Hybridization Studies by Crossing and Protoplast Fusion within the Genus *Schizosaccharomyces* Lindner

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Hybridization studies based on the use of crossing and the protoplast fusion technique revealed the relationship between the various taxa of the yeast genus *Schizosaccharomyces* Lindner. From a series of intra- and interspecific crosses as well as protoplast fusions, we concluded that a revision of the genus is required. Being interfertile, *S. pombe* and *S. malidevorans* have to be included in one species *S. pombe*. From the protoplast fusion results, this new taxon seems to be closely related to *S. octosporus*. The results obtained are discussed in connection with the characteristics of the low temperature spectra of the cytochromes.

INTRODUCTION

Yeast taxonomy which is based almost exclusively on physiological and morphological characteristics (for details, see Lodder, 1970) does not reveal real evolutionary relationships between the various taxa. In many cases the criteria used to differentiate yeast species reflect only small genetic differences rather than a true species delineation (Stahl & Esser, 1979). The shortcomings of the classification criteria have recently resulted in the revival of interfertility, introduced by Winge & Laustsen (1939) as a criterion for conspecificity. It has fruitfully been used in the delimitation of species in a number of ascomycetous genera, for instance *Hansenula* (Wickerham, 1970), *Saccharomyces* (Winge & Laustsen, 1939; Neumann, 1972) and *Kluyveromyces* (Johannsen & van der Walt, 1978; Johannsen, 1980). Johannsen (1981) recently reported hybridization studies within the genus *Schizosaccharomyces* Lindner, but failed to detect interfertility between the species.

The purpose of our report is to present further data concerning interfertility within the genus *Schizosaccharomyces*, and to introduce the protoplast fusion technique in studies on the relatedness between the species. According to Sloof (1970), the genus contains four species, including two varieties, which can be distinguished on the basis of some morphological properties and their different abilities to utilize three sugars (maltose, raffinose and melibiose). Later, an additional species was described by Kumbhojkar (1972), who found that two strains of the Centraalbureau voor Schimmelcultures (CBS) previously thought to be *S. octosporus* Beijerinck do not ferment maltose and can therefore be considered as comprising a new species. He suggested the name *S. slooffii* for this taxon. Although our studies have been restricted to the four species previously described (Sloof, 1970), it can nevertheless be concluded that the genus must be reconsidered. Since all of the four species possess marked sexuality (Lindner, 1893; Beijerinck, 1894; Wickerham & Duprat, 1945; Leupold, 1950; Suminoe & Dukmo, 1963; Rankine & Fornachon, 1964), hybrids can be formed within any species. This provides a possibility of demonstrating interfertility between the phenotypically delimited species, as attempted by Johannsen (1981). In contrast to her findings we have observed that *S. pombe* and *S. malidevorans* can be hybridized, and recombination also occurs between the two genomes. From a series of intra- and interspecific crosses as well as protoplast fusions, we concluded that *S. pombe* and

S. malidevorans in all probability constitute one common species and are closely related to *S. octosporus*.

METHODS

Strains. The *Schizosaccharomyces* strains used in this study are listed in Table 1. All species except *S. pombe* Lindner originated from the Czechoslovak Collection of Yeasts (CCY) in Bratislava. The genetically marked strains of *S. pombe* were obtained from the Institute of General Microbiology of the University of Bern (Switzerland) or from the Institute of Genetics of the University of Munich (F.R.G.). *Saccharomyces cerevisiae* DP1-1D originated from the Microbiological Institute of Wrocław University (Poland).

Media. YEL, YEA, MEA, MMA and MMAK were described previously (Sipiczki & Ferenczy, 1977). YEL contained 0.5% (w/v) yeast extract and 1% (w/v) glucose. YEA was YEL supplemented with 2% Bacto-agar (Difco). MEA contained 3% (w/v) malt extract and 2% (w/v) Bacto-agar. MMA was synthetic minimal medium containing 1% (w/v) glucose, 2% Bacto-agar, 0.5% (w/v) (NH₄)₂SO₄, 0.01% (w/v) KH₂PO₄, 0.005% (w/v) MgSO₄ and vitamins according to van der Walt (1970). Where the experiment required this, the MMA was supplemented with amino acids and bases, each at 50 µg ml⁻¹. MMAK was MMA supplemented to contain 0.6 M-KCl. SPA was synthetic sporulation medium, containing 1% (w/v) glucose, 0.1% (w/v) KH₂PO₄ and vitamins as in MMA (Leupold, 1970). GCM was glycerol complete medium containing 0.5% (w/v) yeast extract, 3% (v/v) glycerol and 2% (w/v) Bacto-agar (Wolf *et al.*, 1971). Drug media were prepared by adding ethanolic solutions of erythromycin (Antibiotika Fabrik, Jasi) and diuron (Riedel-De Haen AG) in final concentrations of 6 mg ml⁻¹ and 0.01 mg ml⁻¹, respectively, to GCM at 40 °C. YPG contained 1% (w/v) yeast extract, 1% (w/v) Bacto-peptone, 2% (w/v) glucose and 2% (w/v) Bacto-agar.

Table 1. *Species and strains of Schizosaccharomyces used in this study*

Organism	Origin of culture
1. <i>Schizosaccharomyces octosporus</i> Beijerinck CCY 44-2-1 (CBS 1804)	CCY
2. <i>Schizosaccharomyces octosporus</i> Beijerinck <i>met-105</i>	This study; UV-induced mutant from 1.
3. <i>Schizosaccharomyces octosporus</i> Beijerinck <i>ade-110</i>	This study; UV-induced mutant from 1.
4. <i>Schizosaccharomyces japonicus</i> var. <i>japonicus</i> Yukawa et Maki CCY 44-5-1 (CBS 354)	CCY
5. <i>Schizosaccharomyces japonicus</i> var. <i>japonicus</i> Yukawa et Maki <i>ura-106</i>	This study; UV-induced mutant from 4.
6. <i>Schizosaccharomyces japonicus</i> var. <i>japonicus</i> Yukawa et Maki <i>ura-110</i>	This study; UV-induced mutant from 4.
7. <i>Schizosaccharomyces japonicus</i> var. <i>versatilis</i> (Wickerham et Duprat) Sloof CCY 44-3-1 (ATCC 9987)	CCY
8. <i>Schizosaccharomyces japonicus</i> var. <i>versatilis</i> (Wickerham et Duprat) Sloof <i>lys-103</i>	This study; UV-induced mutant from 7.
9. <i>Schizosaccharomyces japonicus</i> var. <i>versatilis</i> (Wickerham et Duprat) Sloof <i>glu-126</i>	This study; UV-induced mutant from 7.
10. <i>Schizosaccharomyces malidevorans</i> Rankine et Fornachon CCY 44-6-1 (CBS 5557)	CCY
11. <i>Schizosaccharomyces malidevorans</i> Rankine et Fornachon <i>ura-103</i>	This study; UV-induced mutant from 10.
12. <i>Schizosaccharomyces malidevorans</i> Rankine et Fornachon <i>arg-121</i>	This study; UV-induced mutant from 10.
13. <i>Schizosaccharomyces pombe</i> Lindner <i>his7-366 h⁻</i>	UB
14. <i>Schizosaccharomyces pombe</i> Lindner <i>his7-366 h⁺</i>	UB
15. <i>Schizosaccharomyces pombe</i> Lindner <i>leu2-120 h⁺</i>	UB
16. <i>Schizosaccharomyces pombe</i> Lindner <i>his7-366 arg-1 leu2-120 h⁻</i>	This study; haploid spore from crosses between 13, 15 and 19
17. <i>Schizosaccharomyces pombe</i> Lindner <i>ura5-294 h⁻</i>	UB
18. <i>Schizosaccharomyces pombe</i> Lindner <i>lys4-95 diu^r-301 ery^r-100 h⁻</i>	UM
19. <i>Schizosaccharomyces pombe</i> Lindner <i>arg-1 diu^r-122 h⁻</i>	UM

Sources: CCY, Czechoslovak Collection of Yeasts, Bratislava, Czechoslovakia; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; ATCC, American Type Culture Collection, Washington, D.C., U.S.A.; UB, Institute of General Microbiology, University of Bern, Switzerland; UM, Institute of Genetics, University of Munich, F.R.G.

Mutant induction and characterization. UV mutagenesis, enrichment and isolation of auxotrophic mutants and determination of the auxotrophic characters were as described previously for *S. pombe* (Sipiczki & Ferenczy, 1978).

Hybridization by crossing. Sexual hybridization of strains was achieved by the procedure of Wickerham & Burton (1956) with slight modifications. Auxotrophic parents were mass-mated on MEA slants. After 1 week at 30 °C, the growth from the slants was taken up in sterile tap-water, washed three times by centrifugation and resuspended in 1.0 ml sterile tap-water. For estimation of the total number of cells and the frequency of prototrophic hybrids and/or recombinants, samples were taken from the suspension, diluted and spread on to YEA and MMA plates. The plates were incubated at 30 °C and the colonies were counted after 10 d. Samples (0.5 ml) of the suspension were transferred on to fresh MEA slants. The whole procedure was then serially repeated at 7 d intervals over a period of 4 weeks. In order to detect the possible back-mutations, similar culturing of each auxotrophic parent strain was carried out in parallel with the crosses.

Hybridization by protoplast fusion. Protoplasts from the auxotrophic strains were prepared using *Trichoderma* enzyme. The methods of enzyme production and protoplast formation were similar to those described in a previous paper (Sipiczki, 1979). Fusion was induced using polyethylene glycol (PEG, mol. wt 4000) as for *S. pombe* (Sipiczki & Ferenczy, 1977).

Genetic analysis of hybrids. Random spore analysis and tetrad analysis were performed according to Leupold (1970). For studies on the transmission and recombination of mitochondrial markers, the methods of Seitz-Mayr *et al.* (1978) were used with slight modifications. As all *S. malidevorans* mutants isolated in this study were of a mating type resembling the h^+ type of *S. pombe*, the favourable features of *meil-102* could not be exploited in the production of stable diploids. Therefore, each *S. malidevorans* strain was mixed with a *S. pombe* mutant of type h^- instead of *meil-102*. The mixed suspension was then pipetted on to the surface of SPA and incubated at 25 °C to allow zygote formation. On SPA, which does not contain any nitrogen source, no vegetative growth occurs, but the cells of opposite mating types copulate if they are in direct contact. After 18–24 h the mating mixture was suspended in sterile tap-water and plated on to MMA. The plates were incubated at 30 °C for 4 d and the colonies with cell morphology resembling the diploid cell shape of *S. pombe* were picked off and suspended separately in sterile tap-water. Samples were then taken from these suspensions, diluted, spread on to MMA plates and incubated to allow colony formation. After 4 d the colonies were replica-plated on to GCM plates supplemented with the respective drugs.

Cytochrome absorption spectra. The cytochrome absorption spectra of intact cells harvested from YPG plates grown at 28 °C were determined on a cell paste. The cells were scraped off the plates, reduced by mixing with powdered $\text{Na}_2\text{S}_2\text{O}_4$, pressed into specially prepared cuvettes giving a final cell paste thickness of 1 mm, and chilled in liquid nitrogen. The spectra were produced with a recording spectrophotometer Specord UV-vis (Carl Zeiss, Jena), using parafilm 'M' (American Can Company) as a reference blank.

Sugar fermentation test. Fermentative utilization of maltose was tested according to van der Walt (1970).

RESULTS

Mutants

From each species and variety (except *S. pombe*) auxotrophic mutants were isolated by UV treatment and subsequent enrichment by snail enzyme, and tested for reversion. All strains with a genetic reversion rate higher than 10^{-9} were discarded. Two of the stable ones were then chosen from every *Schizosaccharomyces* for crosses and fusion experiment. The *S. pombe* mutants used either originated from genetic collections (see Table 1) or were isolated as haploid spores from their crosses.

Intraspecific crosses

Prototrophic colonies appeared in all intraspecific crosses (Table 2), including those between the varieties of *S. japonicus*. Most of them had a lower sporulating ability than that of the original wild-type strains. As expected, in *S. pombe* the frequency of prototrophs was much higher when strains of different mating types were crossed than when two h^- strains were mass-mated. The appearance of prototrophs in the latter case could be a consequence of the instability of mating characters occasionally emerging in heterothallic strains (Egel, 1976). In *S. malidevorans* and *S. pombe* an increased cell size was peculiar to about 10% of the prototrophic colonies, which led us to believe that they are diploid hybrids produced from zygotes by vegetative growth, for in *S. pombe* diploid and polyploid cells are known to be systematically larger than haploid cells (Leupold, 1956). The colonies with normal cell size presumably resulted from recombinant

Table 2. *Intra- and interspecific crosses between auxotrophic mutants of Schizosaccharomyces*

Crosses were carried out as described in Methods. The number of viable cells was determined by plating after crossing. Counts of prototrophs were determined by plating on to MMA plates.

Parental strains	10 ⁻⁷ × Number of viable cells scored	Frequency of prototrophs
<i>S. pombe</i> his7-366 h ⁻ × <i>S. pombe</i> leu2-120 h ⁺	53.2	8.9 × 10 ⁻⁴
<i>S. pombe</i> his7-366 h ⁻ × <i>S. pombe</i> ura5-294 h ⁻	60.7	8.9 × 10 ⁻⁸
<i>S. malidevorans</i> ura-103 × <i>S. malidevorans</i> arg-121	30.0	1.1 × 10 ⁻⁴
<i>S. octosporus</i> met-105 × <i>S. octosporus</i> ade-110	8.2	3.4 × 10 ⁻⁵
<i>S. japonicus</i> var. <i>japonicus</i> ura-106 × <i>S. japonicus</i> var. <i>japonicus</i> ura-110	11.3	6.7 × 10 ⁻⁵
<i>S. japonicus</i> var. <i>versatilis</i> lys-103 × <i>S. japonicus</i> var. <i>versatilis</i> glu-126	10.7	1.6 × 10 ⁻⁶
<i>S. pombe</i> his7-366 h ⁻ × <i>S. malidevorans</i> ura-103	47.0	5.7 × 10 ⁻⁴
<i>S. pombe</i> his7-366 h ⁻ × <i>S. malidevorans</i> arg-121	37.0	2.5 × 10 ⁻⁵
<i>S. pombe</i> his7-366 h ⁺ × <i>S. malidevorans</i> ura-103	37.6	2.9 × 10 ⁻⁸
<i>S. pombe</i> his7-366 h ⁺ × <i>S. malidevorans</i> arg-121	36.4	3.1 × 10 ⁻⁷
<i>S. pombe</i> his7-366 h ⁻ × <i>S. octosporus</i> met-105	22.7	0
<i>S. pombe</i> his7-366 h ⁻ × <i>S. octosporus</i> ade-110	26.3	0
<i>S. pombe</i> his7-366 h ⁺ × <i>S. octosporus</i> met-105	19.1	0
<i>S. pombe</i> his7-366 h ⁺ × <i>S. octosporus</i> ade-110	31.0	0
<i>S. pombe</i> his7-366 h ⁻ × <i>S. japonicus</i> var. <i>japonicus</i> ura-106	17.0	0
<i>S. pombe</i> his7-366 h ⁻ × <i>S. japonicus</i> var. <i>japonicus</i> ura-110	26.8	0
<i>S. pombe</i> his7-366 h ⁺ × <i>S. japonicus</i> var. <i>japonicus</i> ura-106	22.9	0
<i>S. pombe</i> his7-366 h ⁺ × <i>S. japonicus</i> var. <i>japonicus</i> ura-110	19.9	0
<i>S. pombe</i> his7-366 h ⁻ × <i>S. japonicus</i> var. <i>versatilis</i> lys-103	15.3	0
<i>S. pombe</i> his7-366 h ⁻ × <i>S. japonicus</i> var. <i>versatilis</i> glu-126	17.2	0
<i>S. pombe</i> his7-366 h ⁺ × <i>S. japonicus</i> var. <i>versatilis</i> glu-126	20.5	0
<i>S. pombe</i> his7-366 h ⁺ × <i>S. japonicus</i> var. <i>versatilis</i> lys-103	19.7	0
<i>S. malidevorans</i> ura-103 × <i>S. octosporus</i> met-105	21.6	0
<i>S. malidevorans</i> ura-103 × <i>S. octosporus</i> ade-110	24.0	0
<i>S. malidevorans</i> arg-121 × <i>S. octosporus</i> met-105	19.5	0
<i>S. malidevorans</i> arg-121 × <i>S. octosporus</i> ade-110	22.2	0
<i>S. malidevorans</i> ura-103 × <i>S. japonicus</i> var. <i>japonicus</i> ura-106	26.3	0
<i>S. malidevorans</i> ura-103 × <i>S. japonicus</i> var. <i>japonicus</i> ura-110	31.2	0
<i>S. malidevorans</i> arg-121 × <i>S. japonicus</i> var. <i>japonicus</i> ura-106	62.3	0
<i>S. malidevorans</i> arg-121 × <i>S. japonicus</i> var. <i>japonicus</i> ura-110	47.4	0
<i>S. malidevorans</i> ura-103 × <i>S. japonicus</i> var. <i>versatilis</i> lys-103	32.9	0
<i>S. malidevorans</i> ura-103 × <i>S. japonicus</i> var. <i>versatilis</i> glu-126	21.0	0
<i>S. malidevorans</i> arg-121 × <i>S. japonicus</i> var. <i>versatilis</i> lys-103	28.3	0
<i>S. malidevorans</i> arg-121 × <i>S. japonicus</i> var. <i>versatilis</i> glu-126	34.1	0
<i>S. octosporus</i> met-105 × <i>S. japonicus</i> var. <i>japonicus</i> ura-106	7.3	0
<i>S. octosporus</i> met-105 × <i>S. japonicus</i> var. <i>japonicus</i> ura-110	10.7	0
<i>S. octosporus</i> ade-110 × <i>S. japonicus</i> var. <i>japonicus</i> ura-106	3.9	0
<i>S. octosporus</i> ade-110 × <i>S. japonicus</i> var. <i>japonicus</i> ura-110	11.2	0
<i>S. octosporus</i> met-105 × <i>S. japonicus</i> var. <i>versatilis</i> lys-103	14.6	0
<i>S. octosporus</i> met-105 × <i>S. japonicus</i> var. <i>versatilis</i> glu-126	8.9	0
<i>S. octosporus</i> ade-110 × <i>S. japonicus</i> var. <i>versatilis</i> lys-103	11.6	0
<i>S. octosporus</i> ade-110 × <i>S. japonicus</i> var. <i>versatilis</i> glu-126	9.9	0
<i>S. japonicus</i> var. <i>japonicus</i> ura-106 × <i>S. japonicus</i> var. <i>versatilis</i> lys-103	10.5	1.7 × 10 ⁻⁶
<i>S. japonicus</i> var. <i>japonicus</i> ura-106 × <i>S. japonicus</i> var. <i>versatilis</i> glu-126	10.2	4.9 × 10 ⁻⁷
<i>S. japonicus</i> var. <i>japonicus</i> ura-110 × <i>S. japonicus</i> var. <i>versatilis</i> lys-103	18.0	1.3 × 10 ⁻⁷
<i>S. japonicus</i> var. <i>japonicus</i> ura-110 × <i>S. japonicus</i> var. <i>versatilis</i> glu-126	6.1	0

haploid spores. In crosses of *S. octosporus* and *S. japonicus* no similar conclusion could be drawn because of their less regular cell shape and size.

Interspecific crosses involving auxotrophic mutants

In order to detect interfertility between species, at least two mutants per species were mass-mated in all possible combinations, and tested for production of prototrophs. Data relating to the individual crossings are given in Table 2. Colonies arose only from crosses between *S. pombe*

and *S. malidevorans*. Their frequencies ranged from 2.9×10^{-8} to 5.7×10^{-4} . No prototrophs (that is no interfertility) were detected in the other combinations.

To return to the crosses between *S. pombe* and to *S. malidevorans*, it is worth noting that the prototroph frequency depends to a great extent on the mating type of the *S. pombe* partner. If strains of h^- type were involved, the frequency was nearly as high as in the intraspecific $h^- \times h^+$ cross. In the combinations *S. pombe his 7-366 h⁺* $\times$ *S. malidevorans ura-103* and *S. pombe his 7-366 h⁺* $\times$ *S. malidevorans arg-121*, the prototroph frequency remained at a level three orders of magnitude lower. These differences suggest that the *S. malidevorans* mutants used are presumably of a heterothallic mating type resembling the h^+ type of *S. pombe*. The average percentage of colonies with increased cell size was 43.94% when h^- was crossed with *S. malidevorans*. Since most of these sporulated on MEA and formed azygotic asci (Leupold, 1970), they can be regarded as 'interspecific' diploids.

No significant differences were found between the prototroph frequencies of samples taken at various time intervals. The data in Table 2 depict only the situation after the fourth passage.

Twenty prototrophic colonies presumed to be diploid were isolated from the MMA plates and examined for maltose fermentation. All of them could utilize maltose fermentatively, which strongly indicates that their genotype was mal^-/mal^+ and that the mal^+ character was dominant. Since the taxonomic distinction between these species is based mainly on their different abilities to ferment maltose (*S. pombe* is mal^+ , while *S. malidevorans* is mal^-), the further fate of mal^+ during sporulation of hybrids was also of great interest. To study its segregation, a strain of *S. pombe* carrying markers in all chromosomes was constructed and crossed with *S. malidevorans ura-103*. The *S. pombe his7-366 arg-1 leu2-120 h⁻* partner carried the *leu-2*, *his-7* and *arg-1* markers on chromosomes I, II and III, respectively (Kohli *et al.*, 1977), and in addition possessed the mal^+ allele. The *S. malidevorans* was known to have the *ura* marker and the mal^- character, but both in unknown positions. A prototrophic diploid hybrid was picked off and sporulated, and a random analysis of the spores was carried out. Colonies (298) arising from haploid spores were isolated and characterized for auxotrophy as well as for maltose fermentation. The results are summarized in Table 3. (Data on wild-type colonies are not given, because it was difficult to judge which ones originated from spores.) The following conclusions can be drawn from the results. (i) The hybrid studied must have been diploid, because all markers involved could be reisolated. (ii) During meiosis and spore formation the chromosomes of both parents showed independent reassortment, which is expressed in the nearly equal frequencies of single and double auxotrophs. (iii) The mal^+ character is very probably linked to *leu-2*. Consequently, the *mal* gene for *S. pombe* is located in chromosome I. (iv) Recombination took place within two chromosomes, as can be inferred from the segregation of markers in chromosome I (*leu-2* and mal^+) and chromosome II (*his-7* and h^-).

For a more precise localization of *mal* in chromosome I the above-mentioned cross was repeated and asci were isolated. However, tetrad analysis was not possible, because of the low viability of the spores: no complete tetrad was found among 50 asci dissected. The presumable reason for the low proportion of viable spores is the physiological differences between the parents. With the intention of reducing their influence in subsequent crosses, a new partner was chosen for *S. pombe*. This was a segregant spore designated 179 *his7-366 arg-1 ura mal⁻* from the cross discussed above. As regards the genotype, it was thought to have two chromosomes (carrying *his-7* and *arg-1*) from *S. pombe* and the remaining part of its genome (carrying *ura*) from *S. malidevorans*. Since the mating character of *S. malidevorans* was also retained, this strain could be crossed with *S. pombe his7-366 arg-1 leu2-120 h⁻*. However, the viability of the spores was not higher in this case either, and so the tetrad analysis again proved impossible. Nevertheless, segregation of mal^+ character in the viable spores allowed certain inferences concerning the localization of the *mal* gene. Leaving aside the *his-7* and *arg-1* markers, of 910 spores isolated 254 were prototrophs, 224 were *leu ura*, 212 were *ura* and 220 were *leu*. The nearly equal numbers of the four types, characteristic of zygotes heterozygous for two markers, favours the supposition that none of the spore classes exhibited any differential viability. The leucine auxotrophic spores were also tested for maltose fermentation, and it turned out that 94% of them were mal^+ . Hence, mal^+ and *leu-2* are probably closely linked.

Table 3. *Random spore analysis of the cross S. pombe Lindner his7-366 arg-1 leu2-120 h⁻ × S. malidevorans Rankine et Fornachon ura-103*

Crossing and analysis by replica-plating on to supplemented MMA plates was performed according to Leupold (1970). Data of wild-type spores are not included.

Total auxo-trophs	ura			leu			arg			his			leu			arg			his			ura			leu			arg			his		
	ura	leu	arg	his	leu	ura	arg	ura	arg	ura	his	leu	his	leu	arg	his	ura	leu	his	ura	leu	arg	ura	leu	his	ura	leu	arg	his				
Total spores (%)	21	26	4	19	19	11.2	8	4.6	21	20	8	4	7	2	3	5	3	1.8	3.1	1.8	3	5	3	1.8	3.1	1.8	3	5	3	1.8			
mal ⁺ spores (%)*	2	25	1	6	19	100	12.5	19.0	4	20	8	0	7	2	0	5	3	100	100	100	100	100	100	100	100	100	100	100	100	100			
h ⁻ spores (%)†	3	1	0	18	3	15.8	0	95.2	20	18	1	3	6	0	3	5	2	85.7	100	100	100	100	100	100	100	100	100	100	100	66.7			

* Percentage of mal⁺ spores in relation to the total number of spores in the individual auxotrophic classes.

† Percentage of h⁻ spores in relation to the total number of spores in the individual auxotrophic classes.

Fig. 1. Transmissional pattern of mitochondrial markers in 100 individual zygote clones from the cross *S. pombe lys4-95 diu^r-301 ery^r-100 h⁻* × *S. malidevorans ura-103*. Crossing and isolation of diploid clones were carried out as described in the Methods. Cells of diploid clones were spread on to MMA plates and resistance of the colonies appearing was determined by replica-plating on to GCM plates supplemented with the respective drugs. The transmissional pattern is plotted in intervals of 5% transmission of *diu^r*, *ery^r* and double resistance. ■, *diu^r*-transmission; ▨, *ery^r*-transmission; □, *diu^rery^r*-transmission.

Transmission of mitochondrial genomes in zygote clones from a cross of *S. pombe* × *S. malidevorans*

Cells of *S. pombe lys4-95 diu^r-301 ery^r-100 h⁻* conferring mitochondrially inherited resistance to diuron [3(3,4-dichlorophenyl)-1,1-dimethylurea] and erythromycin were mass-mated with cells of *S. malidevorans ura-103*. Zygotes were isolated as described in the Methods, resuspended individually and plated on MMA. Colonies appearing were then replica-plated on MMA supplemented with drugs. For each zygote clone about 200 colonies were analysed for resistance. Figure 1 shows the transmissional pattern of 100 individual zygote clones, plotted in intervals of 5% transmission of *diu^r*, *ery^r* and double resistance. Most of the clones belonged to the classes of 0–5% and 95–100% resistance transmission and very few consisted of cells of different phenotypes. Erythromycin resistance was transmitted more frequently than diuron resistance and only a comparatively low proportion of clones showed double resistance. The excess of *ery^r* and *diu^r* over the parental *ery^r diu^r* phenotype indicates that frequent recombinations occurred between mitochondria of *S. pombe* and *S. malidevorans* during copulation and/or multiplication of the zygotes.

Somatic hybridization by protoplast fusion

Cells of auxotrophic strains used for crosses and listed in Table 2 were converted into protoplasts by use of a lytic enzyme produced by *Trichoderma viride*. Protoplasts were mixed in all possible combinations, fused using PEG, and prototrophic fusion products were selected on MMAK. After 14 d the resulting fusion colonies were counted, and their frequencies compared to the total number of protoplast pairs used were calculated. In all intraspecific combinations, prototrophs occurred with frequencies comparable to those observed in crosses. As expected, the same was found between *S. pombe* and *S. malidevorans*. In addition, however, slowly growing colonies were also formed when *S. malidevorans* or *S. pombe* was fused with *S. octosporus* (Table 4). In principle, their morphology and segregation were similar to those described in a previous paper (Sipiczki, 1969).

Low-temperature absorption spectra of *Schizosaccharomyces* species

Low-temperature spectra of the cellular cytochromes of *Schizosaccharomyces* species are shown in Fig. 2. For better analysis a wild-type DP1-1D strain of *Saccharomyces cerevisiae* which

Table 4. Frequencies of prototrophic colony formation between *S. pombe*, *S. malidevorans* and *S. octosporus* auxotrophic mutants after PEG-induced protoplast fusion

Protoplasts were prepared, mixed, treated with PEG and plated on to MMAK as described in Methods. The plates were incubated at 30 °C.

Strains fused	Fusion frequency
<i>S. pombe</i> Lindner <i>his7-366 h⁻</i> × <i>S. octosporus</i> Beijerinck <i>ade-110</i>	2.28×10^{-4}
<i>S. pombe</i> Lindner <i>his7-366 h⁺</i> × <i>S. octosporus</i> Beijerinck <i>ade-110</i>	5.19×10^{-5}
<i>S. malidevorans</i> Rankine et Fornachon <i>ura-103</i> × <i>S. octosporus</i> Beijerinck <i>ade-110</i>	1.07×10^{-5}
<i>S. malidevorans</i> Rankine et Fornachon <i>arg-121</i> × <i>S. octosporus</i> Beijerinck <i>ade-110</i>	8.71×10^{-5}

Fig. 2. Low-temperature spectra of aerobically grown cells of *Schizosaccharomyces* species and *Saccharomyces cerevisiae*. Cells were collected after 72 h aerobic growth at 28 °C on YPG plates. Spectra were determined on $\text{Na}_2\text{S}_2\text{O}_4$ -reduced whole cell pastes 1 mm thick versus neutral filter. Samples were chilled in liquid nitrogen. 1, *S. pombe* Lindner *his7-366 h⁻*; 2, *S. octosporus* Beijerinck CCY 44-2-1; 3, *S. malidevorans* Rankine et Fornachon CCY 44-6-1; 4, *S. japonicus* var. *versatilis* (Wickerham et Duprat) Sloof CCY 44-3-1 (ATCC 9987); 5, *S. japonicus* var. *japonicus* Yukawa et Maki CCY 44-5-1 (CBS 354); 6, *Saccharomyces cerevisiae* DP1-1D.

contains cytochromes *a* + *a*₃, *b*, *c*₁ and *c* was compared with them. The strains of *S. pombe*, *S. malidevorans* and *S. octosporus* contained all cytochromes, but in reduced amounts. Unexpectedly, *S. japonicus* var. *versatilis* lacked all cytochromes *a* + *a*₃, *b*, *c*₁ and *c*. In the case of the respiratory chain of *S. japonicus* var. *japonicus* cytochromes *a* + *a*₃ and *c* were absent, as deduced from the lack of peaks for cytochrome *c* in the α (548 nm) and β (520 nm) regions and for cytochromes *a* + *a*₃ at 607 nm. Finally, a close correlation was found between the absence of these cytochromes and the ability to grow on unfermentable carbon sources. Thus, strains of *S. japonicus* var. *versatilis* and *S. japonicus* var. *japonicus* which lack cytochromes *a* + *a*₃ and *c*, or *a* + *a*₃, *b*₁, *c*₁ and *c* are unable to grow on medium containing glycerol as the sole source of energy.

DISCUSSION

The data in Table 2 demonstrate that prototrophs appeared in all intraspecific crosses, including those between *S. japonicus* var. *japonicus* and *S. japonicus* var. *versatilis*, with a frequency in the range 2.9×10^{-8} – 8.9×10^{-4} . One might attribute the marked differences in frequency to the different mating abilities of the species. Although this supposition must be correct to a certain extent, it has to be taken into account that formation of prototrophs can be affected by a number of factors which have nothing to do with the mating potency. *Schizosaccharomyces* has a haplontic life cycle (Leupold, 1950; Sloof, 1970), i.e. copulation is followed immediately by meiosis and haploid spores are formed within the cell wall of the original zygote (zygotic asci). However, zygotes can sometimes germinate into diploid cell clones which are distinguishable from haploids because they have larger cells, are unstable and form azygotic asci on sporulation medium; this is known in *S. pombe* (Leupold, 1970). Since the latter phenomenon is rather rare, the number of prototrophs in a mass combination of complementing auxotrophic mutants does not necessarily reflect the proportion of zygotes. All viable diploids form colonies on the selection medium, but only those spores do so which are recombinant. Since recombination leading to prototrophy is dependent upon the degree of linkage between the genes involved (Perkins, 1953), the number of prototrophic spores in mass-mated population is proportional to the degree of linkage of the markers used as well as to the number of zygotes. To avoid the effect of linkage, the interspecific crosses in *S. pombe* were made with strains carrying unlinked markers. The big differences between the frequencies were therefore due virtually only to the different mating type combinations.

A problem which may be encountered is that the growth rate of zygotes and/or recombinants can differ from that of the parents, whereby the proportion of prototrophs in a sample does not reflect the frequency of zygote formation. Of course, failure to detect prototrophs can also be due to the reduced fertility of the mutants used or to unfavourable combinations of mating types (e.g. both parents are of like mating types). In view of all these eventualities in *Schizosaccharomyces* it seems inadvisable, in contrast to Johanssen's (1980) suggestion for *Kluyveromyces*, to accept any arbitrary minimal recombinant rate as a criterion of the genetic relatedness of the parents.

Only *S. pombe* and *S. malidevorans* proved interfertile in interspecific crosses (Table 2). Although all species except *S. pombe* were presumed to be homothallic (Sloof, 1970; Wickerham & Duprat, 1945; Beijerinck, 1894), both h^- and h^+ strains of *S. pombe* were used in these experiments to avoid the possible influence of mating types. This precaution proved to be justified, because *S. malidevorans* showed a higher prototroph frequency with h^- than with h^+ . It can therefore be assumed that *S. malidevorans* too is heterothallic, and its mutants were of a mating type resembling h^+ of *S. pombe*. This finding is in disagreement with the report of Sloof (1970), who presumed the homothallism of this species.

Crossing of *S. malidevorans* with a *S. pombe* strain carrying markers in every chromosome enabled us to analyse the recombination of nuclear genes. It is evident from the results in Table 3 that chromosomes of both parents segregated independently and recombination also took place within two chromosomes. These data, together with those revealing recombination between mitochondrial genomes, suggest the strong genetic relatedness of *S. pombe* and *S. malidevorans*. Since this may give rise to more or less free exchanges of genes in their mixed populations, they can not be considered as distinct species. The considerable similarity of their respiratory chains, suggested by the cytochrome spectra, also supports this conclusion.

Johannsen (1981) recently reported hybridization studies within the genus *Schizosaccharomyces*, but she failed to recover recombinants or hybrids between the species. The disagreement of her findings with ours is probably due to the fact that the *S. pombe* strains used in the crosses were of different origins. Johannsen's strains (*S. pombe* Lindner CBS 5682 and CSIR Y 469) originated from the Yeast Collection of the Centraalbureau voor Schimmelcultures (CBS) or from the Council for Scientific and Industrial Research (CSIR), while our mutants were obtained from a genetic collection. It has to be stressed, however, that the original wild-type strain of the latter mutants [*S. pombe* Lindner str. *liquefaciens* (Osterwalder)] was also from CBS (Leupold, 1950).

Hybridization of *S. pombe* and an eight-spored species was attempted by Kossikov & Medve-

deva (1977). It is not clear, however, whether *S. japonicus* Yukawa et Maki [*Octosporomyces japonicus* (Yukawa et Maki) Kudriawzew] or *S. octosporus* Beijerinck [*Octosporomyces octosporus* (Beijerinck) Kudriawzew] was the partner of *S. pombe*: they used an adenine-requiring strain isolated from *S. octosporus* (Kossikhov & Zapadnaya, 1974) but treated this strain as a mutant of *Octosporomyces japonicus* in the hybridization experiment. Nor was the hybrid, or recombinant, nature of the prototrophs recovered proved beyond doubt: the confirmation was based primarily on the morphological characteristics, without reference to the fermentation of melibiose and without the exclusion of genetic reversion to leaky forms.

By means of protoplast fusion further information could be obtained about the relatedness of the taxa. As shown in Table 4, prototrophic somatic hybrids were formed between the four-spored strains and *S. octosporus*. Although the fusion products were not stable and possessed a disturbed genetic balance reflected in low viability, formation of incomplete cell wall, and osmotic sensitivity (Sipiczki, 1979), the two genomes could nevertheless exist together, which is taken as good evidence of their close relatedness. As such hybrids could be constructed and maintained only under laboratory conditions, the fusion partners still have to be regarded as separate species.

The results reported in this work show that a revision of the genus is required. Being interfertile, *S. pombe* Lindner and *S. malidevorans* Rankine et Fornachon have to be included in one species. As regards Art. 57 of the International Code of Botanical Nomenclature (Stafleu *et al.*, 1972), the oldest legitimate descriptive expression, i.e. the term *pombe* introduced by Lindner (1893), has to be retained for this new species. In spite of the interfertility, there are still a number of morphological and physiological differences, such as a rough outer membrane on the spores of *S. malidevorans*, its inability to ferment maltose, and its increased ability to decompose L-malic acid and to produce hydrogen sulphide (Rankine & Fornachon, 1964), which all offer a possibility to distinguish these two taxa. It therefore seems justified to relegate them [in terms of Art. 60 of the International Code of Botanical Nomenclature (Stafleu *et al.*, 1972)] to the status of two varieties of *Schizosaccharomyces pombe* Lindner as follows:

1. *Schizosaccharomyces pombe* Lindner var. *pombe*; synonym, *Schizosaccharomyces pombe* Lindner (1893).
2. *Schizosaccharomyces pombe* Lindner var. *malidevorans*; synonym, *Schizosaccharomyces malidevorans* Rankine et Fornachon (1964).

To legalize the proposed taxonomical situation the authors give the following descriptions.

Schizosaccharomyces Lindner 1893. emend.

Genus of the class Endomycetes of Fungi. Mononucleate, rectangular box-shaped or cylindric cells with rounded ends multiplying by 'fission' (annelloconidiogenesis on a broad basis at both ends of the cell); occurrence of true mycelium was observed. Cell wall consists of galactomannans, α -glucans and β -glucans; chitin is absent. Extracellular polysaccharides staining blue with iodine may occur. Homothallic or heterothallic conjugation of two vegetative cells, one vegetative cell and spore or two spores produce zygote in which after meiotic division (2)-4-8 globose, rectangular box-shaped or reniform spores are produced. Spore wall is smooth or verrucose. Vegetative multiplication of the zygotes by fission may occur; diploid cells form spores without previous conjugation. Fermentation and assimilation of some sugars and assimilation of inulin and glycerol was observed. During utilization of sugars a more or less intensive decomposition of malic acid into carbon dioxide and ethanol may occur. Assimilation of nitrates is negative. Inositol as growth factor is always needed. Coenzyme Q is of the CoQ₁₀ or of the CoQ₉ type. Type species: *Schizosaccharomyces pombe* Lindner.

Genus Fungorum de classe Endomycetes. Cellulae uninucleate, oblongae aut cylindricae obtusae multiplicantes per 'fissionem': processus qui est annelloconidiogenesis performatus ad ambos extremibus cellulae cum base lata. Productio mycelii veri accidere potest. Paries cellulae constat ex galactomannanis, α -glucanis β -glucanisque; quitinum abest. Productio extracellularis compositorum amylo similium adest. Zygota formatur per conjugationem heterothallicam aut homothallicam duae cellulae vegetativae, unae cellulae et unae sporae aut duae sporae. In zygotia per divisionem meioticam (2)-4-8 sporae globosae, oblongae vel reniformes formantur. Paries sporae levis aut verrucosus. Multiplicatio vegetativa zygotae per fissionem accedit; in cellulis

diploidois sporae formantur sine coniguatione precedente. Fermentatio assimilatioque aliquorum saccharorum, casu assimilatio inulini et glyceroli. Tempore utilisationis saccharorum decompositio plus minusve fortis acidi malici in dioxido carboni et ethanolo. Assimilatio nitratis negativa. Inositolum ad multiplicationem semper necessarium. Coenzymum Q est de typo CoQ₁₀ vel CoQ₉. Species typica: *Schizosaccharomyces pombe* Lindner.

Schizosaccharomyces pombe Lindner 1893. emend.

Species of the genus *Schizosaccharomyces*. Assimilates glucose, sucrose and raffinose. Assimilation of maltose, α -Me-glucoside, inulin and glycerol variable. Ferments glucose and sucrose; fermentation of maltose and raffinose variable. Coenzyme Q is of the CoQ₁₀ type.

Species generis *Schizosaccharomyces*. Assimilat glucosum, saccharosum et raffinolum. Assimilatio maltosi, α -Me-glucosidi, inulini et glyceroli variabilis. Fermentat glucosum et saccharosum; fermentatio maltosi et raffinosi variabilis. Coenzymum Q est de typo CoQ₁₀.

Schizosaccharomyces pombe Lindner var. *pombe*

Variety of the species *Schizosaccharomyces pombe*. Assimilates glucose, sucrose, maltose and raffinose; assimilation of α -Me-glucoside, inulin and glycerol variable. Ferments glucose, sucrose and maltose. At 37 °C growth occurs. Type strain: CBS 356.

Varietas speciei *Schizosaccharomyces pombe*. Assimilat glucosum, saccharosum, maltosum et raffinolum; assimilatio α -Me-glucosidi, inulini et glyceroli variabilis. Fermentat glucosum, saccharosum et maltosum. Multiplicat ad 37 °C. Cultura typica: CBS 356.

Schizosaccharomyces pombe Lindner var. *malidevorans* Rankine et Fornachon (1964)

Variety of the species *Schizosaccharomyces pombe*. Assimilates and ferments glucose, sucrose and raffinose; assimilation of inulin variable; assimilation of maltose, α -Me-glucoside and glycerol negative. No growth at 37 °C. Type strain: CBS 5557.

Varietas speciei *Schizosaccharomyces pombe*. Assimilat et fermentat glucosum, saccharosum et raffinolum; assimilatio inulini variabilis; assimilatio maltosi, α -Me-glucosidi et glyceroli negativa. Non multiplicat ad 37 °C. Culture typica: CBS 5557.

The results obtained in this work are in good agreement with the proposal of Yamada *et al.* (1973) that the two four-spored species should be included in one species. They were led to this conclusion by their findings on the coenzyme Q systems of 16 strains. It was found that the four-spored *pombe-malidevorans* group possessed the CoQ₁₀ system, while the eight-spored *octosporus-japonicus* group was characterized by the CoQ₉ system. The introduction of a new genus, *Octosporomyces*, suggested by Kudriawzew (1960) and supported by Yamada *et al.* (1973), which would include *S. japonicus* and *S. octosporus*, seems unjustified, however, especially in view of the ability of the latter to undergo somatic hybridization both with *S. pombe* and with *S. malidevorans* and the strong similarities of its cytochrome spectra to those of the four-spored group (Fig. 2 and Claisse & Simon-Becam, 1978). The homogeneity of the genus is supported by the results of Spencer & Gorin (1969), who reported that the proton magnetic resonance spectra of the cell wall galactomannans of the four species were very similar. Nevertheless, a detailed DNA-DNA reassociation analysis of nucleic acid homologies would be of great help in further revealing the degrees of relatedness between the taxa of this genus. GC (guanine and cytosine) contents of DNA so far published, 40–44.8% and 42% for *S. pombe* and *S. octosporus*, respectively (Rose & Venner, 1964; Storck, 1966) do not allow a reliable conclusion.

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