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Linear Mitochondrial DNAs of Yeasts: Frequency of Occurrence and General Features

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In most yeast species, the mitochondrial DNA (mtDNA) has been reported to be a circular molecule. However, two cases of linear mtDNA with specific termini have previously been described. We examined the frequency of occurrence of linear forms of mtDNA among yeasts by pulsed-field gel electrophoresis. Among the 58 species from the genera *Pichia* and *Williopsis* that we examined, linear mtDNA was found with unexpectedly high frequency. Thirteen species contained a linear mtDNA, as confirmed by restriction mapping, end labeling, and electron microscopy. The mtDNAs from *Pichia pijperi*, *Williopsis mrakii*, and *P. jadinii* were studied in detail. In each case, the left and right terminal fragments shared homologous sequences. Between the terminal repeats, the order of mitochondrial genes was the same in all of the linear mtDNAs examined, despite a large variation of the genome size. This constancy of gene order is in contrast with the great variation of gene arrangement in circular mitochondrial genomes of yeasts. The coding sequences determined on several genes were highly homologous to those of the circular mtDNAs, suggesting that these two forms of mtDNA are not of distant origins.

Mitochondria have their own DNA which specifies several proteins of the respiratory complexes as well as mitochondrion-specific rRNAs and tRNAs. While the majority of these genomes seem to be circular, some organisms have been shown to have a linear mitochondrial DNA (mtDNA). Well-known examples are those of the ciliates of the genera *Paramecium* (7) and *Tetrahymena* (17). The first case of a linear mtDNA in yeasts was found in 1981 in *Williopsis mrakii* (formerly called *Hansenula mrakii*) (19). A second case was reported for a yeast identified as *Candida parapsilosis* (8; see also reference 1). Occurrence of linear DNAs in yeast mitochondria raises questions about their origin and mode of replication. In a number of other organelle genomes, linear forms of DNA have also been described, although in some cases, the reality of the linearity might be questioned because the terminal structure of these forms has not been defined. The evolutionary and taxonomical positions of these linear genomes with respect to the circular forms need to be clarified. These linear DNAs may replicate as a linear molecule, or they may replicate through circular intermediates. In this study, we examined (i) the frequency of the linear forms of mtDNAs among yeast species and (ii) whether they have any basic structures in common. In the accompanying report (4), we examine their possible modes of replication by analysis of their terminal structure.

MATERIALS AND METHODS

Yeast strains. Yeast strains were obtained from Centraalbureau voor Schimmelcultures (CBS), Delft, The Netherlands. They are independent isolates of known origins. As a rule, the type strain of each species, as indicated in the CBS 1990 catalog, was chosen for analysis unless otherwise stated. The culture medium contained 1% yeast extract (Difco), 1% Bacto Peptone (Difco), and 2% glucose. Cells were grown on a rotary shaker at 28°C until early stationary phase of growth.

Isolation of mtDNA. All strains examined were found to be sensitive to Zymolyase (Kirin Brewing Ltd., Tokyo, Japan). Cells were transformed into protoplasts by Zymolyase treatment, and crude mitochondria were prepared according to the conventional procedure used for *Saccharomyces cerevisiae* (2). The mtDNA was separated from the nuclear DNA by two cycles of isopycnic centrifugation in CsCl in the presence of bisbenzimide (13). Because of the high adenine-thymine content, the mtDNA from all species examined could be efficiently separated from the nuclear DNA in the CsCl gradient.

PFGE of cell lysates. Each strain was grown in 2 ml of medium and lysed in an agarose block (16). Pulsed-field gel electrophoresis (PFGE) of the lysates was performed by using a LKB Pulsaphor apparatus in the contour-clamped homogeneous electric field configuration (16). The migration conditions were optimized for the detection of mtDNA: pulse time of 5 to 30 s (exponential variation) for 28 h at 160 V in 1.5% agarose gel.

Other DNA procedures. DNA was analyzed by standard techniques (11). Radiolabeled nucleotides were purchased from New England Nuclear (Les Ulis, France). DNA was sequenced by a dideoxy-chain termination method, using either [α-^{35}S]dATP and a commercial kit (Pharmacia France) or an automated DNA sequencer (Applied Biosystem model 373A). DNA sequence data analysis was performed as described by Sor et al. (15).

Probes of mitochondrial genes. Mitochondrial genes cloned from *S. cerevisiae* (18) and *Kluyveromyces lactis* (12) were used as hybridization probes for localization of equivalent genes on the restriction maps. The probes were labeled with [α-^{32}P]dCTP by using a commercial nick translation kit (Amersham). Hybridization of probes with Southern blots of

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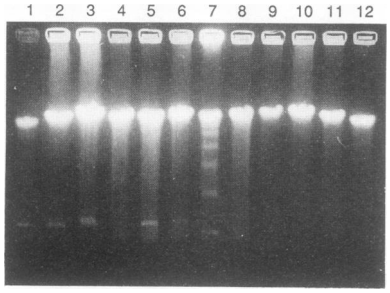

FIG. 1. Detection of linear mtDNA by PFGE. After electrophoresis of lysed cells, the gel was stained with ethidium bromide and treated with RNase. Lanes: 1, *T. pyriformis* GL; 2, *C. utilis* CBS 621; 3, *P. jadinii*; 4, *P. salmenticensis* CBS 5121; 5, *W. beijerinckii* CBS 2564; 6, *W. suaveolens* CBS 1670; 7, lambda ladder; 8, *P. farinosa* CBS 185; 9, *P. henricii* CBS 5765; 10, *P. fabiani* CBS 5642; 11, *P. salictaria* CBS 5456; 12, *P. ohmeri* CBS 5367. All the strains to the right of the lambda ladder have a circular mtDNA (which cannot be seen in PFGE).

mtDNA digests was carried out at 60°C for 18 h in the presence of 0.6 M NaCl and 1 mM EDTA. Hybrids were washed in 0.3 M NaCl at room temperature, dried, and revealed by autoradiography.

Electron microscopy. Native or denatured mtDNA, and restriction fragments from DNA termini, were spread as described by Davis et al. (3) except that the hypophase was 40% formamide. DNA denaturation was performed by the formamide-glyoxal method. The grids were rotary shadowed with Pt-C and examined in a Siemens Elmiskop 1 transmission electron microscope (accelerating voltage, 60 kV). DNA length was calibrated with the double-stranded circular bacteriophage ϕX174 RFII DNA (5,386 bp) or with the single-stranded circular DNA from bacteriophage fd (6,408 nucleotides). Details of procedures have previously been described (14).

Nucleotide sequence accession numbers. The sequence data reported have been assigned EMBL accession numbers X66593, X66594, and X66595.

RESULTS

PFGE of cell lysates detects linear mtDNA. Among some 600 yeast species, we surveyed all known species of the genus *Williopsis* (because the first linear mtDNA in yeasts was found in *W. mrakii*) as well as species from the large genus *Pichia*. The survey was not restricted within a narrow range of related species, as shown by a large variation of the GC content of chromosomal DNA (37 to 50%). Each culture was lysed in an agarose block, and the DNA was examined by PFGE. Selected examples are shown in Fig. 1. Lanes 1 to 6 show the DNAs from known (*Tetrahymena pyriformis*) or supposed linear mtDNAs (see below); lanes 8 to 12 show lysates from the species known to have a circular mtDNA. Linear mtDNAs usually formed a discrete band migrating far ahead of the chromosomal DNA (the latter is not resolved under these PFGE conditions). Circular mtDNAs were not detected as a discernible band. The mitochondrial origin of the supposed linear DNA bands was confirmed by hybridization with labeled purified mtDNA from each species (data not shown). In a few cases, the mtDNA band was found close to the front of the migrating materials, resulting in an ambiguous interpretation (e.g., lane 4). Either by electrophoresis of a new preparation of DNA or by restriction mapping, the ambiguities could be resolved. Among 58 species examined (mostly type strains of the species), 16 species gave a distinct mtDNA band in PFGE, suggesting its linearity; all others gave no distinct band. Among the 16 species, 13 were confirmed to be linear by restriction mapping and later by enzymatic digestions of termini (see the accompanying report [4]). Three turned out to be circular, indicating that visualization by PFGE is not an absolute criterion of linearity, though highly indicative. In the species which did not show a distinct mtDNA band in PFGE, 24 were also examined by restriction mapping of purified mtDNA, and all were confirmed to be circular. For others, the analysis has not been completed.

Linearity deduced from restriction site mapping. For all of the mtDNAs considered to be linear by the electrophoretic test, restriction maps were constructed by the standard double-digestion and overlapping hybridization procedures. Maps of several circular mtDNAs were also established for comparison. Examples of the linear restriction maps are shown in Fig. 2. Hybridization of mitochondrial gene probes from *S. cerevisiae* and *K. lactis* with Southern blots of mtDNA allowed us to identify and locate their homologous sequences on the restriction maps. The unambiguous hybridization signals indicated that there is a high degree of conservation of coding sequences in all species examined.

Constancy of gene order in the linear mtDNAs of yeasts. The arrangement of genes in the circular mtDNAs is known to be highly variable among yeast species (18). In relatively related species (mtDNAs of *K. lactis*, *Torulopsis glabrata*, and *S. cerevisiae*), blocks of genes are translocated to other positions, while the local gene order is more or less conserved. All of the linear DNAs examined in this study showed a remarkable constancy of the gene order, independent of the large variation in genome size (24 to 58 kbp). The small rRNA (15S) gene was always found near the right terminus, and the cytochrome oxidase subunit 2 gene (*COX2*) was found at the opposite end; between them, all of the coding genes were aligned in the same order.

Coding sequences. To determine whether there is any bias in the coding system of linear mtDNAs, sequences of several protein-coding genes were examined. The sequence analysis (*CYTB* of *P. pijperi* and *W. mrakii* and *COX2* of *W. mrakii* [data not shown]; EMBL accession numbers X66593 to X66595) indicated that the DNA-deduced amino acid sequences were highly homologous to the known yeast mitochondrial protein sequences when the standard mitochondrial code is applied (UGA for tryptophan, CUN for leucine). With respect to *S. cerevisiae* proteins, the *CYTB* gene product showed 75% (*W. mrakii*) to 78% (*P. pijperi*) identity, and the *COX2* product showed 78% (*W. mrakii*) identity. Five tRNA genes from *W. mrakii* and three tRNA genes from *P. pijperi* showed 60 to 80% sequence identity with the *S. cerevisiae* counterparts (5) (EMBL accession numbers X66437 to X66444). In cases for which the map resolution was sufficient, the orientation of transcription of rRNA could be determined. It was always in the direction from the small-subunit rRNA (15S) to the large-subunit rRNA (23S) gene, as determined by the use of probes derived from different parts of the rRNA genes and by partial sequencing. Similarly, all protein genes so far sequenced ran from right to left on the maps, except for two unassigned open reading frames (ORF1 and ORF2) found in *W. mrakii* mtDNA. In some circular mtDNAs, there are many repetitive GC-rich clusters (e.g., in *S. cerevisiae* and several strains of *K. lactis* [12]). In the case of *S. cerevisiae*, these sequences are known to be the sites of frequent recombina-

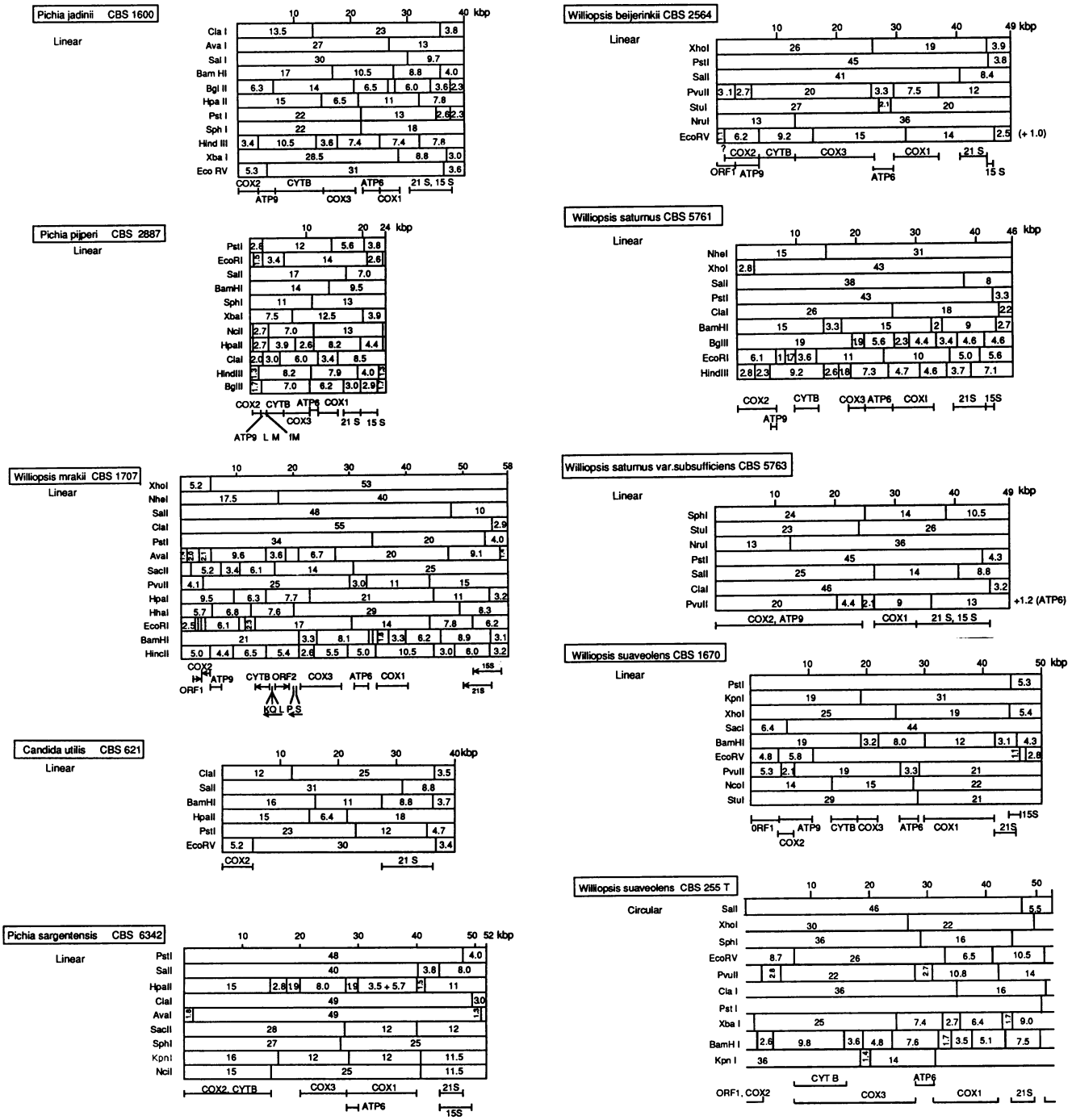

FIG. 2. Genes and restriction sites of linear mtDNAs. Purified mtDNAs were digested with various combinations of restriction enzymes, and the molecular weights of fragments were determined by agarose gel electrophoresis. DNA fragments containing known mitochondrial genes from *S. cerevisiae* and/or *K. lactis* were used as radioactive probes to delimit the position of homologous sequences by hybridization (see Materials and Methods). A part of the *W. mrakii* mtDNA map has previously been published (19), but the sizes of restriction fragments were corrected according to the confirmed lengths of the bacteriophage lambda DNA. In *P. pijperi* and *W. mrakii*, the positions of identified tRNA genes are indicated by one-letter amino acid symbols. The circular map of mtDNA from *W. suaveolens* CBS 255^T is included for comparison (see text).

tion leading to deletions and rearrangements. The presence of these sequences, usually indicated by an abnormally high frequency of *Hpa*II (CCGG) or *Sac*II (CCGCGG) restriction sites, could not be detected in any of the linear mtDNAs examined.

Electron microscopy. Biochemical scale preparations of mtDNA from different yeasts generally suffer from fragmentation to various degrees. When we examined by electron microscopy the preparations of the large mtDNA from *W. mrakii* (58 kbp long), we found only a single molecule (the

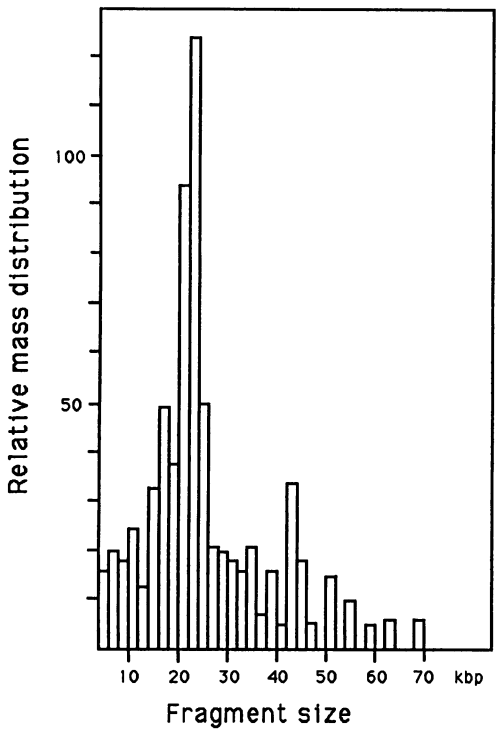

FIG. 3. Length histogram of *P. pijperi* mtDNA. An aliquot of *P. pijperi* mtDNA, used for enzymatic analyses, was spread by the procedure of Davis et al. (3). The peak lengths are 22.02 ± 0.15 and 43.67 ± 0.39 kbp for supposed monomer and dimer molecules, respectively. The length was calibrated with bacteriophage ϕX174 RFII DNA.

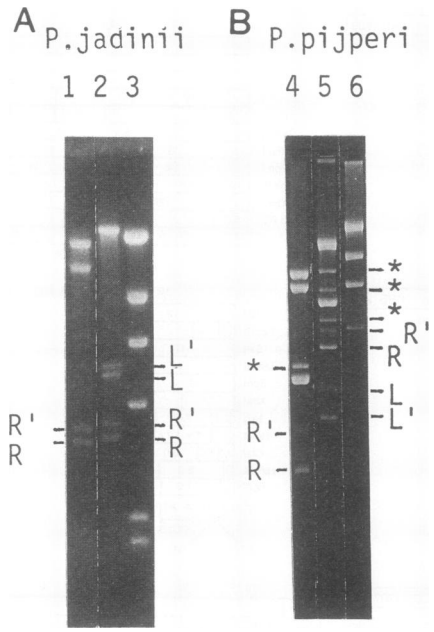

FIG. 4. Abnormal restriction fragments from the termini of linear mitochondrial DNAs. (A) *P. jadinii* mtDNA was digested with *Cla*I (lane 1) and *Eco*RV (lane 2). After electrophoresis, DNA fragments were visualized by ethidium bromide staining. R, R′, L, and L′ indicate the right terminus and its shadow and the left terminus and its shadow, respectively. In the *Cla*I digest, L and L′ are not resolved because of their high molecular weight. (B) *P. pijperi* mtDNA was digested with *Bgl*II (lane 4) and *Pst*I (lane 5). In the *Bgl*II digest, R and L are superposed because the enzyme cut the two termini symmetrically. * indicates the junction fragment of the termini (R+R, L+L, and R+L superposed). In the *Pst*I digest, the three types of terminal junctions are visible (*). Lanes 3 and 5 are molecular weight markers (lambda phage DNA *Hin*dIII digest). See Fig. 2 for restriction maps.

largest) with the expected size. Other molecules were all considerably smaller. No circles were found. For *P. pijperi* mtDNA (24 kbp), length histograms showed a highest frequency of linear pieces at the expected length (24 kbp versus ϕX174 RFII DNA), although longer molecules were also observed with a small peak at the dimer size (Fig. 3). In *P. jadinii* (40 kbp), the largest linear molecules measured approximately 13 μm, consistent with the restriction map size.

Microheterogeneity of the terminal structure. The construction of restriction maps shown in Fig. 2 was initially somewhat complicated by the fact that the restriction digests contained several minor components derived from the termini. First, restriction fragments from both termini were most often accompanied by a shadow fragment, usually weakly stained, that migrated more slowly than the main terminal bands. This was seen typically with *P. pijperi* and *P. jadinii* mtDNAs. In *W. mrakii*, however, these faint bands are not visible because the shadow bands constitute the majority species of the terminal fragments (see the accompanying report [4]). The intensity of these shadow bands varied among the DNA preparations. Figure 4 shows some of the most visible examples. Second, there were additional minor bands. Hybridization with appropriate probes later showed that they represented various junction structures derived from the termini (right-right, left-left, and right-left terminal junctions; see below). They were hardly visible in *P. jadinii* by ethidium bromide staining but were clearly seen in *P. pijperi* (Fig. 4). As we will show (see below and reference 4), these fragments arise from several types of association of the terminal sequence, for example (i) joining of right and left ends by circularization or (ii) *Paramecium* mtDNA-like dimer formation creating right-right and left-left palindromic dimer molecules. This heterogeneity of terminal bands is not due to the presence of any associated protein or RNA, since the restriction patterns did not change after treatment of mtDNA with proteinase K or RNase A (data not shown).

Right and left termini share homologous sequences. Hybridization experiments showed that the radioactive probe made of one terminal fragment (the main band fragment as opposed to the shadow band) hybridized with the terminal fragments from both ends of mtDNA, indicating the presence of homologous sequences at the two termini (sequence analysis in the accompanying report [4] shows that they are inverted repeats). Furthermore, the minor components mentioned above hybridized with the terminal probes. Figure 5 shows the example of *P. jadinii* mtDNA. We found the following. (i) The shadow bands gave a strong hybridization signal, demonstrating that they are indeed derivatives of the terminal sequences. (ii) Three additional components were also detected. One had the size of a dimer of the right terminal fragment, another had the size of a dimer of the left terminal fragment, and the third corresponded to the sum of the right and left terminal fragments. The proportions of these three components varied in different preparations of DNA, but they always represented a minor fraction of mtDNA, as they could be only weakly stained with ethidium

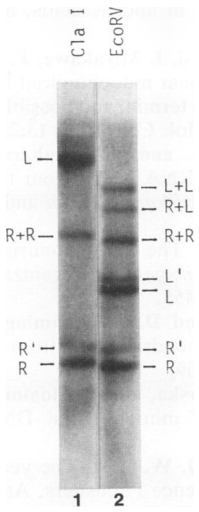

FIG. 5. Presence of homologous sequences at both termini. *P. jadinii* mtDNA was digested with *Cla*I (lane 1) and *Eco*RV (lane 2), electrophoresed, and blotted on nitrocellulose filters. The blotted DNA was hybridized with a radioactive *Bgl*II 2.3-kbp fragment (right terminus) and autoradiographed. In lane 1, the right end (R, 3.8 kbp), its shadow (R'), and the left end (L, 13.5 kbp) fragments as well as a junction fragment (R+R, 7.6 kbp) are visible (the shadow fragment L' and large junction fragments do not show up clearly at the top of the gel). In lane 2, one detects, from the bottom, the right end (R, 3.6 kbp), its shadow (R'), the left end (L, 5.3 kbp), and its shadow (L'), as well as the junction fragments (R+R, 7.2 kbp; R+L, 8.9 kbp; and L+L, 10.6 kbp).

bromide staining (Fig. 4). Existence of these minor bands suggested that the termini may be involved in reactions specifically joining them. As these minor components may be a key to understanding the replication mechanism, their nature is examined in more detail by sequence analysis in the accompanying report (4). The restriction maps presented in Fig. 2 have in fact been fully established only after confirmation of the nature of these components.

DISCUSSION

Linear mtDNAs are frequent among yeasts. To assess the frequency of occurrence of linear mtDNA, it was necessary to devise a simple method that can be applied to the analysis of many strains and that can distinguish linear DNAs from circular forms. We had noticed that the killer plasmids of *K. lactis* (linear double-stranded DNA of 8.8 and 13.4 kbp) could be detected directly in the cell lysates by PFGE, while under the same conditions, none of the many circular mtDNAs tested formed a visible band. We therefore applied PFGE analysis for a systematic survey of linear mtDNAs. Table 1 lists the mtDNAs for which the molecular form could be established by several criteria (PFGE, restriction sites, and gene mapping). Although the analysis is not yet complete, 13 of the 58 species examined contained a linear mtDNA, an unexpectedly high proportion. It remains to be seen whether the linearity of mtDNA is a species-specific trait. We do not know what genetic basis determines the linear-circular difference of mtDNA.

Constancy of gene order. The constancy of the gene order in the linear mitochondrial genomes is intriguing in view of the great variability of gene arrangement in the circular genomes in yeasts. It is possible that gene shuffling could be minimized by the linear structure of the genome. Among the known circular genomes, only *P. petersonii* (synonym: *Hansenula petersonii*) CBS 5555 (6) and *W. suaveolens* CBS 255^T (T indicates type strain) mtDNAs (Fig. 2) had a gene order similar to that described here, except that *COX2* and 15S rRNA genes were brought next to each other to form a circle. The DNA sequences in the junction regions are under study.

TABLE 1. Forms and sizes of various yeast mitochondrial DNAs

Species	Strain	Nuclear mol% GC[a]	Mitochondrial DNA		
			Form	Size (kbp)	Group[b]
Williopsis mrakii	CBS 1707^T	44.3	Linear	58	A
W. suaveolens	CBS 1670	44.7	Linear	50	B
W. beijerinckii	CBS 2564^T	44.4	Linear	49	B
W. saturnus var. *saturnus*	CBS 5761	44.4	Linear	46	A
W. saturnus var. *subfaciens*	CBS 5763^T	43.5	Linear	49	A
Pichia pijperi	CBS 2887^T	42.5	Linear	24	
P. sargentensis	CBS 6342^T	44.1	Linear	52	A
P. jadinii	CBS 1600^T	45.5	Linear	40	C
Candida utilis	CBS 621^T	45.0	Linear	40	C
W. californica	CBS 252^T	43.0	Circular	50	D
W. californica	CBS 5762	42.6	Circular	54	D
W. pratensis	CBS 7079^T	39.9	Circular	50	
W. suaveolens	CBS 255^T		Circular	50	
W. salcorniae sp. nov.	CBS 8071		Circular	42	
P. henricii	CBS 5765^T	49.6	Circular	50	
P. fabiani	CBS 5640^T	45	Circular	53	
P. wickerhamii	CBS 4107^T	46.5	Circular	46	
P. fermentans	CBS 187^T	43.1	Circular	35	
P. minuta var. *minuta*	CBS 6502	46.7	Circular	55	
P. minute var. *nonfermentans*	CBS 5764^T	45.3	Circular	40	
P. salictaria	CBS 5456^T	37.8	Circular	52	
P. farinosa	CBS 185^T	42.5	Circular	42	
P. ohmeri	CBS 5367^T	44.5	Circular	32	
P. petersonii	CBS 5555^T	44.9	Circular	42	

[a] From references 9 and 10.
[b] Strains under the same letter showed obviously related restriction maps.

We noted that the linear mtDNA from *C. parapsilosis* SR23 has a completely different gene map, as described by Kovac et al. (8). We confirmed this map by examining the type strain of the species (CBS 604). This finding suggests that there exist distinct groups of linear mtDNAs with different gene orders. Structure of linear mtDNAs from a few other *Candida* species are currently under investigation.

Taxonomical considerations. By DNA-DNA hybridization experiments, Kurtzmann (10) has estimated the chromosomal DNA relatedness of several yeast species, especially those of the *Pichia-Williopsis* group. The results of mtDNA restriction mapping shown here are consistent with some of his conclusions. (i) *P. jadinii* and *C. utilis* had been considered conspecific; their mtDNAs indeed showed highly related restriction maps and both were linear. (ii) The relatedness between *P. sargentensis* CBS 6342 and *W. mrakii* CBS 1707 was confirmed by mtDNA restriction site mapping and by electrophoretic karyotyping; *W. saturnus* CBS 5761 has also an mtDNA clearly related to this group. (iii) *W. beijerinckii* CBS 2564 and *W. suaveolens* CBS 1670 mtDNAs also appeared to be related to each other. However, we found some apparent inconsistencies between mtDNA analysis and the current classification. For example, *W. suaveolens* CBS 1670 has a linear mtDNA of 50 kbp, while the type

strain of the species, CBS 255, has a circular mtDNA with similar gene order (Fig. 2).

Possible relationship between linear and circular forms of mtDNA. If the linear mtDNAs described here indeed replicate as a linear molecule, implying a replication mechanism very different from that of the circular DNAs, these two forms of mtDNA may be thought to have diverged early from each other. The available data do not seem to support this view. First, the protein-coding sequences as well as rRNA and tRNA genes are highly conserved with respect to those in circular mtDNAs. Second, two closely related strains according to the standard criteria of yeast taxonomy can have either linear or circular mtDNA, as mentioned above in the case of *W. suaveolens*. These observations suggest that the linear and circular forms of mtDNA in yeasts do not represent radically different life styles, in the sense that the conversion from one form to another may occur through a relatively simple mechanism. For example, after replication as a circle, the mtDNA molecules may be cut at specific points so that the majority of molecules remain linear with defined termini until the next replication cycle. Such a hypothesis will have to explain how these linearized molecules can recircularize for replication and why, in most species, the circular mtDNA remains circular. These questions are examined in the companion report (4).

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