Genome analysis of Candida subhashii reveals its hybrid nature and dual mitochondrial genome conformations

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Running title: Genome of C. subhashii
Abstract

*Candida subhashii* belongs to the CUG-Ser clade, a group of phylogenetically closely related yeast species that includes some human opportunistic pathogens, such as *Candida albicans*. Despite being present in the environment, *C. subhashii* was initially described as the causative agent of a case of peritonitis. Considering the relevance of whole-genome sequencing and analysis for our understanding of genome evolution and pathogenicity, we sequenced, assembled and annotated the genome of *C. subhashii* type strain. Our results show that *C. subhashii* presents a highly heterozygous genome and other signatures that point to a hybrid ancestry. The presence of functional pathways for assimilation of hydroxyaromatic compounds goes in line with the affiliation of this yeast with soil microbial communities involved in lignin decomposition. Furthermore, we observed that different clones of this strain may present circular or linear mitochondrial DNA. Re-sequencing and comparison of strains with differential mitochondrial genome topology revealed five candidate genes potentially associated with this conformational change: MSK1, SSZ1, ALG5, MRPL9 and OYE32.

Key-words: *Candida subhashii*, genome assembly, mitochondria, hybrid, metabolism of hydroxyaromatic compounds

Introduction

*Candida* species represent a non-monophyletic group of yeasts which comprises important pathogens for human health. *Candida* infections (i.e. candidiasis) are mostly caused by *Candida albicans*, *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*, which combined account for more than 90% of the cases worldwide\(^1\). However, over the last years the epidemiology of this disease has been shifting, with non-albicans *Candida* species increasing their relative incidence and with the emergence of new rare *Candida* species that are responsible for several cases of infection or even outbreaks\(^2\). The mechanisms involved in the emergence of new pathogens are still unknown, but hybridization has been suggested as a possible evolutionary path for the appearance of novel species with ability to cause infection in humans\(^3\)–\(^5\). Indeed, a majority of hybrid clinical isolates, or a hybrid origin of the species have been described in important pathogenic lineages such as *Candida orthopsilosis*, *Candida metapsilosis*, *Candida inconspicua*, and *C. albicans*\(^6\)–\(^10\). The comparatively low abundance or absence of the parental species among clinical isolates for many of these hybrids has led to the hypothesis that this pattern results from a higher ability of the hybrids to colonize and infect humans\(^1\)\(^4\). Of note, hybridization in these lineages only became evident after next-generation sequencing data analysis, showcasing the importance of whole-genome sequencing for our understanding of the evolution of human pathogens.

*Candida subhashii* was first described in 2009 in Canada as a novel species causing peritonitis and reported as a non-fermentative yeast member of the CUG-Ser clade\(^1\)\(^1\). Later on, this species was isolated from agricultural soil in Switzerland\(^1\)\(^2\) and Japan\(^1\)\(^3\), as well as from peatlands in Poland\(^1\)\(^4\). Although its first
description was as a fungal pathogen\textsuperscript{11}, it was suggested that the soil can possibly be its natural habitat, particularly considering that it is a highly competitive yeast, behaving as antagonist for several filamentous fungi\textsuperscript{12}. \textit{C. subhashii} has been reported to have a linear mitochondrial genome and it was described as the yeast species with the highest GC content on its mitochondrial DNA (52.7\%)\textsuperscript{15}. Moreover, mitochondrial sequence conservation between Canadian and European \textit{C. subhashii} isolates was reported, suggesting a recent and fast global spreading\textsuperscript{12}.

Considering the relevance of genomic analysis for our understanding of the evolution of human pathogens, we decided to sequence and analyze the genome of \textit{C. subhashii} type strain (CBS10753). Our analysis reveals genomic signatures characteristic of yeast hybrids: high levels of heterozygosity organized in non-homogeneously distributed blocks interspersed by regions with low levels of heterozygosity. Thus, \textit{C. subhashii} represents another case in the growing list of hybrid yeast pathogens. We also discovered that this species can harbor both linear and circular mitochondrial genome (see Results section for more details) including in two clonal descendants of the same strain, and we used a comparative genomics analysis to identify possible genomic alterations related to the different mitochondrial genome conformation. Moreover, we demonstrate that \textit{C. subhashii} has functional gentisate pathway and two branches of the 3-oxoadipate pathway indicating that this yeast participates in degradation of hydroxyaromatic compounds resulting from lignin decomposition in soil.

\section*{Materials and Methods}

\subsection*{Strains}

The \textit{C. subhashii} type strain (FR-392-06, CBS10753) and derived clones (see below) were cultivated in yeast extract-peptone-dextrose (YPD) medium (1\% (w/v) yeast extract, 1\% (w/v) peptone, 2\% (w/v) glucose) at 28 °C. The original strain has been subcloned as follows. A culture grown overnight in a YPD medium was diluted in water and plated on a YPD medium containing 2\% (w/v) agar. Several clones were obtained from single colonies growing on the plate. The clones dubbed SUB1, SUB3 and SUB10 were used in further experiments.

The assimilation tests were done in synthetic media (0.17\% (w/v) yeast nitrogen base w/o amino acids and ammonium sulfate (Difco), 0.5 \% (w/v) ammonium sulfate, 2\% (w/v) agar) supplemented with an appropriate carbon source (\textit{i.e.} 2\% (w/v) glucose, 2\% (w/v) glycerol, 2\% (v/v) lactate or 10 mM hydroxyaromatic compound dissolved in dimethyl sulfoxide (DMSO)).

\subsection*{Mitochondrial genome conformation}

Mitochondrial genome conformation has been assayed by pulsed-field gel electrophoresis (PFGE), restriction enzyme mapping of isolated mitochondrial DNA (mtDNA) and Sanger sequencing essentially as described in Fricova et al. (2010)\textsuperscript{15}. PFGE separations were carried out in a Pulsaphor apparatus (LKB).
using a contour-clamped homogeneous electric field (CHEF) configuration. DNA samples prepared in agarose plugs were separated in 45 mM Tris-borate, 1 mM EDTA using following conditions/settings: (i) 0.8% (w/v) agarose gel, pulses 60-600s (linear interpolation), 100 V, 72 h, 9°C (separation of chromosomal DNA) and (ii) 1.0% (w/v) agarose gel, pulses 5-50s (linear interpolation), 150 V, 24 h, 9°C (separation of mtDNA). For Southern blot hybridization, the PFGE separated DNA samples were transferred onto a Hybond N+ membrane (Amersham) using a VacuGene XL Vacuum Blotting System (GE Healthcare) and hybridized overnight at 50 ºC with 5’ [32P] end-labeled oligonucleotide (5’-TCAGTAGGATCAGTCCCTCTGATAGTCAT-3’) derived from the C. subhashii mitochondrial gene nad3 in a buffer containing 5x SSC (750 mM sodium chloride, 75 mM trisodium citrate, pH 7.0), 5x Denhardt’s solution (0.1% (w/v) Ficoll 400, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) bovine serum albumin fraction V) and 0.5% (w/v) sodium dodecyl sulfate (SDS). After hybridization, the membrane was washed in 2x SSC, 0.1% (w/v) SDS at room temperature for 5 min, followed by two washes at 45 ºC for 15 min each. The membrane was exposed to a storage phosphor screen (Kodak) and the signal was detected using a Personal Molecular Imager (Bio-Rad). Restriction enzyme digestion of isolated mtDNAs was performed by PvuI endonuclease (New England Biolabs) according to the manufacturer’s instructions.

Sanger sequencing of circular mitochondrial genome of the SUB1 clone was performed by primer-walking approach on isolated mtDNA template as described by Valach et al. (2008).10

**Genomic DNA sequencing**

A modified protocol from the MasterPure™ Yeast DNA Purification Kit was used to extract the DNA. In brief, samples were grown overnight in liquid YPD at 30°C. Cells were pelleted and lysed with RNase treatment at 65°C for 15 min. After 5 min of cooling down on ice, samples were purified by the kit reagent by mixing, centrifugation and removal of the debris as described in the kit protocol. Further, samples were left at -20°C with absolute ethanol for at least 2 h after which the DNA was precipitated for 30 min at 4°C. The pellet was washed in 70% (v/v) ethanol and left to dry. TE buffer (10 mM Tris.Cl, 1 mM EDTA, pH 7.5) was used to resuspend the DNA. Genomic DNA Clean & Concentrator kit was used for the final purification.

Whole-genome sequencing was performed at the Genomics Unit from CRG. Libraries were prepared using the NEBNext® DNA Library Prep Reagent Set for Illumina® kit (New England BioLabs) according to the manufacturer’s protocol. Briefly, 1 µg of genomic DNA was fragmented by nebulization in Covaris to approximately 600 bp and subjected to end repair, addition of “A” bases to 3′ ends and ligation of Truseq adapters. All purification steps were performed using QIAquick PCR purification columns (Qiagen). Library size selection was done with 2% low-range agarose gels. Fragments with average insert size of 700 bp were cut from the gel, DNA was extracted using QIAquick Gel extraction kit (Qiagen) and eluted in 30 µl EB. 10 µl of adapter-ligated size-selected DNA were used for library amplification by PCR using the Truseq Illumina primers. Final libraries were analyzed using Agilent DNA 1000 chip to estimate the
quantity and check size distribution and were then quantified by qPCR using the KAPA Library Quantification Kit (KapaBiosystems, ref. KK4835) prior to amplification with Illumina’s cBot. Libraries were loaded at a concentration of 2 pM onto the flow cell and were sequenced 2 x 125bp on Illumina’s HiSeq 2500.

For *C. subhashii* SUB3 clone, a mate-pair library was also sequenced. For that, DNA was fragmented to sizes between 1 and 20 kb using a transposase that binds biotinylated adaptors at the breaking point. Strand displacement was performed to “repair” the nicks left by the transposase. Fragment sizes of 3 to 6 kb were then selected on a 0.8% agarose gel and were then circularized. Non-circularized DNA was removed by digestion. The circular DNA was then mechanically sheared to fragments of 100 bp to 1 kb approx. and the fragments containing the biotinylated ends were pulled down using magnetic streptavidin beads and submitted to a standard library preparation. A final size selection on 2% agarose gel was done and fragments of 400 to 700 bp were selected for the final library. Final libraries were analyzed using Agilent High Sensitivity chip to estimate the quantity and check size distribution and were then quantified by qPCR using the KAPA Library Quantification Kit (ref. KK4835, KapaBiosystems) prior to amplification with Illumina’s cBot. Libraries were sequenced 2 x 125 bp on Illumina’s HiSeq 2500.

**Genome assembly**

Next-Generation Sequencing data was inspected with FastQC v0.11.5 ([http://www.bioinformatics.babraham.ac.uk/projects/fastqc/](http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)). Paired-end reads were filtered for the presence of adapters and for quality below 10 or 4 bp sliding-windows with average base quality of 15, setting a minimum read size of 31 bp with Trimmomatic v0.36\(^{17}\). Mate-pair reads were filtered with NxTrim v0.4.1-53c2193\(^{18}\). After filtration, only reads clearly identified as paired end or mate pair by the respective programs were used for further work. The K-mer Analysis Toolkit (KAT)\(^{19}\) was used to count k-mer frequency and estimate the expected genome size using default parameters (*k* = 27). SOAPdenovo v2.04\(^{20}\) and SPAdes v3.9 in both SPAdes and dipSPAdes modes\(^ {21,22}\) were used separately to perform the genome assembly of the paired-end and the mate-pair reads. Afterwards, redundant contigs were removed from each assembly with Redundans v0.13c\(^ {23}\). The quality of the different assemblies was inspected with Quast v4.5\(^ {24}\). Genome annotation was performed with Augustus v3.5 using *C. albicans* as model organism\(^ {25}\). The assembly completeness was estimated with KAT\(^ {19}\) and BUSCO v3\(^ {26}\) (Ascomycota database). The best assembly was chosen based on the assembly completeness, genome size, N50 and number of scaffolds. Mitochondria-related genes were identified with TargetP v1\(^ {27}\). Phylome reconstruction was performed as previously described\(^ {28}\) and deposited in the PhylomeDB database ([http://beta.phylomedb.org](http://beta.phylomedb.org))\(^ {29}\). Phylome results were used to infer orthology and paralogy relations and determine the associated GO terms. Enrichment analysis was performed with FatiGO\(^ {30}\).

**Read mapping and variant calling**
Read mapping and variant calling were performed with HaploTypo pipeline v1.0.1\(^1\) using default parameters. Briefly, BWA-MEM\(^2\) v0.7.15 was used for read mapping. GATK v4.0.2.1\(^3\) was used to sort the reads by coordinate and to mark duplicates. Freebayes\(^4\) v1.1.0-50-g61527c5 was used for variant calling considering a minimum depth of coverage of 30 reads. Vcffilter (https://github.com/vcflib/vcflib#vcffilter) set with "TYPE = snp & QUAL > 1 & QUAL / AO > 10 & SAF > 0 & SAR > 0 & RPR > 1 & RPL > 1" was used to filter variants. Ploidy estimation was performed with nQuire histotest\(^5\). To confirm the mitochondrial conformation of C. subhashii clones, SUB1, SUB3 and SUB10 sequencing reads were aligned in the linear mitochondrial genome sequence available in NCBI database (accession number: NC_014337.1).

**Heterozygous and loss of heterozygosity block definition**

To determine the presence of heterozygous and loss of heterozygosity (LOH) blocks, heterozygous and homozygous variants were separated. Then, the procedure applied and validated by Pryszcz et al. 2015\(^6\) was used. Briefly, bedtools\(^7\) merge v2.25.0 with a distance of 100 bp was used to define heterozygous regions, and by opposite, LOH blocks would be all non-heterozygous regions in the genome. Moreover, the minimum heterozygous and LOH size was established at 100 bp.

**Data availability**

Data generated by this project can be found under the BioProject PRJNA691204 in NCBI database and under the phylome ID 777 in PhylomeDB (http://beta.phylomedb.org)\(^8\).

**Results and Discussion**

**Genome assembly of C. subhashii type strain and phylome reconstruction**

To obtain a better understanding of C. subhashii genome evolution, we analyzed the type strain of this species (CBS10753), which was isolated in 2006 from a patient with peritonitis in Canada\(^9\). As mentioned above, C. subhashii was previously reported to have a linear dsDNA mitochondrial genome with unusually high GC content (52.7%) and proteins covalently bound to the 5' ends of linear mtDNA molecules\(^10\). Nevertheless, the PFGE analysis of several clones derived from the original culture of CBS10753 revealed that clones harboring circular mitochondrial genome also exist in this particular strain (Supplementary figure 1). Restriction enzyme mapping and Sanger sequencing demonstrated that the circular mtDNA represents a mutant presumably formed by end-to-end fusion of linear molecules which was accompanied by deletion of a substantial part of terminal sequences present in the linear mtDNA\(^11\) (see Materials and Methods for more details, and Supplementary figure 1). Of note, this alteration of mitochondrial genome topology in C. subhashii did not impair assimilation of respiratory substrates such as glycerol and lactate, but has possibly contributed to a slightly weaker growth of the circular mtDNA clones (Supplementary figure 2). Considering this dual mitochondrial genome topology, we initially selected two subclones derived from the type strain for whole-genome sequencing, which differ in their mitochondrial genome...
conformation (SUB1 and SUB3 with circular and linear mtDNA, respectively, Supplementary figure 1). Both samples were sequenced with Illumina paired-end sequencing strategy, and SUB3 was additionally sequenced with Illumina mate-pair sequencing strategy (see Materials and Methods for more details). During our analyses, we identified read coverage issues in the paired-end sequencing data of SUB3. As this subclone was no longer available in the laboratory, we sequenced a third clone (SUB10) that had been isolated in parallel with SUB3 by plating from the same original culture, and that also presented a linear mitochondrial genome (Supplementary figure 3). K-mer frequency analysis of the genomic reads of CBS10753 revealed the presence of two peaks of coverage (Figure 1a), one with half coverage of the other, corresponding to heterozygous and homozygous regions, respectively. This suggests that C. subhashii has a highly heterozygous genome. Indeed, nQuire histotest\textsuperscript{35} estimates that this is a diploid species ($r^2 = 0.92$).

After applying different assembly strategies (see Materials and Methods for more details), the best assembly was chosen based on different quality parameters such as genome completeness, percentage of mapped reads, N50, and fragmentation. The best genome assembly obtained for C. subhashii was the one generated with SPAdes\textsuperscript{21} and comprises 15.4 Mb in 333 contigs and an N50 of 108,455 bp. This assembly had high completeness as judged by high sequencing reads mapping rates (98.6% and 98.8% for SUB1 and SUB10, respectively, Table 1). As expected for an unphased hybrid genome, the assembly comprised only 58.81% of the k-mers present in the sequencing libraries (Table 1). Although at first this number may seem low, the comparison of the k-mers present in C. subhashii sequencing reads with those present in the genome assembly reveals that all the k-mers of the homozygous regions (second peak) are represented in the assembly, and that the missing portion of the genome actually corresponds to approximately 50% of the first peak, thus 50% of the k-mers of the heterozygous regions (Figure 1a). Therefore, this value indicates the expected consequence of a successful reduction of the heterozygous regions, with only one of the haplotypes being represented in the assembly. Finally, genome annotation predicted 6,178 protein-coding genes, corresponding to an estimated proteome completeness of 99.3% (see Table 1 and Materials and Methods for more details).

We next reconstructed the phylome, i.e. the complete collection of gene phylogenies\textsuperscript{37}, of C. subhashii including other 26 species (Supplementary file 1), and assessed orthology and paralogy relationships (see Materials and Methods for more details). Our results revealed that C. subhashii has 501 orphan genes, and that genes specifically duplicated in this species are enriched in transmembrane transport activity (Supplementary file 2). This result is similar to what was previously obtained for other Candida pathogens, such as C. inconspicua, Diutina (Candida) rugosa and Trichomonascus (Candida) ciferrii\textsuperscript{7,28}, and reinforces the previously proposed hypothesis that cell wall composition may play a role in the adoption of an opportunistic pathogenic behavior by Candida species\textsuperscript{38}. As previous reports suggest that the human body is not the main habitat of C. subhashii\textsuperscript{12}, we hypothesize that the duplication of cell wall proteins...
must have been advantageous in non-human environments while perhaps secondarily facilitating opportunistic colonization of humans. Similar exaptation processes (i.e. traits that provide an advantage in niches different from those in which they were originally selected) may be common in other opportunistic pathogens.

Assimilation of hydroxyaromatic compounds in *C. subhashii*

As soil appears as a natural niche for *C. subhashii*, we investigated whether this yeast is able to contribute to decomposition of lignin by assimilation of compounds such as hydroxyderivatives of benzene and benzoic acid. An assimilation test demonstrated that *C. subhashii* utilizes a range of hydroxybenzenes and hydroxybenzoates as a sole carbon source (Figure 2a). In other yeasts from the CUG-Ser clade such as *C. parapsilosis* and *C. albicans*, these compounds are metabolized via the 3-oxoadipate and gentisate pathways. The hydroxyhydroquinone (HHQ) branch of the 3-oxoadipate pathway is common to both *C. albicans* and *C. parapsilosis*, which is reflected by their ability to grow with hydroquinone and resorcinol as sole carbon sources. Moreover, as *C. parapsilosis* has longer version of this pathway it also assimilates various hydroxybenzoates (i.e. 4-hydroxybenzoate, 2,4-dihydroxybenzoate and 3,4-dihydroxybenzoate). In contrast, the catechol branch of this pathway is absent in *C. parapsilosis*, and for this reason this species is not able to grow when phenol or catechol are the exclusive carbon sources. Nevertheless, *C. parapsilosis* utilizes the gentisate pathway for catabolism of 3-hydroxybenzoate and 2,5-dihydroxybenzoate, which does not occur in *C. albicans*. In the particular case of *C. subhashii*, the assimilation assays demonstrated that, similarly to *C. parapsilosis*, this species is able to utilize 3-hydroxybenzoate and 2,5-dihydroxybenzoate, suggesting the existence of the gentisate pathway, which is confirmed by the presence of the metabolic gene cluster for this pathway (Supplementary file 3) previously described for *C. parapsilosis*. Nevertheless, despite being able to grow with phenol, resorcinol, 4-hydroxybenzoate, 2,4-dihydroxybenzoate and 3,4-dihydroxybenzoate as sole carbon sources, which is indicative of the occurrence of both branches of the 3-oxoadipate pathway, *C. subhashii* is not able to grow exclusively in catechol and hydroquinone (Figure 2a). Therefore, we searched for orthologs of the relevant genes and identified that *C. subhashii* presents orthologs for all the genes involved in both branches of the pathway, except for *PHH2* encoding a phenol monoxygenase (Supplementary file 3). As *C. subhashii* assimilates both phenol and resorcinol, we assume that monoxygenase encoded by the orthologue of *MNX3/PHH1* has a broader substrate specificity and catalyzes hydroxylation of both substrates. In this reaction, phenol and resorcinol/hydroquinone are converted to catechol (catechol branch) and HHQ (HHQ branch), respectively. Moreover, decarboxylation of 4-hydroxybenzoate by monoxygenase Mnx1 produces hydroquinone (Figure 2b). Therefore, it is intriguing that *C. subhashii* does not grow in media containing catechol and hydroquinone as carbon sources. Nevertheless, substrate assimilation assays (i.e., the growth on phenol and 4-hydroxybenzoate) and the presence of corresponding gene orthologs (Supplementary file 3) indicate that the growth impairment on these two substrates could rather reflect their inefficient uptake from medium than a defect in the pathway. Taken together, *C. subhashii* has
functional catechol and HHQ branches of the 3-oxoadipate pathway as well as the gentisate pathway, which may belong to ancestral inventory of the CUG-Ser clade species. The importance of metabolism of hydroxyaromatic compounds for this yeast is also underlined by the presence of two copies of *MNX1* as well as by two and four paralogues of *HBT2* and *HBT1*, respectively, coding for hydroxybenzoate transporters.

**Genomic variability in *C. subhashii***

Based on read mapping and variant calling analysis (see Materials and Methods for more details), we determined that *C. subhashii* SUB1 and SUB10 have 218,606 and 221,394 SNPs, respectively. Consistently with the high levels of heterozygosity inferred from the k-mer frequency analysis and nQuire estimations, from these SNPs, 218,242 (14.23 per kb) and 220,958 (14.37 per kb), respectively, are heterozygous (Table 1). Of note, these heterozygous variants are not homogeneously distributed across the genome, but rather form blocks of heterozygosity separated by homozygous regions. These heterozygous blocks have an estimated nucleotide sequence divergence of 4.3% with a single density peak (Figures 1b and 1c), suggesting that they all originate at a single time point. These results resemble the genomic patterns previously described for other *Candida* hybrids, indicating that the heterozygous blocks of *C. subhashii* do not result from the accumulation of mutations with time, but are instead the footprints of a past hybridization. For the same reason, we conclude that the homozygous regions of this genome actually correspond to blocks of LOH, a common trace of genome shaping after hybridization, which in this case represents 70% of the genome. Despite the fact that the estimated parental divergence of *C. subhashii* (4.3% current haplotype divergence) and the absence of known parental lineages do not allow us to determine if we are in presence of an intra- or interspecific hybrid, it is important to note that the genomic variability and haplotype divergence observed in *C. subhashii* are similar to what was previously described for other hybrids of the CUG-Ser clade, as *C. metapsilosis* and *C. orthopsilosis*. *C. metapsilosis* and *C. orthopsilosis* represent two hybrid pathogens, for which all sequenced strains thus far correspond to clinical isolates. The absence of known parental lineages for *C. metapsilosis* among clinical isolates has led to the hypothesis that they are possibly non-pathogenic and that the hybridization event contributed to the emergence of a new pathogenic lineage. However, it remains unclear whether hybrid clones also exist in the environment or whether they are exclusively found in association with humans. Contrary to these hybrid lineages, *C. subhashii* has mostly been isolated from environmental sources, with the only clinical isolate of the species corresponding to the type strain. Therefore, despite the absence of known parental lineages, in this case we can infer that hybridization occurred in the environment. Yet, as in the previous clade, it is still possible that some traits in the hybrids may have also facilitated opportunistic colonization of humans. Therefore, *C. subhashii* adds to an increasing list of hybrid lineages which can adopt a pathogenic behavior, and raises once again the question of the role of hybridization to the emergence of pathogenic lineages.
Genomic differences between clones with linear and circular mitochondrial genomes

The topology of the mitochondrial genome is not the same in all the species of the CUG-Ser clade. For instance, while C. albicans or C. tropicalis have circular mitochondrial genomes, members of the C. parapsilosis species complex have linear mitochondrial genomes terminating with telomeric structures composed of long tandem repeats. More interestingly, even within the C. parapsilosis clade, specifically in C. metapsilosis, two isogenic strains present different mitochondrial genome conformation. However, the existence of substantial differences between the nuclear genomes of these two isogenic strains has never allowed the identification of possible regions involved in the different mitochondrial genome conformation. To identify genomic alterations potentially related to the change of mitochondrial genome topology in C. subhashii we analyzed the genomes of two related strains differing in this trait (SUB1 and SUB10 clones). Our comparative genomics analysis revealed the presence of 317 and 618 exclusive non-synonymous SNPs in SUB1 (circular) and SUB10 (linear), respectively. From these polymorphisms, only one in SUB1 (scaffold81, position 22,972) overlaps a mitochondria-related gene, namely, MSK1 (Table 2). This gene is involved in tRNA processing and mitochondrial translation, but to the best of our knowledge has not been associated with any mechanism that could lead to alterations in mtDNA conformation. However, in baker’s yeast Saccharomyces cerevisiae, functional mitochondrial translational machinery is required for the maintenance of intact organellar genome.

Considering that C. subhashii is a hybrid and LOH blocks represent traces of haplotype shaping to achieve a stage of genome stabilization, we decided to inspect the differences between the two C. subhashii clones in terms of LOH. Jaccard metric between LOH blocks of SUB1 and SUB10 revealed that they share 99.4% of their LOH regions. Indeed, we only identified three LOH blocks that are not shared by the two clones, and therefore may correspond to relevant targets. One of such blocks is exclusive of SUB1 (circular mtDNA) and overlaps SSZ1 (Table 2), which is a Hsp70 protein that interacts with Zuo1p (a DnaJ homolog). The second block overlaps both ALG5 and MRPL9 genes in SUB10 (linear mtDNA, Table 2). This last gene is a component of the large subunit of the mitochondrial ribosome, which mediates translation in the mitochondrion, and identified in our analysis as a mitochondria-related gene (see Materials and Methods for more details). Important to note, in this case, the LOH does not affect the promoter region. A third and last LOH event, also exclusive of SUB10 (linear mtDNA), involves OYE32 (Table 2), which encodes NAD(P)H oxidoreductase family protein whose homolog in S. cerevisiae Oye2 is associated with mitochondria. Interestingly, SUB1 (circular mtDNA) has four non-synonymous SNPs covering the same gene. This suggests that the event of LOH may indeed have led to important differences between the two clones in OYE32, and this gene is a possible good target for future studies addressing such a question. Noteworthy, by mapping the mate-pair reads of SUB3 clone to C. subhashii genome assembly, we confirmed that SUB3 and SUB10 share the same allelic sequences in these regions, reinforcing the possible role of these genes in the alteration of the mitochondrial conformation of SUB1 (Supplementary file 4).
In summary, *C. subhashii* is a new *Candida* species, whose main niche is likely environmental and as a common member of soil microbial communities it is involved in degradation of lignin-derived hydroxyaromatic compounds. Despite this, *C. subhashii* was the causative agent of a case of fatal peritonitis, indicating that this species can adopt a pathogenic behavior. This work represents the first genome analysis of this species and reveals that *C. subhashii* results from a hybridization event. The role of hybridization on the emergence of lineages with ability to infect humans is not yet understood, nevertheless next-generation sequencing analyses have allowed the identification of an increasing number of hybrids with possible pathogenic behavior, and we showed that *C. subhashii* is one of them. Furthermore, similarly to what was observed in *C. metapsilosis* hybrids, two clones of the same strain of *C. subhashii* present different mitochondrial genome conformation. In this regard, through a comparative genomics analysis we were able to identify five genes with potential role on mitochondrial genome conformation, namely, *MSK1, SSZ1, ALG5, MRPL9* and *OYE32*. Unfortunately, given the difficulty of genetic manipulation of non-model species, it was not possible to test this association in the laboratory, but future studies may profit from this analysis and other datasets provided by this study as the genome assembly, and the complete collection of gene phylogenies of *C. subhashii*.

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Table 1. Summary of the genomic features of *C. subhashii* type strain, with indication of estimated and obtained genome size, number of contigs, assembly N50, percentage of GC content, assembly and proteome completeness, number of predicted proteins, percentage of mapped reads, number of SNPs per kilobase (kb), number of LOH blocks, average haplotype divergence in heterozygous blocks, and percentage of loss of heterozygosity (LOH).

*C. subhashii* genome assembly with SPAdes

|                                | SUB1                    | SUB10                   |
|--------------------------------|-------------------------|-------------------------|
| Estimated genome size (Mb)*    | 15.61                   | 15.61                   |
| Assembly size (Mb)             | 15.40                   | 15.40                   |
| Contigs                        | 333                     | 333                     |
| Contigs > 50kb                 | 252                     | 252                     |
| N50                            | 108,455                 | 108,455                 |
| GC                             | 34.56 %                 | 34.56 %                 |
| Assembly completeness (KAT)    | 58.81 %                 | 58.81 %                 |
| Proteome completeness (BUSCO)  | 99.30 %                 | 99.30 %                 |
| Number of proteins             | 6,178                   | 6,178                   |
| Mapped reads                   | SUB1 - 98.63%           | SUB10 - 98.82%          |
| SNPs/kb                        | SUB1 - 14.26 (14.23 heterozygous) | SUB10 - 14.40 (14.37 heterozygous) |
| LOH blocks                     | SUB1 - 22,705           | SUB10 - 22,679          |
| Divergence (%)                 | SUB1 - 4.32%            | SUB10 - 4.34%           |
| LOH (%)                        | SUB1 - 70.02%           | SUB10 - 69.73%          |

*Estimated with KAT\(^9\)
**Table 2.** List of genes identified as having a potential role on the different mitochondrial genome conformation observed in *C. subhashii*, with indication of the mitochondrial genome topology where the relevant genomic alteration was observed, the type of genomic feature associated, and the indication if the gene was identified as a mitochondria-related gene by our analysis.

| Gene      | Mitochondrial genome topology | Genomic feature | Predicted mitochondrial localization (TargetP) |
|-----------|-------------------------------|-----------------|-----------------------------------------------|
| MSK1      | linear                        | Non-Syn SNP     | Yes                                           |
| SSZ1      | circular                      | LOH event       | No                                            |
| ALG5 and MRPL9 | linear                      | LOH event       | Yes                                           |
| OYE32     | linear                        | LOH event*      | No§                                          |

*Non-Syn mutations were identified in the clone harboring circular mitochondrial DNA, suggesting the relevance of the LOH event.

§Homolog in *S. cerevisiae* is associated with mitochondria.

Figure legends

Figure 1. Genomic patterns of *C. subhashii* support its hybrid ancestry. A) 27-mer frequency in raw sequencing data of CBS10753. The x-axis represents the k-mer coverage, and the y-axis the k-mer frequency. The density of 27-mers present in the genome assembly is represented in red, and the absence is represented in black\(^1\). For diploid genomes, two peaks of coverage are expected, the first one (heterozygous) with half coverage of the other (homozygous). When only one of the haplotypes of the heterozygous regions is represented in the assembly, only half of the k-mers of the first peak are present. B) Heterozygous SNP density in heterozygous blocks. C) IGV screenshot of the genomic patterns of *C. subhashii* in scaffold2.

Figure 2. *C. subhashii* utilizes a range of hydroxyaromatic substrates metabolized via the 3-oxoadipate and gentisate pathway. A) Assimilation test of the strain CBS10753. These results indicate that *C. subhashii* has active two branches of the 3-oxoadipate pathway as well as the gentisate pathway. The assimilation test of the strain CBS10753 was performed in synthetic media containing indicated substrates as a sole carbon source at 28 °C. The cells were pre-grown in a YPD medium, washed with water and serial fivefold dilutions were spotted onto the test plates. B) A simplified scheme of the 3-oxoadipate (catechol and HHQ branches) and gentisate pathways in *C. subhashii*. Biochemical reactions operating in *C. albicans*, *C. parapsilosis* and both species are shown in blue, red and magenta, respectively. The names of genes for enzymes catalyzing corresponding biochemical reactions are based on the nomenclature from *C. albicans* and *C. parapsilosis* (see Supplementary file 3 for the complete list of *C. subhashii* orthologs). Note that although *C. subhashii* does not grow on plates containing catechol and hydroquinone the corresponding biochemical reactions should be functional as it assimilates phenol and 4-hydroxybenzoate. We assume that the growth on catechol and hydroquinone is impaired due to a defect in the substrate uptake.
Supplementary Material

Supplementary figure 1. The clones SUB1 and SUB3 of *C. subhashii* FR-392-06 (CBS10753) differ by mitochondrial genome conformation. A, B) The PFGE analysis revealed that the clone SUB1 lacks a band typical for the presence of linear mtDNA\(^5\), which is clearly visible in both the original strain (CBS10753) and the clone SUB3. Two different conditions were used to demonstrate the regular migration of band corresponding to the linear mtDNA form. The identity of this band was also confirmed by Southern blot hybridization with radiolabeled mtDNA-derived probe (for details see Materials and Methods). M – Low Range PFG Marker (New England Biolabs), 1 – the original culture of *C. subhashii*, 2 – SUB3, 3 – SUB1. C) Digestion of mtDNAs isolated from the clones SUB1 and SUB3 by endonuclease *Pvu*I shows that SUB1 mtDNA lacks two restriction fragments corresponding to the right and left telomere, but it contains a new fragment (Right+Left) corresponding to the fused termini of the linear mtDNA. Note that the joint fragment is shorter than a sum of the terminal fragments from the SUB3 mtDNA as the fusion was accompanied by deletion of substantial part of the terminal sequences as demonstrated by Sanger sequencing (GenBank accession numbers: GU126492 and KC993184 for the linear (29,795 bp) and circular (28,562 bp) mtDNA, respectively). M – λ DNA / *Pst*I, 1 – SUB1 mtDNA / *Pvu*I, 2- SUB3 mtDNA / *Pvu*I. Note that there is an additional difference between the SUB1 and SUB3 mtDNAs. The SUB1 mtDNA has an insertion of a 135-bp long fragment between the genes *trnQ* and *nad5*. This difference between both clones has been confirmed by polymerase chain reaction (PCR) using 5’-AGGGTATCTTGGGTGTGC-3’ and 5’-AACGACTGGCACGGTAC-3’. M – λ DNA / *Pst*I, 1 – SUB1 PCR product, 2- SUB3 PCR product. D) Note that the identical insertion is present in the mtDNA of another *C. subhashii* strain (FGA 2.2; accession numbers: KX781248\(^{12}\)).

Supplementary figure 2. Alteration of mitochondrial genome topology in *C. subhashii* does not impair assimilation of respiratory substrates such as glycerol and lactate. The clones SUB1, SUB3 and SUB10 were isolated from individual colonies of the *C. subhashii* CBS10753 culture. While both SUB3 and SUB10 harbor linear mtDNA, SUB1 is a mutant with circularized mitochondrial genome. (A, C) The clones harboring circular (SUB1) and linear (SUB3, SUB10) mtDNA were grown overnight in a YPD medium, washed with water and spotted in serial fivefold dilutions onto plates with synthetic media containing indicated carbon source and cultivated at 28 °C. Note that although the clones utilize all three substrates, the SUB1 clone exhibits a slightly weaker growth compared to SUB3 or SUB10. This is even more pronounced by comparison of the growth curves (B, D). The clones SUB1 (red) and SUB3/SUB10 (blue) were grown in liquid synthetic medium containing 2% glucose at 28 °C and the cell density was determined by measuring OD\(_{660}\). Values plotted represent the mean of three biological replicates.

Supplementary figure 3. Whole-genome sequencing provides evidence that the clones SUB3 and SUB10 harbor linear mitochondria, while SUB1 has circular mitochondria. IGV screenshot of the...
alignment of the sequencing reads of SUB1, SUB3 and SUB10 to the linear mitochondrial genome sequence of *C. subhashii* (accession number: NC_014337.1). Red boxes highlight the extremities of the linear mitochondrial genome, showing the presence of these regions in SUB3 and SUB10, and a deletion in the SUB1 clone, which has circular mitochondria.

**Supplementary file 1.** List of species and respective proteome IDs used for *C. subhashii* phylome reconstruction.

**Supplementary file 2.** GO enrichment analysis results for genes specifically duplicated in *C. subhashii*.

**Supplementary file 3.** Complete list of *C. subhashii* orthologs to *C. albicans* and *C. parapsilosis* genes involved in hydroxyaromatic substrates metabolism. Orthology relations were obtained from phylome data.

**Supplementary file 4.** Gene sequences of SUB1, SUB3 and SUB for the five genes identified as possibly related to alteration in mitochondrial conformation: MSK1, SSZ1, ALG5, MRPL9 and OYE32. Genomic regions differing between clones harboring linear and circular mitochondria are highlighted in yellow.
Figure 1

169x124mm (300 x 300 DPI)
Figure 2

169x99mm (300 x 300 DPI)