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NADH Dehydrogenase Subunit Genes in the Mitochondrial DNA of Yeasts

JOZEF NOSEK[1] AND HIROSHI FUKUHARA[2]*

Institut Curie, Section de Biologie, Centre Universitaire Paris XI, 91405 Orsay, France,[2] and Department of Genetics, Comenius University, 84215 Bratislava, Slovakia[1]

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The genes encoding the NADH dehydrogenase subunits of respiratory complex I have not been identified so far in the mitochondrial DNA (mtDNA) of yeasts. In the linear mtDNA of *Candida parapsilosis*, we found six new open reading frames whose sequences were unambiguously homologous to those of the genes known to code for NADH dehydrogenase subunit proteins of different organisms, i.e., ND1, ND2, ND3, ND4L, ND5, and ND6. The gene for ND4 also appears to be present, as judged from hybridization experiments with a *Podospora* gene probe. Specific transcripts from these open reading frames (*ND* genes) could be detected in the mitochondria. Hybridization experiments using *C. parapsilosis* genes as probes suggested that *ND* genes are present in the mtDNAs of a wide range of yeast species including *Candida catenulata*, *Pichia guilliermondii*, *Clavispora lusitaniae*, *Debaryomyces hansenii*, *Hansenula polymorpha*, and others.

The NADH dehydrogenase complex (or mitochondrial respiratory complex I) catalyzes the oxidation of NADH by ubiquinone. This reaction is linked to proton transfer across mitochondrial membranes. Complex I is composed of a large number of subunits, several of which are encoded by mitochondrial DNA (mtDNA). These genes (for short, *ND* genes) have been identified in a variety of organisms including mammals (1–3, 7), amphibians (45), insects (9, 20), protozoa (29, 43, 53), plants (38), and algae (34), as well as filamentous fungi (4, 13, 36). In the plants liverwort (40), tobacco (50), and rice (26, 54), equivalent genes in the chloroplast genome have been identified. Various multiple disorders of humans are associated with mutations of the mitochondrial *ND* genes (55). In yeast mitochondria, the presence of *ND* genes has not been reported so far. No sequences related to the ND-encoding genes could be found in the total mtDNA sequences of *Saccharomyces cerevisiae* (15) and *Schizosaccharomyces pombe* (47), and site 1 phosphorylation seems to be absent in *S. cerevisiae* (30, 32, 48 [see, however, reference 39]). It is not clear whether the absence of mtDNA-encoded ND subunits in these species constitutes an exceptional case for yeasts. In *Candida utilis* (anamorph—asexual form—of *Pichia jadinii*), site 1-linked phosphorylation seems to occur and NADH dehydrogenase synthesis appears to be under growth-related control. *Candida parapsilosis* (6) and *Candida tropicalis* (19) have also been reported to have NADH dehydrogenase activity coupled with site 1 phosphorylation. Assuming that site 1 phosphorylation depends on the presence of mtDNA-encoded ND subunits, one may expect that the mtDNA of some aerobic yeasts possesses the *ND* genes, as in the case of the filamentous fungi so far studied (members of the genera *Neurospora*, *Podospora*, and *Aspergillus*). However, the search for *ND* genes in yeast mtDNAs with gene probes from filamentous fungi has not been successful, probably because of a large divergence of nucleotide sequences. During sequencing work on the mtDNA of *C. parapsilosis*, we found a set of new open reading frames which turned out to be clearly homologous to the NADH dehydrogenase subunit genes known in other organisms. This report describes the nucleotide sequences of six ND-encoding genes and their map positions in the mtDNA of *C. parapsilosis*. Use of these sequences as hybridization probes allowed us to examine how the mitochondrial genes for NADH dehydrogenase subunits are distributed among yeast species.

* Corresponding author. Mailing address: Institut Curie, Section de Biologie, Bâtiment 110, Centre universitaire Paris XI, 91405 Orsay, France. Phone: (33 1) 6986 3063. Fax: (33 1) 6986 9429.

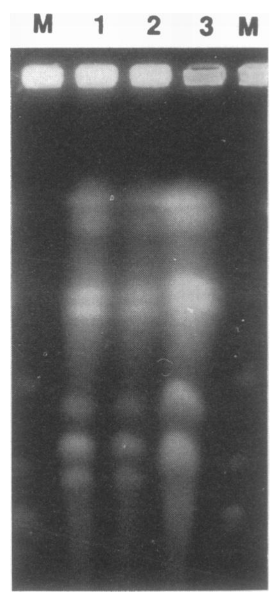

FIG. 1. Pulsed-field gel electrophoresis of chromosomal DNAs from strain SR23 and the *C. parapsilosis* type strain CBS 604. Cells of each culture were lysed in an agarose block by treatment with Zymolyase and proteinase K and electrophoresed in a 1% agarose gel (52) with 0.5× Tris-borate EDTA buffer (46). Electrophoresis was carried out on an LKB pulsaphor apparatus in contour-clamped homogeneous electric field configuration. Pulse conditions were the following: phase 1, 100 s, 17 h; phase 2, 130 s, 33 h; and phase 3, 360 s, 20 h; 130 V and 9°C throughout. DNA bands were stained with ethidium bromide and visualized under UV light. Lanes 1 and 2, CBS 604; lane 3, SR23; M, *S. cerevisiae* MH41-7B as molecular weight markers.

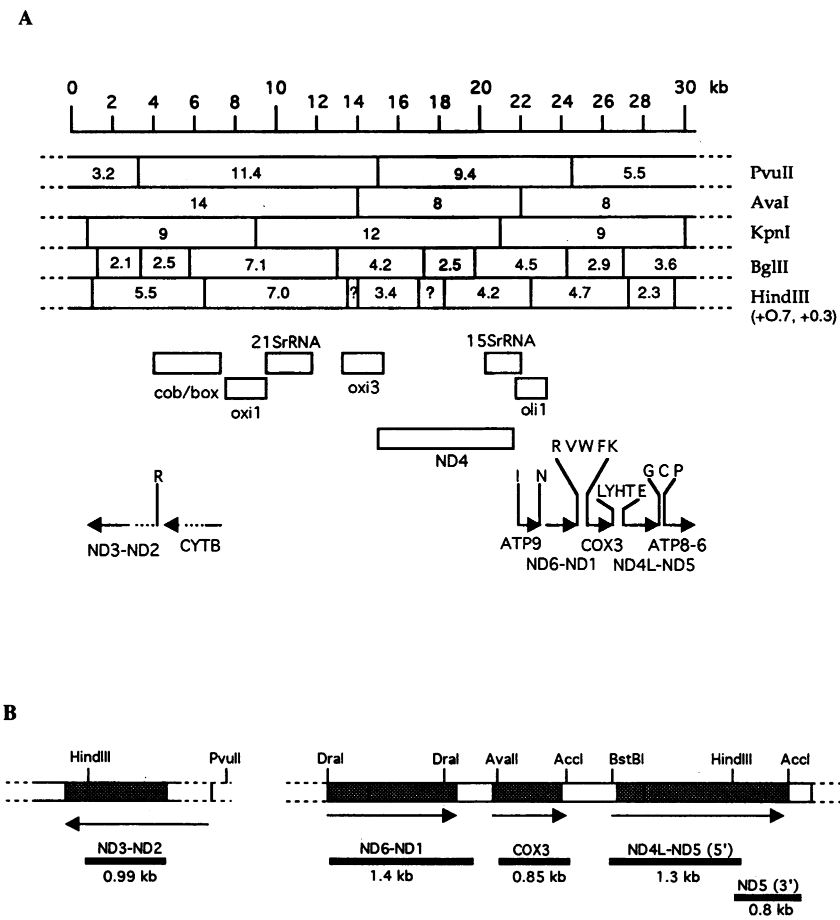

FIG. 2. Structure of the mitochondrial DNA of *C. parapsilosis* SR23. (A) Restriction map of the linear mitochondrial DNA, about 30 kbp long. Only relevant sites are shown. The details of the telomeric regions are to be described in a separate article. The mitochondrial DNA of the type strain of the species (CBS 604) showed the same restriction patterns. Approximate gene locations based on the data published by Kovac et al. (31) are shown by open boxes. Each gene is indicated by the locus name of the *S. cerevisiae* gene used as a probe. The position of the putative *ND4* gene, on the basis solely of our hybridization experiments with *Podospora ND4* probe, is also included. In the lower part, the positions of genes actually sequenced are shown. tRNA genes are indicated by the one-letter amino acid code. The positions of prolyl tRNA and the *ATP6-ATP8* genes are from Guélin et al. (23, 24). The positions of all other tRNA genes are from Nosek and Fukuhara (37). Arrows indicate the directions of transcription. (B) Detailed map of *ND* genes sequenced in this work. The sizes of the cloned fragments used as probes are indicated under the gene names. The 5′ part of the *ND2* gene has not yet been sequenced.

MATERIALS AND METHODS

Yeast strains. All the strains, including the type culture of *C. parapsilosis* (Ashford) Langeron et Talice, CBS 604, were obtained from Centraalbureau voor Schimmelcultures (CBS), Delft, The Netherlands. The *C. parapsilosis* strain SR23 (CBS 7157), on which the sequencing work was carried out, is a stock from L. Kovac, Comenius University, Bratislava, Slovakia. Since species identification of asexual yeasts often raises ambiguity, we reexamined the identity of strain SR23 by comparing the restriction patterns of mtDNA with those of the type strain. Identical results were obtained, in agreement with the previous report from Camougrand et al. (5). Furthermore, the electrophoretic karyotypes were also similar between these two strains, as shown in Fig. 1. These data confirmed the taxonomical definition of the strain SR23 as *C. parapsilosis*. All other strains are the type cultures representative of each species, except that *S. cerevisiae* MH41-7B is a laboratory strain of known genotype.

Preparation of mtDNA. mtDNA of *C. parapsilosis* was purified from a total DNA preparation by two cycles of isopycnic centrifugation in CsCl in the presence of bisbenzimide (H33258; Hoechst) (21) and used in DNA cloning procedures. For dot blot hybridization, minipreparations of mtDNA from various yeasts were obtained according to a previously described procedure (12).

Dot blot hybridization. Samples (approximately 100 ng) of mtDNA from various yeasts were denatured and dot blotted on Nylon$^+$ membranes (Appligène, Illkirch, France). Hybridization of the membrane-bound DNA with ^{32}P-labelled probes was performed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M Na_3-citrate) at 50°C, overnight. The membranes were then washed in 3× SSC–0.1% sodium dodecyl sulfate (SDS) at room temperature (low-stringency condition) or in 0.1× SSC–0.1% SDS at 50°C (high-stringency condition). The *ND* gene probes were the restriction fragments isolated from cloned mtDNA pieces. These probes contained *ND6-ND1*, *ND2-ND3*, *ND4L-ND5* (5′ part), or *ND5* (3′ part) sequences. Their sizes and map positions are indicated in Fig. 2B. The probe for gene *ND4* was a 1.9-kbp *Pst*I-*Eae*I fragment from the *Pst*I-6 clone of *Podospora anserina* mtDNA (10). To prepare labelled probes,

Sequence alignment of ND1

```
ND1_CANPA   MQY------IELLIMFLSVLLAVAFLTVAERKTMAYMQRRTGPNAVGYLGVLMAIADAAKLLLKEIITPTHADKFILFISPMIALISSLLCWSIIPFAPG
ND1_PODAN   MYYSIIISLIEVVLVLVPALLGIAYVTIAERKTMASMQRRLGPNFVGYYGLLQAFADALKLLLKEYVAPTQANIILFFLGPVITLIFSLLGYAVIPYGPS
ND1_MARPO   MRIYLIGLVAKMLGIMMPLLLGVAFLVLAERKVMASMQRRKGPNVVGILGLLQPLADGLKLMMKEPILPSSANIFIFLMAPVLTFTLALCAWAVIPFDYG
ND1_HUMAN   MPM------ANLLLLIVPILIAMAFLMLTERKILGYMQLRKGPNVVGPYGLLQPFADAMKLFTKEPLKPATSTITLYITAPTLALTIALLLWTPLPMPNP
            *          .. . .. *...*.. ..*** .. ** * *** **  *.* ..**. **  ** . *. .    . . .* ...  .*   . .*

ND1_CANPA   VTIYDSNYGFILMLAISSVGVFGPLLAGW-SGNSKYSLLGSIRATAQLISYELILTTIVLICILLAGTMNLSKYVELQESIWIGIPLLPLSIMWYIGCVA
ND1_PODAN   LAINDFSLGIYYILAVSSLATYGILLAGWGSANSKYAFLGSLRSTAQLISYELVLSSAILLVIMLTGSLNLSVNIESQRAIWNIFPLLPVFIIFFIGSVA
ND1_MARPO   MVFSDLNIGVLYLFAMSSLGVYGIITAGWAS-NSKYAFLGALRSAAQMVSYEVSMGLLLMSVMLCAGSCNFSEIVLAQTRMWFVFPLFPVFLMFFISCLA
ND1_HUMAN   LV--NLNLGLLFILATSSLAVYSILWSGWAS-NSNYALIGALRAVAQTISYEVTLAIILLSTLLMSGSFNLSTLITTQEHLWLLLPSWPLAMMWFISTLA
            .   . . *    ..* **.. .. . .** * **.*...*..*. ** .***. .    ..  .. .*. *.*  .  *  .*  .*  *. .. .*. .*

ND1_CANPA   ETARPPMDEVEAESELVSGHMTEYSSSIFVLFFLSEYASILFLSTLTAILFFGG-----------------------------------------------
ND1_PODAN   ETNRAPFDLAEAESELVSGFMTEHAAVVFVFFFLAEYGSIVLMCILTSILFLGGYLSINSLDVFNFFYSILFNIGFIDLNFFNIFYYFYKEIFVNNSIIE
ND1_MARPO   ETNRAPFDLPEAEAELVAGYNVEYSSMGFALFFLGEYANMILMSSLCTLLFLGGWLPILDIPIFQ---------------------------------VIP
ND1_HUMAN   ETNRTPFDLAEGESELVSGFNIEYAAGPFALFFMAEYTNIIMMNTLTTTIFLGTTYDALSPELY-------------------------------------
            ** * * *  *.*.***.*   *  ..  * .**..** ... .  * . .*.*

ND1_CANPA   --GTGVILALKANIFAYTYIWIRATLPRIRYDRLIHLCWMVFLPILFAVAIFIPVLIYSLD--AIILL
ND1_PODAN   GLIYGLTIGLKSSILIFLFIWVRASFPRIRFDQLMAFCWTVLLPLLFALIVLLPCILYSYNILPVNVL
ND1_MARPO   GSIW---FSMKVLFFLFVYMWVRAAFPRYRYDQLMRLGWKVFLPLSLAWVVFVSGVLVAFEWLP----
ND1_HUMAN   -TTY---FVTKTLLLTSLFLWIRTAYPRFRYDQLMHLLWKNFLPLTLALLMWYVSMPITISSIPPQT-
                  .  *   ..   ..*.*.. ** *.*.*. . *  .**. .*  .    .  .    .
```

Sequence alignment of ND3

```
ND3_CANPA   M-FTA---YLILCPIIAVVLVGLNWLLATSNS--YIEKDGPFECGFTSF-QQSRSAFSVAFILVAILFLPFDLEISSILP--FVISPYSNGLYGLVILVI
ND3_PODAN   MSSMTLFILFV--SIIALLFLFINLIFAPHNP--YQEKYSIFECGFHSFLGQNRTQFGVKFFIFALVYLLLDLEI--LLTFPFAVSEYVNNIYGLIILLG
ND3_MARPO   MEFAPIFVYLVISLLLSLILIGVSFLFASSSSLAYPEKLSAYECGFDPF-DDARSRFDMRFYLVSILFIMFDLEVTFLFP--WAVSLNKIGLFGFWSMMV
ND3_HUMAN   MNFALI---LMINTLLALLLMIITFWLPQLNG--YMEKSTPYECGFDPM-SPARVPFSMKFFLVAITFLLFDLEIALLLPLPWALQTTNLPLMVMSSLLL
            *         ..   ....... .   ..  .    * **   .**** .      *   *  . *  . .. .. .***.  ..      .      .    ..

ND3_CANPA   FLILLIVAFVVEIQLKALQLNRTYTNDLPHTELYDININ----D
ND3_PODAN   FITIITIGFVYELGKSALKIDSRQVITMTRFN-YSSTIEYLGKI
ND3_MARPO   FLFILTIGFVYEWKKGALDW----------------------E
ND3_HUMAN   -IIILALSLAYEWLQKGLDWT---------------------E
             . .. ...  *    .*
```

Sequence alignment of ND4L

```
ND4L_CANPA   M-----LALISILLL-----FYISQNNLITLLIAIEILLLTVTVKLIYMGSVYDDIYGTIFSIVIIIILAGAESAIGLSILVSY---YRLRGKVGHTI------
ND4L_PODAN   MNI------TLILFLIGILGFVLNRKNIILMLISIEIMLLAITFLILVSSLNMDDIIGQTYAIYIIVVAGAESAIGLGILVAF---YRLRGSISIEYK-----
ND4L_MARPO   MDLVKYLTFSMILFLLGIWGIFLNRKNILIMLMSIELMLLAVNLNFLVFSVYLDDMMGQLFALFVLTVAAAESAIGLAILVIT---FRIRGTIAVEFINCMKG
ND4L_HUMAN   MPLI-YMNI-MLAFTISLLGMLVYRSHLMSSLLCLEGMMLSLFIMATLMTLNTHSLLANIVPIAMLVFAACEAAVGLALLVSISNTYGLDYVHNLNLLQC---
             *          . .         . . ...  *.  .*  ..*..             . . .    .. ..   *.  *.*.**..**       . .
```

Sequence alignment of ND5

```
ND5_CANPA   MFQYL-------------YYIW------------------------------------EEPISISLGNWLNIGDIIIPYGLSLDSLAMTVMIPVGIVTLCV
ND5_PODAN   MYLSIIILPL-LGCIVSGFLGRKVGV-KGAQLITCSNVVITTILSILAFIEVGFN--NIPVTINLFRWIDSEWFNIIWGFQFDSLTVSMLIPVLIISSLV
ND5_MARPO   MYLLMVILPL-IGSFAAGFFGRFLGS-RGVAVVTTTCVSLSSIFSCIAFYEVALC--ASACYMKIAPWIFSELFDAAWGFLFDSLTVILLLVVTIVSSLV
ND5_HUMAN   MTMHTTMTTLTLTSLIPPILTTLVNPNKKNSYPHYVKSIVASTFIISLFPTTMFMCLDQEVIISNWHWATTQTTQLSLSFKLDYFSMMFIPVALFVTWSI
            *                                                           .       *            .. .* ...  .      ... .

ND5_CANPA   LLYAIEYMSHDPNRNTFYIILSVFAIFMTILVVSDNYIMMFIGWEFVGVISYLLISFWSTRIAAMKAALSAILLNRMGDTFFMLALGIF--LSYFHAVDF
ND5_PODAN   HIYSISYMSSDPHNQRFFSYLSLFTFMMIILVTANNYLLMFVGWEGVGVCSYLLVSFWFTRIAANQSSISAFLANRVGD--CFLTIGMFAILWSLGNLDY
ND5_MARPO   HIYSISYMSEDPHSPRFFCYLSIFTFFMLMLVTGDNFIQLFLGWEGVGLASYLLMNFWFTRIQANKAAIKAMLMNRVGD--FGLALGIMGCFTIFQTVDF
ND5_HUMAN   MEFSLWYMNSDPNINQFFKYLLIFLITMLILVTANNLFQLFIGWEGVGIMSFLLISWWYARADANTAAIQAILYNRIGDIGFILALAWF----ILHS---
             ... **. **.    *.   * .*  .  * .**  ..*  .  .*.*** **.  *.**..  *  .*   *    ...  * *  **.**     *...        .

ND5_CANPA   DTL-SLAAPYTNTLI-----------LNILSLLLLLAATAKSAQLGLHAWLLQAMEGPTPVSALLHAATMVCAGVYVLVRSYFILEYAPSLLIGICWLGG
ND5_PODAN   STVFS-LAPYINSKVVLII---------GICLL--IGAMAKSSQVGLHVWLPMAMEGPTPVSALIHAATMVTAGVYLLMRSSPLIEYNSTVLLLCLWLGA
ND5_MARPO   STIFACASAFSEPHHYFLFCNMGFHAMTVICILVFIGAVGKSAQIGLHTWLPDAMEGPTPVSALIHAATMVTAGVFMMARCSPLFEYSPNALIVITFVGA
ND5_HUMAN   -------NSWDPQQMALLNANPSLTPL----LGLLLAAAGKSAQLGLHPWLPSAMEGPTPVSALLHSSTMVVAGIFLLIRFHPLAENSPLIQTLTLCLGA
                   .                        .    ..* .**.*.*** **   ***********.*..*** **.... *      . *   .          .*.

ND5_CANPA   VTTLVSGLIAIVTNDIKKVIALSTMSQLSIMVLAIGISSYDLAIYHLYCHAFFKALLFMGAGSVIHSYISETQDMRKYGGLVNYLPFSYTAILIASLSLM
ND5_PODAN   ITTVFSSLIGLFQQDIKKVIAYSTMSQLGMMVIAIGLSSYNVALFHLINHAFYKALLFLGAGSVIHA-VADNQDFRKYGGLINFLPLTYSVMLIASLSLV
ND5_MARPO   MTSFFAATTGMLQNDLKRVMAYSTCSQLGYMIFACGISNYSVSVFHLMNHACFKALLFLSAGSVIHA-MSDEQDMRKMGGLASLLPFTYAMMLMGSLSLI
ND5_HUMAN   ITTLFAAVCALTQNDIKKIVAFSTSSQLGLMMVTIGINQPHLAFLHICTHAFFKAMLFMCSGSIIHN-LNNEQDIRKMGGLLKTMPLTSTSLTIGSLALA
            .*.   ..   ..  .*.*...* ** ***. *.  . *..      ..   *.   ** .**.**. .**.**   .  .  ** ** ***    .*.. .  .  . ..**.*

ND5_CANPA   AIPGLTGYYSKDIIIESLYGTYTFSGYILYYMAVGSATLTSLYSIRVLYLTFYNNPNSNKATYQHIHENIR-------MLIPMIILVIYSIFIGF-NRDN
ND5_PODAN   AFPFMTGFYSKDFILESAYGQFSFSGVAVYIIATIGAIFTTLYSVKVLYLTFLSNPNGTRAYYNYKPAL----EGDLFLTLPLIILAIFSIFFGFITKDI
ND5_MARPO   GFPFLTGFYSKDVILELAYTKYTISGNFAFWLGSVSVFFTSYYSFRLLFLTFLAPTNS------FKRDLSRCHDAPILMAMPLILLAFGSIFVGYLAKDM
ND5_HUMAN   GMPFLTGFYSKDHIIET--ANMSYTNAWALSITLIATSLTSAYSTRMILLTLTGQPRF--------PTLTNINENNPTLLNPIKGLAAGSLFAGFLITNN
            . * .**.**** *.*        . .       .     .   .*. **  ... **.                                  .  *.    *     *.* *.     .

ND5_CANPA   VIG---HY-----AMSLPANNSFIETEFTLPWYIKLLPLILGLSLSLLLV----YIYEYAYKVRPSS----IYNYFNNKI-YYDQLLNNVIIRKTLIFGG
ND5_PODAN   FIGLGSNFFGDNSLFIHPTHEIMIDTEFAVPVLFKLLPFIFTISFSIIALTISEFLSELVIYFKLSRLGYNIFGFFNQRF-LIELFYNKYITNLILDLGG
ND5_MARPO   MIGLGTNFWAN-SLFMLPKNEILAESEFATPTIIKLIPILFSTLGSFVAYSVNFVVNPLIFALKTSTFGNRLYCFFNKRW-FFDKVFNDFLARSFLRFGY
ND5_HUMAN   -ISPASPF-----------------QTTIPLYLKLTALAVTFLGLLTALDLNYLTNKLKMKSPLCTF------YFSNMLGFYPSITHRTIPYLGLLTSQ
             *.    .                  . . *  .** ..       .                          .*..           .    .       *   .

ND5_CANPA   YLN-TYIDNGLLKVLGSTGISRAL----------------TY-----INIGIFLNLL----YL--------FFFYR----------
ND5_PODAN   QMT-KILDKGSIELFGPFGLEKGLVNFSKNISSLSTSHVTTYALYILVAFILYL----LYNYLSF-----NFLPFLIAGLTILTTI
ND5_MARPO   EVSFKALDKGAIEILGPYGISYTIRKMAQQMSKIQSGFVYHYAFVMLLGLTMFISVMGLWDFISFWVDNRLYFIYMVSFL--FINI
ND5_HUMAN   NLPLLLLDLTWLEKLLPKTISQHQIS-TSIITSTQKGMIKLY------------------FLSF------FFPLI---LTLLLIT
             .    .*  .  .  .  .                      *                    ..         ..
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Sequence alignment of ND6

```
ND6_CANPA   M------FLI----------------SGISSILAIGLL----SPVQSIVCLIVLFVSAAISLYSNGFVLMGILYVLIYVGAIAILFLFILSLLNIE-----
ND6_PODAN   MNNNYPLFWMNEILTNGFVEYMLDMFSMMAFTTGMYVILTKNPIVSVLFLMTLFGGISSYLNMMGTNFMGLSYMMVYMGAVSMLFLFILMLMNIRTSETQ
ND6_MARPO   MM----LFYV----------------FVVLALVSGAMVMRAKNPVHSVLFLILVFCNTSGLLVLLGLDFFAMIFLVVYVGAIAVLFLFVVMMLHMRMEEIH
ND6_HUMAN   MM--YALFLLSVGLVMGFVGF----------------SSKPSPIYGGLVLIVSGVVGCVIILNFGGGYMGLMVFLIYLGGMMVVFGYTTAMAIEEYPEAW
            *        * .                              .*. . . *.       .    *      ..      ..*.*.. ..*  .     .

ND6_CANPA   -----YNYKGTIHPLIFTILI--------ICLIPLDLS--YET---YGIVENVN----------IAYPFNSLLDW---D------LELTTVGSLLYTEYA
ND6_PODAN   SNTSNSIPLTMFIGIMFSNFLFPMLP--YDIVMLSNFYNNYFSEDFYTMDVNMNDNNLNNLYNNVTY-FMTSVMW---DGSVMDFNHMTAMGNMMYTIYN
ND6_MARPO   ENVLRYLPVGGIIGLIFLLEIFLMVDNDYIPMLPTKLSATYLT---YTV-----------------YA-GKMHSW---T-------NLETLGNLLYTTYF
ND6_HUMAN   GSGVEVL-VSVLVGLAMEVG-FVLWVKEYDGVV------------------------------VVVNFNSVGSWMIYEGEGSGFIREDPIGAGALYDYG
                        .   .                 ..                                                 *                    .*        *

ND6_CANPA   IPMILIGLILILSVIGAIAIT---------------------------K
ND6_PODAN   MWLMIASFMTLTAMVGSIVMTMKQ--------------------RKI-
ND6_MARPO   FLFLVSSLILLVALIGAMVLTMHKTTKVKRQDVFMQNAMDFQNTIKKVR
ND6_HUMAN   RWLVVVTGWPL--FVGVYIVI----------------------EIARGN
               ..     .   .*    .
```

FIG. 3. Comparison of deduced amino acid sequences of the mitochondrial NADH dehydrogenase subunits of *C. parapsilosis* (CANPA) with those from other organisms (PODAN, *P. anserina*; MARPO, *Marchantia polymorpha*). Amino acid sequences deduced from the gene sequences were compared with available data chosen from a wide range of organisms. For *C. parapsilosis* mtDNA, AUA and CUN codons were read as isoleucine and leucine, respectively. Sequences were aligned by the CLUSTAL V program. Asterisks indicate amino acid residues that are identical in all the sequences compared. Dots indicate homologous residues.

DNA fragments were excised from agarose gels, purified with the GeneClean II kit (Bio 101 Inc., La Jolla, Calif.), and labelled with [α-^{32}P]dCTP by use of the Oligolabelling kit (Pharmacia).

DNA sequencing. mtDNA *Hin*dIII restriction fragments were cloned in the pTZ18R/19R vector system (Pharmacia). DNA was sequenced on a double-stranded plasmid template by the dideoxy chain termination method with α-^{35}S-dATP and the T7 Sequencing kit (Pharmacia).

Detection of gene transcripts. Mitochondrial RNA was extracted from partially purified mitochondria, electrophoresed in 1.2% agarose gels containing formaldehyde, and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The blotted RNA was hybridized with the ^{32}P-labelled *ND* gene probes described above in a solution containing 50% formamide, 5× SSC, 50 mM Na-phosphate (pH 7.0), 5× Denhardt's solution, and 100 μg of denatured salmon sperm DNA per ml at 42°C, overnight (see reference 46). Membranes were washed in 2× SSC–0.1% SDS and then in 0.1× SSC–0.1% SDS at 50°C, dried, and autoradiographed. The mitochondrial RNA preparations were contaminated with cytoplasmic RNA, as judged from the presence of cytoplasmic ribosomal RNAs, but its presence did not interfere with specific hybridization of *ND* probes with mitochondrial RNA species, as this had been checked by the use of an RNA preparation purified from ribonuclease-treated mitochondria (data not shown).

Nucleotide sequence accession numbers. The DNA sequences discussed in this report have been deposited in the EMBL Sequence Data Library under accession numbers X75674 (*ND4L-ND5*), X75675 (*ND6-ND1*), X75676 (*ND2* [partial]-*ND3*), X75679 (*COX3*), and X74411 (*ATP9*).

RESULTS

Detection of NADH dehydrogenase subunit genes by DNA sequencing. A physical and genetic map of the linear mtDNA of *C. parapsilosis* is shown in Fig. 2A. The structure of the telomere regions has not been described (37a). While sequencing the left terminal region, we noticed the presence of an open reading frame which showed a convincing homology with the NADH dehydrogenase subunit ND3 gene previously described for several mitochondrial genomes. Since this was the first case of a likely *ND* gene sequence found in yeast, other *ND* genes were searched for by further sequencing. In total, six open reading frames whose deduced amino acid sequences were clearly homologous to the known NADH dehydrogenase subunits (ND1, ND-2, ND-3, ND-4L, ND-5, and ND-6) were discovered. Figure 3 shows comparisons with the sequences from a filamentous fungus, a plant, and the human species. The homology was dispersed over the total sequence of each protein, with stretches of identical residues. The identity scores, as summarized in Table 1, were relatively low, with the highest value of 52.1% for ND1 with respect to its *P. anserina* counterpart and the lowest for ND6 with respect to all homologs. The hydropathy profiles (not shown) generated by an available program (Kyte-Doolittle algorithm; program DNA Strider version 1.2) also reproduced the characteristics of the highly hydrophobic patterns of mtDNA-encoded ND proteins (17), confirming the functional identity of these open reading frames.

The *ND* gene sequences have been precisely localized on the restriction map (Fig. 2b). They are scattered over the linear

TABLE 1. Divergence of *C. parapsilosis* mitochondrial *ND* gene products from known homologs produced by other organisms

Product of *Candida parapsilosis*	% Identity with the homologous product of:			
	Podospora anserina	*Marchantia polymorpha*	*Homo sapiens*	*Saccharomyces cerevisiae*
ND1	52.1	43.4	39.5	
ND3	35.9	33.6	29.8	
ND4L	51.2	35.7	28.6	
ND5	46.1	43.2	38.9	
ND6	29.7	33.5	23.2	
ATP6[a]	47.2	40.7	31.7	56.5
ATP8[a]	43.8		29.2	58.3
ATP9		55.3		75.0
COX3	53.5	42.4	42.8	59.1

[a] For ATP6 and ATP8, gene sequences from references 23 and 24 were used.

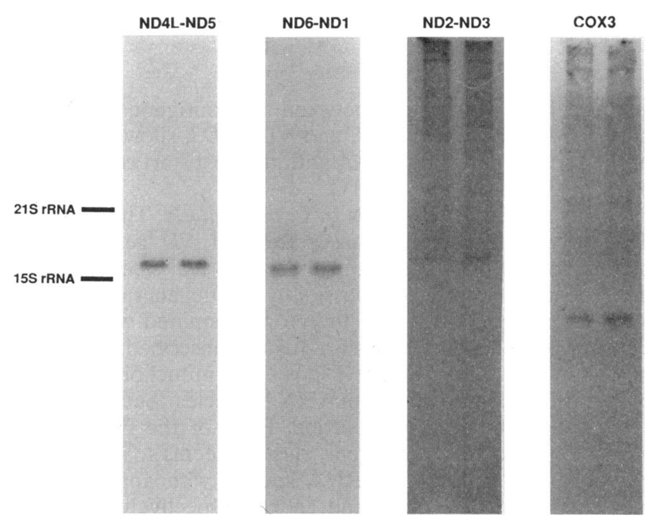

FIG. 4. Transcripts of *ND* genes. *C. parapsilosis* was grown in glucose complete medium until early stationary phase, and mitochondrial RNA was isolated. After electrophoresis, RNA was blotted onto nitrocellulose membranes and hybridized with each *ND* gene probe as indicated. The ^{32}P-labelled probes were the gene fragments indicated in Fig. 2B. Hybrids were detected by autoradiography. See Materials and Methods for the details of the procedure. The molecular weights of the hybridized RNA species was estimated by comparison with the positions of the two mitochondrial rRNA bands (indicated at the left), on the assumption that their sizes are close to those of their *S. cerevisiae* counterparts (3.4 and 1.7 kb). The hybridization probes were *ND4L-ND5* (5′ half), *ND6-ND1*, *ND2-ND3*, and *COX3* (included for comparison). The probe *ND5* (3′ half) gave a result (not shown) similar to that of *ND4L-ND5*.

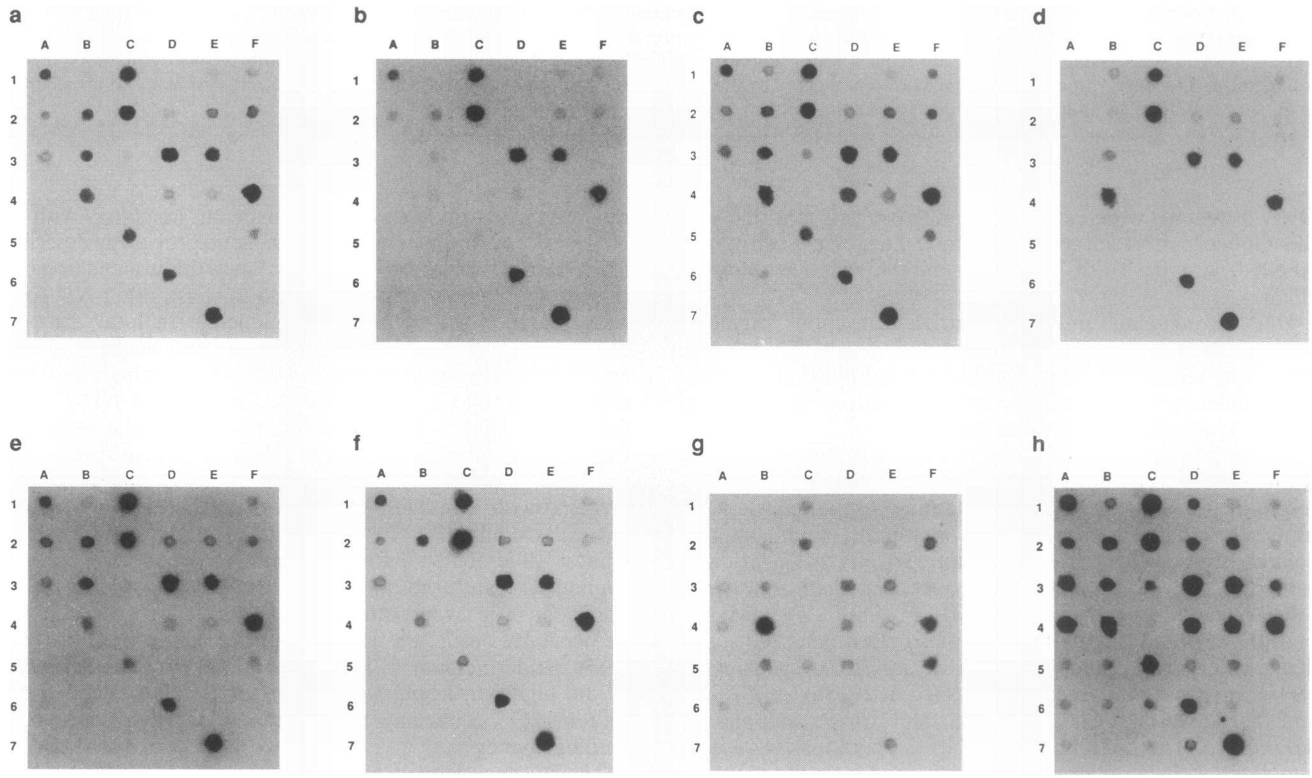

FIG. 5. Hybridization of *C. parapsilosis ND* gene probes with mtDNAs from various yeast species. Minipreparations of mtDNAs from various species were alkali denatured and dot blotted onto Nylon$^+$ membranes as detailed in Materials and Methods. Each DNA spot was identified by a letter-number coordinate (example: A1 corresponds to *Candida albicans* mtDNA) (Table 2). The following probes were used: *ND4L-ND5* (5′ half) at low and high stringency, respectively (a and b); *ND5* (3′ half) at low and high stringency, respectively (c and d); *ND6-ND1* at low stringency (e); *ND2-ND3* at low stringency (f); *ND4* of *P. anserina* at low stringency (g); *COX3* at low stringency (h).

genome, but the junctions between the contiguous gene couples, *ND4L-ND5*, *ND6-ND1*, and *ND2-ND3*, showed a fusion of the termination codon TAA with the next start codon ATG, forming the sequence TAATG.

Transcription of *ND* genes in *C. parapsilosis*. The relatively low degree of sequence conservation of *ND* genes among organisms and their absence from the *S. cerevisiae* mitochondrial genome raised the possibility of degenerate *ND* sequences in some cases. We therefore examined whether the putative ND-encoding genes are being transcribed in *C. parapsilosis* mitochondria. Figure 4 shows that mitochondrial RNA preparations contained various RNA species that hybridized specifically with each gene probe. Each of the *ND4L-ND5*, *ND6-ND1*, and *ND2-ND3* gene clusters seems to be cotranscribed into a major large RNA species with approximative molecular sizes of 2.0, 1.7, and 1.9 kb, respectively. A second minor species, with a slightly higher molecular weight, was observed in each case. They may represent unprocessed primary transcripts but have not been studied further. The sizes of these minor transcripts largely exceeded those expected for the transcripts of the individual genes. None of the gene sequences revealed any introns.

The RNA extracted from mitochondria may contain low variable amounts of cytoplasmic contaminants. This leaves the possibility that the RNA species detected by hybridization with *ND* gene probes might be due to contaminating cytoplasmic species. Such a possibility was shown to be unlikely by two kinds of experiments (data not shown): (i) RNA preparations extracted from ribonuclease-treated mitochondria (which indeed retained much-reduced amounts of cytoplasmic contaminants) still showed unchanged intensity of hybridization signals with an *ND* gene probe, and (ii) a cloned mitochondrial *ND* gene probe did not hybridize to any of the blotted chromosomal DNAs. The latter experiment was aimed at detecting possible nuclear *ND* gene homologs, because such a case is known for the ATPase subunit 9 gene in *Neurospora crassa*.

Detection of *ND* genes in other yeast species. The genetic organization of a multigenic system, such as respiratory complex I, can be thought to be one of the most significant criteria of relatedness between species. Since the use of hybridization probes from filamentous fungi has not provided convincing evidence of the presence of *ND* gene homologs in yeast mtDNAs, the *C. parapsilosis* genes offered the probes of choice in the search for their homologs in other species. Crude mtDNAs were prepared from a series of selected yeast species, including those which had often been the object of NADH dehydrogenase studies. These DNAs were blotted onto nylon membranes and hybridized with the *C. parapsilosis* probes under high- and low-stringency conditions. Figure 5 illustrates these experiments. According to the intensity of hybridization signals, the yeast species could be classified into three categories: (i) strains showing intense signals with high stability of hybrids, (ii) strains showing no detectable hybridization, and (iii) strains with intermediate, relatively unstable hybrids. The results are summarized in Table 2. For any given strain, not all the *ND* gene probes gave similar efficiencies of hybridization, suggesting either that the whole set of tested genes were not

TABLE 2. Detection of *C. parapsilosis ND* gene homologs in other yeast species[a]

Species	Strain	Zoo blot[b]	Hybridization of indicated gene probe with mtDNA from indicated yeast species[c]					
			ND4L-ND5 (5′)[d]	*ND5* (3′)[d]	*ND2-ND3*	*ND6-ND1*	*ND4*	*COX3*
Candida albicans	CBS 562 (NT)	A1	++/+	++/+−	++	++	+−	+++
Candida boidinii	CBS 2428 (T)	B1	+−/−	+/+−	+−	+−	+−−	+
Candida catenulata	CBS 565 (T)	C1	+++/++	+++/+++	+++	+++	+	+++
Candida glabrata	CBS 138 (T)	D1	−/−	−/−	−	−	+−	++
Candida magnoliae	CBS 166 (T)	E1	+/+−	+−/−	+−	−	+−	+−
Candida maltosa	CBS 5611 (T)	F1	+/+−	+/+−	+	+	+−	+−
Candida membranaefaciens	CBS 1952 (T)	A2	+/+−	+/−	+	++	+−	++
Candida oleophila	CBS 2219 (T)	B2	++/+−	++/+−	++	++	+	++
Candida parapsilosis	CBS 604[e] (T)	C2	+++/+++	+++/+++	+++	+++	++	+++
Candida rugosa	CBS 613 (T)	D2	+/−	+/+−	+	+	+−−	++
Candida sake	CBS 159 (T)	E2	+/+−	+/+	+	+	+	++
Candida salmanticensis	CBS 5121 (T)	F2	++/+−	++/+−	+	+	++	+−
Candida shehatae	CBS 5831 (T)	A3	+/−	+/−	+	+	+	++
Candida tropicalis	CBS 94 (T)	B3	++/+−	++/+	+−	++	+	++
Candida utilis	CBS 621	C3	+/−	+/−	+−	+−	+−	+
Clavispora lusitaniae	CBS 6936 (T)	D3	+++/+++	+++/++	+++	+++	+	+++
Debaryomyces hansenii	CBS 767 (T)	E3	+++/+++	+++/++	+++	+++	+	+++
Kluyveromyces marxianus	CBS 397	F3	−/−	−/−	−	−	+−	+
Kluyveromyces lactis	CBS 2359	A4	−/−	−/−	−	−	+	+++
Pichia angusta (Hansenula polymorpha)	CBS 4732	B4	++/+−	++/++	+	+	+++	+++
Pichia anomala	CBS 5759 (NT)	C4	−/−	−/−	−	+−	−	+−
Pichia canadensis	CBS 1992 (T)	D4	+/+−	++/−	+	+	+	+++
Pichia fermentans	CBS 187 (T)	E4	+/−	+/−	+	+	+	+++
Pichia guilliermondii	CBS 2030 (T)	F4	+++/++	+++/+++	+++	+++	++	+++
Pichia jadinii	CBS 1600 (T)	A5	+−/−	+−/−	−	+−	+	+
Pichia membranaefaciens	CBS 107 (T)	B5	+−/−	+−/−	−	+−	+	+
Pichia pastoris	CBS 704 (T)	C5	++/+−	++/−	+	+	+	+++
Pichia philodendra	CBS 6075 (T)	D5	−/−	−/−	−	−	+	+
Pichia pijperi	CBS 2887 (T)	E5	−/−	−/−	−	−	−	+
Williopsis mrakii	CBS 1707 (T)	F5	+/+−−	+/−	+−	+	++	+−
Williopsis saturnus	CBS 5761	A6	+−/−	+−/−	−	+−	+	+
Yarrowia lipolytica	CBS 599	B6	+−/−	+−/−	−	+−	+	+
Zygosaccharomyces rouxii	CBS 732 (NT)	C6	−/−	−/−	−	−	−	+
Candida parapsilosis	CBS 604[e]	D6	+++/+++	+++/+++	+++	+++	+	+++
Dekkera anomala	CBS 8139	E6	−/−	−/−	−	+−	−	+−
Dekkera anomala	CBS 77	F6	−/−	−/−	−	−	−	−
Dekkera custersiana	CBS 4805	A7	−/−	+−−/−	−	−	−	+−
Dekkera bruxellensis	CBS 72	B7	−/−	−/−	−	−	−	−
Dekkera bruxellensis	CBS 74	C7	−/−	+−−/−	−	−	+−−	+−
Saccharomyces cerevisiae	MH 41-7B	D7	−/−	−/−	−	−	−	+
Candida parapsilosis	SR 23	E7	+++/+++	+++/+++	+++	+++	++	+++

[a] The data from Fig. 5 are assembled.
[b] See Fig. 5.
[c] The intensities of hybridization signals are expressed by combinations of + and − signs (+−, a very weak signal; −, absence of signal). Hybrids were washed at low-stringency conditions except as indicated in footnote *d*. Details of hybridization and wash conditions are described in Materials and Methods.
[d] Intensities at low-stringency and high-stringency conditions are indicated before and after the shill, respectively.
[e] Two different stocks of CBS 604.

always present together on the mtDNA or that each gene has a different degree of sequence divergence. The species which showed very strong hybridization with all of the probes under the stringent conditions were *Candida catenulata*, *Clavispora lusitaniae*, *Debaryomyces hansenii*, and *Pichia guilliermondii*. *Hansenula polymorpha* also showed strong hybridization with most of the probes. The species which gave completely negative hybridization, even under low-stringency conditions, were *S. cerevisiae*, *Zygosaccharomyces rouxii*, *Candida glabrata*, and *Pichia pijperi*. Several important groups of yeast have not been included in the present survey, because these strains, often resistant to Zymolyase treatment, were not suited for efficient extraction of mtDNA by the routine procedure (for example, strains from the genera *Cryptococcus*, *Rhodotorula*, *Sporobolomyces*, *Tremella*, and *Trichosporon*, etc.)

DISCUSSION

Occurrence of *ND* genes in *C. parapsilosis* and other yeasts. In *C. parapsilosis* mtDNA we have found, so far, six genes, encoding ND1, ND2, ND3, ND4L, ND5, and ND6. Since more than one-half of this mitochondrial genome remains unsequenced, more *ND* genes may still be discovered. For example, gene *ND4* is probably present, because a probe from *P. anserina* showed a weak but significant level of hybridization. Sequencing of the putative *ND2* gene has not yet been completed. This set of seven-ND-encoding genes are those most frequently found in the mtDNAs of a variety of organisms (mammals, *Xenopus laevis*, *Drosophila melanogaster*, and plants). They have also been identified in the mtDNAs of several filamentous fungi (*N. crassa*, *P. anserina*, *Aspergillus*

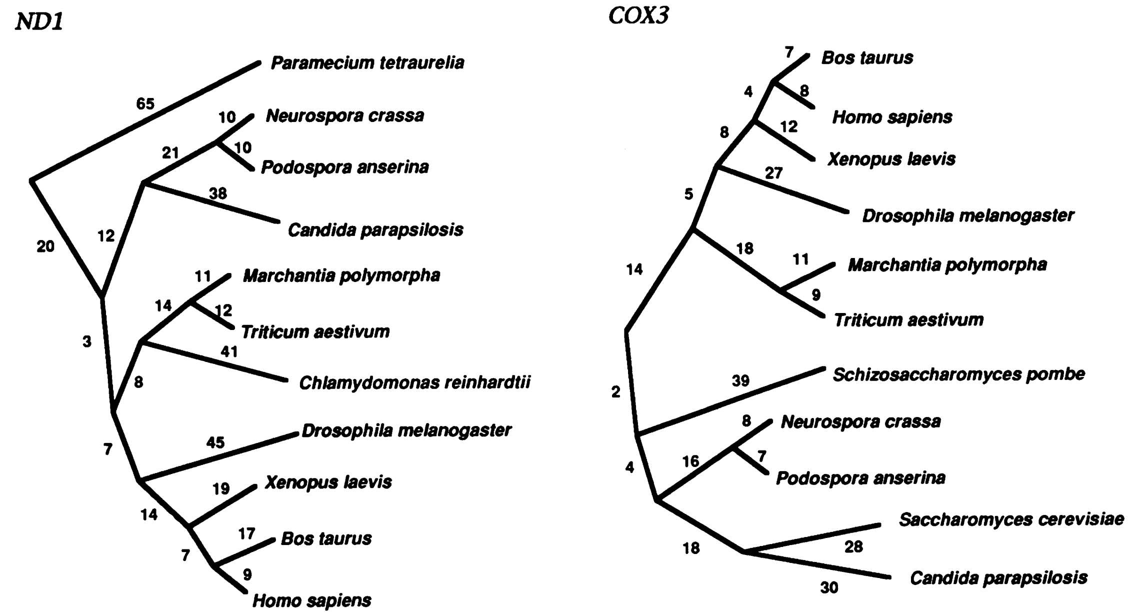

FIG. 6. Phylogenetic trees. Amino acid sequences of ND1 (left) and cytochrome oxidase subunit 3 (COX3; right) were compared among different organisms. Phylogenetic trees were constructed by using a server of Computational Biochemistry Research Group (CBRG), Zürich, Switzerland, via Internet electronic mail. The values above each branch indicate the estimated PAM distances.

nidulans, *Trichophyton rubrum* [11], and *Cephalosporium acremonium* [42]), but not in the mtDNAs of the familiar species of yeast so far examined. In *S. cerevisiae* and *S. pombe*, for which almost the entire mtDNA sequences are known, no *ND*-like genes could be identified, and these mitochondria may completely lack a complex I-like NADH dehydrogenase (14). In one case (27), the presence of gene *ND1* in the species *Brettanomyces custersianus* and *Brettanomyces anomalus* (they were recently moved to the genus *Dekkera*) had been suggested by a weak but positive hybridization with a URF1 (*ND1*) probe from *A. nidulans*. We have also identified and mapped *ND* gene homologs in the mtDNA of *Endomyces magnusii* (37b).

The results of the survey of mitochondrial *ND* gene homologs suggest that genes of this set are likely to be widespread among yeast species. Such a survey has not been often made, perhaps because *S. cerevisiae*, which has none of these genes, has been the most popular source of mitochondrial gene probes in mapping studies. Most of the weak hybridization signals we found with many species probably reflect the real presence of *ND* gene homologs, since, in a case examined in more detail, unambiguous hybridization was seen with specific restriction fragments of purified mtDNA (e.g., *C. utilis* [data not shown]). Also, the mtDNA of *Williopsis suaveolens* CBS 255, which showed only a weak hybridization signal, did contain gene *ND4*, as revealed by partial DNA sequencing (not shown). The large majority of yeasts, which are aerobic species, might have *ND* genes as standard components of the mitochondrial genome.

Of particular interest is the probable presence of *ND* genes in *C. utilis*, as judged from the weak signals obtained in the hybridization experiments with both the *C. parapsilosis* and the *P. anserina* probes. The synthesis of the NADH dehydrogenase activity in *C. utilis* has been reported to be largely variable depending on the growth phase (22, 28, 32). Regulatory aspects of complex I biogenesis have also been described for *Aspergillus niger* (44). With the possibility of identifying mitochondrial *ND* genes by the use of *C. parapsilosis* probes, regulatory studies may be extended to various yeasts, especially those suited for mutational analyses.

Transcription of *ND* genes. In many yeasts so far studied, including those carrying a linear mtDNA (18), the mitochondrial genome uses a common signal, ATATAAGTA (8, 16, 41, 57), or more generally (A/C)TA(T/A)A(A/C)G(A/T/C)(A/T)(A/G) (33), that marks the transcriptional start sites. Such signals were not found in *C. parapsilosis* mtDNA. The fused arrangement of *ND* genes, as seen for *ND4L-ND5*, *ND6-ND1*, and *ND2-ND3*, seems to be characteristic of this group of genes, since similar junctions have often been found in other fungal mtDNAs (10, 11, 36). The transcripts we found were indeed quite large, consistent with the idea that these genes probably give rise to bicistronic messenger RNAs which are not further processed to individual messages. In the downstream region of the genes, no consensus motifs which could be the sites of RNA processing could be seen. Since tRNA genes are present between genes coding for proteins, these might serve as RNA processing sites, as in the case of animal mitochondria. *C. parapsilosis ND* gene sequences have no introns, in contrast to most of their homologs, especially those of filamentous fungi. Open reading frames of the RF1, RF2, and RF3 type known in *Saccharomyces* species (35, 49) and in some *Williopsis* species (16) have not been found so far in *C. parapsilosis* mtDNA.

Divergence of *ND* gene sequences. Published data (8, 16, 25, 56, 57) indicate that the mitochondrial genes known in various yeasts (several species of the genera *Williopsis*, *Kluyveromyces*, and *Pichia*, as well as *C. glabrata*) generally have a high degree of sequence conservation. *C. parapsilosis* mitochondrial genes appear, in contrast, significantly more divergent. The *ATP6* gene, which was not detectable by hybridization with an *S. cerevisiae* probe, could only be identified by DNA sequencing (23). The cytochrome oxidase subunit 3 gene (*COX3*) also confirmed the high degree of divergence from *S. cerevisiae* (Table 1). The sequences of mitochondrial tRNA genes of *C. parapsilosis* are as much related to (or distant from) those of the genus *Podospora* as those of *Saccharomyces* counterparts (37). The extent of divergence was not the same for all the *ND* gene products; for example, ND6 seems to diverge much faster than ND1, in agreement with an observation by Fearnley and Walker (17) regarding other organisms. In some mtDNAs, part of the usual set of seven *ND* genes can even be missing; for example, *Chlamydomonas reinhardtii* mtDNA has only five of these genes (34). Perhaps some genes might have moved to the nucleus. *S. cerevisiae* chromosome III contains an open reading frame of unknown function (YCR007C) whose potential product shows some sequence homology with ND3 from *Asterina pectinifera* mitochondria (51).

Despite the sequence divergence, the *ND* genes of *C. parapsilosis* do not show a significantly modified pattern of codon selection compared with other yeasts. The fact that *C. parapsilosis* mtDNA has *ND* genes may mean either that this species among yeasts is particularly close to the filamentous fungi, as suggested by physiological features, or that the majority of yeast species may possess these genes and *C. parapsilosis* would then not be an exception. In the latter case, comparison of the gene sequences of this set may be an interesting tool in yeast identification and classification.

We may attempt to see the relatedness of various organisms in terms of mtDNA-encoded gene products. Phylogenetic trees generated from ND1 and cytochrome oxidase subunit 3 sequences are shown in Fig. 6. The results with both proteins (and other ND subunits, not shown) were similar and consistent with current classification.

Wide occurrence of ND subunit genes among yeast species. The mitochondrial *ND* gene sequences were used as hybridization probes to detect the possible occurrence of their homologs in a wide range of yeast species. Since crude mtDNA preparations have been used in these experiments, the quantitative interpretation of the results is limited, although highly indicative. These experiments are also made difficult by the fact that mitochondrial *ND* gene sequences tend to diverge much faster than other mitochondrial genes. However, there are reasons to suppose that the group of species showing rather low levels of hybridization signals do contain *ND* genes or related sequences, because (i) the mtDNAs that are known to lack these genes completely gave no hybridization, and (ii) when one of the weakly hybridizing mtDNAs was purified, its restriction digests showed unambiguous hybridization with specific mtDNA fragments, and partial sequencing confirmed the presence of an *ND* gene (unpublished data). Therefore, the results of the blotting experiments should be further verified on selected species of physiological interest.

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