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Genomic Exploration of the Hemiascomycetous Yeasts: 14. *Debaryomyces hansenii* var. *hansenii*

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Abstract By analyzing 2830 random sequence tags (RSTs), totalling 2.7 Mb, we explored the genome of the marine, osmo- and halotolerant yeast, *Debaryomyces hansenii*. A contig 29 kb in length harbors the entire mitochondrial genome. The genes encoding Cox1, Cox2, Cox3, Cob, Atp6, Atp8, Atp9, several subunits of the NADH dehydrogenase complex 1 and 11 tRNAs were unambiguously identified. An equivalent number of putative transposable elements compared to *Saccharomyces cerevisiae* were detected, the majority of which are more related to higher eukaryote *copia* elements. BLASTX comparisons of RSTs with databases revealed at least 1119 putative open reading frames with homology to *S. cerevisiae* and 49 to other genomes. Specific functions, including transport of metabolites, are clearly over-represented in *D. hansenii* compared to *S. cerevisiae*, consistent with the observed difference in physiology of the two species. The sequences have been deposited with EMBL under the accession numbers AL436045–AL438874.

Key words: Non-conventional yeast; Ribosomal DNA; Mitochondrial DNA; Retrotransposon

1. Introduction

Debaryomyces (*Torulaspora*) *hansenii*, and its anamorph *Candida famata*, is a cryotolerant, marine yeast, which can tolerate salinity levels up to 24%, whereas *Saccharomyces cerevisiae* growth is inhibited when salinity reaches 10%. These properties account for its important role in several agro-food processes.

D. hansenii is the most common yeast species found in all types of cheese, in contrast to other yeast species such as *Candida vini* or *C. zeylanoides*, the prevalence of which depends on the type of cheese considered [1]. *D. hansenii* is also common in dairies and in brine [2,3], consistent with its ability to grow in the presence of salt at low temperature. It is able to metabolize lactic and citric acids. It also provides proteolytic and lipolytic activities during cheese ripening. A survey of yeasts associated with chilled food revealed that *D. hansenii* was one of the most frequently found species, due to its ability to grow at low temperature [4]. Although *D. hansenii* is considered as non-pathogenic, one case of bone infection associated with this yeast has been reported [5], and several clinical isolates were identified as *D. hansenii* [6].

The genetics and the molecular biology of *D. hansenii* are poorly developed. Most strains are haploid, mate very rarely and diploidize transiently by somatogamous autogamy to form asci containing generally a single spore [7,8]. Seven nuclear genes, one mitochondrial and two plasmidic ones, are documented in GenEMBL database. Links between glycerol metabolism and osmoregulation in *D. hansenii* were first observed in a glycerol non-utilizing mutant [9]. Salt stress provokes changes in the ATP pool and enhances polyols production, mainly glycerol and to some extent arabitol, which act as compatible solutes [10,11]. Osmosensing signalling and glycerol production have not been documented. The *S. cerevisiae HOG1* homologue (encoding a MAP kinase involved in the transduction of the osmosensing signal) was recently cloned [12]. However, this gene has no effect on the osmotolerance of *S. cerevisiae*.

An autonomously replicating sequence, ARSD, was cloned and was shown to have strong sequence similarity to ARSs from other yeasts [13]. Two groups showed the presence of linear plasmids in *D. hansenii*, pDHL1, 2 and 3 [14] and pDH1A and B [15]. Sequence similarity was found between a pDHL1 putative protein and the α-subunit of the *Kluyveromyces lactis* killer toxin, although no killer activity was detected in *D. hansenii* [16]. *D. hansenii* is an alkane-assimilating yeast and two genes, *DH-ALK1* and *DH-ALK2*, were cloned and sequenced showing a close relationship to those of *C. maltosa* [17].

Sequence of the 18S ribosomal DNA unambiguously placed *D. hansenii* within the hemiascomycete subdivision [18]. Recent phylogenetic analysis based on the comparison of the sequence of the 5′-end of the 26S ribosomal DNA [19,20] has shown that the *Debaryomyces* genus is not monophyletic. *D. hansenii* now defines one of the four clades that constitute the *Debaryomyces* genus.

2. Materials and methods

2.1. Yeast strain

The type strain *D. hansenii* var. *hansenii* CBS767, isolated on sherry in Denmark, was used in this study.

2.2. Nucleic acid sequences

The *D. hansenii* DNA library was prepared as described in [21]. The

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library is made of 1632 clones. A total of 2830 random sequence tags (RSTs) with an average size of 957 bp were obtained [22]. The number of inserts that had both ends sequenced was 1320. The average size of the inserts was experimentally determined to be 3.08 kb (standard deviation, 0.51 kb). Only four clones contained overlapping RSTs.

Assembly of the RSTs was performed as described in [24]. A total of 1304 RSTs were included in 295 contigs. Most contigs (89%) were composed of two RSTs (231) or three RSTs (33). The contigs were subsequently used to define repeated sequences within the nuclear genome and extra-chromosomal sequences. Annotations were performed as described in [23].

3. Results and discussion

3.1. Ribosomal DNA

The 8548 bp long contig 408 (36 RSTs) carries the ribosomal RNA genes (rDNA): 18S (bp 3072–4871), 5.8S (bp 5062–5225) and 26S (bp 5425–8455). The extremities of this contig overlap by 843 bp, the deduced size of the rDNA unit is therefore 7705 bp. The 5S gene was found to extend from bp 982 to bp 1102 with a best match to the *Hansenula jadinii* sequence. The 5S gene is in the opposite orientation relative to the other genes, like in *S. cerevisiae*.

3.2. Transposable elements

Transposable elements are found in all eukaryotic organisms. In fungi, they participate to a large extent to genome plasticity through transposition and homologous recombination. Five classes of retrotransposons have been characterized in *S. cerevisiae*: Ty1, 2, 4 and 5 (*copia* family), whereas Ty3 is a member of the *gypsy* family.

In *D. hansenii*, we only found matches to *copia*-like elements. Interestingly, only two contigs revealed high scores with *S. cerevisiae* Ty2B and Ty5. A third contig had a similarly high score with *S. paradoxus* Ty5 and tobacco PolX. In this way we were able to define at least one homologue of *S. cerevisiae* Ty2 and at least two homologues of *S. paradoxus* Ty5. All other RSTs or contigs displayed better scores with *copia* elements from non-yeast origin clearly showing that most of the retrotransposons identified in *D. hansenii* are more related to those found in higher eukaryotes. For these, we defined eight different RNase H domains. The number of retrotransposons is therefore expected to be at least 40–50 in our genome survey, assuming a 20% genome coverage. *D. hansenii* seems to carry as many retrotransposons as *S. cerevisiae*.

One RST (BC0AA002A08D1), carrying a putative *copia*-

Table 1
Structure of the *D. hansenii* mitochondrial genome

Gene or open reading frame (ORF)	Introns	Size (nt or aa)	Match in gene or protein	BLAST expected value	Nt or aa identity (%)	Or
ATP6[a]		259 aa	11–259	2^{e-56}	50	>
ND5[c]		555 aa	7–535	0.0	66	>
tRNA_gln(TTG)		76 nt	1–76		76.3	<
tRNA_ile(GAT)[b]		71 nt	2–71		67.1	<
COX3		269 aa	1–251	1^{e-70}	53	<
tRNA_trp1(TCA)		74 nt	1–71		70.4	<
COB1		385 aa	1–132	2^{e-55}	75	<
	i.COB1.1					
COB1		385 aa	132–141	0.1	57	<
ScbI2	i.COB1.2	280 aa	19–280	5^{e-55}	44	<
COB1		385 aa	144–162	0.002	78	<
	i.COB1.3					
COB1		385 aa	167–381	7^{e-67}	67	<
I-SceII		316	49–294	2^{e-82}	62	<
ND3[b]		189 aa	75–179	1^{e-18}	38	<
ND2[b]		608 aa	375–498	6^{e-11}	29	<
ND1[b]		351 aa	3–347	1^{e-50}	40	<
COX2		251 aa	16–251	1^{e-100}	72	<
tRNA_ala(TGC)		76 nt	1–76		69.7	<
ND4[b]		511 aa	130–511	2^{e-79}	44	<
tRNA_M2(CAT)[b]		71 nt	2–71		85.7	<
tRNA_L2(TAG)[b]		85 nt	1–85		88.2	<
COX1		534 aa	2–127	5^{e-39}	57	<
	i.COX1.1					
COXI		534 aa	128–240	1^{e-32}	60	<
SceII	i.COX1.2	316 aa	49–294	2^{e-82}	62	<
COX1		534 aa	241–373	3^{e-50}	63	<
	i.COX1.3					
COX1		534 aa	373–528	5^{e-35}	50	<
ATP9		76 aa	1–76	5^{e-16}	52	<
tRNA_leu(TAA)		85 nt	1–83		62.7	>
tRNA_his(GTG)		75 nt	8–73		77.3	>
tRNA_met3(CAT)[b]		75 nt	1–73			>
tRNA_met(CAT)		76 nt	2–68		74.6	>
tRNA_thr2(TGT)		76 nt	1–75		73.3	>
ATP8		48 aa	1–48	3^{e-10}	60	>

The expected values of the matches with tRNA genes are not indicated. Or: gene transcription orientation relative to the contig.
[a]ATP6 is located beyond the extremities of contig 409 (see text).
[b]Match only with *P. canadensis*.
[c]Best match with *C. parapsilosis* ND5 protein.

like element and considerably rearranged, is reminiscent of transposition hot spots of *S. cerevisiae* [25].

To identify LTRs, we compared one *copia* homologue-carrying RST (BC0AA002A05T1) to the RST library as a bait. Several matches helped to define a common 457 bp sequence bound by the inverted repeats TGTTG and CAACA. A shorter version of the LTR may start with the canonical 5 bp TGTTG 150 bp downstream and was found in 13 RSTs. Since all these 19 RSTs also have a match with internal sequences similar to *copia*, this indicates that no solo LTRs might occur.

3.3. Mitochondrial DNA (mtDNA)

The largest, 29 878 bp contig 409 (520 RSTs) has overlapping extremities over 410 bp with 98.3% identity, making a complete circle of 29 462 bp. Comparisons (Table 1) with various databases led to the unambiguous identification of the following mitochondrial genes: *COX1*, *COX2* and *COX3* (cytochrome *c* oxidase subunits), *COB* (apocytochrome *b*), *ATP9*, *ATP8* and *ATP6*, encoding ATP synthase subunits 9, 8 and 6, respectively. The *ATP6* gene is located on the overlapping ends of the contig. We obtained significant matches to five subunits (ND1–ND5) of the NADH dehydrogenase complex 1 of *Pichia canadensis*, consistent with these *D. hansenii* genes having been detected by hybridization using the *Candida parapsilosis* genes as probes [26]. Further comparisons revealed matches with *Candida albicans* subunit 1, NU5M, and the bacterium *Paracoccus denitrificans* subunit 4, NQOD.

In the *COB* gene, three introns are present compared to five introns in the *S. cerevisiae* gene. A significant match to the *S. cerevisiae COB* intronic ORF *Scb*I2 was found in the second intron of the *D. hansenii* gene. Two large introns were detected in the *COX1* gene. The first intron has a strong match to two *S. cerevisiae* intronic ORFs: the endonuclease I-*Sce*II in intron COX1.4B and the maturase *Scb*I4 in intron COB.4. A significant match with the I-*Sce*I endonuclease encoded by the *S. cerevisiae* LSU intron was found to lie in a part of the mtDNA large enough to harbor the LSU rDNA. Nevertheless, we could not unambiguously identify the LSU in this region. Comparison with rRNA genes gave matches varying between 50 and 60% identity, indicating that mitochondrial rRNA genes are poorly conserved in *D. hansenii*, whereas a high degree of conservation exists between *S. cerevisiae* and *P. canadensis* genes. This lack of conservation was also observed for tRNA genes, since only 11 tRNA genes could unambiguously be identified. We deduced the (G+C) content of mtDNA to amount to 26.9% vs. 35.7% for nuclear DNA not including rDNA.

Overall, *P. canadensis* and *D. hansenii* possess the most closely related mitochondrial genome sequences. Their sizes are also quite similar: 27 694 bp for *P. canadensis* vs. 29 462 bp for *D. hansenii*. However, hardly any synteny was observed among the mitochondrial genes. In contrast to other yeasts, a series of genes including five tRNA genes, *ATP8*, *ATP6* and *ND5*, seem to be transcribed from the opposite strand in *D. hansenii*. Further work is needed to confirm this observation.

3.4. Others

D. hansenii harbors linear DNA plasmids but no match with these plasmid sequences was detected. This is not so surprising since some of these plasmids, such as pDHL, require osmotic pressure to be maintained in the cells and can be cured by growing cells in normal culture medium [14]. Nevertheless, a match was obtained with a hypothetical killer plasmid pGKL-2 protein from *K. lactis* (29% identity over 196 aa).

The 7713 bp contig 407 (27 RSTs) displays a match (89% identity over 136 bp) with the 130 bp of the autonomously replicating sequence, ARSD, isolated by Govind and Banaszak [13], so that we have very likely identified a new ARS in *D. hansenii*. Interestingly, the contig also carries a number of direct 11 bp repeats, TCTGACAACGG, at the 5′-end.

3.5. Identification of nuclear genes

RSTs (depleted of the 573 RSTs corresponding to rDNA, mtDNA and transposable elements) were systematically compared to databases as described in [23]. All comparisons were carefully examined to avoid defining a too high threshold for homology significance which would have eliminated good matches on the basis of their BLASTX expected value since this variable depends on the size of homology detected. Indeed, 105 significant matches to *S. cerevisiae* spanned less than 50 amino acids (aa). The expected value of the BLASTX comparison (E value) ranged from 0.96 to 8^{e-21} and percent of amino acid identity ranged from 32 to 94 for these 105 matches.

By comparison with the *S. cerevisiae* genome, the minimal number of newly identified genes in *D. hansenii* is 1119 and the maximal number 1258. Further comparison with various other genomes revealed 49 new genes (see below). Assuming that the genomes of *S. cerevisiae* and *D. hansenii* are similar in size and in gene number, we consider that the 1168 genes we identified represent about 20% of the genes of *D. hansenii*, not including mitochondrial and tRNA genes.

3.6. Nuclear tRNA genes

A total of 26 tRNA genes were detected, corresponding to 16 different acceptor tRNAs. Overall, the presence of introns was conserved between genes of *S. cerevisiae* and *D. hansenii*, but neither the sequence nor the size of these introns was conserved in any case. The number of tRNA genes we have identified in *D. hansenii* is much less than expected if we assume a coverage of the genome of 20%. This could be due to the poor sequence conservation already observed for mitochondrial tRNA genes. Nevertheless, some *D. hansenii* tRNA genes show remarkable sequence conservation with those of *S. cerevisiae*. The CUG codon was shown to be read as serine for *Candida* species, including *C. famata*, the anamorph of *D. hansenii* [27]. This was confirmed in this study [23], but we did not find a corresponding acceptor tRNA in our search.

3.7. Orthologues with no equivalent in S. cerevisiae

Comparison of the RSTs with databases other than from *S. cerevisiae* [23] led to 49 extra matches (Table 2). Only 10 homologues to bacteria and archaeal ORFs were found. A total of nine homologues to higher eukaryote ORFs (including five *Caenorhabditis elegans* ORFs) were found. The majority of the matches were obtained with fungal genes, mainly *Schizosaccharomyces pombe*. Considering the 20% coverage of the *D. hansenii* genome in our study, the total number of genes with no equivalent in *S. cerevisiae* could amount to 250.

Except for matches to hypothetical *S. pombe* proteins, most of the matches deal with proteins involved in metabolism of

Table 2
Potential functions encoded by *D. hansenii* RSTs having no validated homologues in the genome of *S. cerevisiae*

Kingdom	Species	Gene name	Accession no.	Function
Archaea	*Thermotoga maritima*	–	TM00087	uncharacterized acr
		–	TM01960	not found in database
	Pyrococcus abyssi	*glpA*	PAB0183	glycerol-3-phosphate dehydrogenase
	Methanococcus jannaschii	*napA*	MJ1275	Na(+)/H(+) antiporter
Bacteria	*Bacillus subtilis*	*abfA*		α-l-arabinofuranosidase A
		ybbD		hypothetical lipoprotein
		ywrD		similar to γ-glutamyl transpeptidase precursor, ggt protein
	Mycobacterium tuberculosis		MTRv1592c	hypothetical protein
	Rhodococcus erythropolis	*THCF*	O05691	non-heme haloperoxidase
	Streptomyces clavuligerus	*PAH*	P37819	possible agmatinase
Ascomycetes	*C. albicans*	*HEX1*	P43077	β-hexosaminidase
	K. lactis	*YKP1*	P05467	hypothetical killer plasmid pGKL-2 protein 1
		LAC4	P00723	β-galactosidase
		LAC12	P07921	lactose permease
	Kluyveromyces marxianus	*BGLA*	P07337	β-glucosidase
	Saccharomycopsis fibuligera	*BGL1*	P22506	β-glucosidase 1
	S. pombe	*SAK1*		
		SPBC354.15		putative fructosyl amino acid oxidase
		SPAC1F8.03C		putative major facilitator superfamily protein
		SPAC1327.01C		putative transcriptional regulator
		SPCC965.12		renal dipeptidase
		SPAC19G10.13		hypothetical protein
		SPAC1B9.05C		hypothetical protein
		SPAC26A3.14C		hypothetical protein
		SPAC3C7.05C		hypothetical protein
		SPAC57A7.01		hypothetical protein
		SPAC57A7.05		hypothetical protein
		SPAC637.03		hypothetical protein
		SPBC16H5.12C		hypothetical protein
		SPBC337.02C		hypothetical protein
		SPCC1494.01		hypothetical protein
		SPCC613.11C		hypothetical protein
		SPAC4A8.06C		hypothetical protein
	Trichosporon cutancum	*SLC21A2*	P15245	phenol 2-monooxygenase
	Aspergillus niger	*PHOA*	P34724	acid phosphatase precursor
	Fusarium solani	*DHC1*	P78716	dynein heavy chain, cytosolic
Other eukaryotes	*C. elegans*	*CEF18F11.1*		
		CEF43C1.3		
		CEF54C8.4		protein tyrosine phosphatase
		CEW02D9.2		yeast Yip1 protein-like
		CEY25C1A.a		putative enoyl-CoA hydratase
	Gallus gallus	*TSN*	P79769	translin
	Bos taurus	*ASMT*	P10950	hydroxyindole *o*-methyltransferase
		PRKCSH	Q28034	protein kinase C substrate, heavy chain
	Brassica juncea		Q39287	Ω-6 fatty acid desaturase

sugars, amino acids and fatty acids. In particular, homologues to two β-glucosidases were detected, consistent with *D. hansenii* ability at metabolizing cellobiose. *S. cerevisiae* does not possess β-glucosidase activity, whereas this activity was found in all the *D. hansenii* strains tested [28]. One of these had exocellular cell wall bound and intracellular β-glucosidase activities, and two paralogues were found in the type strain. Homologues to β-galactosidase and lactose permease (match to Lac4p and Lac12p of *K. lactis*, respectively) were detected. Various *D. hansenii* strains are able to assimilate lactose whereas the type strain CBS767 is not. Two peptidases were also detected, consistent with *D. hansenii*'s important proteolytic activities.

3.8. Duplicated genes

The systematic sequencing of the *S. cerevisiae* genome revealed that about 40% of the genes are members of paralogous gene families [29,30]. From our analysis, the duplicated genes in *D. hansenii* amounted to 43 corresponding to 18 single copy genes and 25 multi-copy genes in *S. cerevisiae*. It must be pointed out that genes encoding permeases, transporters and dehydrogenases were frequent in our search. Overall, 97 newly identified genes (8.3% of the entire set) belong to paralogous gene families in *D. hansenii*. Among these, 23 genes (24%) have a putative role in transport, indicating an important over-representation of this type of activities in *D. hansenii* compared to *S. cerevisiae* (see below).

3.9. Functional classification of D. hansenii orthologues

We classified the functions associated with the orthologues of *S. cerevisiae* in *D. hansenii* according to the MIPS functional catalog as modified in [31].

When the expected number of *D. hansenii* orthologues within a functional class was lower than in *S. cerevisiae*, discrepancies between the two species were not analyzed since this may reflect a lower degree of sequence conservation between

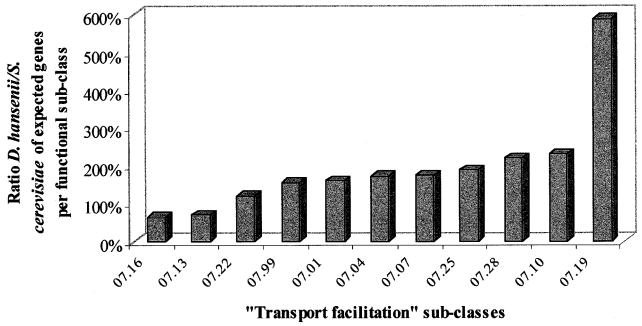

Fig. 1. Over-representation of genes involved in the 'transport facilitation' class (07) in *D. hansenii* compared to *S. cerevisiae*. The ratio of expected *D. hansenii* genes over *S. cerevisiae* genes is plotted against the sub-classes within the functional class 'transport facilitation' as described in [31]. Sub-classes: 07.16, purine and pyrimidine transporters; 07.13, lipid transporters; 07.22, transport ATPases; 07.99, other transport facilitators; 07.01, ion channels; 07.04, ion transporters; 07.07, sugar and carbohydrate transporters; 07.25, ABC transporters; 07.28, drug transporters; 07.10, amino acid transporters; 07.19, allantoin and allantoate transporters.

orthologues. On the other hand, a larger number of orthologues in *D. hansenii* indicates an over-representation within a functional class, which might reflect physiological differences between the two yeasts. Over-representation in *D. hansenii* was observed for several functional classes: 'metabolism of cyclic and unusual nucleotides' (239% vs. *S. cerevisiae*), 'β-oxidation of fatty acids' (341%), 'amines metabolism' (358%) and to a lesser extent, 'other energy generation activities' (159%), 'phosphate utilization' (184%), and 'peroxisomal organization' (196%).

The most striking feature of this comparison can be seen with the orthologues involved in 'transport facilitation' (Fig. 1). Except for two sub-classes ('purine and pyrimidine transporters' and 'lipid transporters') (see legend to Fig. 1), the nine remaining sub-classes are over-represented. They show a 120% increase or higher with most of the samples involving more than 10 orthologues. The largest excess (583%) was observed for the 'allantoin and allantoate transporters' class. Interestingly, other sub-classes involved in the transport of metabolites also show a significant excess: 'nitrogen and sulphur transport' (239%), 'amino acid transport' (187%), 'carbohydrate transport' (175%), 'lipid and fatty acid transport' (179%). On the other hand, little increase (140%) was observed for the genes involved in osmosensing. The latter is linked to glycerol transport and probably due to the fact that two systems exist (H^+/glycerol and Na^+/glycerol) in halotolerant yeasts including *D. hansenii*. Increased transport activities might be needed in addition to the maintenance of a high osmotic pressure within the cell.

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