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Complete DNA sequence of the linear mitochondrial genome of the pathogenic yeast *Candida parapsilosis*

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Abstract The complete sequence of the mitochondrial DNA of the opportunistic yeast pathogen *Candida parapsilosis* was determined. The mitochondrial genome is represented by linear DNA molecules terminating with tandem repeats of a 738-bp unit. The number of repeats varies, thus generating a population of linear DNA molecules that are heterogeneous in size. The length of the shortest molecules is 30,922 bp, whereas the longer molecules have expanded terminal tandem arrays (n×738 bp). The mitochondrial genome is highly compact, with less than 8% of the sequence corresponding to non-coding intergenic spacers. In silico analysis predicted genes encoding fourteen protein subunits of complexes of the respiratory chain and ATP synthase, rRNAs of the large and small subunits of the mitochondrial ribosome, and twenty-four transfer RNAs. These genes are organized into two transcription units. In addition, six intronic ORFs coding for homologues of RNA maturase, reverse transcriptase and DNA endonucleases were identified. In contrast to its overall molecular architecture, the coding sequences of the linear mitochondrial DNA of *C. parapsilosis* are highly similar to their counterparts in the circular mitochondrial genome of its close relative *C. albicans*. The complete sequence has implications for both mitochondrial DNA replication and the evolution of linear DNA genomes.

Keywords *Candida parapsilosis* · Linear mitochondrial DNA · Telomeric circles (t-circles) · DNA replication · Evolution

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Introduction

Although the mitochondrial genome of baker's yeast (*Saccharomyces cerevisiae*) is represented mainly by a population of polydisperse linear DNA molecules lacking specific terminal structures, which includes only a small proportion of circular molecules and branched structures, genetic and physical approaches resulted in a circular map of the mitochondrial DNA (mtDNA; reviewed in Williamson 2002). This type of mtDNA architecture is now referred to as a circular-mapping mitochondrial genome (Jacobs et al. 1996). The mitochondrial genomes of several yeast species have been sequenced completely (Lang 1984; Sekito et al. 1995; Foury et al. 1998; Kerscher et al. 2001; Petersen et al. 2002; Bullerwell et al. 2003; Koszul et al. 2003; Langkjaer et al. 2003; Jones et al. 2004) and presumably have similar structures. In contrast, the molecular architecture of the mtDNA of the petite negative yeast *Candida parapsilosis* is quite different. Restriction enzyme analysis and 5′ end labeling indicate that the mitochondria of *C. parapsilosis* contain uniform, linear DNA molecules of about 30 kb (Kovac et al. 1984). Moreover, it has been demonstrated that linear mtDNA retains its structural and functional integrity in the presence of ethidium bromide and acridine orange,

a feature which may underlie the ability of *C. parapsilosis* to grow in the presence of high levels of these intercalating agents (Maleszka 1994). A series of analyses has confirmed the linear nature of this mitochondrial genome and uncovered specific terminal structures (mitochondrial telomeres) consisting of tandem arrays of a 738-bp unit and a 5′ single-stranded protrusion of about 110 nt (Nosek et al. 1995), which is protected by a protein cap (Nosek et al. 1999; Tomaska et al. 2001). Moreover, it has been demonstrated that mitochondrial telomeres adopt a higher-order structure (telomeric loop, t-loop; Tomaska et al. 2002), like that found in the nuclear chromosomes of eukaryotes (Griffith et al. 1999). Importantly, the molecular mechanisms involved in the maintenance of mitochondrial telomeres may represent evolutionarily earlier and/or independent strategies for replication of the telomeric arrays. This idea is supported by structural similarities between nuclear and mitochondrial telomeres (reviewed in Nosek and Tomaska 2002).

Our previous studies have generated fragmentary sequence data for *C. parapsilosis* mtDNA (Nosek and Fukuhara 1994a, 1994b; Nosek et al. 1995). To systematically elucidate the detailed genetic organization of the linear mitochondrial genome of *C. parapsilosis*, the mechanism of its replication, and evolutionary relationships between mitochondrial genomes with different molecular architectures, we have now sequenced and analyzed the entire mitochondrial genome of this yeast species. In silico analysis indicates that mitochondria of *C. parapsilosis* harbour a highly compact genome which, in spite of its different molecular architecture, shares several common features with the mtDNAs of other yeasts. The complete sequence of *C. parapsilosis* mtDNA provides the basis for addressing problems related to the replication and evolutionary emergence of linear genomes in yeast mitochondria.

Materials and methods

DNA sequencing, assembly and data analysis

DNA was isolated from mitochondria of *Candida parapsilosis* SR23 (CBS 7157) using a protocol described previously (Casey et al. 1974). The restriction enzyme map of the mtDNA of this strain is identical to that of the type strain of the species CBS 604/ATCC 22019 (Camougrand et al. 1988; Nosek et al. 1995, 2002). Libraries of mtDNA fragments were prepared in pUC/pTZ vectors and the DNA sequence was determined using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Sequence assembly and analysis was done using the Vector NTI Advance v. 8.0 software package (Informax). For comparison with other fungal mitochondrial genomes see http://www.cbs.dtu.dk/services/GenomeAtlas/show-kingdom.php?kingdom = Mitochondria&sortKey = ORGANISMSORT&phyla = Fungi.

Nucleotide sequence accession numbers

The complete sequence of *C. parapsilosis* mtDNA was deposited in the GenBank data library under the Accession No. AY423711, and in the EMBL database as an update of the X74411 entry. The sequence (32,744 bp) represents a DNA molecule flanked by *Eco* RI sites and overlaps the fragments deposited under the following EMBL database entries: X74411, X75674-X75681, X76196, X76197.

Results and discussion

The linear mitochondrial genome of *C. parapsilosis* has a compact organization with two putative transcription units

The complete sequence of the mtDNA from *C. parapsilosis* strain SR23 (CBS 7157) was determined. The mitochondrial genome is represented by a population of linear double-stranded DNA molecules terminating with inverted repeats consisting of a 554-bp subtelomeric region and an array of tandem repetitions of a 738-bp unit (Fig. 1). Variation in the number of repeats generates mtDNA molecules that differ in the size of the telomeric array. The length of the shortest molecule, which possesses only a portion of the tandem unit, is 30,922 bp, while longer molecules terminate with integral multiples of the 738-bp repeat motif at both ends.

The sequence is relatively rich in adenine and thymine residues (A + T). Whereas the shortest molecule contains about 75.7% A + T, the proportion of A + T bases within the telomeric repeat unit is as high as 84.3%. With the exception of the subtelomeric regions (positions 1645–1702 and 31043–31100), where the guanine and cytosine (G + C) content exceeds 60%, no significant GC-rich stretches or clusters were detected within the sequence. Almost two-thirds of the shortest molecule codes for protein subunits of the respiratory-chain complexes and ATP synthase (excluding intronic sequences), ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs). The introns detected within the reading frames of the genes *cob* and *cox1* (see below) represent approximately 27% of the mtDNA sequence. Less than 8% of the sequence corresponds to intergenic spacers, indicating a compact organization of the genome.

The coding sequences in *C. parapsilosis* mtDNA (Fig. 2) seem to be organized into two transcription units, *rrnL-nad3* and *cox1-atp6*, both of which may be transcribed from the center of the molecule toward the left and right telomeres, respectively. Computer analysis of this region revealed a sequence, TTATAAGTA, on the upper strand (12427–12435) which corresponds to the canonical WTATAAGTA nonanucleotide promoter motif identified in the mtDNA of *S. cerevisiae* (Osinga et al. 1984). In addition, two copies of a related sequence (AATAAAGTA) were found on the complementary strand

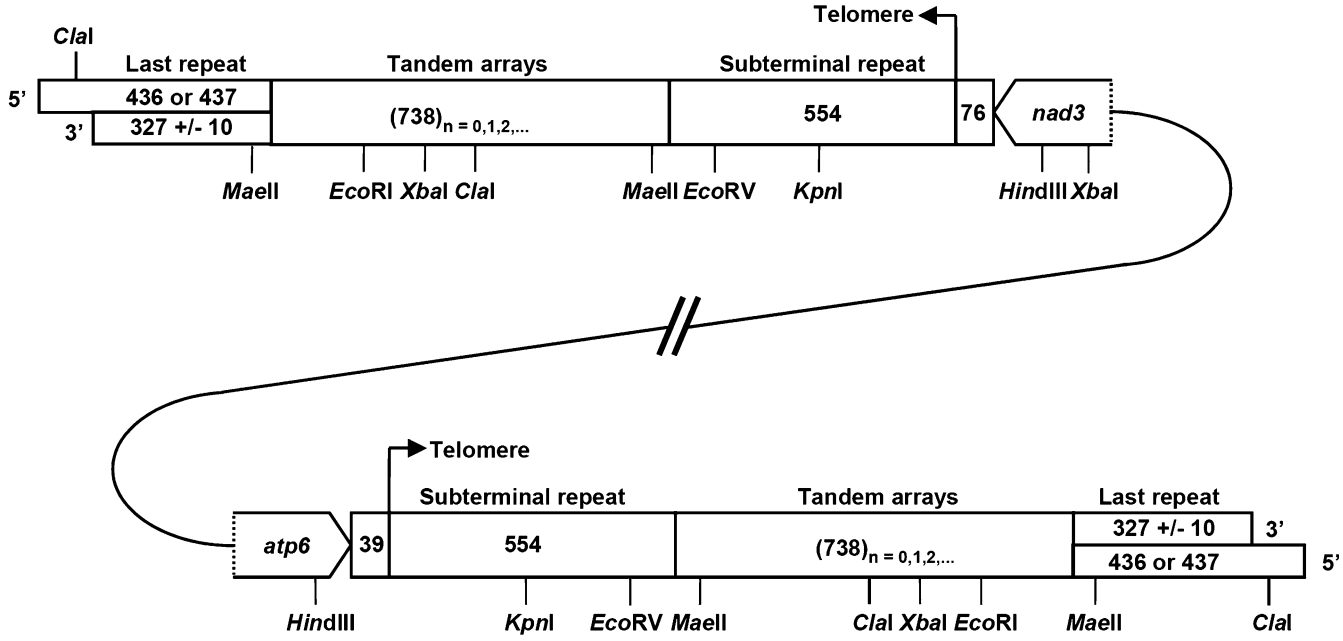

Fig. 1 Architecture of the terminal sequences of the *C. parapsilosis* mtDNA. Mitochondrial telomeres consist of the subterminal repeat (554 bp) and a tandem array of the 738-bp units repeated. Linear molecules terminate with an incomplete repeat and a 5′ single-stranded extension of about 110 nt. The positions of sites for relevant restriction enzymes are shown

(11935–11943 and 12208–12216). These motifs may function as promoters for the right and left arms, respectively. Their absence in intergenic regions indicates that the transcription of both mtDNA strands proceeds from a promoter located upstream of *rrnL* (the left arm) and *cox1* (the right arm), respectively, toward the corresponding telomere. As in the mtDNAs of human and *Schizosaccharomyces pombe* (Anderson et al. 1981; Lang 1984), the genes coding for tRNAs are localized either individually or in small clusters that separate most of the sequences coding for proteins and rRNAs. This indicates that processing of primary transcripts involves the excision of tRNAs and rRNAs, leading to the generation of mono- and bi-cistronic mRNAs that have been identified in the mitochondria of *C. parapsilosis* (Nosek and Fukuhara 1994b; M. Anderkova and J. Nosek, unpublished data). The occurrence of promoter-like motifs (TAATAAGTA and AATAAAGTA) within the telomeric repeats suggests the possibility that the telomeres may also be transcribed.

Putative bi-directional origin of replication

In contrast to the *ori*/*rep* elements containing GC-rich sequence motifs found in baker's yeast (Baldacci et al. 1984; de Zamaroczy et al. 1979), in silico analysis of *C. parapsilosis* mtDNA did not reveal any canonical *ori*/*rep* elements, and, with the exception of the telomeric regions, the sequence lacks GC-rich clusters. Since mtDNAs of several yeast species (e.g., *Yarrowia lipolytica*, *Saccharomyces castellii*; Kerscher et al. 2001; Petersen et al. 2002) also do not contain such motifs, the presence of a GC-rich cluster does not seem to be an evolutionarily conserved feature of mitochondrial DNA replication. Since the analysis of the asymmetry of base distribution in DNA strands, termed GC skew analysis, has been shown to be highly predictive for the identification of origins of replication in many prokaryotic genomes (Lobry 1996; Picardeau et al. 2000), we employed this approach for *C. parapsilosis* mtDNA. The results indicate that, in contrast to *S. cerevisiae*, the mtDNA of *C. parapsilosis* may contain a single bi-directional replication origin localized at position 12289 within the region between the genes *rrnL* and *cox1* (Fig. 3). The occurrence of promoter motifs in this region (see above) that could control the synthesis of RNA primers strongly supports this possibility.

DHE-like elements

The sequence of the subtelomeric GC-rich clusters (1645–1702 and 31043–31100) found in the *C. parapsilosis* mtDNA can be folded into secondary structures that are reminiscent of the double-hairpin elements (DHEs) observed in the mitochondrial genomes of chytridiomycetes and certain zygomycete as well as ascomycete fungal species. DHEs are considered to be active mobile elements that seem to be implicated in mtDNA rearrangements, and may contribute to recombinational transactions (Paquin et al. 2000; Bullerwell et al. 2003). In *C. parapsilosis* DHE-like elements occur specifically within sub-terminal regions of mtDNA, suggesting their participation in recombination-dependent maintenance of mitochondrial telomeres, as previously proposed for *Tetrahymena* species (Morin and Cech 1988).

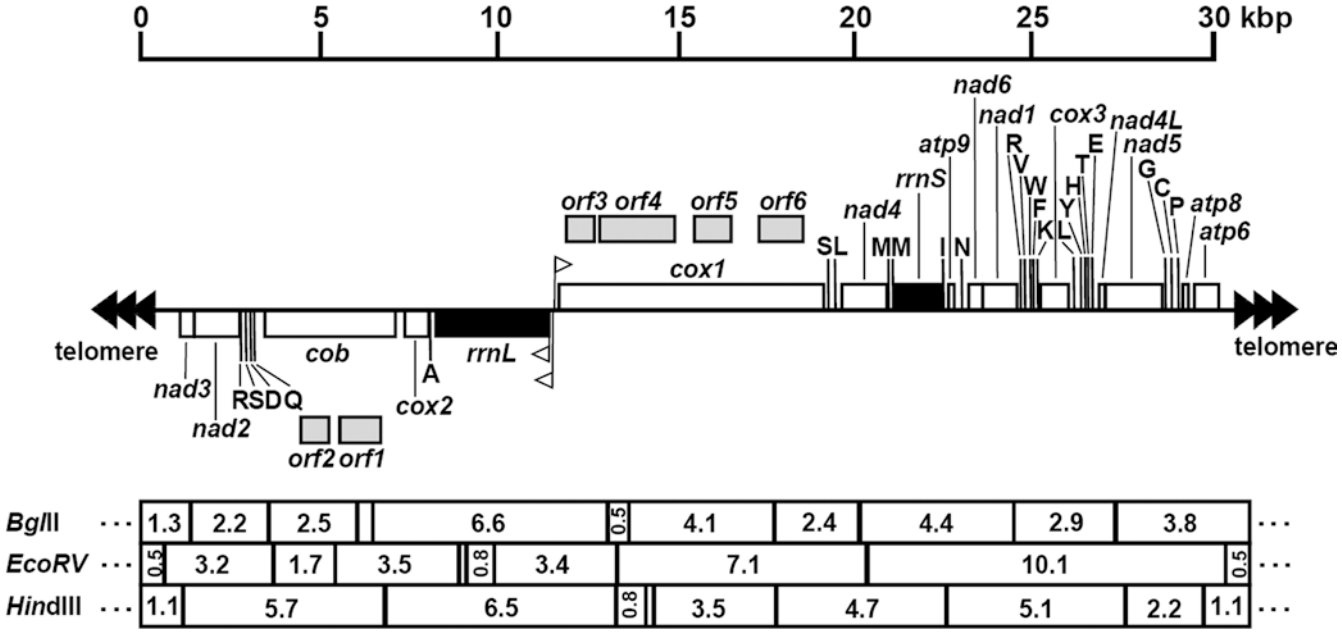

Fig. 2 Genetic organization of the linear mitochondrial genome of the yeast *C. parapsilosis*. Sequences coding for proteins (*open rectangles*), rRNAs (*black rectangles*) and tRNAs (labeled by the single-letter codes for their cognate amino acids) are shown. Note that sequences of *cox1* and *cob* contain three and two introns, respectively, which contain *orf1-orf6* (shown as *grey rectangles*). The *open triangles* indicate the positions of promoter motifs identified within the putative bi-directional origin of mtDNA replication. The arrays of *black triangles* represent mitochondrial telomeres. The *Bgl* II, *Eco* RV and *Hin* dIII restriction maps are shown *below* the genetic map

Genes encoded by linear mtDNA

Computer analysis revealed that the linear mtDNA of *C. parapsilosis* encodes the standard set of mitochondrial genes usually found in yeast mtDNAs. These include genes encoding 14 protein subunits of the respiratory chain complexes (*nad1–nad6, nad4L, cob, cox1–3*) and ATP synthase (*atp6*, *8* and *9*), which display relatively high similarity to their counterparts encoded by circular-mapping mtDNAs. The complete mtDNA sequence contains 24 putative *trn* genes, identified on the basis of conserved sequence motifs and "cloverleaf" structures. The set comprises two tRNAs each for arginine, leucine and serine, an initiator $tRNA^{fMet}$, and $tRNA^{Trp}$ for the UGA codon. Sequences encoding the RNAs of the large and small subunits of the mitochondrial ribosome were detected on the basis of sequence similarity to their counterparts from other species, although their 5′ and 3′ ends have not been mapped precisely. The *rrnS* is flanked by *trnM2* and *trnI*, and the boundaries of *rrnL* are defined by the *trnA* at the 3′ end and one of the putative promoter motifs localized upstream of *rrnL* (12208–12216 and 11935–11943).

The compactness of the genome is emphasized by the presence of overlapping coding sequences. In the gene pairs *nad2-nad3*, *nad6-nad1* and *nad4L-nad5*, the last adenine residue corresponding to the termination codon UAA of the upstream ORF represents the first residue of

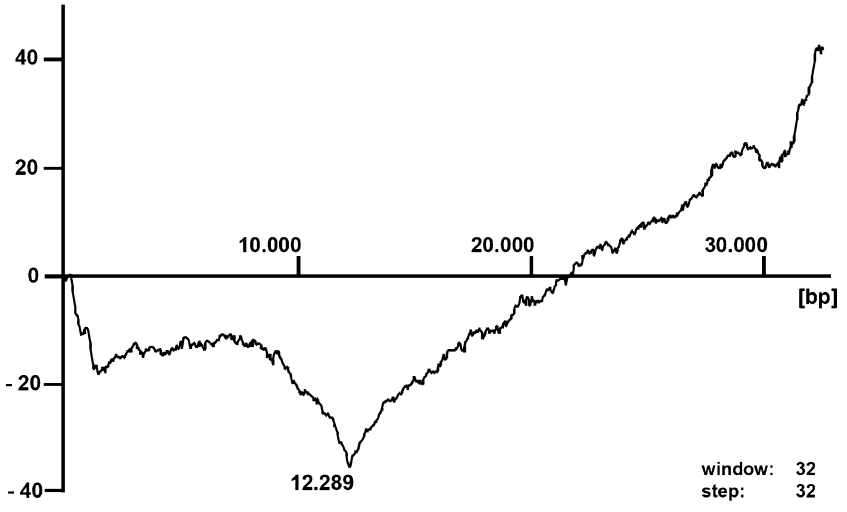

Fig. 3 Identification of a putative replication origin of the *C. parapsilosis* mtDNA. The cumulative GC skew analysis performed using the Genome Skew software v. 1.0 (Technical University of Munich, Germany) predicts a potential origin of replication at nucleotide position 12289 (see text for details)

the initiation codon AUG of the downstream ORF resulting in a 1-nt overlap in the coding sequences. Similar arrangements of the genes for respiratory complex I subunits have also been found in other species (e.g., *Neurospora crassa, C. albicans, Debaryomyces occidentalis*; Nelson and Macino 1987; Fernet et al. 2003; Jones et al. 2004). Moreover, sequences coding for $tRNA^{Arg}$ and $tRNA^{Ala}$ extend beyond the 5′ ends of the ORFs of *nad2* and *cox2* by 57 and 36 nt, respectively, suggesting that, in both cases, translation initiation may precede excision of the tRNA. An alternative possibility is that the translation of both proteins starts at the second AUG codon localized downstream of the 3′ end of the tRNA, resulting in slightly shorter polypeptides. Interestingly, the *trnR1-nad2* overlap is also conserved in the mtDNA of *C. albicans*. However, although the sequence coding for $tRNA^{Ala}$ is also followed by the *cox2* gene in *C. albicans* mtDNA, in this case the sequences do not overlap.

Strains of *C. parapsilosis*, including SR23 (CBS7157) used in the mtDNA sequence analysis, are known to be resistant to various inhibitors of oxidative phosphorylation (Camougrand et al. 1986). Studies on baker's yeasts revealed that substitutions within ATP synthase subunits 6 and 9 confer resistance to oligomycin (reviewed in Nagley 1988). Analysis of *C. parapsilosis atp6* did not uncover potential mutation sites (Guelin et al. 1991); however, inspection of the deduced protein product of the *atp9* revealed an alanine residue in position 23. The counterpart of baker's yeast has a glycine at this position and the substitution Gly23 → Ala is known to confer oligomycin resistance (oli^R). Although we cannot rule out the possibility that the oli^R phenotype is due to an active pleiotropic drug resistance system, our data suggest that it may be mitochondrially encoded within the *atp9* ORF.

Introns and intronic ORFs

The reading frames of *cob* and *cox1* are interrupted by two (bI1 and bI2) and three (aI1–aI3) intronic sequences, respectively. Their positions and splice junctions (Table 1) were identified by comparison of *C. parapsilosis* sequences with known homologues from *S. cerevisiae* (Foury et al. 1998), *S. douglasii* (Tian et al. 1991) and *C. albicans* (Jones et al. 2004). All introns contain ORFs that are in frame with the preceding exons. Deduced protein products encoded by these ORFs show similarity to RNA maturase, reverse transcriptase and endonuclease, respectively, and are presumed to be involved in the splicing of primary transcripts or in intron mobility.

Codon usage

Analysis of the codon usage indicates that all 64 codons are utilized, although there is a strong bias against guanine- and cytosine-containing codons. Thus, the codons UCC, UGG, CUC, CUG, CCC, CGC and GGC occur only within intronic ORFs. As in the mitochondria of other yeast species, UGA is interpreted as tryptophan. However, in contrast to the genetic code of *S. cerevisiae* mitochondria, comparison of conserved protein domains by multiple alignments and analysis of an N-terminal sequence of the Atp6 protein (Guelin et al. 1991) indicate that AUA is decoded as isoleucine and CUN as leucine.

Linear and circular mitochondrial genomes in evolutionary perspective

Yeast species which possess linear mitochondrial genomes are almost randomly distibuted on the phylogenetic tree (Nosek et al. 1998). The structure of mitochondrial telomeres, the gene order and the organization of transcription units observed in linear mitochondrial genomes found in yeast species from the *Williopsis-Pichia* group (Fukuhara et al. 1993; Drissi et al. 1994) differ substantially from that described here. Detailed analysis of the genetic organization of mtDNAs may shed some light on the molecular events that led to the evolutionary emergence of organellar genomes with different molecular architectures. *C. parapsilosis* belongs to the same cluster of species on the phylogenetic tree as *Lodderomyces elongisporus*, *C. sojae*, *C. tropicalis*, *C. maltosa*, *C. viswanathii*, *C. lodderae*, *C. dubliniensis* and *C. albicans* (Kurtzman and Robnett 1998). Except in the case of *C. albicans*, information

Table 1 Properties of introns and intronic ORFs identified in *C. parapsilosis* mtDNA

Intron	5' and 3' exon-intron junctions[a]	ORF	Predicted product
bI1	... tttatgggt-TATAAAACAA-... ... GTTTATTCCG-tattgcttgg-...	*orf1*	GIY...YIG-type endonuclease
bI2	... tcactgaggt-AGTCTTATTG-... ... TTTTATATTG-gcaactgtaa-...	*orf2*	RNA maturase with LAGLIDADG motif
aI1	... aggtgcattt-TTACGACGTG-... ... CATCTCTAGT-ggaaatttct-...	*orf3*	Unknown function
		orf4	Reverse transcriptase with HNH/HNHc endonuclease in C-terminal domain
aI2	... catttatttt-ATATAATATG-... ... TCAAGGTAGG-gattctttgg-...	*orf5*	LAGLIDADG-type endonuclease
aI3	...-agtttgaagt-TGACATACTA-... ...-GATAAATTTG-catcacatgt-...	*orf6*	LAGLIDADG-type endonuclease

[a]Exon sequences are shown in *lower case*, intron sequences in *upper case* letters

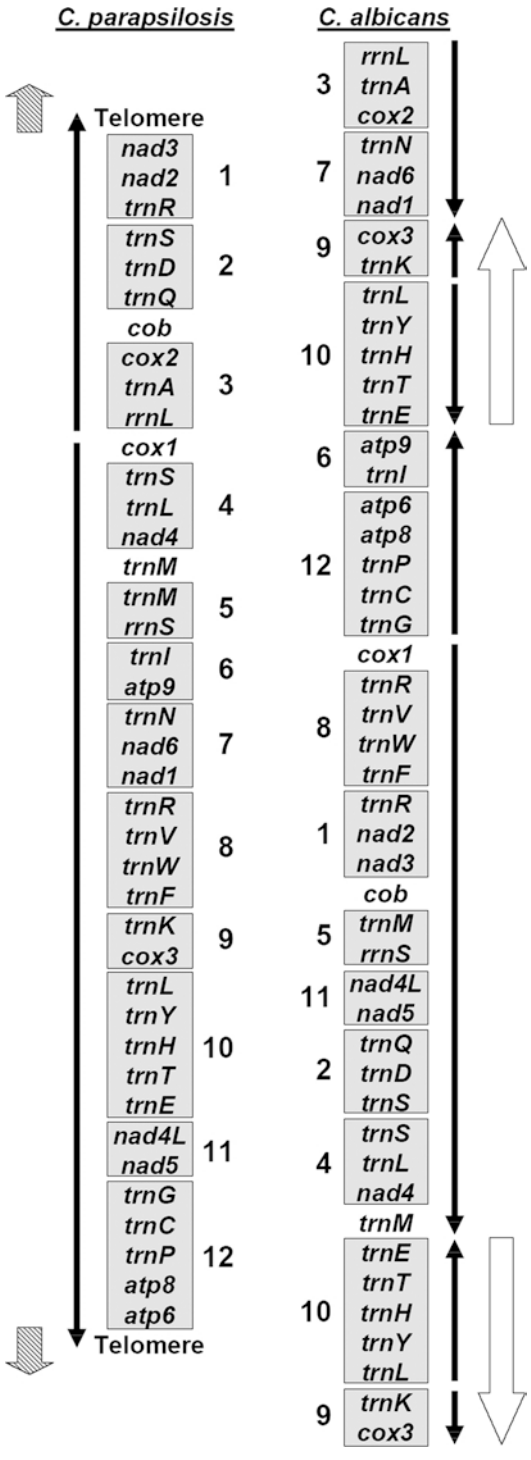

Fig. 4 Comparison of genetic organization of the mitochondrial genomes of *C. parapsilosis* and *C. albicans*. Conserved gene clusters are shown as *grey rectangles* and *numbered*. The *open* and *hatched arrows* represent inverted repeats and telomeres, respectively. The *black arrows* indicate the direction of transcription

about the genetic organization of their mtDNA is not yet available. Comparison of the linear mtDNA of *C. parapsilosis* with the circular-mapping genome of *C. albicans* (Jones et al. 2004) reveals several conserved gene clusters (Fig. 4) which may allow us to trace the molecular form and gene order of mtDNA back to a common ancestor. In contrast to *C. albicans*, the mtDNA of *C. parapsilosis* does not contain large inverted repeats comprising gene duplications. Moreover, while genes on the *C. parapsilosis* mtDNA are arranged into two transcription units, which are subsequently processed into mono- and bi-cistronic mRNAs, the mtDNA of its close relative *C. albicans* possesses essentially the same set of genes but these are organized in multiple transcription units. This suggests that recombination and gene shuffling within an ancestral mtDNA resulted in the emergence of mitochondrial genomes with different molecular architectures. However, delineation of a possible evolutionary scenario that accounts for the generation of the linear genome with a highly compact genetic organization observed in *C. parapsilosis* will require more data on the mtDNAs of closely related species.

Although no circular mtDNA molecules were observed in *C. parapsilosis* SR23 (Kovac et al. 1984; Maleszka 1994; Nosek et al. 1995), its mitochondria have been shown to contain extragenomic telomeric circular DNAs (t-circles), which seem to be involved in telomere maintenance (Tomaska et al. 2004). The t-circles were also detected in mitochondria of two phylogenetically unrelated yeast species, *C. salmanticensis* and *Pichia philodendri*, which have mitochondrial telomeres with a similar molecular architecture (Tomaska et al. 2000). Surprisingly, a recent survey of clinical isolates of *C. parapsilosis* revealed that strains that lack t-circles harbor circular-mapping derivatives of the genome formed by fusion of the termini of the linear molecules (Rycovska et al. 2004). The analysis of the complete sequence indicates that the t-circles are not derived from internal parts of the mtDNA. Moreover, t-circles do not hybridize with nuclear chromosomes (data not shown). These findings are compatible with the recent hypothesis (Nosek and Tomaska 2003) that mitochondrial telomeres derived from mobile elements, such as transposons or plasmids, that invaded mitochondria, contributed to the formation of the linear DNA genophore and provided a solution to the problem of telomere maintenance.

In conclusion, the complete sequence of *C. parapsilosis* mtDNA provides an opportunity to address the problems concerning mtDNA replication and evolutionary relationships between linear- and circular-mapping genomes in mitochondria mentioned above. In addition, as we have recently shown (Nosek et al. 2002; Rycovska et al. 2004), it provides the basis for further improvement of mtDNA-derived markers for molecular diagnostics and the identification of *C. parapsilosis* in clinical samples.

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