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Mitochondrial Evolution in the Fission Yeasts

par

Charles Edward Bullerwell

Département de Biochimie

Faculté de Médecine

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présenté par:

Charles Edward Bullerwell

a été évalué par un jury composé des personnes suivantes:

Président-rapporteur: Léa Brakier-Gingras

Directeur de recherche: Franz Lang

Membre du jury: Stephen Michnick

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ABSTRACT

Schizosaccharomyces pombe is an important model eukaryote, but its placement within the fungal division Ascomycota has been questioned since its discovery more than a century ago. In addition, the relationships among known fission yeast species are uncertain. In order to resolve these questions, as well as provide needed molecular data from other members of this unique group of microorganisms, we have undertaken the complete sequencing of the mitochondrial DNA (mtDNA) of two fission yeasts: *Schizosaccharomyces octosporus* and *Schizosaccharomyces japonicus* var. *japonicus*. We find that the gene content in these two mtDNAs is almost identical to that in the completely-sequenced *S. pombe* mtDNA, except that *trnI2*(cau) is missing in *S. octosporus* mtDNA (as well as corresponding ATA codons in standard protein-coding genes), and *rps3* and *rnpB* are absent in that of *S. japonicus* var. *japonicus*. Both the genetic code and mechanisms of RNA transcript processing appear to be the same in these three fission yeast mitochondrial genomes. We have identified a homolog of *S. pombe* Orf227 in the mtDNA of *S. octosporus* (Orf248), and provide evidence that these proteins (as well as the Var1 and S5 proteins of *Saccharomyces cerevisiae* and *Neurospora crassa*, respectively) are homologs of Rps3 (ribosomal small subunit protein 3). We have also identified 5 Double Hairpin Elements (DHEs) in the mtDNA of *S. octosporus*, the first report of these elements in an ascomycete. Our molecular phylogenetic analysis based on concatenated Cox1, Cox2, Cox3 and Cob amino acid sequences clearly demonstrates that *S. pombe* and *S. octosporus* are more closely related to each other than either is to *S. japonicus* var. *japonicus*, a result consistent with the gene order conservation observed among these mtDNAs. More mtDNA data from basidiomycete and deeply-diverging ascomycete species will be necessary to reliably determine the branching order within the higher fungi (Ascomycota and Basidiomycota), as the divergence of the basidiomycetes, fission yeasts, budding

yeasts and filamentous ascomycetes appears to have occurred over a relatively short period of time.

RÉSUMÉ

La levure de fission, *Schizosaccharomyces pombe*, a été découverte par P. Lindner, qui l'a isolée d'une bière africaine appelée *Pombe* en 1893. Le nom *Schizosaccharomyces* a été choisi afin de refléter les différences morphologiques entre cette espèce et celles du genre *Saccharomyces*, aussi bien que d'indiquer les caractéristiques communes à ces deux groupes d'organismes. Depuis cette découverte, *S. pombe* est devenue un organisme important dans la recherche scientifique. Par exemple, elle a servi comme organisme modèle dans plusieurs études génétiques et moléculaires du cycle cellulaire. Toutefois, l'origine évolutionaire des levures de fission et leur classification dans la division des champignons Ascomycota, restent incertaines. En plus, les relations entre *S. pombe* et les deux autres espèces de levure de fission connues, *Schizosaccharomyces octosporus* et *Schizosaccharomyces japonicus*, sont incertaines.

Plusieurs séquences complètes de génomes mitochondriaux de champignons (y compris celle de *S. pombe*) sont présentement disponibles, et ces séquences ont déjà démontré leur utilité pour faire la reconstruction de la phylogénie des champignons. Dans le but de clarifier la phylogénèse des levures de fission, et de produire des séquences moléculaires mitochondriales d'autres membres des levures de fission que *S. pombe*, nous avons entrepris le séquençage des génomes mitochondriaux de *S. octosporus* et *S. japonicus* var. *japonicus*. Avec ces nouvelles données, nous avons comparé le contenu génétique, les signaux d'expression des gènes et l'ordre des gènes dans ces génomes. Nous avons aussi reconstruit la phylogénie de ce groupe unique d'organismes eucaryotes.

Nous avons trouvé que le contenu en gènes dans ces génomes mitochondriaux est presque identique à celui de *S. pombe*, sauf que le gène *trnI2*(cau) n'est pas présent chez *S. octosporus*, et que les gènes *rps3* et *rnpB* ne sont pas présents chez *S. japonicus* var. *japonicus*. L'ordre des gènes de *S.*

octosporus est hautement similaire à celui de *S. pombe*, seulement deux transpositions du génome mitochondrial de *S. octosporus* seraient nécessaires pour donné l'ordre observé dans le génome de *S. pombe*. Au contraire, l'ordre des gènes du génome de *S. japonicus* var. *japonicus* est complètement différent de celui des deux autres génomes.

Le code génétique des deux génomes mitochondriaux est le même que chez *S. pombe*: i.e., le code universel est utilisé dans les gènes des protéines mitochondriales standards de *S. octosporus* et *S. japonicus* var. *japonicus* (ainsi que dans *rps3* de *S. octosporus*), et TGA code pour une fraction des résidus tryptophanes dans quelques cadres ouverts de lecture (ORFs). Il est intéressant de remarquer que le génome mitochondrial de *S. octosporus* ne contient pas le gène *trnI2*(cau), l'ARN de transfert aminoacylé avec isoleucine qui reconnaît les codons AUA. L'absence de cet ARN de tranfert est en corrélation avec l'absence des codons ATA dans les gènes des protéines standard, ainsi que *rps3*. Malgré ceci, les ORFs encodés dans les introns contiennent des codons ATA. Il est possible que la traduction de ces protéines se fasse, inefficacement, soit en utilisant une *trnI*(gua) modifié, soit en important du noyau un ARN de tranfert isoleucine reconnaissant des codons AUA; ou encore, ces ORFs ne sont pas traduits en protéines fonctionnelles.

Les signaux pour la maturation des transcrits d'ARN sont aussi les mêmes que chez *S. pombe*. Nous avons trouvé que les ARNs de transfert sont impliqués dans la maturation des transcrits dans les mitochondries des trois espèces, et des régions riches en C (cytosine) sont présentes après tout gène codant pour une protéine (ainsi que pour *rns*) dans les trois génomes. Ces régions riches en C sont peut-être impliquées dans la protection des bouts 3' des ARN contre des exonucléases.

Nous avons identifié un homologue de l'Orf227 de *S. pombe* dans le génome mitochondrial de *S. octosporus* (Orf248), et nous présentons des évidences qui indiquent que ces protéines (ainsi que les protéines mitochondriales Var1 et

S5 de *Saccharomyces cerevisiae* et *Neurospora crassa*, respectivement) sont des homologues de Rps3 (protéine de la petite sous-unité ribosomique 3). Ceci est particulièrement important, puisque l'origine de Orf227, Var1 and S5 est disputée depuis leurs découvertes respectives. Finalement, nous avons identifié cinq éléments structurés (Double Hairpin Elements; DHEs) dans le génome mitochondrial de *S. octosporus*, la première découverte de ces éléments chez un ascomycète. Ces DHEs semblent être impliqués dans le réarrangement du génome mitochondrial de *S. octosporus*.

Notre analyse phylogénétique basée sur les séquences protéiques concatenées de Cox1, Cox2, Cox3 et Cob démontre clairement que *S. pombe* et *S. octosporus* sont plus apparentées que l'une ou l'autre ne l'est avec *S. japonicus* var. *japonicus*. Ce résultat est en accord avec la conservation d'ordre des gènes que l'on observe dans les génomes mitochondriaux des trois espèces. De plus, la divergence marquée de *S. japonicus* var. *japonicus* suggère que cettte espèce serait mieux classée dans un genre (*Hasegawaea*; proposé par Yamada et Banno, 1987) séparé des autres. Il est évident que des données supplémentaires seront nécessaires pour déterminer l'ordre de branchement des champignons supérieurs (Ascomycota et Basidiomycota). Les divergences entre les basidiomycètes, les levures de fission, les levures de l'ordre *Saccharomycétales* et les ascomycètes filamenteux sont sans doute survenues de façon rapprochée dans le temps, et ne peuvent pas être résolues avec les données présentement disponibles.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

INTRODUCTION

ARTICLE

RESULTS AND DISCUSSION

LIST OF TABLES

Page

RESULTS AND DISCUSSION

LIST OF ABBREVIATIONS

A	adenine
T	thymine
C	cytosine
G	guanine
U	uracil
ATP	adenosine triphosphate
Atp6,8,9	protein subunits 6,8,9 of the ATP synthase complex
DHE	double hairpin element
Cob	apocytochrome B
CoQ	coenzyme Q
Cox1,2,3	protein subunits 1,2,3 of the cytochrome oxidase complex
cox1-I1	the first intron (from the 5'-end) in the *cox1* gene
DNA	deoxyribonucleic acid
GTP	guanosine triphosphate
kbp	kilobase pairs
mtDNA	mitochondrial DNA
ORF	open reading frame
RNA	ribonucleic acid
rnl	gene for large subunit ribosomal RNA
rnp_B	the RNA component of ribonuclease P
rns	gene for small subunit ribosomal RNA
Rps3	small ribosomal subunit protein 3
rRNA	ribosomal RNA
RT-PCR	reverse transcription-polymerase chain reaction
tRNA	transfer RNA
trnW(cca)	tRNA gene for tryptophan (anticodon 5'-CCA-3')

LIST OF AMINO ACID CODES

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asp	Aspartic acid
P	Pro	Proline
Q	Gln	Glutamic acid
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryprophan
Y	Tyr	Tyrosine

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Chapter I: INTRODUCTION

The first description of a fission yeast was by P. Lindner (1893) who isolated *Schizosaccharomyces pombe* from an East African millet beer called *Pombe*. The name *Schizosaccharomyces* was chosen to reflect the morphological differences in relation to the genus *Saccharomyces*, as well as to indicate common characteristics, such as spore formation and fermentation capacity, which exist between the two groups. Since its discovery, *S. pombe* has become an important organism for scientific research, but despite numerous studies, the evolutionary history of the fission yeasts remains unclear.

In the introduction to this thesis, I will first describe the defining features of the fission yeasts and highlight the scientific importance of *S. pombe*. Secondly, I will review the current understanding of the phylogenetic relationships within this group and the origin of the fission yeasts within the fungal lineage. Finally, I will address what has been learned from studying the mitochondrial genome of *S. pombe*, and outline the experimental approach and implications of this study.

I.1 What are fission yeasts?

I.1.1 Defining characteristics

The fission yeasts are a small group of primarily unicellular, saprobic organisms classified within the higher fungal division Ascomycota, order *Schizosaccharomycetales* (Prillinger *et al.*, 1990; Erikson *et al.*, 1993; Kurtzman, 1993) in the single genus, *Schizosaccharomyces*. Fission yeast species have generally been isolated from sugar-rich environments such as honey, fruit and fruit products (habitats also common to a number of species of the *Saccharomycetales*), but several characters such as their method of cell division (see below) and cell wall composition (see section I.2.1 and Sipiczki, 1995) have long distinguished the members of this genus from the yeasts of the *Saccharomycetales* (Alexopoulos *et al.*, 1996).

The common name 'fission yeasts' comes from their unique method of asexual cell division after mitosis, by cross-wall formation without constriction (cell fission; see Figure I.1). Newly born haploid daughter cells grow by apical extension. When a cell has reached a mature length, it undergoes nuclear division and produces a septum which partitions the two equal-sized daughter cells (Johnson *et al.*, 1973). In rich media, these new cells will separate to start the haploid cell cycle again, which takes about 2 hours. Under nutrient-deprivation, haploid cells can assume a pseudo-hyphal morphology (haploid daughter remain attached following mitosis), which permits more efficient foraging for nutrients.

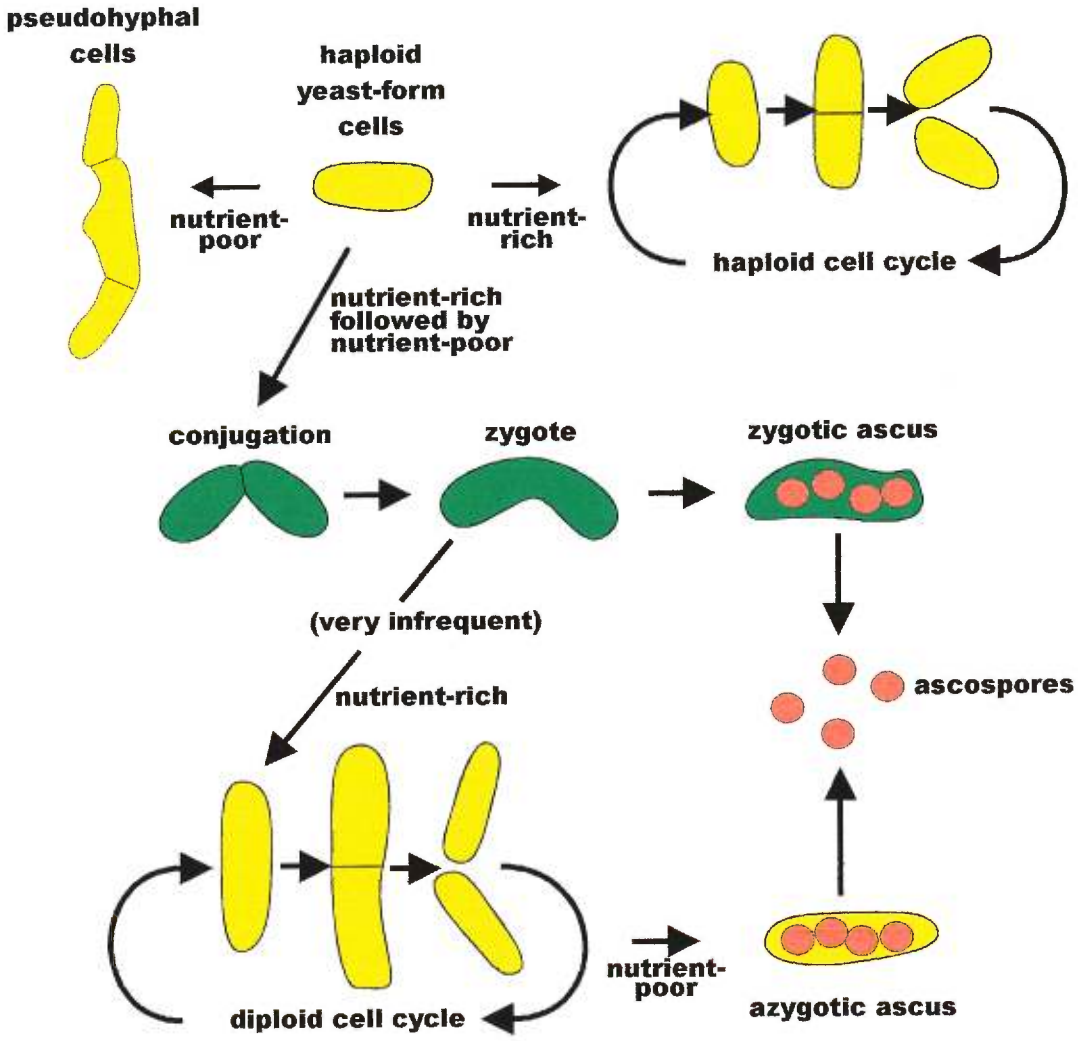
The presence of heterothallic mating behaviour in this group was first confirmed by the results of Leupold (1950) who isolated a homothallic clone (designated h^{90} because about 90% of cells were able to form spores in pure culture), and two heterothallic clones of opposite mating type (designated h^+ and h^-) from a strain of *S. pombe*. h^{90} has subsequently been found to represent the true wild type of *S. pombe*, whereas h^+ and h^- strains arise by rare mutations of the homothallic h^{90} type.

When starved, haploid fission yeast cells enter stationary phase and, if both mating types are present, can undergo pair-wise conjugation with fusion at the cell tips. Following the fusion of nuclei, the diploid zygote undergoes meiosis. Upon sporulation, haploid ascospores are produced contained within an ascus, and subsequently released (Munz *et al.*, 1989). Under nutrient-rich conditions, diploid zygotes can infrequently undergo mitosis instead of meiosis and enter a diploid cell cycle (Leupold, 1955).

I.1.2 *S. pombe* as a model organism

The budding yeast *Saccharomyces cerevisiae* is an important model eukaryote, but exhibits many characteristics uncommon to other eukaryotes. For example, the mitotic spindle is present during much of the *S. cerevisiae* cell cycle, chromosomes fail to condense (Taylor *et al.*, 1993), and actin filaments are used

Figure I.1 The life cycle of *S. pombe*. Yellow indicates asexual phases, green indicates sexual phases. See text for a detailed description.


pseudohyphal
cells
haploid
yeast-form
cells
nutrient-
poor
nutrient-
rich
haploid cell cycle
nutrient-rich
followed by
nutrient-poor
conjugation
zygote
zygotic ascus
(very infrequent)
nutrient-rich
ascospores
nutrient-
poor
azygotic ascus
diploid cell cycle

to transport mitochondria in these cells (Simon *et al.*, 1995). In contrast, most eukaryotes display a distinct G2 phase, chromosomes visibly condense (Taylor *et al.*, 1993) and vertebrate cells use microtubules for mitochondrial transport (Heggeness *et al.*, 1978). Therefore, *S. cerevisiae*, a highly-derived fungus (inferred from branch lengths in molecular phylogenies; e.g., Liu *et al.*, 1999 and Paquin *et al.*, 1995), is not an ideal model system.

In contrast, *S. pombe* has served as an important model organism in genetic and molecular studies of the cell cycle (Forsberg and Nurse, 1991). Unlike *S. cerevisiae*, it displays a distinct G2 phase and visible condensation of its three chromosomes during mitosis (Taylor *et al.*, 1993), and uses microtubules for mitochondrial transport (Yaffe *et al.*, 1996). *S. pombe* is also less-derived (shows shorter branch-lengths in molecular phylogenies based on nuclear data; e.g., Liu *et al.*, 1999 and Paquin *et al.*, 1995) than *S. cerevisiae*, and therefore may retain more ancestral characters.

In addition, *S. pombe* is easily cultured and manipulated in the laboratory, and many molecular genetic techniques are available for this organism (Moreno *et al.*, 1991). For example, mutants can be isolated from haploid strains and systematically tested for dominance or recessiveness, and genes can be cloned by complementation of mutant functions (Hayles and Nurse, 1992). Finally, the sequencing of the nuclear genome of *S. pombe* is nearing completion, which will allow a comprehensive molecular understanding of this organism (see http://www.sanger.ac.uk/Projects/S_pombe/). These results highlight the utility of *S. pombe* as an alternative model system to *S. cerevisiae*.

I.2 Fission yeast phylogeny

In light of the scientific importance of *S. pombe*, it is essential to understand how the fission yeasts are related to other eukaryotes. However, contradictions abound in studies attempting to determine the phylogenetic origin of the fission yeasts, as well as in studies of the interrelationships within this

group. In this section, I will address these controversial topics and attempt to arrive at a consensus.

I.2.1 *S. pombe* is not closely related to either budding yeasts or metazoans

Despite the classification of *S. pombe* within Ascomycota, there are several features which distinguish it from other members of this fungal group. For example, the cell wall composition of *S. pombe* is unique among ascomycetes (Bush *et al.*, 1974; Mateos and Dominguez, 1991; Horisberger and Rosset, 1977; Font de Mora *et al.*, 1990; Kreger-van Rij, 1984; Sipiczki, 1995). In addition, in the budding yeast *S. cerevisiae*, glucosamine builds up large amounts of chitin in the septa that separate mother and daughter cells (Bacon *et al.*, 1966; Molano *et al.*, 1980), whereas the septum of *S. pombe* apparently contains no chitin at all (Horisberger *et al.*, 1978). Similarly, studies based on molecular data (e.g., Lapeyre *et al.*, 1993; Radford, 1993; Naehring *et al.*, 1995; Janbon *et al.*, 1997) support a very distant relationship between *S. pombe* and other ascomycetes.

These and other findings (such as those described in section I.1.2) have influenced the belief that *S. pombe* is, in fact, more closely related to Metazoa than it is to Ascomycota. However, many molecular studies (Walker, 1985; Hendriks, 1992; Wilmotte *et al.*, 1993; Kurtzman, 1993 and 1994) support the monophyly of the ascomycetes including *S. pombe*, although the full range of fungal diversity has rarely been represented in these analyses. More recent studies, using multiple concatenated sequences and including a broad range of species from all fungal lineages (e.g., Paquin *et al.* 1995, Lang *et al.*, 1999 and Keeling *et al.*, 2000), consistently place the fission yeasts with the ascomycetes.

In agreement with these results, the fission yeasts share a set of fundamental features which delimit the division Ascomycota, including life cycle, mode of ascospore formation and non-centric mitosis (Sipiczki, 1989 and 1995). Further, the sexual sporangium of the fission yeasts is unambiguously similar to the asci of other ascomycetes, both in morphology and development (Tanaka and

Hirata, 1982; Sipiczki, 1983). Therefore, based on both morphological and molecular characters, it is reasonable to conclude that the fission yeasts are correctly classified within Ascomycota.

I.2.2 Are the fission yeasts deeply-diverging ascomycetes?

The genus *Schizosaccharomyces* is sometimes included with the archiascomycetes, a diverse assembly of organisms which may represent surviving examples of ascomycetes that diverged prior to the separation of budding yeasts and filamentous ascomycetes (Alexopolous *et al.*, 1996). This group includes the dimorphic genera of plant parasites *Taphrina* and *Protomyces*, the saprobic genus *Saitoella*, and an infective agent of pneumonia, *Pneumocystis carinii*. Unfortunately, the lack of morphological characters commonly used for fungal taxonomy, as well as the paucity of molecular data, has made members of this group difficult to classify.

Some molecular data, however, has supported the relationship of *S. pombe* to members of the archiascomycetes. For example, Nishida and Sugiyama (1993) compared 18S rRNAs from 13 fungi and found that *Taphrina*, *Saitoella* and *S. pombe* form a monophyletic group, and Walker (1985) has provided evidence from 5S rRNA molecules that *Protomyces* and *S. pombe* are specifically related. Furthermore, some molecular phylogenies (Okamoto *et al.* 1996; Janbon *et al.*, 1997) support a more recent divergence of budding yeasts and filamentous ascomycetes than that of either of these groups with *S. pombe*. Together, these data make it tempting to speculate that the fission yeasts represent a deeply-diverging ascomycete lineage.

Although several molecular studies place *S. pombe* specifically with either filamentous ascomycetes (Erickson *et al.*, 1993), or with budding yeasts (Hendriks *et al.*, 1992; Gaertig *et al.*, 1993) in phylogenetic trees, these contradictions may be explained by two factors: (1) high evolutionary rates of fungal sequences result in loss of phylogenetic signal, as changes accumulate in molecular sequences over

time. This saturation of sites makes the branching position of a species or group difficult to determine; (2) the divergence of the ascomycetes and basidiomycetes over a relatively short evolutionary period allowed less time for synaptomorphies (shared-derived characters; the only type of character which gives branching order information in likelihood- and parsimony-based phylogenetic approaches) to develop, thereby lowering the statistical support for the correct branching order.

The general consensus of the current molecular data is that the fission yeasts are members of the higher fungal division Ascomycota, but definitive evidence that they represent deeply-diverging ascomycetes is lacking. In the present study, we will provide mitochondrial DNA sequence data which will aid in determining the position of *S. pombe* within Ascomycota.

I.2.3 Within the genus *Schizosaccharomyces*

Of the many species described since the initial description of *S. pombe*, most have been found to be conspecific with one of three species: *Schizosaccharomyces pombe* Lindner (1893), *Schizosaccharomyces octosporus* Beijerinck (1894) and *Schizosaccharomyces japonicus* Yukawa et Maki (1931) (Sipiczki, 1995). The division of the genus into these three groups is supported by differences in ascospore morphology (Yamada and Banno, 1987) and other characters, described below.

Bridge and May (1984) undertook a numerical classification of the fission yeasts based on 60 strains and 100 characters, and observed three distinct clusters corresponding to *S. pombe*, *S. octosporus* and *S. japonicus*. Similarly, Sipiczki *et al.* (1982) studied the interfertility of numerous fission yeast strains and found interfertility within each species, but no interfertility among them. Yamada *et al.* (1973, 1987) studied the number of isoprene units in the side-chains of coenzyme Q (CoQ) molecules and found that *S. pombe* contained CoQ-10, *S. octosporus* CoQ-9, whereas *S. japonicus* contained no detectable CoQ. Yamada *et al.* (1987) also studied the electrophoretic mobility of five enzymes in various strains of

Schizosaccharomyces, and found that profiles within strains of each species were 40% to >80% identical, whereas 0% identity was observed among species. Finally, Vaughn Martini (1991) studied nDNA reassociation, and found >84% genome reassociation within strains of the same species, whereas <35% genome reassociation was observed among species. Taken together, these results strongly support the separation of the genus *Schizosaccharomyces* into three distinct groups.

Sipiczki (1995) has proposed that *S. japonicus* diverged from the fission yeast lineage before the separation of *S. pombe* and *S. octosporus*. This is supported by several lines of evidence. Jeffrey *et al.* (1997) examined 54 *Schizosaccharomyces* strains and determined that this group is divided into two lineages: (1) *S. japonicus*, which contains high amounts of linoleic acid and low amounts of oleic acid when compared to (2) the group containing *S. pombe* and *S. octosporus*. Low temperature cytochrome spectra indicated that *S. pombe* and *S. octosporus* contain a + a3, b, c1 and c cytochromes, whereas *S. japonicus* var. *japonicus* contains only cytochromes b and c1, and *S. japonicus* var. *versatilis* contains no detectable cytochromes (Sipiczki *et al.*, 1982). The absence of certain cytochromes in both varieties of *S. japonicus* correlates with their inability to grow on glycerol (respiratory deficiency; Bulder, 1963). Finally, a comparison of partial 18S and 26S rRNA sequences by Yamada *et al.* (1993) revealed less sequence divergence between *S. pombe* and *S. octosporus* than either showed with *S. japonicus*. The present study will provide needed molecular data to clearly elucidate the interrelationships among fission yeast species.

I.3 What has been learned from the mitochondrial genome of *S. pombe*?

I.3.1 Gene content and genetic code

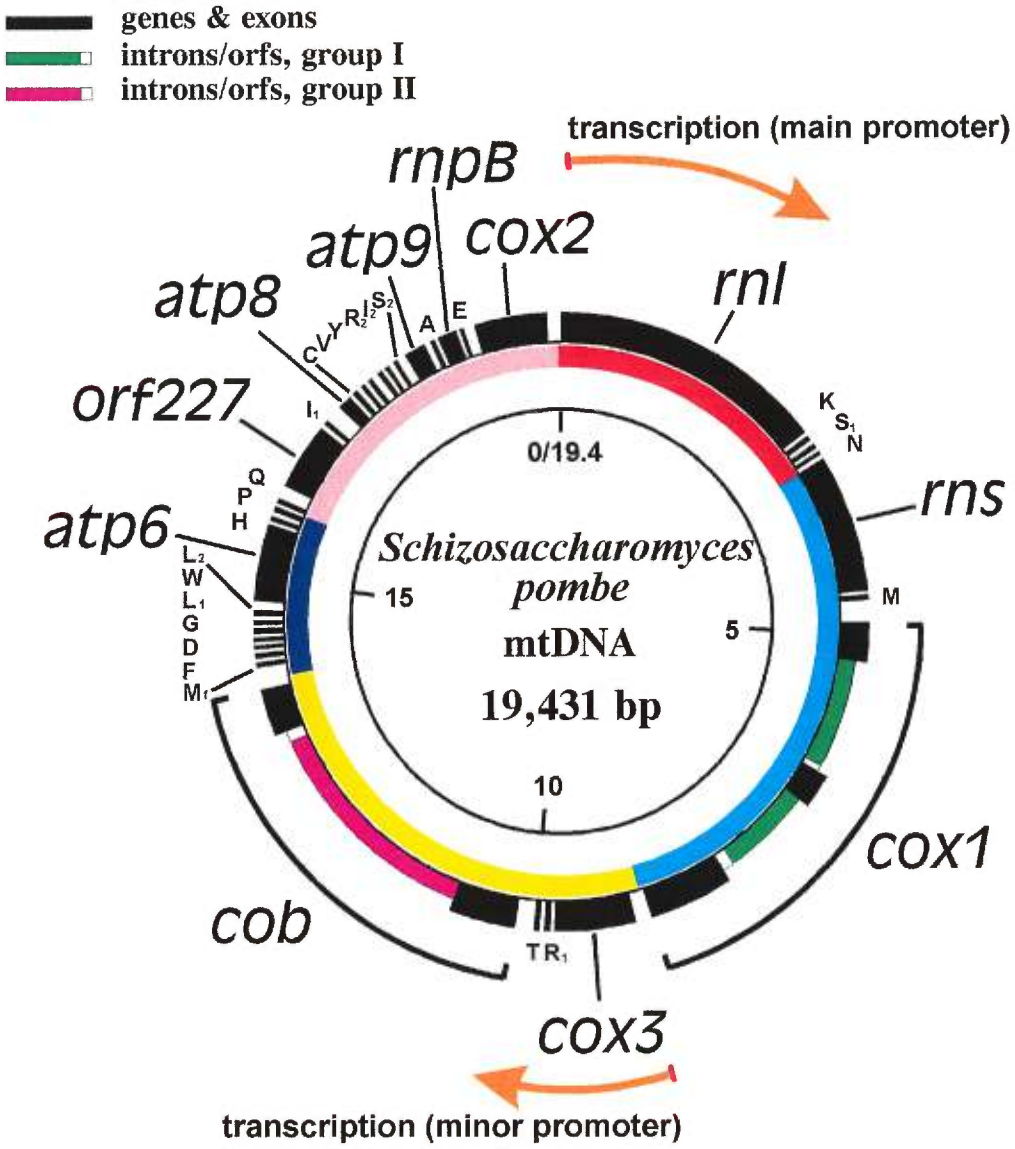
The mitochondrial DNA (mtDNA) of *S. pombe* strain 50 is a circular molecule of 19,431 bp, and has been completely sequenced (Lang *et al.*, 1983;

Lang, 1993) (Figure I.2). This genome is tightly packed with genes, coding for 25 tRNAs (capable of recognizing all codons), the small and large ribosomal RNA subunits (*rns* and *rnl*, respectively), the RNA component of mitochondrial RNaseP (*rnpB*; E. Seif and B.F. Lang, unpublished), cytochrome oxidase subunits 1, 2 and 3 (*cox1*, *cox2* and *cox3*, respectively), apocytochrome b (*cob*), ATP-synthase subunits 6, 8 and 9 (*atp6*, *atp8* and *atp9*, respectively) and an unassigned open reading frame (*orf227*, previously designated *urf a)* (Lang et al., 1983) which encodes a protein of 227 amino acids (discussed in section I.3.3). As in many other fungal mtDNAs, genes are coded on the same DNA strand, and are separated by moderately A+T-rich intergenic sequences.

In this *S. pombe* strain, the *cox1* gene contains two group I introns (Lang, 1984), and the *cob* gene contains one group II intron (Lang et al., 1985). All three introns include open reading frames (ORFs) characteristic of the corresponding intron group, i.e., two highly conserved dodecapeptide motifs in the group I intronic ORFs (Michel et al., 1982) and conserved reverse transcriptase sequence motifs in the group II intronic ORF (Michel and Lang, 1985). Evidence of horizontal transfer of introns has been found: the second intron in the *cox1* gene of *S. pombe* is inserted at the same position where a highly similar intron is found in the *cox1* gene of the distantly-related ascomycete, *Aspergillus nidulans* (Lang 1984, Waring *et al.*, 1984); and the *cob* intron is closely related to the first two introns in the *S. cerevisiae cox1* gene (Lang *et al.*, 1985). Most of the size variation between mitochondrial genomes from naturally-occurring strains (between 17.4 and 24.4 kb) is due to the presence or absence of introns (Zimmer *et al.*, 1987). In fact, these genetic elements have been shown to be superfluous, as an artificial mitochondrial genome of 14.6 kb has been constructed by successive deletion of introns in a strain of *S. pombe*, without affecting cellular respiration (Schäfer *et al.*, 1991).

Contrasting the genetic code deviations regularly found in the mitochondria of other ascomycetes, the universal translation code is used in the standard

Figure I.2 The mtDNA of *S. pombe*. Inner circle gives scale in kbp. Colours on the intermediate circle represent regions of gene order conservation with the mtDNA of *S. octosporus* (see section IV.1.1 for discussion). Outer circle indicates the location of genes, exons (black), introns and intronic orfs (group I, green; group II, magenta) in this mtDNA. Placement of promoters and direction of transcription are indicated by arrows.


genes & exons
introns/orfs, group I
introns/orfs, group II
transcription (main promoter)
rnpB
atp9
cox2
atp8
rnl
orf227
atp6
rns
cox1
cob
cox3
transcription (minor promoter)
Schizosaccharomyces pombe
mtDNA
19,431 bp
0/19.4
5
10
15

protein-coding mitochondrial genes (components of cytochrome oxidase and ATP-synthase complexes) of *S. pombe*. TGA codes for tryptophan instead of termination in *orf227* and two intronic ORFs, whereas in standard mitochondrial protein genes, tryptophan is encoded by TGG only. Contrary to other ascomycetes, the tryptophan tRNA (*trnW*) has the anticodon CCA which specifically recognizes UGG codons, rather than UCA which would recognize both UGA and UGG codons. The *S. pombe trnW*(cca) presumably allows a weak interaction with UGA tryptophan codons, thereby allowing the translation of *rps3* and intronic orf transcripts. Finally, AUA codes for isoleucine in *S. pombe* mitochondria (following the universal translation code) as opposed to methionine, as it does in animal and *S. cerevisiae* mitochondria.

The only data available from the mitochondrial genomes of other fission yeasts is the sequence of the mitochondrial small ribosomal subunit from *Schizosaccharomyces japonicus* var. *versatilis* (GenBank accession number: X72804). It will be interesting to acquire more mitochondrial sequence data and determine whether the features described in this section are present or altered in the genomes of other members of this lineage.

I.3.2 Gene expression

The mitochondrial genome of *S. pombe* is transcribed from the same DNA strand in two units, starting from two promoters situated opposite one another on the circular DNA molecule, one upstream of *rnl* (5'-ATATATGTA-3'), the other upstream of *cox3* (5'-ATATGTGA-3') (Paquin *et al.*, 1997) (see Figure I.2), the only genes which are not preceded by tRNA genes in this mtDNA. Transfer RNA genes are interspersed between all other genes, and S1 nuclease protection experiments of the *cox1* transcript clearly show that the mature 5'-end of this transcript is generated by processing precisely at the 3'-end of the preceding tRNA (Lang *et al.*, 1983). Recent results indicate that the 5'-ends of mitochondrial *rnpB* transcripts in *S. pombe* and *S. octosporus* are also determined by tRNA removal

(E. Seif and B. Franz Lang, unpublished results). This mechanism of RNA precursor processing has previously been proposed for mammalian mitochondria (Ojala *et al.*, 1980; Bibb *et al.*, 1981), as well as for those of *N. crassa* (de Vries *et al.*, 1985; Burger *et al.*, 1985) and *Aspergillus nidulans* (Dyson *et al.*, 1989).

All protein-coding genes, as well as the gene for the small subunit ribosomal RNA (*rns*), are followed by C-rich regions. Several S1 nuclease signals have been observed in the A-rich region following a C-cluster at the 3'-end of the small subunit ribosomal RNA molecule (Trinkl *et al.*, 1989), and in the A+T-rich region following a C-cluster at the 3'-end of the *atp6* transcript (Lang *et al.*, 1983). Further, the mature 3'-end of the mitochondrial *rnpB* transcript of *S. octosporus* has been shown to be immediately adjacent to a C-cluster (E. Seif and B. Franz Lang, unpublished results). Together, these data provide strong evidence that these C-clusters are not only conserved at the sequence level, but are also included in the mature small subunit rRNA, *atp6* and *rnpB* transcripts, and may serve to protect the 3'-ends of RNA transcripts from exonuclease degradation.

One of our interests in the present study was to determine to what extent the signals described here for transcription initiation and RNA processing are conserved in other fission yeast mitochondrial genomes, and whether additional signals may be identified by analyzing fission yeast mtDNA sequence conservation.

I.3.3 The *orf227* gene

Seitz-Mayr and Wolf (1982) demonstrated that mitochondrially-inherited antimycin-resistant mutants of *S. pombe* exhibit a mitochondrial mutator activity which is extrakaryotically inherited. This mutator could be separated genetically from mitochondrially-inherited drug resistance, increased the formation of mitochondrial drug-resistant and respiratory-deficient mutants, and was capable of producing mitochondrial deletions (Ahne *et al.*, 1984, 1988). *orf227* is the only ORF encoded by the mitochondrial genome of *S. pombe* which is not a known

gene for respiratory chain enzymes or the protein-synthesizing machinery, and thus was the prime candidate for the mutator gene. Zimmer *et al.* (1991) later demonstrated that *orf227* is the gene that, when mutated, is responsible for the mitochondrial mutator phenotype.

Neu *et al.* (1998) have more recently shown that the mutator phenotype in strains with mutated *orf227* is completely cured by expression of a universal-code version of *orf227* (the gene transcript contains one UGA tryptophan codon at amino acid position 175) expressed from the nucleus. They concluded that the protein product of this gene is essential for the stable maintenance of the mitochondrial genome. These authors also proposed that the *orf227* gene product may be related to the mtDNA-encoded Var1 protein, which has been shown to be a stoichiometric component of the mitochondrial small ribosomal subunit in *S. cerevisiae* (Terpstra *et al.*, 1979; Groot *et al.*, 1979)

We hoped to identify homologs of *orf227* in the present study in order to analyze amino acid conservation and determine protein sequence motifs, which might indicate the identity of this unique ORF.

I.4 Experimental Approach

As has been shown, *S. pombe* is an important eukaryotic model system, but both the evolutionary origin of the fission yeasts and relationships within this group remain speculative. The small size of mitochondrial genomes allows for efficient sequencing and analysis, and has proven to be a reliable method for elucidating phylogenetic relationships among not only the fungi (Paquin *et al.*, 1997), but also among other eukaryotic organisms (Lang *et al.*, 1999). In addition, a great deal of molecular data is available for *S. pombe*, but very little data is available for other *Schizosaccharomyces* species. In order to develop a more complete understanding of mitochondrial evolution in the fission yeasts, define elements of mitochondrial gene expression within this group and clarify its phylogenesis, we have undertaken the complete sequencing of the mtDNAs of two

members of the fission yeasts, *Schizosaccharomyces octosporus* and *Schizosaccharomyces japonicus* var. *japonicus.*

Chapter II: MATERIALS AND METHODS

II.1 Strains, culture conditions, and preparation of mtDNA

The strains used were *Schizosaccharomyces octosporus* (ATCC 2479; CBS 354) and *S. japonicus* var. *japonicus* (ATCC 10660; CBS 354). Cell cultures of 500 mL were grown using Yeast Standard Medium (SM), consisting of 1% yeast extract, 1 g/L KH_2PO_4, and 3% glycerol (for *S. japonicus* var. *japonicus*, 3% glucose was used). Cultures were allowed to grow for 24-48 hrs with shaking (100 rpm) at 30°C. Purification of mtDNA was performed using 20-30 g (wet weight) of cells, harvested in the early stationary phase by centrifugation. After resuspension in a sorbitol buffer (0.6 M sorbitol, 5 mM EDTA, 50 mM Tris pH 7.4), the cells were broken mechanically by shaking with glass beads, and a crude mitochondrial fraction was isolated by differential centrifugation. The mitochondrial fraction was lysed in the presence of 1% SDS and 100 µg/ml proteinase K, at 50°C for 1 hr. SDS was subsequently eliminated from the lysate by addition of 1 M NaCl, and after 1 hr on ice, the precipitate (SDS-protein complex) was removed by centrifugation. The total nucleic acids were fractionated on a CsCl gradient (1.1 g/ml, 40,000 rpm for 48 hours) in the presence of 10 µg/ml bis-benzimide (Hoechst 33258, Sorval). The upper band (A+T- rich DNA, generally mitochondrial) was extracted and re-centrifuged in one or two subsequent CsCl gradients. Yields of 0.5-3 µg DNA were recovered.

II.2 Cloning and sequencing of mtDNAs

0.5-3 µg of DNA in TE (10mM Tris pH 8, 1mM EDTA pH 8) plus 25% glycerol (final volume = 500 µl) was physically sheared by nebulization for 90 s at 5-10 psi (Okpodu *et al.*, 1994). This gave an average fragment size of about 1.5 kbp. DNAs were precipitated with 100% ethanol plus 0.5 mM ammonium acetate. Broken ends of fragments were repaired by incubation of 1.0 µg DNA for 30 min. at 12°C in the presence of dNTPs, the Klenow fragment of DNA polymerase (Boehringer-Mannheim) and T7 DNA polymerase (Boehringer-Mannheim). This mix was then incubated at 65°C for 5 min to inactivate the enzymes.

DNA fragments were separated on a 1.0% agarose gel, and the smear corresponding to fragments of 1-3 kbp was excised from the gel. DNA was subsequently electro-eluted for 1 hr at 300 V to extract DNA from the agarose block, collected and precipitated as above. These DNA fragments were subsequently phosphorylated for 30 min at 37°C in the presence of ATP and polynucleotide kinase (Pharmacia). The enzyme was inactivated by incubation at 65°C for 10 min. Phosphorylated DNA fragments were then ligated into a modified Bluescript vector cut with SmaI (a restriction enzyme which produces blunt DNA ends; Boehringer-Mannheim), by incubation overnight at 14°C in the presence of ATP and DNA ligase (USB). Transformation was performed, using the products of the ligation reaction, into competent cells of *Escherichia coli* strain XL1.

DNA sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977), using single-stranded DNA as template and S^{35} as label. Labelled DNA fragments were subjected to electrophoresis in 4% polyacrylamide gels, dried onto glass plates (Lang and Burger, 1990), and autoradiographed. Sequences were entered manually into computer files. In addition, automated sequencing was performed on a LiCor 4000L apparatus, using an end-labelled primer and a cycle sequencing protocol (Amersham).

II.3 Data analysis

Sequences were assembled using GAP (Dear & Staden, 1991) and custom-made command line interfaces, and stored in the MasterFile format (http://megasun.bch.umontreal.ca/ogmp/masterfile/intro.html). Sequence analysis was performed on SUN workstations, using software developed by the OGMP (Organelle Genome Megasequencing Project; see http://megasun.bch.umontreal.ca/ogmp/ogmpid.html) and others (such as the Staden sequence analysis package). The FASTA program (Pearson, 1990) was used for searches of local databases. Sequence similarity searches were also performed at the National Center for Biotechnology Information (NCBI), using the

BLAST network service (Altschul *et al.*, 1990). The CLUSTALW (Thompson *et al.*, 1994) program was used for multiple protein alignments, run from the GDE package (Genetic Data Environment; Smith *et al.*, 1994).

II.4 PCR amplification, cloning and sequencing of the *N. crassa S5* gene

Several nanograms of purified mtDNA from *N. crassa* strain 74A (kindly provided by Rick Collins, University of Toronto) was amplified by PCR using oligonucleotides (5'-GGAATGCCCGTGTAATAAAAATTATGG-3' and 5'-CCATCATTCACATAATGGTTTCAG-3') designed to amplify a fragment of 831 bp corresponding to the 3'-region of the *S5* gene. DNA was amplified using a Perkin-Elmer thermal cycler by Taq Expand High Fidelity DNA polymerase (Roche) in the presence of 2mM dNTPs, 25mM Mg^{2+} and the buffer supplied by the company. PCR conditions were as follows: 5 min denaturing at 95°C was followed by 5 min at 88°C, during which time the polymerase was added. 30 cycles, consisting of 30 s at 95°C (denaturing), 30 s at 55°C (primer annealing) and 1 min at 72°C (extension) were then performed, followed by 5 min at 72°C. A PCR product of the expected size was obtained, phosphorylated, cloned into a modified BlueScript vector (cut with SmaI as above) and transformed into *E. coli* strain XL1 competent cells. DNA sequencing and assembly of several clones was performed as described in sections II.2 and II.3.

II.5 Phylogenetic inference

Phylogenetic trees were estimated from an alignment of 1305 amino acids. The alignment consisted of concatenated Cox1, Cox2, Cox3 and Cob protein sequences, inferred from mtDNA data. PROTDIST (Felsenstein, 1990) was used to calculate the distances with rate variation α estimated at 0.2.

PUZZLE version 4.0.2 (quartet puzzling; Strimmer and von Haeseler, 1996) was used on the data set using a gamma distribution (with 8 gamma rate categories) and the mtREV24 model of amino acid substitution (Adachi and

Hasegawa, 1996). 40,000 puzzling steps were performed to infer quartet puzzling trees.

The Neighbor Joining (NJ; Saitou and Nei, 1987), Biological Neighbor Joining (BioNJ; Gascuel, 1997) and Weighted Neighbor Joining (Weighbor; Bruno *et al.*, 2000) phylogenetic programs were used to infer tree topologies from the distances calculated with PROTDIST. 1,000 bootstraps were performed using SEQBOOT (Felsenstein, 1990) on selected trees to estimate the support for the observed branching order.

Chapter III: ARTICLE

A novel motif for identifying Rps3 homologs in fungal mitochondrial genomes

Mitochondrial genomes code for a variable number of mitochondrial ribosomal proteins. For example, the mitochondrial DNA (mtDNA) of the jakobid protist *Reclinomonas americana* encodes 27 of these proteins, that of the angiosperm *Arabidopsis thaliana* seven, whereas animal mtDNAs contain no ribosomal protein genes at all (reviewed in Ref. 1). To date, the only mtDNA-encoded ribosomal protein gene identified by sequence similarity in a fungus is *rps3* (ribosomal small subunit protein 3) in the primitive chytridiomycete *Allomyces macrogynus*[2]. Here we report the identification of a second *rps3* homolog in the mtDNA of the zygomycete fungus *Mortierella verticillata* by sequence similarity to *rps3* of *A. macrogynus*. This suggests that other fungal mitochondrial genomes might also code for Rps3 proteins.

Previous research has shown that *Escherichia coli* Rps3 binds *in vitro* to the small ribosomal subunit at a late step of ribosome assembly and aids in the assembly of other ribosomal proteins[3,4]. These results indicated two candidates for mitochondrial Rps3 homologs: Var1 from *Saccharomyces cerevisiae* and S5 from *Neurospora crassa*. Var1 is the only stoichiometric component of the mitochondrial small ribosomal subunit in *S. cerevisiae* encoded by the mtDNA[5,6]. The absence of this protein results in the defective assembly of the mitochondrial small ribosomal subunit, respiratory deficiency and the appearance of a ribonucleoparticle lacking not only Var1, but also several other proteins[7–9]. Similar phenomena are observed in the ascomycete *N. crassa* in the absence of S5. Like Var1 in *S. cerevisiae*, S5 is the only

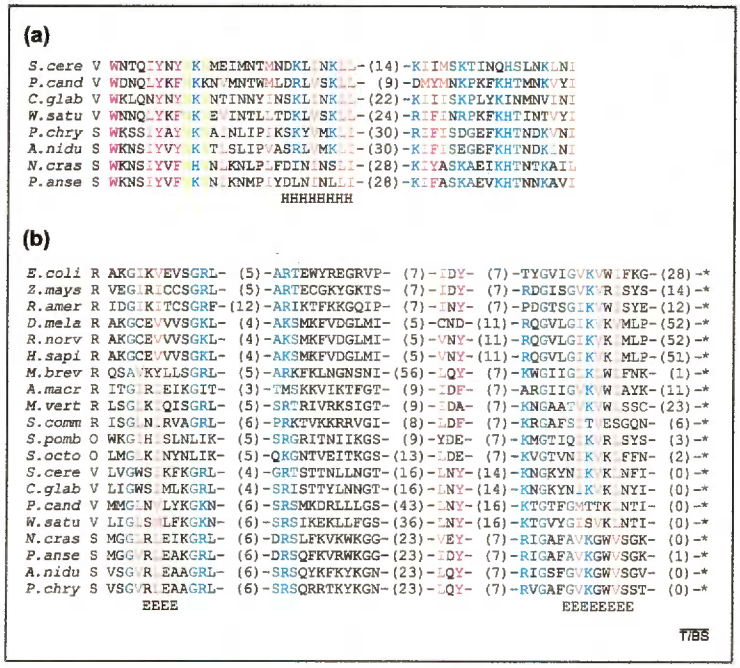


Figure 1

Protein sequence motifs in Rps3 homologs. Multiple protein alignments were determined using CLUSTAL W (Ref. 22) [executed from GDE (Ref. 23)] and adjusted manually thereafter. Numbers in parentheses indicate the number of amino acids not shown. Genes are mtDNA encoded, unless otherwise indicated. A column in the alignment was considered conserved when at least 75% of sequences contained a similar or identical amino acid, and are color coded according to chemical characteristics: basic, blue; aliphatic, orange; aromatic, magenta; small neutral, dark green; small polar, light green. Secondary structure prediction (shown below alignment) was determined using PhDsec (Ref. 24) on the multiple alignments. E denotes a β strand, and H an α helix. Only secondary structure predictions with probability (*P*) scores of at least 8 (maximum = 9) were considered acceptable. The statistical program PRSS (Refs 25,26) was used to determine the significance of similarity of the aligned sequences. Using sequences shown in Fig. 1b (except the C-terminal sequences shown in parentheses) all nonfungal Rps3 representatives ($P < 1.01e^{-5}$), as well as those of the lower fungi *Allomyces macrogynus* ($P = 1.67e^{-8}$) and *Mortierella verticillata* ($P = 4.44e^{-7}$), were shown to be significantly similar to that of *Escherichia coli*. *M. verticillata* Rps3 was shown to be significantly similar to *Schizosaccharomyces pombe* Orf227 ($P = 9.45e^{-5}$), *Schizophyllum commune* Orf1453 ($P = 6.00e^{-7}$) and *Aspergillus nidulans* S5 ($P = 2.35e^{-5}$). Using sequences shown in Fig. 1a, *A. nidulans* S5 was shown to be significantly similar to *Saccharomyces cerevisiae* Var1 ($P = 2.17e^{-5}$). PRSS settings were as follows: scoring matrix = BLOSSOM50; *ktup* = 2; gap open penalty = −12; gap extension penalty = −2; number of shuffles = 1000. PRSS *P* values of <0.001 were considered significant. Abbreviations: O, Orf227; R, Rps3; S, S5; V, Var1. **(a)** Conserved central regions in Var1 and S5. Sequences used (amino acid positions and GenBank accession numbers): *S.cere, Saccharomyces cerevisiae* (ascomycete fungus, 93–151,V00705); *P.cand, Pichia canadensis* (*'Hansenula wingei'*; ascomycete fungus, 48–129, D31785); *C.glab, Candida glabrata* (ascomycete fungus, 63–101, X02893); *W.satu, Williopsis saturnus* (ascomycete fungus, 53–122, CAA47158); *P.chry, Penicillium chrysogenum* (ascomycete fungus, 37–111, D13859); *A.nidu, Aspergillus nidulans* (ascomycete fungus, 37–111, X06961); *N.cras, Neurospora crassa* (ascomycete fungus, 56–128, J01427); *P.anse, Podospora anserina* (ascomycete fungus, 44–116, X14735). A multiple sequence alignment (alignment number ds43184) has been deposited with the European Bioinformatics Institute (ftp://ftp.ebi.ac.uk/pub/databases/embl/align/ds43184.dat). **(b)** Rps3 C-terminal motif. Sequences used (amino acid positions and GenBank accession numbers): *E.coli, Escherichia coli* (eubacterium, 146–205, U18997); *Z.mays, Zea mays* (plant, 486–545, X57445); *R.amer, Reclinomonas americana* (protist, 209–275, AF007261); *D.mela, Drosophila melanogaster* (fruit fly, nuclear, 133–193, X72921); *R.norv, Rattus norvegicus* (mammal, nuclear, 131–191, X51536); *H.sapi, Homo Sapiens* (mammal, nuclear, 131–191, U14990); *M.brev, Monosiga brevicollis* (choanoflagellate, 40–148, AF275274, this study); *A.macr, Allomyces macrogynus* (chytridiomycete fungus, 101–160, U41288); *M.vert, Mortierella verticillata* (zygomycete fungus, 198–260, AF275273, this study); *S.comm, Schizophyllum commune* (basidiomycete fungus, 1386–1447, AF275272, this study); *S.pomb, Schizosaccharomyces pombe* (archiascomycete fungus, 163–224, X54421); *S.octo, Schizosaccharomyces octosporus* (archiascomycete fungus, 181–246, AF275271, this study); *S. cerevisiae* (322–396); *C. glabrata* (265–339); *P. canadensis* (281–386); *W. saturnus* (282–380); *N. crassa* (149–225, AF275270, this study); *P. anserina* (382–458); *A. nidulans* (343–410); *P. chrysogenum* (323–399). A multiple sequence alignment (alignment number ds43185) has been deposited with the European Bioinformatics Institute (ftp://ftp.ebi.ac.uk/pub/databases/embl/align/ds43185.dat).

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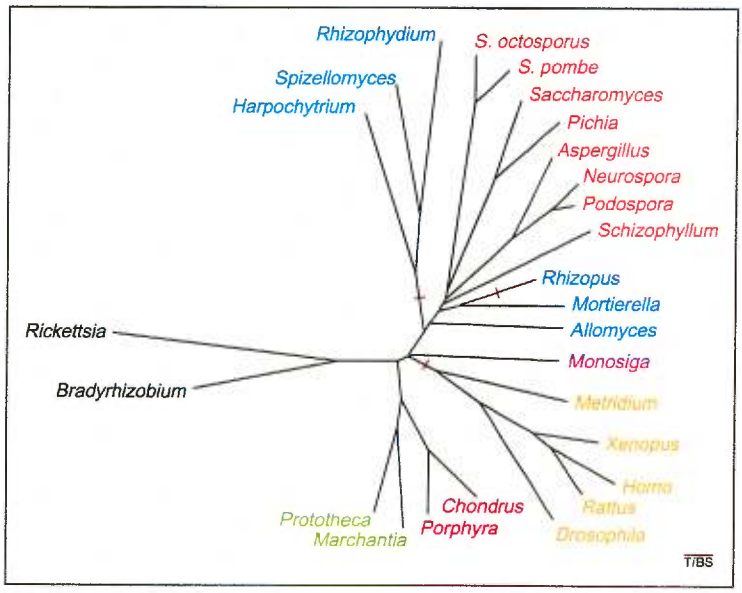

Figure 2

Phylogenetic distribution of mtDNA-encoded Rps3. Schematic tree based on the branching order of a published phylogenetic tree[27] and data presented here. Thick lines indicate lineages containing mtDNA-encoded Rps3, red bars represent loss of this gene. We postulate that mtDNA-encoded *rps3* was present in the common ancestor of fungal and animal mtDNAs (it is indeed present in the choanoflagellate *Monosiga brevicollis* that branches near the animal–fungal divergence[28,29]). It was lost from the animal clade, as well as in one branch of the fungal lineage (including most chytridiomycetes), but was retained in the ascomycetes, basidiomycetes, zygomycetes and the chytridiomycete *A. macrogynus*. The absence of *rps3* in the mtDNA of the zygomycete *Rhizopus stolonifer* indicates independent loss in this lineage. Color code: α-proteobacteria (black), green algae and land plants (green), red algae (red), animals (yellow), choanoflagellates (magenta), lower fungi (blue), higher fungi (orange). Organisms: *Rickettsia prowazekii* (α-proteobacterium), *Bradyrhizobium japonicum* (α-proteobacterium), *Prototheca wickerhamii* (green alga), *Marchantia polymorpha* (liverwort), *Porphyra purpurea* (red alga), *Chondrus crispus* (red alga), *Drosophila melanogaster* (fruit fly), *Rattus norvegicus* (rat), *Homo sapiens* (human), *Xenopus lividus* (frog), *Metridium senile* (cnidarian), *Monosiga brevicollis* (choanoflagellate), *Allomyces macrogynus* (chytridiomycete fungus), *Mortierella verticillata* (zygomycete fungus), *Rhizopus stolonifer* (zygomycete fungus), *Schizophyllum commune* (basidiomycete fungus), *Podospora anserina* (ascomycete fungus), *Neurospora crassa* (ascomycete fungus), *Aspergillus nidulans* (ascomycete fungus), *Pichia canadensis* (ascomycete fungus), *Saccharomyces cerevisiae* (ascomycete fungus), *Schizosaccharomyces pombe* (archiascomycete fungus), *Schizosaccharomyces octosporus* (archiascomycete fungus), *Rhizophydium* sp. (chytridiomycete fungus), *Spizellomyces punctatus* (chytridiomycete fungus), *Harpochytrium* sp. (chytridiomycete fungus).

mtDNA-encoded protein shown to be a stoichiometric component of the mitochondrial small ribosomal subunit in *N. crassa*[10]. We have identified regions of significant amino acid sequence similarity in Var1 and S5 proteins (Fig. 1a). Together, these results provide compelling evidence that Var1 and S5 are homologous proteins necessary for proper assembly of the mitochondrial small ribosomal small subunit. In Fig. 1b, we present a C-terminal protein sequence motif that supports the notion that Var1 and S5 are related to Rps3. Despite the high variability of Rps3 proteins in terms of length and amino acid sequence, this motif is found in all nuclear, bacterial and organellar Rps3 proteins.

This motif is also present in the C-terminal region of Orf227 (formerly Urfa; Fig. 1b), the protein encoded by the unique unidentified open reading frame (ORF) in the mtDNA of the archiascomycete fungus *Schizosaccharomyces pombe*, and in the C-terminal region of its counterpart, Orf248, in *Schizosaccharomyces octosporus*. Interestingly, the Orf227 protein tolerates mutations in its N-terminal region, whereas mutations affecting the C-terminal region cause respiratory deficiency in *S. pombe*[11,12]. Together, these data suggest that Orf227 and Orf248 are also Rps3 homologs.

Unexpectedly, the published *N. crassa* S5 sequence[13] (GenBank accession number J01427.1) lacks the Rps3 C-terminal motif. However, resequencing of this gene revealed (GenBank accession number AF275270) that *N. crassa* S5 does in fact contain this motif (Fig. 1b), and that the original nucleotide sequence contains several insertions and deletions within a GC cluster (nucleotides 495–647; GenBank accession number AF275270) resulting in the inference of a premature termination codon.

This study resolves the long-standing questions surrounding the nature and origin of S5 (*N. crassa*)[13], Var1 (*S. cerevisiae*)[14,15] and Orf227 (*S. pombe*)[12]. Further, the data describe the phylogenetic distribution of mtDNA-encoded Rps3, indicating at least three independent losses of the *rps3* gene from the mitochondrial genome (Fig. 2). These results are also useful for identifying other Rps3 homologs. For example, we were able to identify the Rps3 C-terminal motif in the C-terminal region of an ORF of 1453 amino acids in the mtDNA of the basidiomycete *Schizophyllum commune* (Fig. 1b). We also confirm that Orf1740, encoded by the mtDNA of the cellular slime mold *Dictyostelium discoideum*[16] (GenBank accession number AB000109), is in fact a Rps3 homolog.

Finally, it is interesting to note that mutations affecting the C-terminal region of Orf227 have been shown to be responsible for mitochondrial mutator activity in *S. pombe*[12], possibly as the result of impaired mtDNA repair[17]. Similarly, Rps3 has been shown to have a DNA endonuclease activity, which functions in the repair of oxidative DNA damage in *Drosophila*[18,19] and mammals[20,21]. These data, together with our results, make it tempting to speculate that the conserved C-terminal region of Rps3 plays an essential role, not only in the assembly of the small ribosomal subunit, but also in the DNA repair process.

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CHARLES E. BULLERWELL, GERTRAUD BURGER AND B. FRANZ LANG

Program in Evolutionary Biology, Canadian Institute for Advanced Research, Département de Biochimie, Université de Montréal, 2900 Boul. Edouard Montpetit, Montréal, Qué., Canada H3T 1J4.
Email: Franz.Lang@Umontreal.ca

Box 1. Note added in proof

We have identified a further *rps3* gene in the mitochondrial DNA of the zygomycete fungus *Smittium culisetae*. This gene codes for a putative protein of 231 amino acids, which contains the Rps3 C-terminal motif in its C-terminal region.

Chapter IV: RESULTS AND DISCUSSION

IV.1 The mtDNAs of *S. octosporus* and *S. japonicus* var. *japonicus*

IV.1.1 Genome size, gene content and gene order

We have completely sequenced the mtDNA of *S. octosporus* (Figure IV.1). This genome is a circular molecule of precisely 44,227 bp, i.e., more than twice the size of the mtDNA of *S. pombe*. We have identified 24 tRNA genes, *rns*, *rnl*, *cox1*, *cox2*, *cox3*, *cob*, *atp6*, *atp8*, and *atp9*, as well as *rps3* (see chapter III and section IV.1.2) and *rnpB* in this mtDNA. All of these genes are coded on the same DNA strand. The only observed difference in gene content observed between the genomes of *S. pombe* and *S. octosporus* is the absence of the *trnI2*(cau) gene in the mtDNA of *S. octosporus* (discussed in section IV.1.4). The gene order in this mtDNA is similar to that observed in *S. pombe*, with five conserved blocks of gene order conservation comprising the entire genome (excluding the absent *trnI2* gene in *S. octosporus*; see Figures IV.1 and I.2). Only two transpositions of the *S. octosporus* mtDNA would be necessary to give the gene order observed in the *S. pombe* mitochondrial genome.

We have also sequenced more than 80 kbp of the mtDNA of *S. japonicus* var. *japonicus* (Figure IV.2), a genome which we estimate to be more than four times as large as that of *S. pombe*. We have identified the same set of genes in this genome as in that of *S. pombe*, except that *rps3* and *rnpB* are apparently not present. Although the vast majority (>95%) of this genome has already been sequenced, we cannot state with absolute certainty that these missing genes are absent. The sequencing of this mtDNA is presently incomplete in two regions (indicated by red lines in Figure IV.2). Sequencing will continue until the exact DNA sequence of these regions has been determined. Based on the sequence information available at present, it is clear that the gene order in this mtDNA bears no resemblance to that observed in either *S. pombe* or *S. octosporus* (see figures I.2, IV.1 and IV.2). Further, in contrast to these two mtDNAs, *S. japonicus* var. *japonicus* mitochondrial genes are coded on both DNA strands.

Figure IV.1 The mtDNA of *S. octosporus*. Inner circle gives scale in kbp. Colours on the intermediate circle represent regions of gene order conservation with the mtDNA of *S. pombe* (see Figure I.2). Outer circle indicates the location of genes, exons (black), introns and intronic ORFs (group I, green; group II, magenta) in this mtDNA. Location of DHEs is indicated by asterixs.

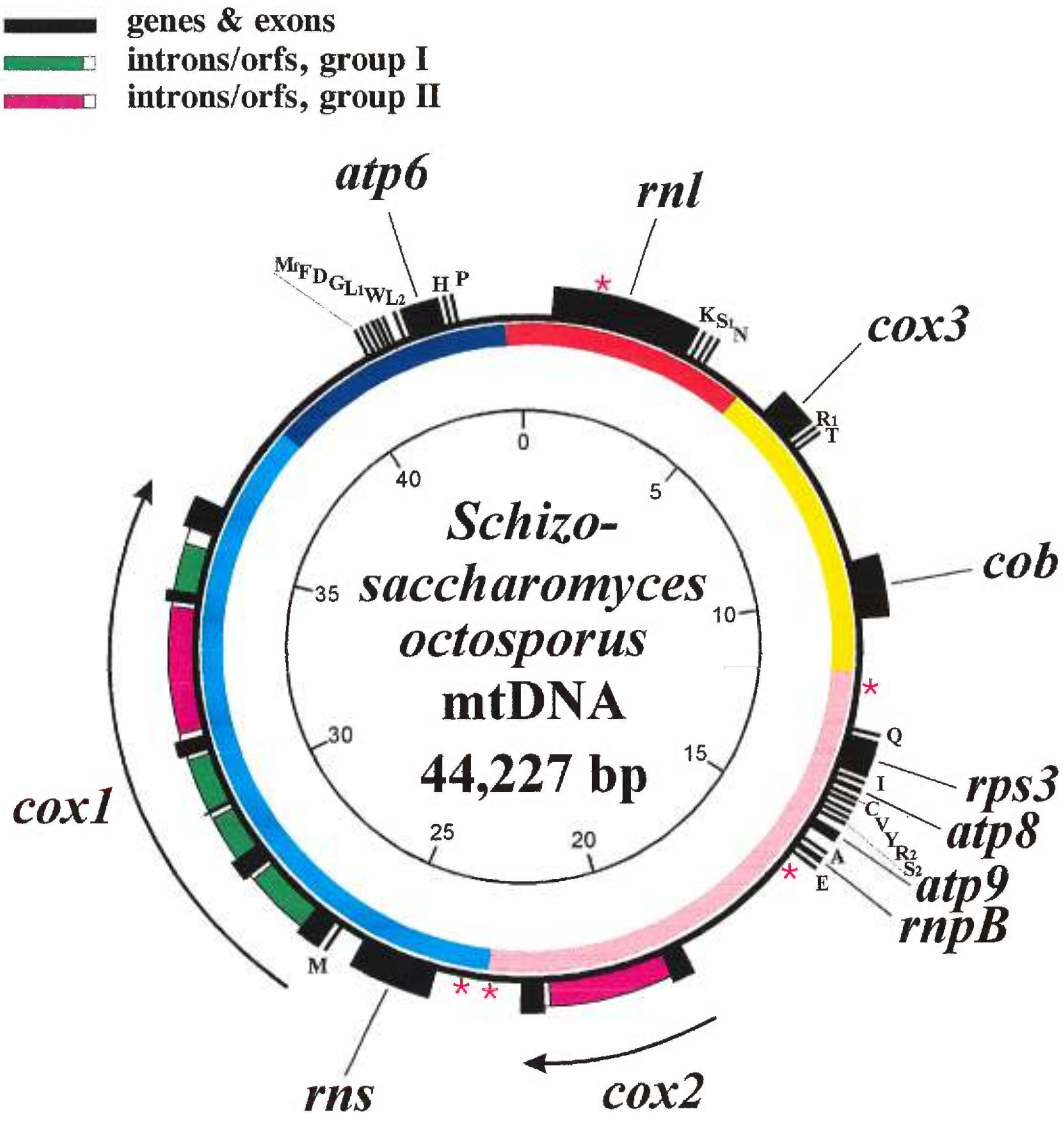
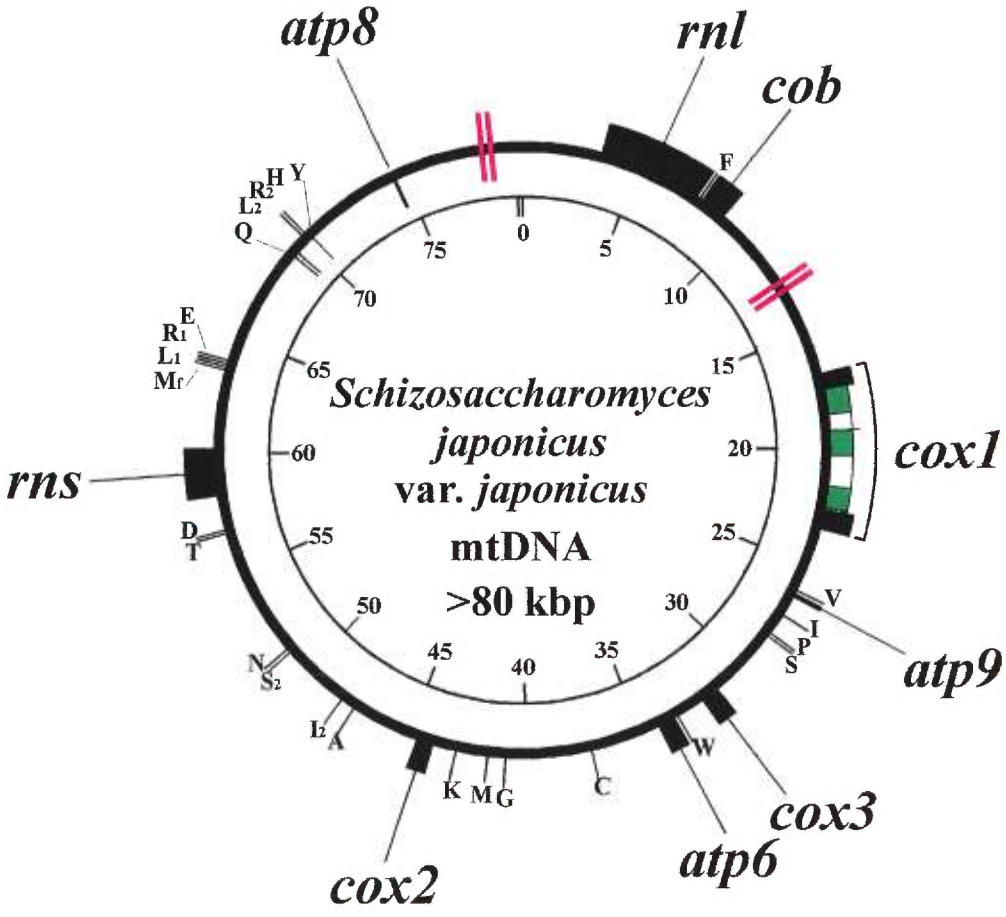

genes & exons
introns/orfs, group I
introns/orfs, group II
atp6
rnl
cox3
cob
rps3
atp8
atp9
rnpB
cox1
rns
cox2
Schizo-
saccharomyces
octosporus
mtDNA
44,227 bp
0
5
10
15
20
25
30
35
40
M
rF
D
G
L1
W
L2
H
P
K
S1
N
R1
T
Q
I
C
V
Y
R2
S2
A
E

Figure IV.2 The mtDNA of *S. japonicus* var. *japonicus*. Inner circle gives scale in kbp. Outer circle indicates the location of genes, exons (black), group I introns and ORFs (green) in this mtDNA. Red lines indicate regions whose DNA sequence is presently incomplete.


genes & exons
introns/orfs, group I
atp8
rnl
cob
cox1
atp9
cox3
atp6
cox2
rns
Schizosaccharomyces
japonicus
var. japonicus
mtDNA
>80 kbp
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
F
V
I
P
S
W
C
K
M
G
A
I2
N
S2
D
T
Mf
L1
R1
E
Q
L2
R2
H
Y

The gene order data derived from the sequencing of these two genomes supports a closer relationship between *S. octosporus* and *S. pombe* than between either of these species and *S. japonicus* var. *japonicus*, as proposed by Sipiczki (1995), and supported by several other lines of evidence (discussed in section I.2.3).

IV.1.2 Orf227 and Orf248 are homologs of Rps3

We have identified a homolog of the *S. pombe* mitochondrial gene *orf227* in the mtDNA of *S. octosporus* (*orf248*), by sequence similarity to its *S. pombe* counterpart (see chapter III for a more thorough analysis). Alignment of the amino acid sequences of these proteins (inferred from the DNA sequences) aided in the definition of a motif, which has allowed us to relate these proteins to Rps3 (ribosomal small subunit protein 3), a gene which had only been previously identified in one fungal mtDNA, that of the chytridiomycete *Allomyces macrogynus*(Paquin and Lang, 1996). Our results indicate that both the mtDNA-encoded proteins Var1 in budding yeasts (e.g., *S. cerevisiae*) and S5 in filamentous ascomycetes (e.g., *N. crassa*) are also derived from this ribosomal protein, and that the *rps3* gene is much more widespread in fungal mitochondrial genomes than was previously appreciated.

This finding is particularly interesting, as the evolutionary origins of *S5*, *var1* and *orf227* have been debated (Burke and RajBhandary, 1982; Butow *et al.*, 1985; de Zamaroczy and Bernardi, 1987; Neu *et al.*, 1998) since their respective discoveries. Although evidence of the homology of these proteins has appeared in the literature on several occasions, a link to *rps3* has been lacking. This study resolves this long-standing question.

IV.1.3 Intron content

The mtDNA of *S. octosporus* contains six introns, five located in the *cox1* gene (four group I, one group II) and one in the *cox2* gene (group II). The

mtDNA of *S. japonicus* var. *japonicus* contains two introns, both group I, located in the *cox1* gene. All of the introns contained within these mtDNAs contain ORFs characteristic of the corresponding intron group (LAGLIDADG motifs in the case of group I intronic ORFs, Belfort and Perlman, 1995; conserved reverse transcriptase sequence motifs in the group II intronic ORFs, Michel and Lang, 1985). The group II intronic ORF of *S. octosporus cox1*-I4 (intron nomenclature: *cox1*-I4 indicates the fourth intron within the *cox1* gene) gave a high BLAST score with that of *Marchantia polymorpha cox2*-I2 ($7x10^{-64}$), and that of *S. octosporus cox2*-I1 gave high BLAST scores with the group II intronic ORFs of *P. anserina cox1*-I1 ($1x10^{-107}$) and *A. macrogynus cox1*-I3 ($1x10^{-105}$). The high similarity of these proteins may indicate common origins of the introns in which they are encoded.

In addition, we have identified fission yeast homologs for five of the six group I introns: *S. octosporus cox1*-I1 and *S. pombe cox1*-I1 are inserted in the same position of the *cox1* gene; *S. octosporus cox1*-I2 is located in the same position of the *cox1* gene as S. *japonicus var. japonicus cox1*-I1; and *S. octosporus cox1*-I3 is located in the same position in the *cox1* gene as *S. pombe cox1*-I2 and *S. japonicus* var. *japonicus cox1*-I2. *S. octosporus* Orf281, located within the group I intron *cox1*-I5, does not appear to have a homolog in other fission yeast mtDNAs. High BLAST scores were obtained against the ORFs located in *cox1*-I15 of *Podospora anserina* ($4x10^{-27}$), *cox1/2*-I3 of *Dictyostelium discoideum* ($5x10^{-25}$), and *cox1*-I8 of *Marchantia polymorpha* ($3x10^{-15}$). Furthermore, all of the introns containing these ORFs are inserted in identical positions within the *cox1* gene (Ogawa *et al.*, 1997; note that the *cox1* and *cox2* genes are fused in *D. discoideum*, therefore the delimitation of Cox1 is inferred by protein sequence alignment). The diversity of these organisms, as well as the high similarity of their encoded proteins, suggests that this group I intron has been involved in numerous horizontal transfers among distantly-related species.

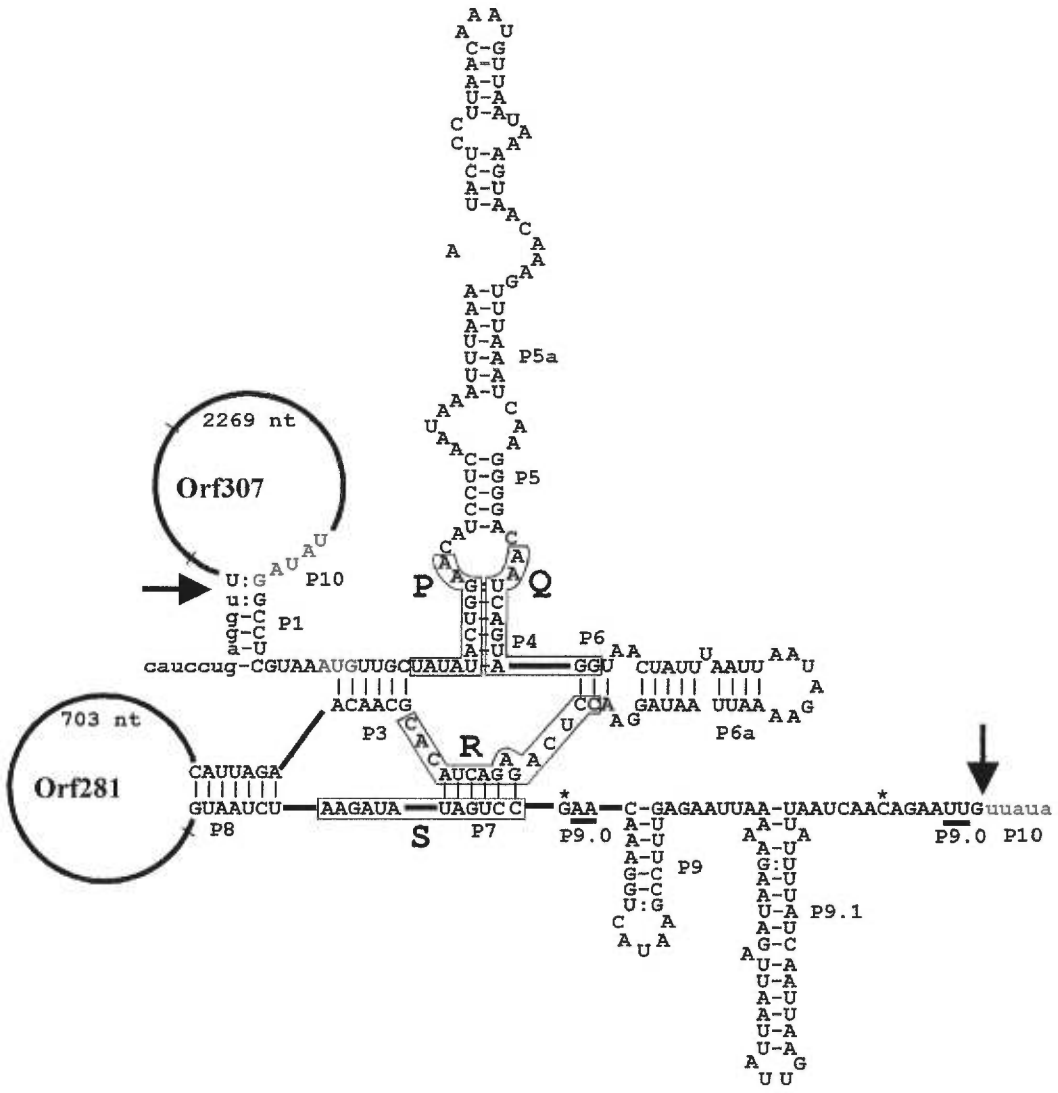
Group I introns encoding two ORFs are rarely observed, but have

previously been described in the mtDNA of *P. anserina* (Cummings *et al.*, 1989) and *D. discoideum* (Ogawa *et al.*, 1997). The second intron (group I) in the *cox1* gene of *S. japonicus* var. *japonicus* (*cox1*-I2) contains two closely-related ORFs, one located within the the P1 loop (Orf307), the other within the P8 loop (Orf281) (Figure IV.3). As mentioned above, both ORFs are related to Orf323, encoded within the P8 loop of *S. pombe cox1*-I2. However, Orf307 shows a higher similarity to this ORF than does Orf281. Furthermore, the two LAGLIDADG motifs in Orf281 appear highly derived (YLAGLIDSSDA and WLTGLAERTIQ), whereas those in Orf307 are canonical (YLAGLIDGDGH and WLAGFSDADAS) and are, in fact, identical to those in the *S. pombe* Orf323. This suggests that Orf307 in *S. japonicus* var. *japonicus* is derived from Orf323 of *S. pombe*, perhaps by horizontal ORF transfer among species. It is possible that Orf281 has assumed a different function from that of Orf307. Interestingly, its start codon is located in a helical region within the conserved RNA secondary structure of *cox1*-I2 (see Figure IV.3), potentially regulating the expression of the protein product of this ORF.

IV.1.4 Genetic code

As is the case in *S. pombe* mitochondria, the universal genetic code is used in the standard mitochondrial protein genes of both *S. octosporus* and *S. japonicus* var. *japonicus* (Table IV.1). TGA codes for a fraction of tryptophan residues in some intronic ORFs in both of these systems, whereas tryptophan is coded by only TGG codons in all standard protein genes (as well as in *rps3* of *S. octosporus*). Accordingly, the tRNA aminoacylated with tryptophan (*trnW*) has the anticodon CCA (rather than UCA). A CCA anticodon might allow weak interactions with UGA tryptophan codons, thereby allowing translation of intronic ORF transcripts to some degree. In fact, a single mutation in the D arm of an amber-suppressing variant of *E. coli trnW* has been shown to significantly increase the efficiency of a CxA wobble pairing (Raftery, L.A., 1986). Alternatively, these intronic ORFs

Figure IV.3 Predicted secondary structure of *S. japonicus* var. *japonicus cox1*-I2. Arrows indicate 5' and 3' splice sites. Sizes of loops and names of ORFs are indicated. Boxed sequences labelled P, Q, R and S indicate core regions, and P1 to P10 refer to the conserved regions of group I introns. Upper-case letters indicate intron sequences, lower-case letters indicate exon sequences. Nucleotides involved in the P10 interaction are shown in blue. The inferred start codon of *orf281* is shown in red. Asterixs indicate a pseudoknot.


2269 nt
Orf307
703 nt
Orf281
P1
P3
P4
P5
P5a
P6
P6a
P7
P8
P9
P9.0
P9.1
P10
P
Q
R
S
cauccug-CGUAAAUGUUGCUAUAU-A
GGU
CUAUU
AAUU
ACAAACG
GAUAA
UUAA
AUCAGG
CAUUAGA
GUAAUCU
AAGAUA
UAGUCC
GAA
C-GAGAAUUAA-UAAUCAACAGAAUUG
uuaua

with non-standard genetic codes may be the result of more recent intron acquisitions by these mtDNAs, and may not be translated into functional proteins. In the latter scenario, these ORFs could not encode essential proteins for intron splicing (the removal of introns from mRNA transcripts being necessary for proper mitochondrial function), and (in the absence of other selective pressures) these ORFs would eventually be eliminated from the genome.

The mtDNAs of both *S. octosporus* and *S. japonicus* var. *japonicus* contain the same set of tRNAs as that of *S. pombe*, with one exception: the mtDNA of *S. octosporus* lacks *trnI2*(cau), the isoleucine-accepting tRNA which recognizes AUA codons. The lack of this tRNA gene correlates with the absence of ATA codons in standard protein-coding genes of this mtDNA, and *rps3*. Despite this, it is interesting to note that intronic ORFs do, in fact, contain ATA codons. An isoleucine tRNA which recognizes AUA codons may be imported into mitochondria from the nucleus to translate these codons. It is also possible that translation of these ORFs occurs, inefficiently, using a modified *trnI*(gua) gene transcript, or (as discussed above) these intronic ORFs may not be translated into functional proteins.

Finally, the mtDNAs of the three available *Schizosaccharomyces* species have a strong tendency for A+T nucleotides in areas of the genome which are not under high selective pressure. This is observed, for example, in the wobble position of codons in all protein-coding genes (including intronic ORFs) in the mitochondria of all three species (see Table IV.1). Interestingly, there is a weak tendency to use TTC codons (as opposed to TTT) to code for phenylalanine in standard mitochondrial protein-coding genes, whereas the opposite trend is observed in *rps3* and intronic ORFs (TTT codons are strongly preferred). The preference for a C in the wobble position of codons in standard proteins may serve to avoid frame-shifting at runs of U residues during translation (previously observed in yeast mitochondria; Fox and Weiss-Brummer, 1980). In support of this, *trnF* has the anticodon GUU, which allows a standard G-C base-pair in the

Table IV.1 Codon usage in the mtDNAs of *S. pombe, S. octosporus* and *S. japonicus* var. *japonicus*. Termination codons are indicated in green, notable features discussed in the text are indicated in red. Actual codon count (percentage count in parentheses) is shown for standard protein-coding gene transcripts (including *rps3*)/intronic ORFs in (from top to bottom) *S. pombe*, *S. octosporus* and *S. japonicus* var. *japonicus* mitochondria.

	73 (44) / 64 (80)		49 (28) / 61 (41)		80 (80) / 58 (81)		15 (100) / 20 (95)
F UUU	88 (49) / 102 (76)	S UCU	55 (34) / 75 (31)	Y UAU	82 (80) / 139 (87)	C UGU	19 (100) / 31 (97)
	80 (47) / 50 (91)		84 (59) / 33 (45)		83 (93) / 48 (94)		12 (100) / 10 (83)
	93 (56) / 16 (20)		0 (0) / 5 (3)		20 (20) / 14 (19)		0 (0) / 1 (5)
F UUC	91 (51) / 32 (24)	S UCC	1 (1) / 13 (5)	Y UAC	20 (20) / 21 (13)	C UGC	0 (0) / 1 (3)
	89 (53) / 5 (9)		4 (3) / 2 (3)		6 (7) / 3 (6)		0 (0) / 2 (17)
	216 (77) / 91 (54)		71 (41) / 43 (29)		8 (100) / 2 (50)		1 (2) / 4 (21)
L UUA	233 (77) / 247 (69)	S UCA	53 (32) / 74 (31)	* UAA	8 (100) / 5 (83)	W UGA	0 (0) / 4 (9)
	196 (71) / 82 (76)		15 (10) / 23 (31)		6 (86) / 3 (100)		0 (0) / 5 (45)
	4 (1) / 15 (9)		0 (0) / 2 (1)		0 (0) / 2 (50)		39 (98) / 15 (79)
L UUG	3 (1) / 16 (4)	S UCG	0 (0) / 6 (3)	* UAG	0 (0) / 1 (17)	W UGG	36 (100) / 40 (91)
	2 (1) / 5 (5)		0 (0) / 1 (1)		1 (14) / 0 (0)		35 (100) / 6 (55)
	35 (12) / 42 (25)		53 (51) / 26 (49)		38 (78) / 30 (81)		0 (0) / 7 (9)
L CUU	44 (14) / 45 (13)	P CCU	66 (65) / 47 (60)	H CAU	39 (85) / 60 (90)	R CGU	0 (0) / 6 (4)
	54 (19) / 6 (6)		67 (77) / 12 (63)		46 (98) / 18 (82)		5 (13) / 3 (7)
	0 (0) / 3 (2)		1 (1) / 4 (8)		11 (22) / 7 (19)		0 (0) / 3 (4)
L CUC	0 (0) / 6 (2)	P CCC	1 (1) / 4 (5)	H CAC	7 (15) / 7 (10)	R CGC	0 (0) / 2 (1)
	2 (1) / 3 (3)		0 (0) / 2 (11)		1 (2) / 4 (18)		0 (0) / 2 (5)
	23 (8) / 18 (11)		49 (47) / 20 (38)		37 (100) / 42 (79)		7 (16) / 13 (16)
L CUA	22 (7) / 40 (11)	P CCA	31 (31) / 25 (32)	Q CAA	36 (97) / 72 (85)	R CGA	2 (6) / 19 (14)
	23 (8) / 12 (11)		20 (23) / 4 (21)		32 (97) / 27 (90)		2 (5) / 4 (9)
	3 (1) / 1 (1)		1 (1) / 3 (6)		0 (0) / 11 (21)		0 (0) / 1 (1)
L CUG	2 (1) / 4 (1)	P CCG	3 (3) / 2 (3)	Q CAG	1 (3) / 13 (15)	R CGG	0 (0) / 2 (1)
	0 (0) / 0 (0)		0 (0) / 1 (5)		1 (3) / 3 (10)		0 (0) / 0 (0)
	128 (58) / 93 (58)		58 (58) / 47 (63)		99 (90) / 95 (86)		48 (27) / 30 (20)
I AUU	161 (83) / 168 (71)	T ACU	59 (59) / 59 (42)	N AAU	113 (86) / 238 (90)	S AGU	49 (30) / 58 (24)
	133 (65) / 54 (56)		79 (82) / 21 (47)		96 (96) / 88 (97)		36 (25) / 13 (18)
	41 (19) / 16 (10)		0 (0) / 5 (7)		11 (10) / 15 (14)		7 (4) / 8 (5)
I AUC	34 (17) / 35 (15)	T ACC	0 (0) / 11 (8)	N AAC	19 (14) / 25 (10)	S AGC	6 (4) / 13 (5)
	40 (20) / 9 (9)		5 (5) / 4 (9)		4 (4) / 3 (3)		4 (3) / 2 (3)
	52 (24) / 51 (32)		41 (41) / 19 (25)		51 (94) / 144 (89)		35 (81) / 52 (65)
I AUA	0 (0) / 32 (14)	T ACA	40 (40) / 62 (44)	K AAA	58 (98) / 280 (85)	R AGA	34 (94) / 99 (74)
	32 (16) / 33 (34)		12 (12) / 20 (44)		50 (100) / 81 (90)		32 (82) / 32 (73)
	56 (100) / 19 (100)		1 (1) / 4 (5)		3 (6) / 18 (11)		1 (2) / 4 (5)
M AUG	57 (100) / 42 (100)	T ACG	1 (1) / 8 (6)	K AAG	1 (2) / 51 (15)	R AGG	0 (0) / 6 (4)
	48 (100) / 14 (100)		0 (0) / 0 (0)		0 (0) / 9 (10)		0 (0) / 3 (7)
	56 (49) / 29 (52)		92 (69) / 41 (72)		47 (96) / 50 (88)		102 (68) / 40 (51)
V GUU	69 (56) / 44 (35)	A GCU	98 (72) / 40 (53)	D GAU	48 (83) / 99 (94)	G GGU	85 (61) / 59 (49)
	56 (64) / 19 (54)		75 (77) / 15 (56)		43 (96) / 30 (97)		87 (78) / 21 (50)
	4 (4) / 3 (5)		5 (4) / 2 (4)		2 (4) / 7 (12)		0 (0) / 3 (4)
V GUC	6 (5) / 9 (7)	A GCC	6 (4) / 3 (4)	D GAC	10 (17) / 6 (6)	G GGC	1 (1) / 6 (5)
	5 (6) / 4 (11)		4 (4) / 1 (4)		2 (4) / 1 (3)		3 (3) / 1 (2)
	52 (46) / 20 (36)		33 (25) / 12 (21)		48 (96) / 42 (79)		45 (30) / 31 (40)
V GUA	47 (38) / 65 (52)	A GCA	31 (23) / 28 (37)	E GAA	48 (96) / 108 (87)	G GGA	45 (32) / 48 (40)
	22 (25) / 11 (31)		17 (18) / 11 (41)		48 (98) / 21 (88)		17 (15) / 19 (45)
	2 (2) / 4 (7)		4 (3) / 2 (4)		2 (4) / 11 (21)		2 (1) / 4 (5)
V GUG	2 (2) / 7 (6)	A GCG	1 (1) / 4 (5)	E GAG	2 (4) / 16 (13)	G GGG	8 (6) / 7 (6)
	4 (5) / 1 (3)		1 (1) / 0 (0)		1 (2) / 3 (12)		4 (4) / 1 (2)

wobble position of UUC codons, thereby reducing the possibility of frame-shifting. The selective pressure to avoid frame-shifting during the translation of *rps3* and intronic ORF transcripts may be significantly reduced compared to that for transcripts of standard protein-coding genes, as the proteins encoded by the former are not likely needed in the same quantities as the latter.

IV.1.5 Transcription and RNA transcript processing

The sequence immediately upstream of the *S. pombe rnl* gene (5'-ATATATGTA-3') has been shown to function as a promoter of mitochondrial transcription (Lang *et al.*, 1987). The *S. octosporus rnl* gene is preceded by a similar sequence (5'-ATAAAAGTA-3') which may also serve as a promoter. In *S. japonicus* var. *japonicus*, at least two promoters must be present, since genes are coded on both strands of this mtDNA (Figure IV.2). However, no sequence similar to that in *S. pombe* and *S. octosporus* was identified. GTP capping experiments should be performed on mitochondrial transcripts from both *S. octosporus* and *S. japonicus* var. *japonicus* in order to determine the exact placement of mitochondrial promoters.

In *S. pombe*, tRNAs have been shown to function as signals for the processing of mitochondrial RNA transcripts (Lang *et al.*, 1983), a mechanism which has also been observed in the mitochondria of mammals (Ojala *et al.*, 1980; Bibb *et al.*, 1981), *Neurospora crassa* (de Vries *et al.*, 1985; Burger *et al.*, 1985) and *Aspergillus nidulans* (Dyson *et al.*, 1989). tRNA genes are located both upstream and downstream of most genes in this genome, except upstream of *rnl* and *cox3*, regions which have been shown to contain promoters. The absence of tRNAs is thus indicative of promoter location. In the mtDNA of *S. octosporus*, all genes with the exception of *rns* have tRNAs upstream and downstream (Figure IV.1). We predict that the *S. octosporus* mitochondrial genome is transcribed from at least two promoters, one upstream *rnl* (see above for a possible promoter motif), and one upstream *rns* (no tRNA upstream). Finally, a similar situation is

observed in *S. japonicus* var. *japonicus* wherein most genes have tRNAs upstream and downstream. No tRNAs were identified upstream *rnl*, *cox1*, or *atp8*, which may also indicate promoters in these regions. However, sequencing of these regions is not complete, and tRNAs may yet be identified in these locations.

C-rich regions are present downstream all genes in *S. pombe* (except *rnl*), and S1 nuclease protection signals are clustered immediately downstream of these regions in the *atp6* (Lang *et al.*, 1983) and *rnl* (Trinkl *et al.*, 1989) transcripts, indicating that they are present in the mature transcripts. Similarly, C-rich regions are also present downstream of all protein-coding genes and *rns* in the mtDNAs of both *S. octosporus* and *S. japonicus* var. *japonicus*, as well as downstream of *rps3* and *rnpB* in *S. octosporus* (Figure IV.4). The consensus sequence of these regions was found to be 5'-AACCCCC-3' in these two genomes, whereas in *S. pombe* the consensus is 5'-CCCCC-3'. Taken together, these results strongly indicate that C-rich regions have a function in determining the mature 3'-ends of RNA transcripts in fission yeast mitochondria. We speculate that they might function as a protection of RNA 3'-ends from exonuclease degradation.

IV.1.6 Respiratory-deficiency in *S. japonicus* var. *japonicus*

S. japonicus var. *japonicus* is unable to grow on non-fermentable substrates (such as glycerol), and cytochrome spectra indicate the absence of cytochromes a + a3 and c in this organism (Sipiczki *et al.*, 1982). The cause and significance of respiratory-deficiency in *S. japonicus* var. *japonicus* remains unclear, as all the expected mtDNA-encoded components of the translation machinery (except *rps3*), as well as subunits of the respiratory chain and ATP synthase complexes, are present (with respect to *S. pombe* mtDNA), and apparently functional in this mtDNA.

The absence of the *rps3* and *rnpB* genes in *S. japonicus* var. *japonicus* is not likely responsible for respiratory-deficiency in this species. For example, the *rps3* gene has been identified in the mtDNA of the zygomycete *Mortierella*

Figure IV.4 Conserved sequences for RNA 3'-end processing in the mtDNAs of *S. pombe*, *S. octosporus* and *S. japonicus* var. *japonicus*. Termination codons are indicated in red, consensus nucleotides are indicated in blue. Additional nucleotides present in 17 of 18 genes in *S. octosporus* and *S. japonicus* var. *japonicus* (as well as 3 of the 9 genes in *S. pombe*) are indicated in yellow. S1 nuclease signals determined for *S. pombe rns* (Trinkl *et al.*, 1989) and *atp6* (Lang *et al.*, 1983) and primer-extension signals for *S. octosporus rnpB* (E. Seif and B.F. Lang, unpublished results) are indicated by red arrows. Numbers in brackets indicate nucleotides not shown.


S. pombe
rns GGTAACCGTAGTGGAGTTGCGGTTGAACTAATTAATTCAGGTTAACCCCCCCCCAAAAAATAAAATACATCCATATCAAAAAGATGTTAATT-trnM
cox1 TAA.....TTTAAAATCTTAACCCCCTTTTATTTTATAAACTTTATTCCAATTTAAAAATT-(114)-cox3
cox2 TAA.....CCTCCCCCCTAAAGTGTTTATTAAATGATGAACTCAAATAGAGAA-(79)-rnl
cox3 TAA.....GGCTTACTTCAATTTTTCCCCCCTTTTTATATCTTATTTCTCTAAAATA.....-trnR1
cob TAA.....TCTCTTCCCCCTTTACACTATATAAAAATATCTTATTTAAACAGTAT-(171)-trnMf
atp6 TAA.....TTTTCTCCCCCTATTTTTTAT.....-trnH
atp8 TAA.....TTTAACCCCTCCAAAAGTATT.....-trnC
atp9 TAA.....TTTCCCCCTTTAAAAAAGAGAATACT.....-trnA
rps3 TAA.....TTGTTTCCCCCTTCAATCATTTAA.....-trnI1
S. octosporus
rns GTAACCGTAGTGGAAATTGCGGTTGAATAATTATTATCAGGATAACCCCCAAAAATAAGAAAACTTTAGAACTAACTAAATAAAAA-(440)-trnM
rnpB GTAATTTCAGTTAATTTTACATAATCCATCTTATAAATTATCAAACCCCCTTTTTAAATATATTAAAATAAAA.....-trnE
cox1 TAA.....GAAACTATCCCCTATTAACCCCCATTTAAATTAAAAATTAACCCCCACCTTAAGAAAC-(4292)-trnMf
cox2 TAA.....ATAATCCAAAAATCTAAAAAATTAACCCCCACAAACCTTAAATAATGAGTACAGTATCAAATCAT-(1813)-rns
cox3 TAA.....TCTAAAAAATAACCCCCATCAGATAAA.....-trnR
cob TAA.....CCCCCATCAAATAAGAACGCTTCAAAAAATATATAAAATG-(2092)-trnQ
atp6 TAA.....ATAAAAATAACCCCCTAATAAATAAATTAT.....-trnH
atp8 TAA.....ACTAAACTTTAAAAACCCCCTATAATAAATTA.....-trnC
atp9 TAA.....GTAAAATAAAGGTAATATAAAGTAACCCCCACATATTTAAAGATTAATAATGAGTAACTTGTTAGT-(135)-trnA
rps3 TAA.....TTTTATCCCCCTATCTTTACTTTT.....-trnI
S. japonicus var. japonicus
rns ATGCTGTAGAGGAATCTGTAGCTGAATTATTATAAAGGAAAATAACCCCCCCCCAAAAAATCCTAATTAATAAATTAAACTAAAT-(3283)-trnMf
cox1 TAA.....TTGTTTTCGATAATCTTTGATTATCTTTAATTTAACCCCCCCCCCACACACACACATACATACACATATTTACAC-(2604)-trnV
cox2 TAA-(201)-AATTCTATTTATTCATCTTTAATACTATTATTTAACCCCCTCCACACTCCTATATATACATACTCTCTCTATCTA-(2594)-trnA
cox3 TAA-(687)-CGACTATTCTTTAATACTTAAATCTATAATTTTAACCCCCCCCTCCCTTTCTAATTATAATTTACCCCTCATATTA-(389)-trnW
cob TAA.....TTTATATTATTTTTTAAACCCCCTACTACCCCCCTCTATAAATATTATATGTAATTATTAATTTA-(?)
atp6 TAA-(297)-AGTTATTCGATAATCTTTGATTATCTTTAATTTAACCCCCCCCTCTATACCACTATATGCCTATATCAGTTATACTGATATT-(?)
atp8 TAG-(94)-ATTCTATTAACTACTATTTGTTATTAACTCATAAACCCCCTCTTCTTATTATATATTTATATTTATATTTAAATT-(4014)-trnY
atp9 TAA-(69)-AGTTCTATTTATCTCTTATTAATTTAATTTATAAACCCCCTCCTCTTAAATCTTTAAATAAGCTACACTTATTTAT-(829)-trnI1

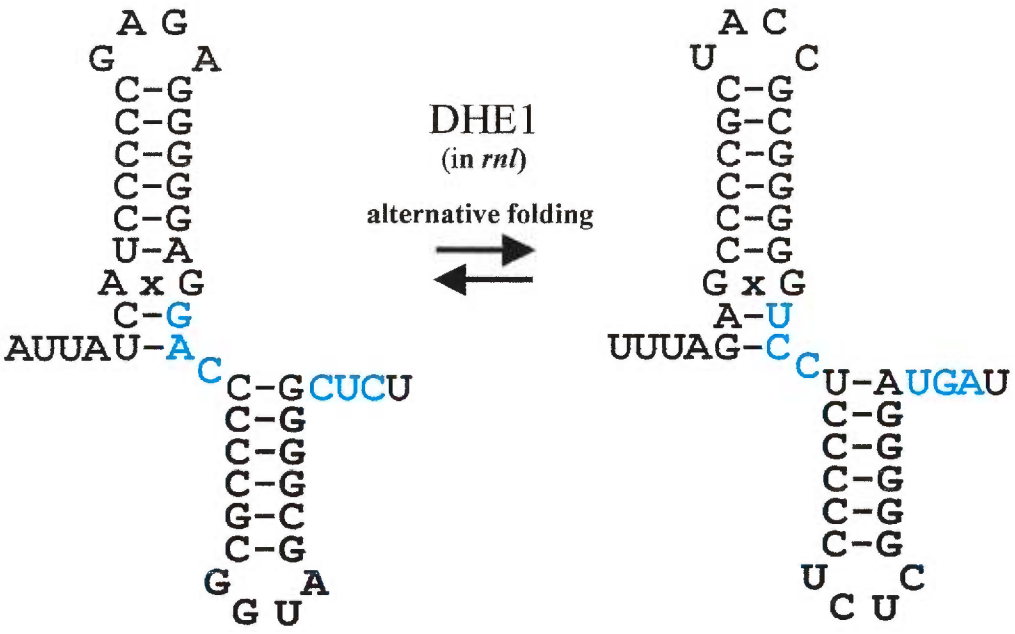
verticillata, whereas that of the closely-related *Rhizopus stolonifer* does not contain this gene (see chapter III), and the *rnpB* gene is absent in the mtDNA of *Podospora anserina*, but present in that of the related filamentous ascomycete *Aspergillus nidulans* (Lee *et al.*, 1996). In these instances, the genes are assumed to have been replaced by nuclear genes, whose products are then imported into mitochondria. We assume that this also applies to *S. japonicus* var. *japonicus*.

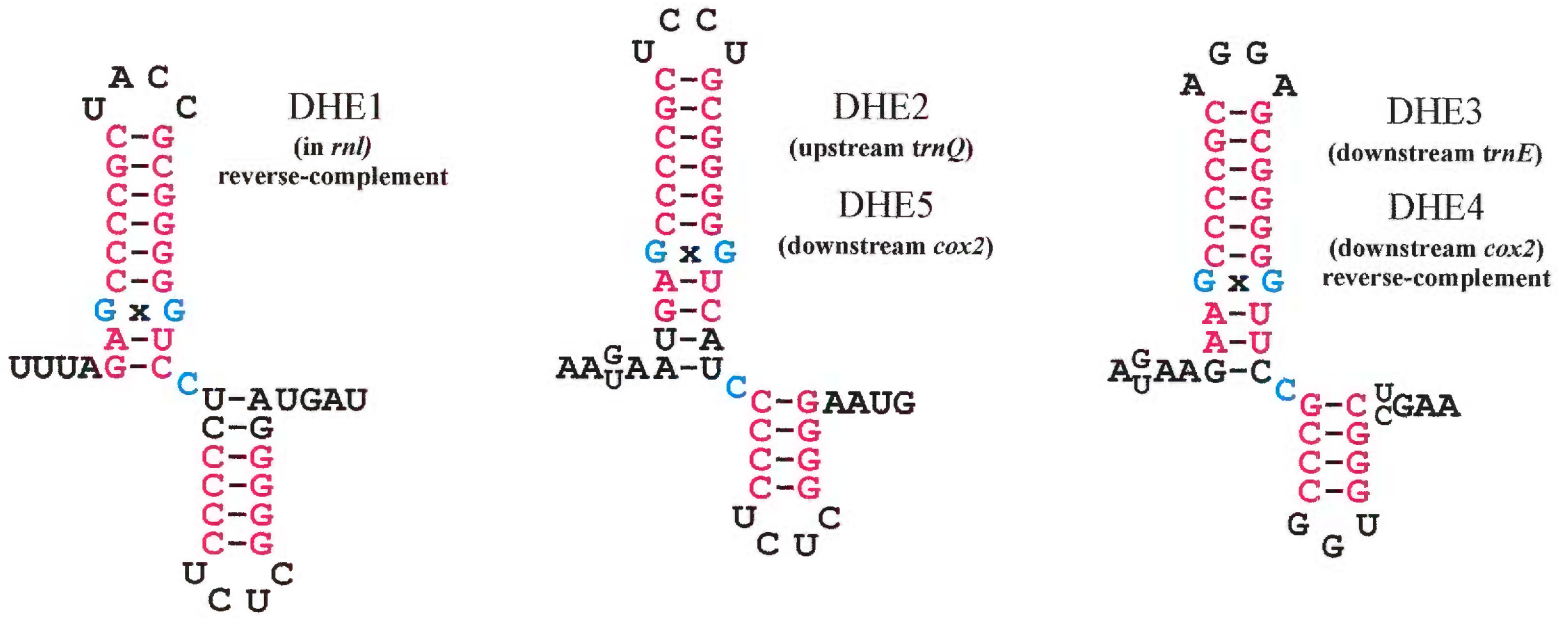
Many nuclear (which cause respiratory-deficiency, e.g., by affecting mitochondrial RNA splicing; Kreike *et al.*, 1987) and mitochondrial mutations (in *rps3*; Zimmer *et al.*, 1991, Neu *et al.*, 1998) have been shown to cause respiratory deficiency in *S. pombe*. It is possible that *S. japonicus* var. *japonicus* has acquired a nuclear mutation which affects the cytochrome oxidase complex (since a + a3 cytochromes are absent). Does the presence of a complete mtDNA in this species reflect a recent nuclear mutational event? The presence of an intact *rns* gene (GenBank accession number: X72804) in the mtDNA of the distantly-related (based on mitochondrial ribosomal small subunit phylogeny; unpublished results) and respiratory-deficient *S. japonicus* var. *versatilis* suggests that this event is actually quite ancient. It is more likely that genes encoded by the mtDNA have an essential function other than efficient ATP synthesis; e.g., maintaining a proton gradient across the inner mitochondrial membrane (which is involved in, for example, protein import into mitochondria; Herrmann and Neupert, 2000) . In any case, it is difficult to understand how respiratory-deficiency could be selected for in a population, since respiratory-competent cells would have an advantage when fermentable substrates are depleted from the environment.

IV.1.7 Double Hairpin Elements (DHEs) in the mtDNA of *S. octosporus*

We have identified 5 Double Hairpin Elements (DHEs) in the mtDNA of *S. octosporus* (Figure IV.5) which meet the consensus model for DHE secondary structure (Paquin *et al.*, 2000). DHEs have previously been described in the mitochondrial genomes of members of the chytridiomycete genus *Allomyces*, in

Figure IV.5 Double Hairpin Elements (DHEs) in the mtDNA of *S. octosporus*. **A** Possible alternative secondary structures of DHE1. Nucleotides potentially involved in other alternative foldings are indicated in blue. **B** Predicted secondary structures of *S. octosporus* DHEs based on DHE sequence alignment. Universally conserved nucleotides are indicated in red. Universally conserved GxG mismatches, as well as the C separating the two hairpins, are indicated in blue. DHEs which are not found in the direction of transcription are labelled reverse-complement. R, purine; Y, pyrimidine; S, G or C.


DHE1
(in *rnl*)
alternative folding
in direction of transcription
reverse-complement



DHE1	uauuua	GAGCCCCGC	(UACC)	GCGGGGGUC	C	UCCCCC	(UCUC)	GGGGGA ugauaa
DHE2	aaaaga	AUGAGCCCCGC	(UCCU)	GCGGGGGUCAU	C	CCCC	(UCUC)	GGGG aaugau
DHE3	aaauaa	GAAGCCCCGC	(AGGA)	GCGGGGGUUC	C	GCCC	(GGU)	GGGC cgaaua
DHE4	uaagaa	GAAGCCCCGC	(AGGA)	GCGGGGGUUC	C	GCCC	(GGU)	GGGC ugaauu
DHE5	gaaaua	AUGAGCCCCGC	(UCCU)	GCGGGGGUCAU	C	CCCC	(UCUC)	GGGG aaugau

consensus RAGCCCCGC → loop ← GCGGGGGUY C SCCC → loop ← GGGS

members of the chytridiomycete order *Monoblepharidales*, and the chytridiomycete *Spizellomyces punctatus* (Paquin *et al.*, 2000), but this is the first report in an ascomycete mtDNA. Four of these elements are located in the intergenic regions of the *S. octosporus* mtDNA (DHE2-5), and one is located in a variable region of the *rnl* gene (DHE1) (see Figure IV.2). Their location is not suggestive of a role in gene expression. Furthermore, the identification of identical DHEs (DHE4 and 5) in distant locations (and on different mtDNA strands) supports DHE mobility. Finally, the absence of DHEs in the mtDNAs of *S. pombe* and *S. japonicus* var. *japonicus* suggests recent lateral acquisitions of DHEs in *S. octosporus*, rather than vertical ancestry.

These 5 DHEs have the potential to form several alternative RNA foldings (Figure IV.5A), and alignments revealed a strong primary sequence consensus (used to predict the secondary structure of these elements; Figure IV.5B). These elements are presumed to form highly-structured RNA molecules, including a conserved GxG mismatch and a C separating the two hairpins. The fact that some DHEs (DHE1 and 4) meet this consensus only when reverse-complemented relative to the general transcription direction, indicates that insertion of these elements does not likely occur *via* an RNA intermediate, although the conserved secondary structure would seem to support an RNA-based mechanism of mobility.

DHEs have been implicated in rearrangements of the mtDNA of *Allomyces* (Paquin *et al.*, 2000). We have evidence that DHEs are also involved in rearrangements of the mtDNA of *S. octosporus*. 3 DHEs (DHE2, 4 and 5) in this genome are located near predicted sites of rearrangement of *S. octosporus* mtDNA with respect to that of *S. pombe* (see Figure IV.2). This may indicate that the highly similar DHE sequences serve as preferential recombination sites (as do GC-rich palindromes in yeast; e.g, Weiller *et al*, 1991). Further, DHE3 is located downstream of *trnE*, a region of the mtDNA which is apparently the result of a duplication event involving the *trnE* gene and the 5' section of the *cox2* gene (not shown). This has resulted in a *cox2* pseudogene (as well as a tRNA which

completely lacks a D-stem, showing high similarity to *trnE*, although it has the anticodon AAA of a *trnF* gene) upstream of the intact *cox2* gene. The correlation of DHE location and predicted sites of rearrangement strongly supports a role of DHEs in mitochondrial genome plasticity.

IV.2 Fission yeast phylogeny

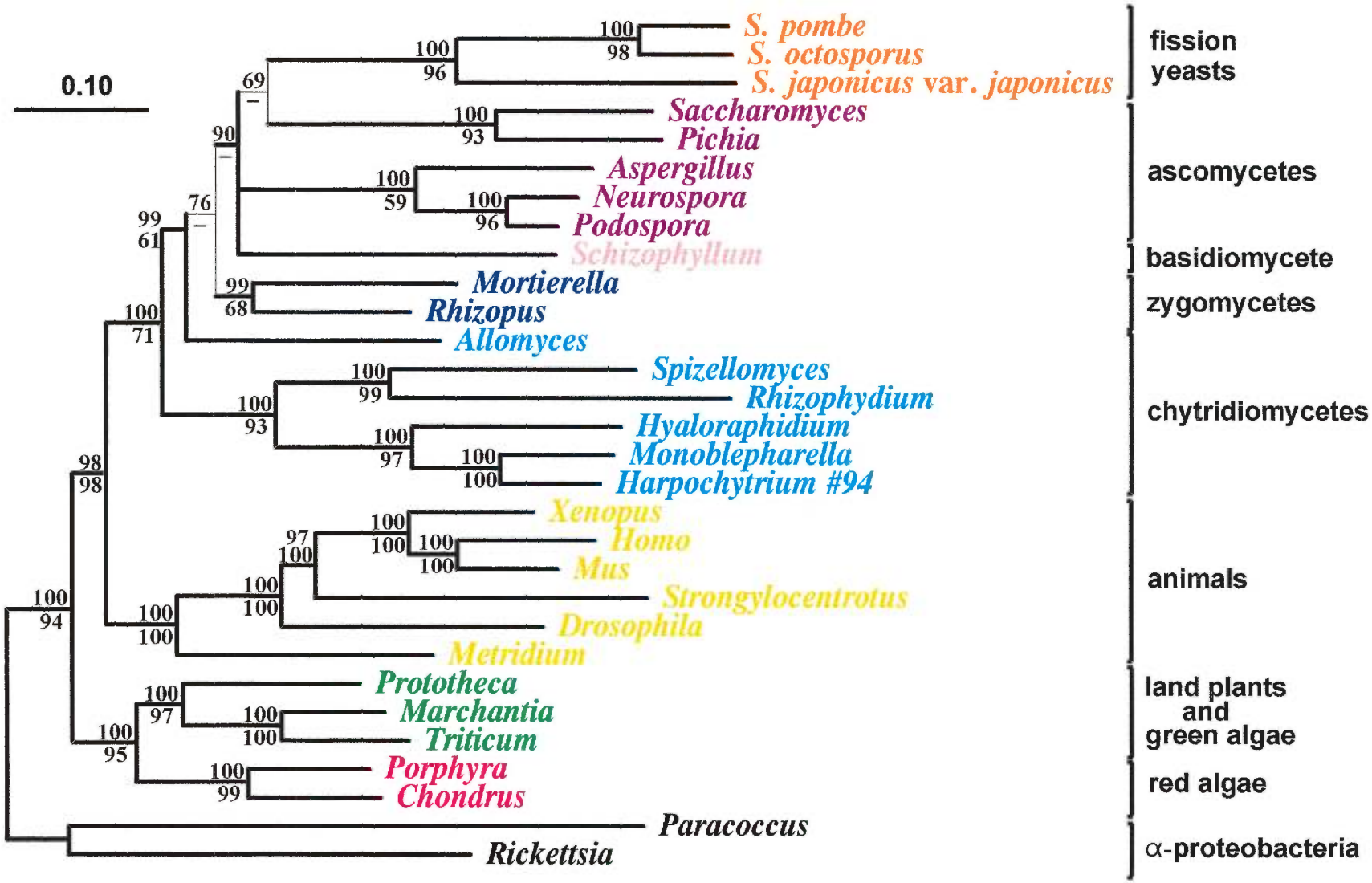
As described in section I.2, the interrelationships among fission yeasts, as well as their placement within Ascomycota has been questioned. Our phylogenetic analysis (using Cox1, Cox2, Cox3 and Cob sequences; Figure IV.6) clearly and consistently demonstrates that *S. pombe* and *S. octosporus* are more closely-related than either is to *S. japonicus* var. *japonicus* (100% bootstrap support), in agreement with the gene order data presented in section IV.1.1. The deep-divergence and complete lack of gene order conservation with both other species suggests that *S. japonicus* var. *japonicus* may best be classified in a second genus (*Hasegawaea*; proposed by Yamada and Banno, 1987) within the order *Schizosaccharomycetales*.

Our phylogenetic analyses support a specific relationship between animals and fungi to the exclusion of plants (98% bootstrap support) (also supported by studies of small subunit ribosomal RNA sequences; Wainright *et al.*, 1993; Paquin *et al.*, 1995), the paraphyly of *Allomyces* (Blastocladiales) and other chytridiomycetes (99% bootstrap support) (Paquin *et al.*, 1997), and the divergence of zygomycetes after *Allomyces* (76% bootstrap support) but before the higher fungi (90% bootstrap support). This branching order of fungal clades is consistent with classical taxonomy (Sparrow *et al.*, 1973; Cavalier-Smith, 1987). Most importantly, these phylogenetic analyses consistently indicate that the fission yeasts are correctly classified as ascomycetes.

However, we were not able to reliably determine the branching order within the higher fungi (Ascomycota and Basidiomycota) by either distance- or likelihood-based approaches, a result which has also been obtained in other

Figure IV.6 Molecular phylogeny of the fission yeasts. Phylogenetic tree created by Weighted Neighbor Joining (Weighbor) based on concatenated Cox1, Cox2, Cox3 and Cob amino acid sequences inferred from mtDNA sequences. The same topology was obtained using the Neighbor Joining (NJ) and Biological Neighbor Joining (BioNJ) methods, as well as the quartet puzzing method (PUZZLE). Numbers at nodes indicate support greater than 50% from 1000 bootstrap resamplings of the data set (top) and percentage occurrence (out of 40,000 puzzling steps) in the quartet puzzling tree (bottom). Scale bar indicates mean number of substitutions per site. Colour-code is as follows: α-proteobacteria, black; red algae, red; land plants and green algae, green; animals, yellow; chytridiomycete fungi, blue; zygomycete fungi, purple; basidiomycete fungi, pink; budding yeasts and filamentous ascomycetes, magenta; and fission yeasts, orange. Organisms used (Genbank accession numbers in parentheses): *Rickettsia prowazekii* (α-proteobacterium, AJ235270 to AJ235273); *Paracoccus dentrificans* (α-proteobacterium, X05829, M17522, X05934, X05828); *Chondrus crispus* (rhodophyte, Z47547); *Porphyra purpurea* (rhodophyte, AF114794); *Triticum aestivum* (wheat, P07747, Y00417, X01108, P15953); *Marchantia polymorpha* (liverwort, M68929); *Prototheca wickerhamii* (chlorophyte, U02970); *Metridium senile* (cnidarian, AF000023); *Drosophila yakuba* (fruit fly, X03240); *Strongylocentrotus purpuratus* (echinoderm, X12631); *Mus musculus* (mouse, J01420); *Homo sapiens* (human, J01415); *Xenopus laevis* (frog, M10217); *Harpochytrium sp.* #94 (chytridiomycete fungus; B. Franz Lang, unpublished); *Monoblepharella sp.* (chytridiomycete fungus; B. Franz Lang, unpublished); *Hyaloraphidium curvatus* (chytridiomycete fungus; B. Franz Lang, unpublished); *Rhizophydium sp.* (chytridiomycete fungus; B. Franz Lang, unpublished); *Spizellomyces punctatus* (chytridiomycete fungus; B. Franz Lang, unpublished); *Allomyces macrogynus* (chytridiomycete fungus, U41288); *Rhizopus stolonifer* (zygomycete fungus; B. Franz Lang, unpublished); *Mortierella verticillata* (zygomycete fungus; B. Franz Lang, unpublished); *Schizophyllum commune*

(basidiomycete fungus; B. Franz Lang, unpublished); *Podospora anserina* (ascomycete fungus, X55026); *Neurospora crassa* (ascomycete fungus, K01181, X01850, K00825, V00668); *Aspergillus* (*Emericella*) *nidulans* (ascomycete fungus, J01387, X15441, X06960); *Pichia conadensis* (*Hansenula wingei*) (ascomycete fungus, D31785); *Saccharomyces cerivisiae* (ascomycete fungus. P00163, V00694, J05007, J01478); *Schizosaccharomyces japonicus* var. *japonicus* (ascomycete fungus; C.E. Bullerwell and B. F. Lang, unpublished); *Schizosaccharomyces octosporus* (ascomycete fungus; C.E. Bullerwell and B. F. Lang, unpublished); *Schizosaccharomyces pombe* (ascomycete fungus, X54421).


0.10
S. pombe
S. octosporus
S. japonicus var. japonicus
Saccharomyces
Pichia
Aspergillus
Neurospora
Podospora
Schizophyllum
Mortierella
Rhizopus
Allomyces
Spizellomyces
Rhizophydium
Hyaloraphidium
Monoblepharella
Harpochytrium #94
Xenopus
Homo
Mus
Strongylocentrotus
Drosophila
Metridium
Prototheca
Marchantia
Triticum
Porphyra
Chondrus
Paracoccus
Rickettsia
fission yeasts
ascomycetes
basidiomycete
zygomycetes
chytridiomycetes
animals
land plants and green algae
red algae
α-proteobacteria

molecular studies (Nishida and Sugiyama, 1993; Kurtzman, 1994). We believe that this reflects the relatively short period of time between the divergences of the basidiomycete, fission yeast, budding yeast and filamentous ascomycete lineages. Finally, the marginal bootstrap support (69%) for the position of fission yeasts with budding yeasts may reflect a specific relationship between these two groups, but we have observed variability in this support under different conditions (not shown). Although rate variation among different lineages was taken into account in our analyses, it is possible that the highly-derived budding yeast and fission yeast mitochondrial sequences are attracted to each other due to the long branch attraction artifact (Felsenstein, 1978).

To resolve the branching order of the higher fungi, more mtDNA sequence will be necessary. Sequence data from a second basidiomycete (preferably distantly-related to *S. commune*) would better represent the diversity of this lineage, and might serve to stabilize higher fungal relationships. In addition, species which branch in intermediate positions between highly-derived species (such as budding yeast and fission yeast) will be needed to improve support (i.e., bootstrap values) for the correct tree topology. Other archiascomycete species (presumed to branch prior to the separation of budding yeasts and filamentous ascomycetes; see section I.2.2) may be helpful in this respect. For example, both *Taphrina* and *Saitoella* have been shown to group close to *S. pombe* in molecular phylogenetic analyses (Nishida and Sugiyama, 1993).

IV.3 Conclusions

In order to acquire molecular data from fission yeast species other than *S. pombe*, as well as to infer a robust phylogeny of this fungal group, we undertook the complete sequencing of the mitochondrial DNA (mtDNA) of two fission yeasts: *Schizosaccharomyces octosporus* and *Schizosaccharomyces japonicus* var. *japonicus*. The gene content in these two mtDNAs is almost identical to that in the completely-sequenced *S. pombe* mtDNA, except that *trnI2*(cau) is missing in

the *S. octosporus* mtDNA (as well as corresponding ATA codons in standard protein-coding genes), and *rps3* and *rnpB* are absent in that of *S. japonicus* var. *japonicus*. Both the genetic code and signals for processing of RNA transcripts appear to be the same in these three fission yeast mitochondrial genomes. Our results also indicate that *S. pombe* Orf227 and *S. octosporus* Orf248 are homologs of Rps3 (ribosomal small subunit protein 3). In addition, we have identified 5 Double Hairpin Elements (DHEs) in the mtDNA of *S. octosporus*, the first report of these elements in an ascomycete. Our molecular phylogenetic analysis based on concatenated Cox1, Cox2, Cox3 and Cob amino acid sequences clearly demonstrates that *S. pombe* and *S. octosporus* are more closely related to each other than either is to *S. japonicus* var. *japonicus*, a result consistent with the gene order conservation observed among these mtDNAs. Finally, we have shown that the fission yeasts are correctly classified as ascomycetes, although we were not able to resolve the branching order of the basidiomycetes, fission yeasts, budding yeasts and filamentous ascomycetes. The divergence of these lineages apparently occurred over a relatively short period of time, and may require additionally molecular sequence data to be reliably resolved.

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