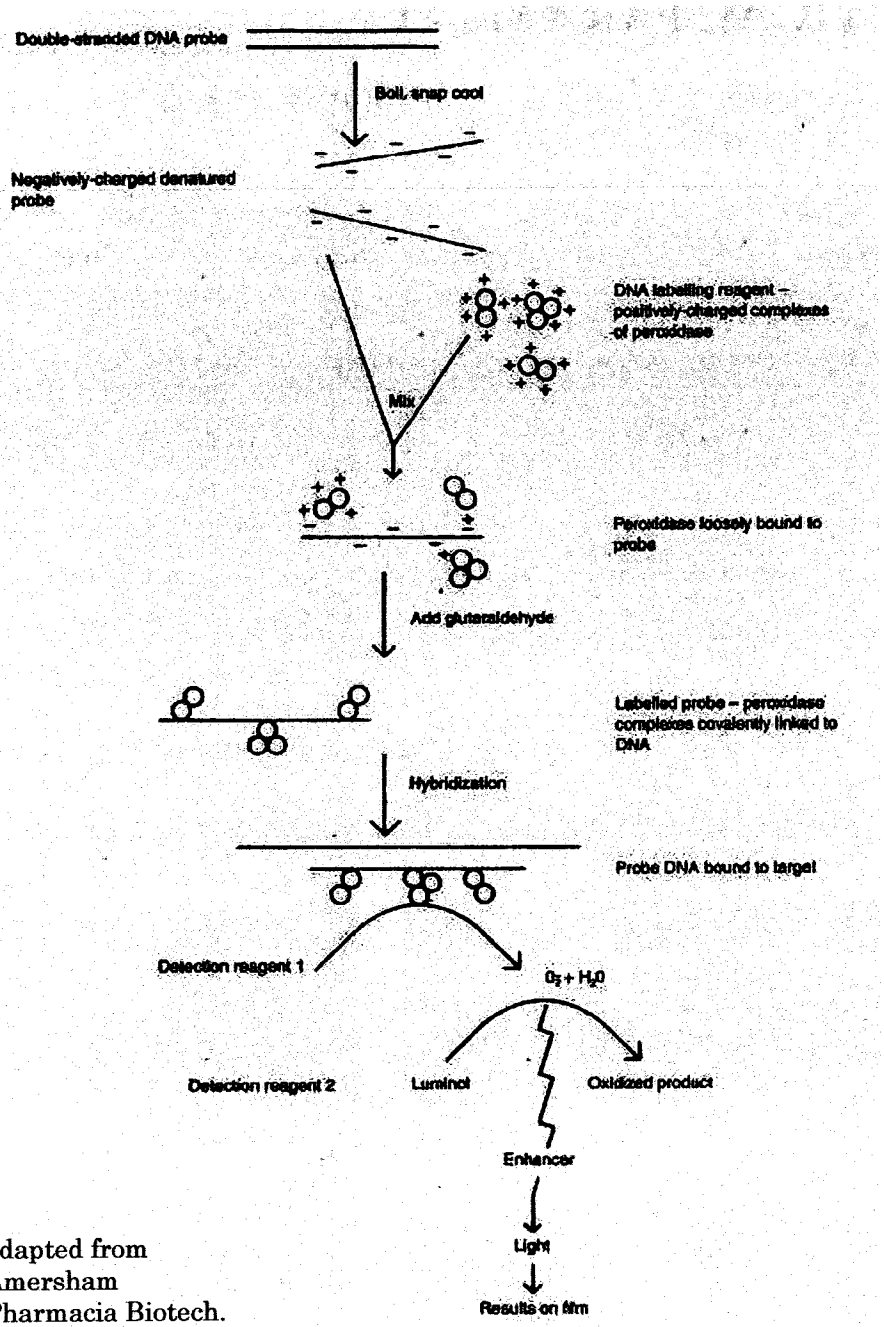
Southern blot method.
The alkaline method to transfer large DNAs is depicted in this figure. Capillary action of the paper towels above the gel pulls solvent buffer through the gel into the towels. The solvent passes through the nylon membrane positioned between the paper towels and the gel where DNA is trapped. Prior to transfer, the gel is UV irradiated to cut the large DNA into smaller pieces thereby making the transfer more efficient. Diagram adapted from adapted from Amersham Pharmacia Biotech.



Adapted from Amersham Pharmacia Biotech

Figure 7.

Principle of direct nucleic acid labeling and detection.
The DNA probe is denatured and labeled with peroxidase. Gluteraldehyde is used to covalently bind the peroxidase to the single stranded DNA. Following hybridization of the probe with the target DNA, detection reagents are added to cause decay of the peroxidase and detectable light is emitted which is captured on light sensitive film. Diagram adapted from Amersham Pharmacia Biotech.



adapted from
Amersham
Pharmacia Biotech.

Figure 8.

Determination of chromosome size based on distance traveled.
By plotting the distance traveled of known size standards, it is possible to derive the representative line equation. By measuring the distance traveled of the unknown chromosome and solving the derived equation, it is possible to determine the unknown chromosome size with some degree of accuracy. These calculations were also verified by using Eagle Eye imaging software, which performs similar calculations upon the obtained gel photograph.

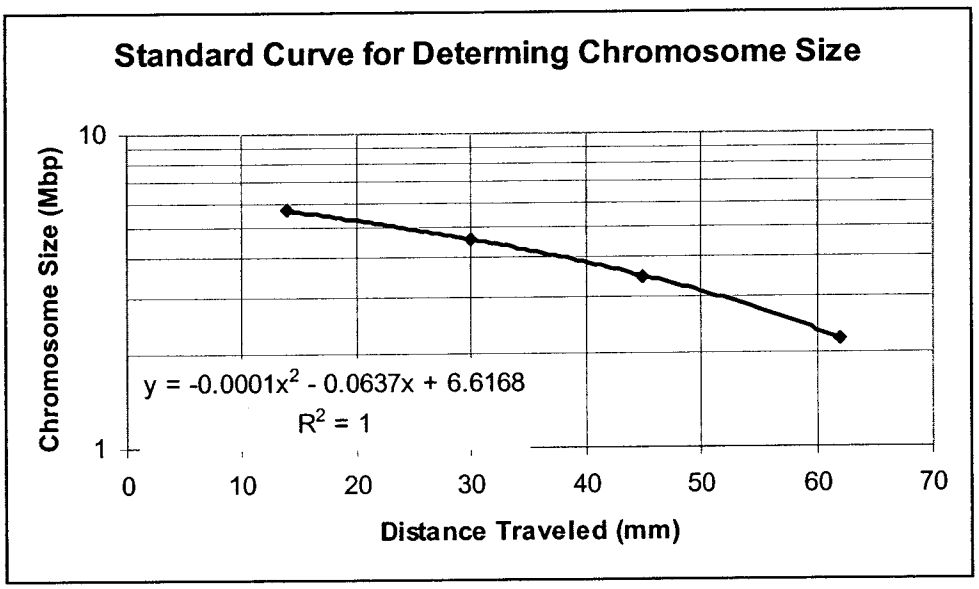

Standard Curve for Determing Chromosome Size
Chromosome Size (Mbp)
10
1
y = -0.0001x2 - 0.0637x + 6.6168
R2 = 1
0
10
20
30
40
50
60
70
Distance Traveled (mm)

Table 1.

Conditions used for CHEF gel electrophoresis.
The table details the conditions employed to separate chromosomal size DNAs.
Condition A was utilized for routine separation.
Condition B was employed to resolve the two least mobile chromosomes.
Condition C was designed to gain greater resolution between chromosomes I and II. This condition employed 3 differing conditions or "blocks" within the program.

Conditions Used for CHEF gel electrophoresis

	% Gel	Buffer and concentration	Temperature (C)	Switch Time (min) Beginning	Switch Time (min) Ending	Running Time (Hours)	Angle of Inclusion	Voltage (V/cm)
Condition A	0.5	0.5 X TBE	8	55	90	168	106	1.3
Condition B	0.8	1.0 X TAE	14	20	30	48	106	2.0
Condition C	0.5	0.5 X TBE	10	50 45 37	50 45 37	70 18 70	106	1.4

Table 2.

Probe sources for *P. marneffei* gene fragments.

This table denotes probe designations and the gene for which it was utilized to locate. The source for each probe is also identified.

Probe sources for *P. marneffei* gene fragments.		
Probe Designation	**Gene**	**Source**
PmICL1	isocitrate lyase	N. Haycocks (2001)
PmICD1	iscitrate dehydrogenase	N. Haycocks (2001)
PmMLS1	malate synthase	N. Haycocks (2001)
PmCHS1	chitin synthase	C.R. Cooper
PmCHS2	chitin synthase	C.R. Cooper
PmCHS3	chitin synthase	C.R. Cooper
PmCHS4	chitin synthase	C.R. Cooper
PmCHT1	chitinase	Patthama Pongpom
PmITS	5.8S rDNA and ITS	This study
23S rDNA	large subunit rDNA	This study

CHAPTER III

RESULTS

PULSED FIELD GEL ELECTROPHORESIS

Initially, agarose plugs containing intact chromosomes from *P. marneffei* were resolved in the shorter running condition described previously and labeled as "Condition A" (Table 1) Under these conditions, the expected karyotype patterns of the *S. pombe* and *S. cerevisiae* standards were resolved (Figure 9; lanes 1 and 4, respectively) (Birren and Lai 1993). Many of the chromosomes from *P. marneffei* strains Pm30 and PmF4 were also resolved using these parameters. Strain PmF4 clearly possesses at least five distinct chromosomes designated I (most mobile) through V (least mobile) (Figure 9; lane 3). In contrast, only four chromosomes of strain Pm30 were visible (Figure 9; lane 2). The sizes of the two most mobile chromosomes of strain Pm30, chromosomes I and II, appear to be equal in size (2.0 Mbp and 3.1

Mbp, respectively) to those resolved in strain PmF4. However, the sizes of chromosomes III, IV, and V in strain PmF4 appear to differ from chromosomes III and IV in strain Pm30. For strain Pm30, the respective chromosomes are 4.2, and 6.1 Mb in size. In contrast, in strain PmF4 these chromosomes are 4.1, 5.1, and 5.9 Mb in size. It should be noted, however, that for both *P. marneffei* strains the mobility of chromosomes I, V (PmF4), and IV (Pm30) fall outside the range covered by the size standards. While the calculated size for chromosome I appears to be reasonably accurate, based upon its migration compared to those of the *S. cerevisiae* chromosomes, the size estimate for larger chromosomes may be in error. The calculation of such high molecular weight chromosomes is difficult to assess since its mobility is limited. Also, this area of the gel is known to be prone to certain physical phenomena that affect accurate estimate of sizes (Birren and Lai 1993). Finally, intact chromosome-like molecules that resolve in this area of the gel are known to sometimes contain more than one chromosome.

To better estimate the size of chromosome V as well as to determine if it consists of a single linkage group, a different series of pulse-field electrophoresis experiments were conducted. In these investigations, agarose-embedded chromosomes of *P. marneffei* were resolved under "Condition B." Using these alternative CHEF electrophoresis conditions, the general pattern of the resolved chromosomes remained the same. However, the differences in size between chromosome V in strain Pm30 and its

counterpart in strain PmF4 were clear (Figure 10). The size estimate for chromosome V in strain PmF4 was the same as previously calculated (5.9 Mbp). Yet, chromosome IV in strain Pm30 showed remarkable disparity having an estimated size of 7.6 Mbp. Again, both of these size estimates must be regarded with some degree of skepticism given that the mobility of this chromosome lies far beyond the range of the size standard. Nonetheless, the results from this series of experiments suggest that the band representing chromosome the largest of both *P. marneffei* strains consists of a single molecule.

TELOMERE NUMBER

To help confirm the number of chromosomes in *P. marneffei*, a telomeric probe was employed in a Southern blot analysis of digested genomic DNA. In such an experiment, two hybridization signals should be detected per chromosome since telomeres represent the ends of these molecules. Given that the molecular evidence suggests that *P. marneffei* is haploid (Borneman, Hynes et al. 2000; Borneman, Hynes et al. 2001; Boyce, Hynes et al. 2001; Borneman, Hynes et al. 2002), and because the restriction endonucleases employed do not cut within the telomeric sequence, the number of chromosomes in this fungus can be estimated by dividing the number of hybridization signals per digest by two. Similar methods were

used to estimate total chromosome numbers in *Ustilago hordei* and *Penicillium paxilli* (Young, Itoh et al. 1998; Abdennadher and Mills 2000).

When probed with the telomeric sequence, between 11 and 12 hybridization signals were detected in *Sal*1 digested DNA of strain Pm30 (Figure 11). These results suggest that *P. marneffei* actually possesses a minimum of six chromosomes. Because the hybridization signal of two or three of the *Sal*1-produced bands appear more intense, it is conceivable that these represent telomere doublets. Hence, strain Pm30 may actually possess seven chromosomes. *However*, only four chromosomes in strain Pm30 can be resolved by CHEF (Figures 7 and 8) or by hybridization of specific gene fragments (see below). A closer examination of the karyotypes resolved by CHEF shows that chromosomes I and II fluoresces more intensely following ethidium bromide staining as compared to chromosomes III and IV. This may indicate that either or both of the resolved bands labeled as chromosomes I and II may actually possess two different chromosomes of nearly equal size. Hence, attempts were madeto more fully separate chromosomes I and II under "Condition C." Surprisingly, this experiment produced evidence that chromosome III is actually two similarly sized chromosomes (Figure 12). Chromosomes I and II remained as single, brightly stained bands. Under these conditions chromosome III gains some greater resolution and appears as two possible chromosomes, now designated IIIa

and IIIb. This suggestion is consistent with the hybridization patterns of various gene probes (see below).

GENE LOCALIZATION

To assess the chromosomal location of known *P. marneffei* genes, various CHEF gels derived by conditions producing the karyotype depicted in Figure 9 (Condition A) were subjected to Southern blot analysis (Figure 13). The chromosomal location of specific genes is summarized in Table 3. Each chromosome was determined to hybridize at least one of the probes employed. Analogous results were obtained by hybridization analysis of the karyotype derived by the second CHEF electrophoresis condition (Condition B) (data not shown).

Of particular interest are the hybridization patterns of the chitin synthase and chitinase probes. The chitin synthase gene fragments, represented by *PmCHS1*, *PmCHS2*, and *PmCHS3*, bound to multiple chromosomes at different intensities depending upon the level of the stringency washes employed. These gene fragments are highly homologous at the nucleotide sequence level (Cooper 2001, unpublished data), thus explaining the presence of the multiple hybridization signals even under high stringency conditions. Based upon the intensity of the signal from the

individual probes, it is postulated that *PmCHS1*, *PmCHS2*, and *PmCHS3* are located on chromosomes II, I, and III (IIIa/b unresolved complex in Pm30), respectively. The fourth chitin synthase gene fragment used as a probe in this study, *PmCHS4*, also bound to multiple chromosomes. Again, based upon the intensity of hybridization, *PmCHS4* was located on the smallest chromosome (IV) in strain Pm30, whereas this gene was found on a larger chromosome (IV) in strain F4. It is significant to note that *PmCHS4* represents an entirely different class of chitin synthases and has no homology to the other chitin synthase probes employed in this study (Cooper 2001, unpublished data). The multiple signals observed with this probe probably result from homology with chitin synthase genes of other classes not represented by *PmCHS1*, *PmCHS2*, or *PmCHS3*. Multiple classes of *PmCHS4*-like genes are known to exist in *Aspergillus*, a fungus closely-related to *P. marneffei* (Mellado, Aufauvre-Brown et al. 1995; Specht, Liu et al. 1996).

Similarly, multiple hybridization signals were noted when *PmCHT1*, a DNA sequence that encodes a putative of the *P. marneffei* chitinase gene (Cooper, Pongpom et al. 2001), was used as a probe. Previous laboratory work suggests that *P. marneffei*, like other fungi (Yanai, Takaya et al. 1992; McCreath, Specht et al. 1995; Pishko, Kirkland et al. 1995; McCreath, Specht et al. 1996), possesses multiple chitinase gene homologues (unpublished data). The results from the present study indicate that *PmCHT1* binds to

chromosomes I and II in both strains, thereby supporting speculation that there are at least two chitinase genes in *P. marneffei.*

Many of the gene fragments used as probes in this study hybridized to various chromosomes (Table 3). Several of these genes hybridized to either or both chromosomes I and II. By definition, genes that hybridize to the same chromosome are linked. However, this may not be entirely true. Based upon the greater staining intensity of chromosomes I and II as compared to the other chromosomes, as well as the results from the telomere hybridization experiments (see above), each of the two most mobile chromosomes (I and II) may actually consist of two chromosomes. These molecules may be so close in size and composition that they cannot be resolved under the electrophoretic conditions employed in this study. Clarification of this possible caveat will require further mapping studies following the physical isolation of chromosomes I and II from *P. marneffei.*

Figure 9

Resolution of *P. marneffei* chromosomes under "Conditions A".
The ethidium bromide-stained gel depicts agarose-embedded chromosomes of strains Pm30 and PmF4 subjected to CHEF electrophoresis. Standard sizes are depicted to establish *P. marneffei* chromosome sizes.
Sc – *S. cerevisiae* standard
30 – Pm30
F4 – PmF4
Sp – *S. pombe* standard

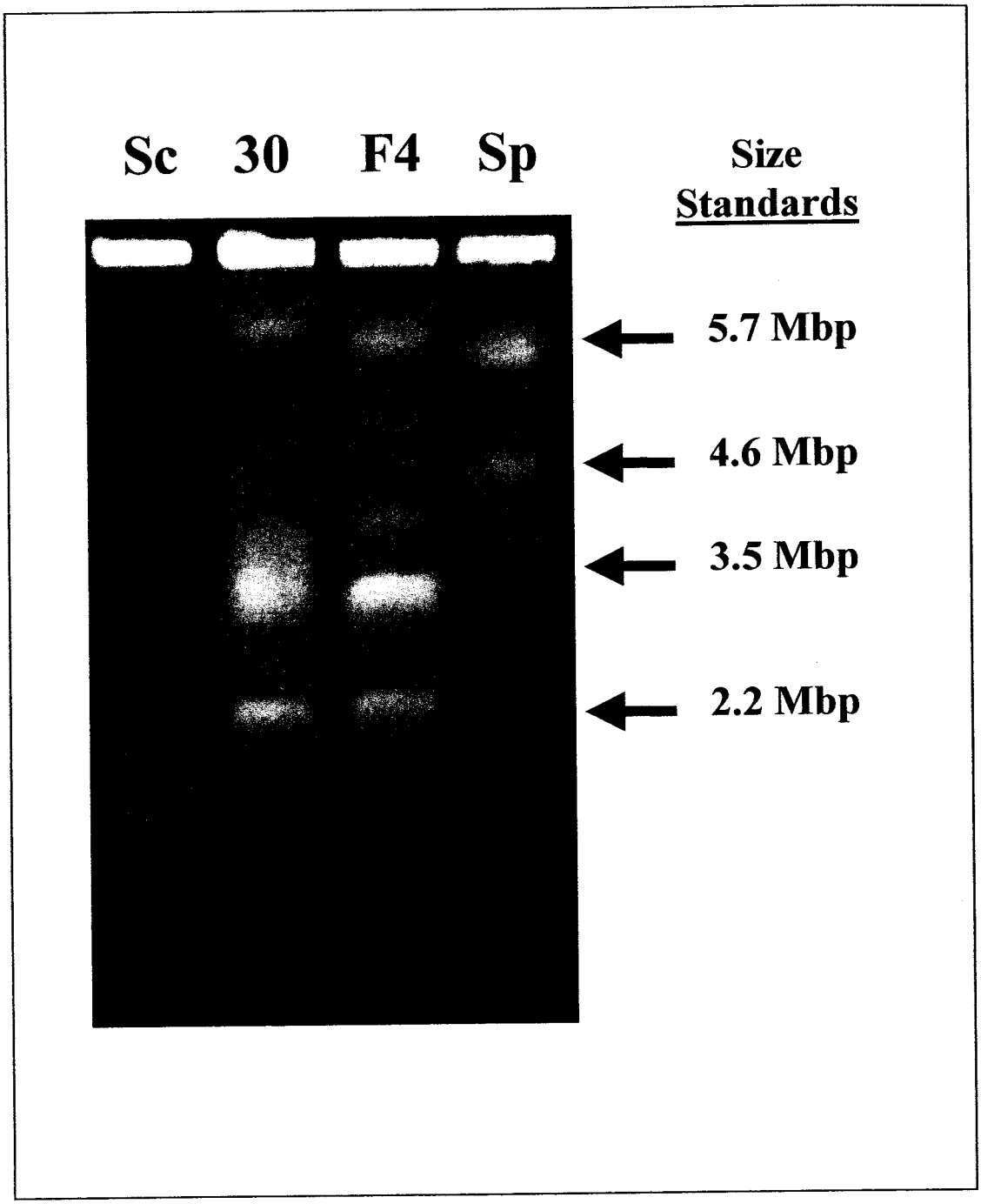

Sc
30
F4
Sp
Size
Standards
5.7 Mbp
4.6 Mbp
3.5 Mbp
2.2 Mbp

Figure 10.

Resolution of *P. marneffei* chromosomes under "Condition B"
This extended run makes it possible to estimate the sizes of the two least mobile chromosomes as 7.6 Mbp and 6.0 Mbp. It further demonstrates that these two large chromosomes are resolved and do not contain additional similarly sized chromosomes.
Sp – *S. pombe*
F4 – PmF4
30 – Pm30

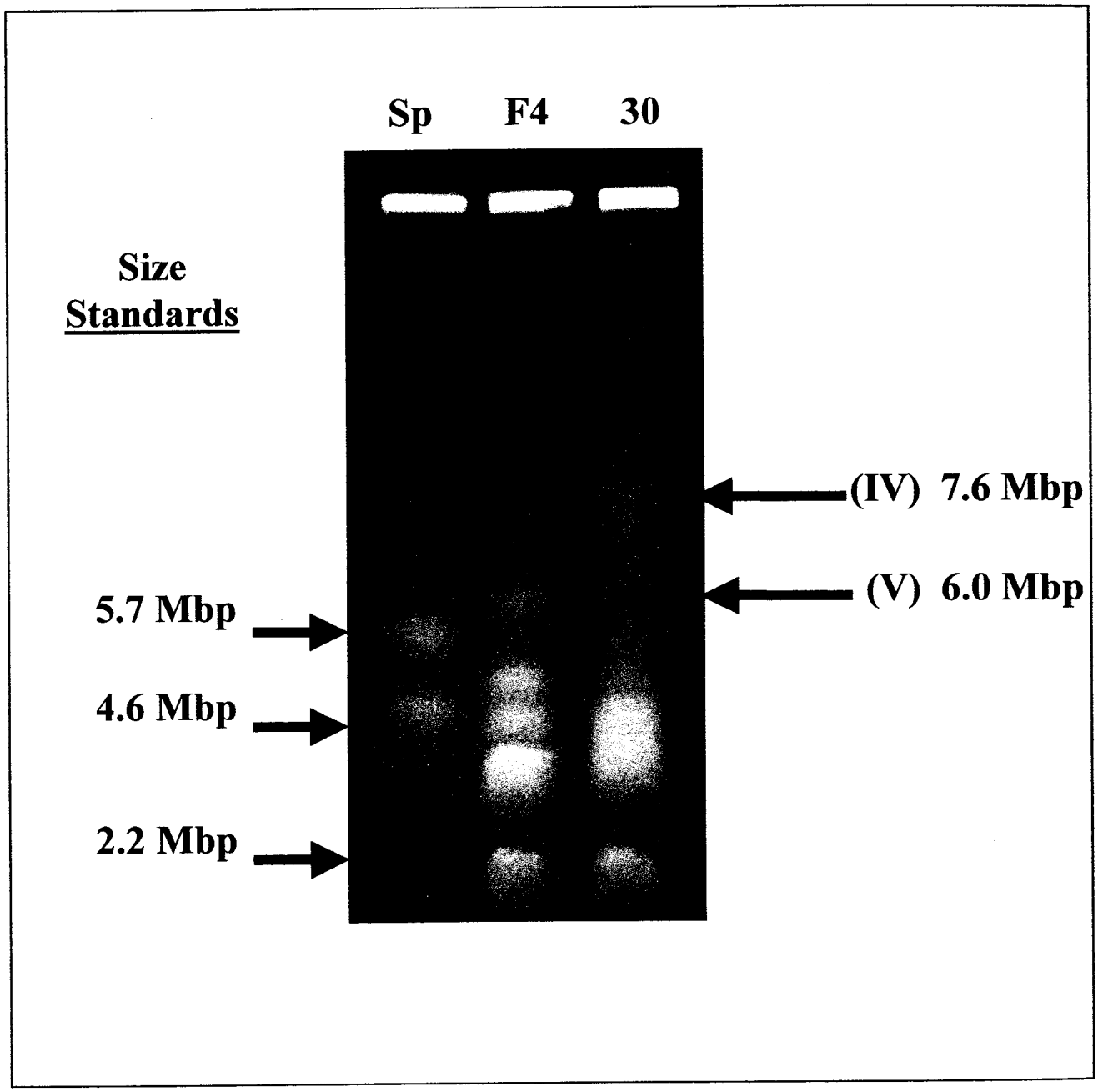

Sp
F4
30
Size
Standards
(IV) 7.6 Mbp
(V) 6.0 Mbp
5.7 Mbp
4.6 Mbp
2.2 Mbp

Figure 11.

Restriction digest and telomeric probing of *Penicillium marneffei*
Strain Pm 30 genomic DNA was digested with nine different restriction enzymes. The resulting DNA fragments were probed with telomeric repeats to confirm the total number of chromosomes present. Lane designations: 1, *Xho*I; 2, *Sal*I; 3, *Xba*I; 4, *Eco*RI; 5, *Pst*I; 6, *Hin*D III; 7, *Bam*HI; 8, *Pvu*II; 9, *Eco*RV; 10, undigested DNA. Numbered arrows identify at least 12 telomeric labels in the *Sal*I restriction. As two telomere segments are present within each whole chromosome, this indicates at least six total chromosomes are present.

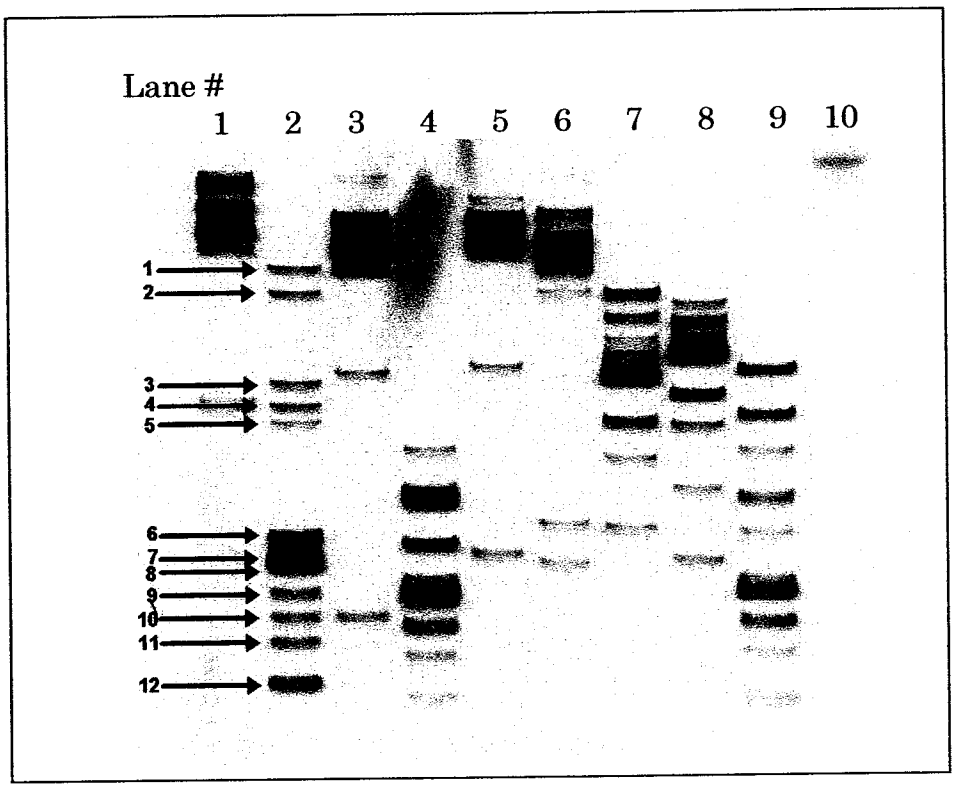

Lane #
1 2 3 4 5 6 7 8 9 10
1
2
3
4
5
6
7
8
9
10
11
12

Figure12.

Resolution of *P. marneffei* chromosomes under "Condition C"
This condition shows two important pieces of data. First, it clearly shows the elusive chromosome IV (which previously was only detected by Southern blot analysis). Additionally, chromosome II is resolved into a wider band then would be expected of a band containing a single chromosome. This supports the conclusion that this band is composed of at least two chromosomes of similar size.
30 – Pm30
Sp – *S. pombe*
Hw – *Hansenula wingei*
* indicates 2 incompletely resolved chromosomes.

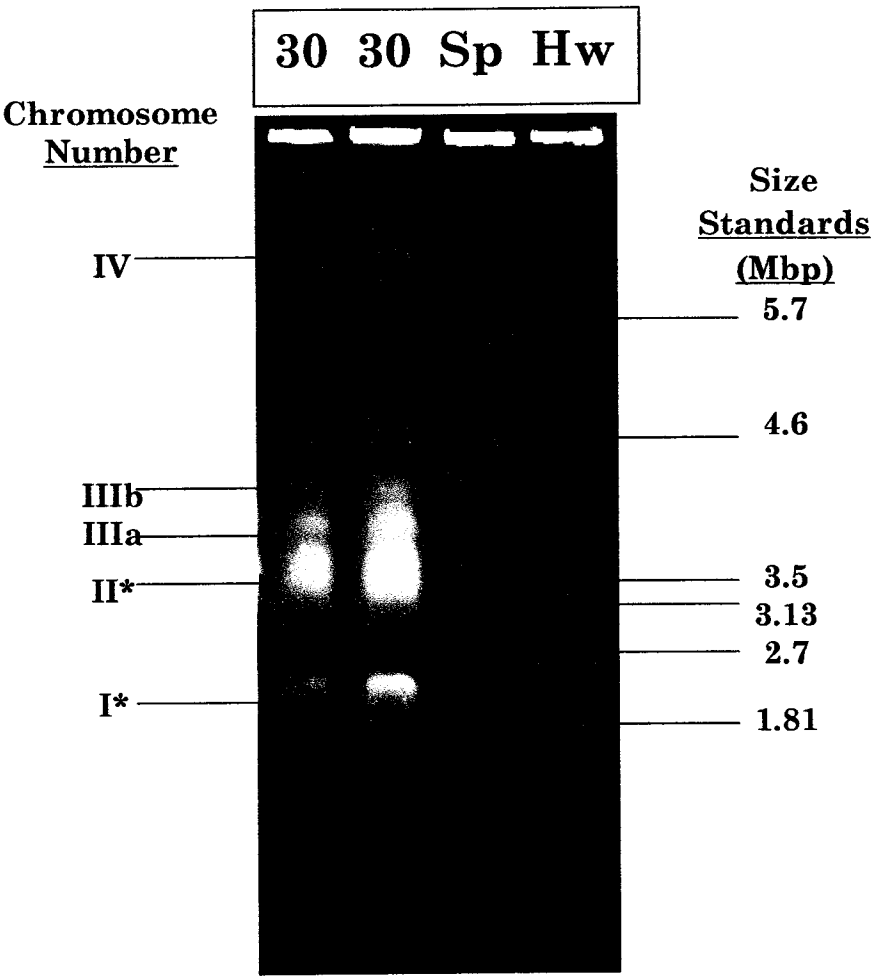

30 30 Sp Hw
Chromosome Number
IV
IIIb
IIIa
II*
I*
Size Standards (Mbp)
5.7
4.6
3.5
3.13
2.7
1.81

Figure 13

Chromosomal location of selected *P. marneffei* genes.
The migration of CHEF-resolved chromosomes for each particular strain is indicated by the arrows. Lanes marked "30" and "F4" contain chromosomes from strains Pm30 and PmF4, respectively. Gene designations can be found in table 3.

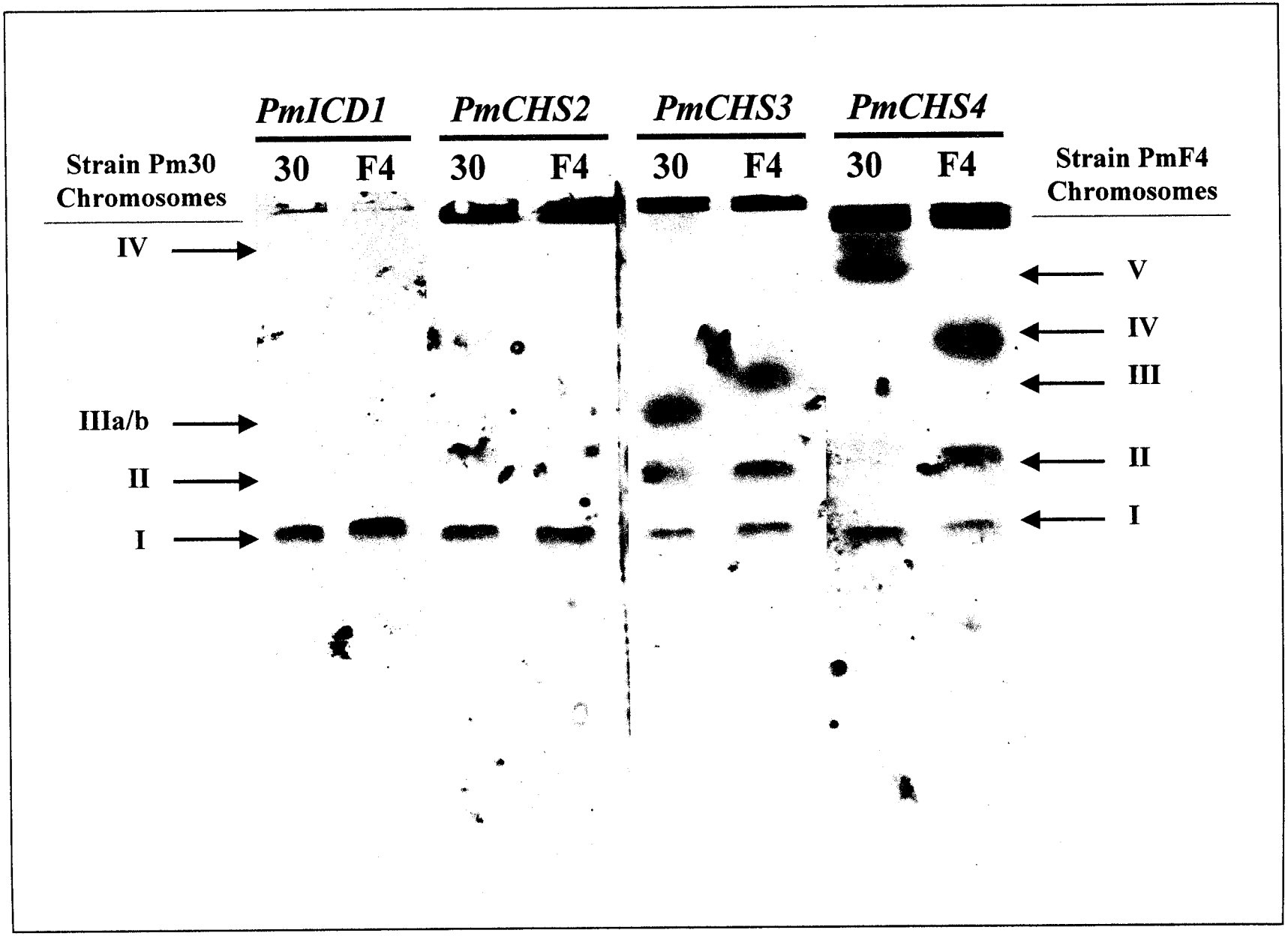

PmICD1
PmCHS2
PmCHS3
PmCHS4
Strain Pm30 Chromosomes
30
F4
30
F4
30
F4
30
F4
Strain PmF4 Chromosomes
IV
IIIa/b
II
I
V
IV
III
II
I

Table 3.

Chromosomal location of *P. marneffei* genes.
Gene designations; *23S rDNA*, large subunit ribosomal RNA; *PmITS*, internal transcribed spacers with *5.8S rDNA*; *PmICD1*, isocitrate dehydrogenase; *PmMLS1*, malate synthase; *PmICL1*, isocitrate lyase; *PmCHT1*, chitinase; *PmCHS2, PmCHS3,* and *PmCHS4,* chitin synthase homologues.
* Indicates presumed chromosomal location of gene based upon intensity of hybridization signal.

Chromosomal Location of Genes

Gene	*P. marneffei* strain:	
	Pm30	PmF4
23S rDNA	IIIa/b	IV
PmITS	IIIa/b	IV
PmICD1	I	I
PmCHT1	II*, I	II*, I
PmMLS1	IV	V
PmICL1	IIIa/b	V
PmCHS1	II	II
PmCHS2	I*, II	I*, II
PmCHS3	IIIa/b*, I, II	III*, I, II
PmCHS4	IV*, I	IV*, I, II

CHAPTER IV

DISCUSSION

Collectively, these results are significant for several reasons. First, they provide the first unequivocal karyotype of *P. marneffei.* In a previously published study, only three chromosomes were reported using two entirely different sets of CHEF electrophoresis conditions (Wu, Guo et al. 1996). Also, the sizes of the chromosomes in this previous report, 4.6, 3.5, and 1.0 Mbp, were significantly different from those found in these experiments. It is difficult to assess the results from the prior study since no photograph was included in the report. Moreover, the electrophoresis condition used by those investigators that was closest to the parameters employed herein contained a run time of only 24 hours. This is probably a significant source of experimental error for those earlier investigators since those conditions do not permit the resolution of each *P. marneffei* chromosome. In contrast, an

unpublished report using CHEF electrophoresis conditions similar to those employed in this study resolved four *P. marneffei* chromosomes having the following sizes: 5.7, 4.2, 3.0, and 2.2 Mbp (Szaniszlo 1996). These estimates are remarkably close to those derived in these experiments, save for the largest chromosome.

Second, although few karyotyping studies have been conducted, comparisons can be made to other *Penicillium* species (Table 4). From this work it has been determined that at least five chromosomes comprise the genome of *P. marneffei* having a total genome ranging from 20.7 to $\geq$ 21.6 Mbp. In contrast, other *Penicillium* species possess different numbers of chromosomes and total genome size. In *P. paxilli*, there is telomeric evidence for 8 chromosomes, but CHEF electrophoresis has resolved only 6 molecules ranging in size from 2.5 to 6.0 Mb (Itoh, Johnson et al. 1994; Young, Itoh et al. 1998). These results are analogous to those obtained here for *P. marneffei.* In studies using standard strains of *P. chrysogenum* and *P. notatum*, four chromosomes were observed in each species using CHEF electrophoretic conditions (Fierro, Gutierrez et al. 1993; Banuelos, Casquiero et al. 1999; Fierro and Martin 1999). The chromosomes in these species ranged from 5.4 to 10.8 Mb in size with the genome size being 32.1 Mb and 33.4 Mb for *P. notatum* and *P. chrysogenum*, respectively. Moreover, mutants of *P. chrysogenum* exhibited different individual chromosome sizes due to structural rearrangements. Using different pulsed-field systems, the

karyotypes of *P. nalgiovense* and *P. janthinellum* have been resolved as well (Kayser and Schulz 1991; Farber and Geisen 2000). The 26.5 Mb genomes of four *P. nalgiovense* strains were resolved into four chromosomes ranging in size from 4.1 to 9.1 Mb. In contrast, the genome size of *P. janthinellum* is estimated to range from 39 to 46 Mb divided among a minimum of 8 chromosomes.

Third, the present study has assigned the locations of particular genes to specific chromosomes. The results confirm the presence of multiple homologues of chitin synthase and chitinase genes in *P. marneffei.* Furthermore, some of the genes probed may be linked. Overall, the assignment of genes to specific chromosomes is an important first step in the molecular characterization of *P. marneffei.*

Finally, chromosomal polymorphisms are clearly present in the two strains of *P. marneffei* we studied. Notably, these strains come from the same region, but were isolated nearly a decade apart. The results indicate that CHEF electrophoresis may provide yet another tool to investigate the molecular epidemiology of penicilliosis. Recently, restriction endonuclease treatment of agarose-embedded chromosomes and subsequent PFGE separation of the resulting fragments was used to study a group of *P. marneffei* clinical isolates (Trewatcharegon, Sirisinha et al. 2001). Past studies previously used restriction endonucleases or amplification-based techniques (Vanittanakom, Cooper et al. 1996; Mekha, Poonwan et al. 1997;

Hsueh, Teng et al. 2000). Conceivably, by combining all three methods, as well as clinical and environmental data, it may be possible not only to trace outbreaks of disease caused by *P. marneffei*, but also to finally pinpoint the reservoir of this fungus. To date, the discovery of this reservoir and how humans acquire *P. marneffei* infection remains a mycological mystery (Restrepo, Baumgardner et al. 2000).

Table 4.

Comparison of genomes between various *Penicillium* species.
The chromosome number and size of various *Penicillium* species studied by pulse-field gel electrophoresis indicates that the genome of *P. marneffei* most closely resembles that of *P. purpurogenum*. Additional data from previously published sources. (Wu, Guo et al. 1996, Fierro, Gutierrez et al. 1993, Kayser and Schulz, 1991, Farber and Geisen, 2000, Young, Itoh et al. 1998, Chavez, Fierro et al. 2001.)

Genome Sizes of Various *Penicillium* Species as Determined by Pulsed-Field Gel Electrophoresis

Penicillium Species/Strain	Number of Chromosomes	Sizes of Individual Chromosomes (Mbp)	Total Genome Size (Mbp)
P. marneffei			
Pm30	5	2.2, 3.3, 3.8, 4.2, 6.1	19.6
PmF4	5	2.2, 3.3, 4.1, 5.1, 5.9	20.6
Not specified	3	1.0, 3.5, 4.6	9.1
P. chrysogenum			
NRRL-1951	4	6.8, 7.3, 9.6, 10.4	34.1
Wis 54-1255	4	6.3, 7.2, 9.7, 10.3	33.5
npe10	4	5.8, 7.1, 9.7, 10.2	32.8
AS-P-78	4	5.6, 7.5, 9.4, 11.0	33.5
P2	4	5.6, 6.7, 10.5, 10.5	33.3
P. jantinellum	8	2.0, 3.3, 3.9, 4.4, 4.7, 5.2, 6.0, 7.5	37.0
P. nalgiovense	4	4.1, 5.4, 7.9, 9.1	26.5
P. notatum	4	5.4, 6.3, 9.6, 10.8	32.1
P. paxilli	6	2.5, 3.0, 3.2, 3.7, 5.0, 6.0	23.4
P. purpurogenum	5	2.3, 2.9, 3.7, 5.2, 7.1	21.2

CHAPTER V

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