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PHYLOGENETIC ANALYSIS OF THE YEAST COX2 GENE *DEKKERA BRUXELLENSIS*

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Abstract: The yeast *Dekkera bruxellensis*, a teleomorph of *Brettanomyces bruxellensis*, is the biggest contaminant in distilleries that use sugarcane juice around the world, causing a decrease in ethanol productivity and, consequently, causing losses to the industry. Despite its importance, few genetic studies have been published in the scientific literature. Recent work by our group has shown that this yeast is highly adaptable to the industrial process and we propose a broad genomic analysis to identify the factors responsible for this characteristic. In this study, we evaluated the polymorphism of the *COX2* gene, which encodes the cytochrome oxidase II enzyme. The results showed an unexpected greater similarity between the *COX2* gene sequences of industrial isolates of *D. bruxellensis* with its orthologue in *D. custersii* than with the *COX2* sequence of the type strain of *D. bruxellensis* deposited in GenBank. *In* addition, we started an *in silico* comparative analysis of the mitochondrial genome of ascomycete yeasts that have their mitochondrial genome sequenced and deposited in GenBank. This made it possible to construct a physical map of the mitochondrial genome of this clade. Six species with nuclear genomic similarity to *D. bruxellensis* were subjected to multiple alignments using the computer program Mega v. 4.0. The gene order was defined as L-rRNA COII COIII S-rRNA COI ATPase 8 ATPase 6 Cyt b ATPase 9 Var 1, based on the *Saccharomyces cereviseae* genome. The CODEHOP and Codon Usage programs were used to refine the design of degenerate primers in order to amplify the orthologous genes of *D. bruxellensis*. The alignments proved to be representative for primer construction, since a high degree of variability was observed between the syntenic gene sequences of the aforementioned structural genes. These data provide the basis for future analyses of the genetics and evolution of the *D. bruxellensis* population, which will serve as a basis for establishing correlations between the variability and genetics and the physiological capacities of different industrial strains *of D. bruxellensis* in search of a better understanding of this yeast's -competitive fitness‖ in the industrial environment.
Keywords: microbiology, yeast, bioinformatics, molecular genetics.

INTRODUCTION

Yeasts occupy a prominent position in the field of industrial biotechnology due to their use in traditional fermentation processes. It is predicted that the traditional production of products by breweries, wineries, distilled spirits and fuel industries, and the production of biomass by the food industry, will continue to provide the largest amount of fermented products in the world.

From a physiological point of view, two groups of yeasts have developed the ability to grow even in the absence of oxygen: the *Saccharomyces* and *Dekkera/Brettanomyces* clades. This suggests that the ancestor of modern ascomycetes must have been completely dependent on the presence of oxygen for growth, demonstrating an adaptive advantage over the other clades.

Comparative genomic studies with the various other species of hemiascomycetes whose genomes have already been sequenced have made it possible to place the fungi in their respective clades. The data produced by the UFPE group shows a high degree of genetic variability among *S. cereviseae* isolates from fuel alcohol, similar to what has been shown for isolates from the wine industry. The same is not true for the *Dekkera/Brettanomyces* group, which has very few published sequences. Currently, our research group has used data from the partial sequencing of the *Dekkera bruxellensis* genome provided by Prof. Jure Piskur, from Lund University in Sweden, to identify genes involved in various aspects of

metabolism and response to environmental conditions in this species. However, information on mitochondrial genes would be very important in the context of the mechanisms of adaptability of this yeast to the industrial environment, as well as in the exploitation of its fermentative capacity, which has also been demonstrated in recent work by the group.

The aim of this study was to analyze the *COX2* mitochondrial gene in order to reconstruct its phylogeny among hemiascomycetes, evaluate the pattern of evolution of the mitochondrial genome in comparison with the nuclear genome and help in future molecular physiology studies of this yeast.

LITERATURE REVIEW

ALCOHOLIC FERMENTATION

History

Yeasts are linked to mankind, taking part in the processes of baking, wine production, beer production, the production of fuel alcohol and other fermented beverages. In the 1970s, the fermentation process became more important, commercially and socially, at a national level with the implementation of the federal government's Pro-Alcohol Program. This program stimulated not only the production of ethanol as an environmentally clean fuel, but the entire Brazilian automotive industrial park. From this perspective, there was a growth in demand for alcohol fuel, which led to an increase in ethanol production. As a result, various scientific and technological development initiatives were supported with public and private funds.

The yeast *S. cerevisiae* is traditionally the most widely used microorganism in industrial fermentation processes. This yeast has important physiological characteristics for the industrial process, such as metabolism type positive -Crabee-tree||, producing ethanol even in the absence of oxygen, with high fermentation efficiency and high ethanol tolerance. Several strains of this yeast are present in industrial fermentation processes (Silva-Filho *et al.,* 2005a; Silva-Filho *et al.,* 2005b).

Fermentative metabolism

Cellular metabolism can be understood as a set of highly coordinated reactions, interconnected in such a way that the product of a first reaction becomes the substrate for a second reaction, and so on. Metabolism consists of two phases: catabolism, also known as the degradative phase, and anabolism, known as biosynthesis. In the catabolic pathways, chemical energy is released in the form of ATP and NADH, which are used in the anabolic pathway to convert small precursor molecules into cellular macromolecules.

From a biochemical point of view, fermentation is an anaerobic catabolic process that does not involve the respiratory chain or cytochromes.

Alcoholic fermentation is characterized as a catabolic pathway in which sugar molecules (glucose or fructose) are broken down inside the S. cereviseae cell to form ethanol and CO2, releasing chemical and thermal energy.

The phenomenon of anaerobic growth occurs when the enzymes of the respiratory chain and the Krebs cycle have their synthesis repressed, a phenomenon called catabolic repression by glucose, so when this is consumed there is a depletion of sugars in the medium favoring the enzymes of respiratory metabolism which are then synthesized.

Microbiology of the fermentation process

Traditionally, fuel alcohol producers used baker's yeast to start the fermentation process. Today there are even specific commercial strains for this fermentation, such as PE-2, CAT-1 and BG-1 (Lallemand Inc., Canada). Currently, another strain has been added to

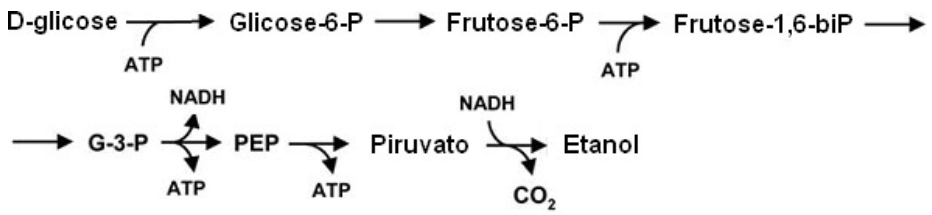

Figure 1 Schematic of alcoholic fermentation in *S. cerevisiae*. PEP = Phosphoenolpyruvate. Adapted.

this list, called JP1, which was isolated and sequenced by Silva- Filho *et al.* (2005b) and is currently marketed under the brand name Fermol Distiller® (AEB Group, Brazil). On the other hand, the industrial process also has a population of *non-S. cereviseae* called contaminants due to the fact that industrial alcoholic fermentation for the production of fuel alcohol is carried out without prior sterilization of the culture medium, and the yeast population used (*Saccharomyces cerevisiae*) is recycled into the process after centrifugation. Bacterial contamination in the industrial process is controlled by keeping the pH low (around 3.5) or, in many cases, by using antibiotics. However, these measures are not effective in combating process contamination by non-S. *cerevisiae* yeasts.

Occasionally, operational problems such as a drop in yield and delays in fermentation associated with high counts of these contaminating yeasts are observed. Extensive monitoring work in distilleries in the Northeast showed that *Dekkera bruxellensis* is the most frequent contaminating yeast in distilleries that use raw sugarcane juice in fermentation systems, and is also responsible for the most severe contamination episodes (Basílio et al., 2005).

Sherata (1960) identified the presence of 14 non-S. *cereviseae* yeast species in sugarcane juice belonging to the genera *Candida, Endomyces, Hansenula, Kloekera, Pichia, Saccharomycodes, Schizosaccharomyces* and *Torulopsis*. Soon after, species of *Debaromyces, Rhodotorula* and *Cryptococcus* were also identified in this substrate (de Azeredo *et al.*, 1998). In recent years, more species from the genera *Candida, Hanseniaspora, Kloekera, Kluyveromyces, Schizosaccharomyces* and *Pichia* have been identified in the industrial fermentation process for both fuel ethanol and cachaça production (Cabrine & Gallo 1999; Guerra *et al.*, 2001; Schwan *et al.*, 2001; Gomes *et al.*, 2002; Olasupo *et al.*, 2003). Recently, Basílio *et al.* (2008) used molecular techniques for typing and identification for the first time in Brazil and showed the presence of more than 30 yeast species in the fermentation process in various distilleries in the Northeast region (Basílio *et al.,* 2008), among which the *Dekkera bruxellensis*, *Candida tropicalis* and *Pichia galeiformis* species stand out.

DEKKERA BRUXELLENSIS: BIOLOGY AND PHYSIOLOGY OF THE SPECIES

Ecology

In 1960 van der Walt and van Kerken reported the formation of ascospores in yeast strains previously classified as *Brettanomyces* yeasts, considered to be non-sporulating, and proposed the new genus *Dekkera* to accommodate this teleomorphic phase. Barnett *et al.* (2002) recognized four species in this genus: *D. bruxellensis, D. anomala, D. curstesianus* and *D. naardenensis*.

Under the microscope, the cells of this yeast are spheroidal to ellipsoidal, often ogival (Figure 1), or they can also be cylindrical or elongated, and they can also exhibit pseudomycelium. Vegetative reproduction occurs by budding.

In sexual reproduction, the asci are evanescent and have one to four ascospores. The

ascospores are hat-shaped or spherical with a tangential edge. When released, the spores tend to group together. As general characteristics, in addition to those mentioned above, we can also list their slow growth, short life span on plates, characteristic aroma, strong production of acetic acid from glucose, stimulation of fermentation by molecular oxygen and requirement for an external source of vitamins.

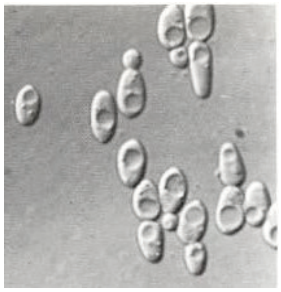

Figure 1. Cells of the yeast Dekkera bruxellensis CBS74.

Source: http://www.ncyc.co.uk/photo-ncyc-CBS74.html

D. bruxellensis is able to metabolize various carbon sources, including glucose, fructose, galactose, sucrose, maltose and ethanol. The nitrogen sources used by this yeast include ammonia, proline, arginine and nitrate (Conterno *et al.,* 2006). In addition, like *S. cerevisiae, D. bruxellensis* is ethanol tolerant, has facultative anaerobiosis, is petite positive (able to survive without mitochondrial DNA) and Crabtree positive, i.e. it has fermentative metabolism when high concentrations of glucose are present in the medium even under aerobic conditions (Woolfit *et al.,* 2007; Piskur *et al.,* 2006).

This type of metabolism produces certain components that have phenolic odors and acetic acid, reminiscent of "leather" or "horse urine" in red wines, among others (Licker *et al.*, 1998; Olsen, 2002). The increase in the production of acetic acid and phenolic compounds inhibits the growth of *S. cerevisiae* and therefore reduces the yeast's ability to produce ethanol (Lema *et al.*, 1996; Gerós *et al.,* 2000).

Dekkera bruxellensis as an industrial contaminant

Although *S. cerevisiae* is the main fermenting microorganism, other species have been identified in these processes. Although most of the yeasts found do not seem to have any effect, some can act as (Basílio *et al.,* 2005). A study carried out in several distilleries in the northeast of Brazil showed that the species *D. bruxellensis* is the main contaminating yeast in fermentation systems. This same study showed that the *D. bruxellensis* subpopulation replaces that of *S. cerevisiae*, even with repeated instances of total cell biomass exchange (de Souza Liberal *et al.*, 2007). Because of this ability to replace *S. cerevisiae* in the yeast population, *D. bruxellensis* represents one of the most significant contaminants in distilleries that ferment sugarcane juice in continuous systems. In the production of fuel ethanol, when *D. bruxellensis* counts are increased, there is a decrease in the volumetric productivity of ethanol, accompanied by significant economic damage (de Souza Liberal *et al.*, 2007). In addition, most studies have shown that *D. bruxellensis* is the prevalent species in contamination events of fermentation products (de Souza Liberal *et al.*, 2007; Röder *et al.,* 2007; Renoulf & Lonvaud-Funel, 2007; Phister & Mills, 2003; Miot-Sertier & Lonvaud-Funel, 2007).

Although there is a perception that *D. bruxellensis*, mainly in its anamorphic form *B. bruxellensis*, represents an industrial contaminant (the concept of contamination was reviewed by Loureiro & Malfeito-Ferreira, 2003), recent data shows that this yeast is capable of producing ethanol in yields very close to those presented by *S. cerevisiae* (de Souza Liberal *et al.*, 2007) and there is even a proposal to use this yeast in fermentation processes in association with the bacterium *Lactobacillus vini* (Passoth *et al.*, 2007). On the other hand, the problem caused by the

presence of this yeast in high counts in the medium is the longer fermentation time needed to convert the sugar into ethanol (de Souza Liberal *et al*., 2007), which causes delays in production and in the daily yield of the process. This low productivity may be related, for example, to the lower specific fermentative capacity due to the lower metabolization of sucrose (Basílio *et al*., 2008).

To become predominant in the yeast population, *D. bruxellensis* must grow at a higher rate than *S. cerevisiae* under the same conditions. One hypothesis suggested is that *D. bruxellensis* probably has greater resistance to ethanol than *S. cerevisiae*, thus managing to overcome the latter's population in the final stages of fermentation (Renouf *et al.,* 2006). In addition, in a continuous fermentation system with cell recycling, high cell densities are reached and the supply of nutrients for cell growth is higher than in a continuous fermentation system with cell recycling becomes limited. In these environments, competition is mainly determined by the ability to utilize the growth-limiting factor. If *D. bruxellensis* is able to metabolize an available nutrient more efficiently, or if it is able to capture it with greater affinity than *S. cerevisiae*, its growth rate can be much higher in this particular condition.

Among the obvious metabolic differences between *S. cerevisiae* and *D. bruxellensis* is the latter's ability to use different metabolic pathways, using the tricarboxylic acid pathway and the genes associated with it to produce ethanol.

DEKKERA GENETICS

Dekkera's genetics are little known

The genus *Dekkera* belongs to the Saccharomycetacea family and is considered a distant relative of *Saccharomyces*, both genera being part of the hemiascomycetes group. The genus *Dekkera* was validated using ribosomal DNA restriction analysis, which unequivocally established the equivalence between the genera *Brettanomyces* (anamorph) and *Dekkera* (teleomorph) and their species (Molina et al. 1993). Subsequently, the separation of the two species *D. bruxellensis* and *D. anomala* was validated on the basis of phylogenetic analyses of the 18S ribosomal DNA *locus* (Cai et al. 1996). Sequencing of the D1/D2 variable region of the 26S ribosomal gene also showed that industrial isolates can be unequivocally identified with *D. bruxellensis*, being differentiated from the species

D. anomala. Of the four species recognized in the genus, *D. bruxellensis* is able to grow in the absence of oxygen (Viser *et al.,* 1990), and has *Crabtree-positive* metabolism, i.e. it ferments hexoses producing ethanol even in the presence of oxygen (Renouf *et al.,* 2006). These characteristics make it very similar to the *S. cerevisiae* species.

In fact, in nature only two groups of yeasts seem to have developed the ability to grow in the absence of oxygen: one belongs to the *Saccharomyces* clade and the other to the *Dekkera/Brettanomyces* clade (Piskur and Langkjaer, 2004). This indicates that the ancestor of modern ascomycetes must have been completely dependent on the presence of oxygen for growth. Following the speciation events, some yeast strains have progressively reduced their dependence on oxygen by remodeling metabolic pathways and by the appearance of new genes. For the *Saccharomyces* group, studies comparing mitochondrial genes with the various other

species of hemiascomycetes whose genomes have already been sequenced have made it possible to pinpoint the main events in this process. This is not the case for the *Dekkera/ Brettanomyces* group, which has very few published sequences (Woolfit et al., 2007).

Based on the discovery made by Ephrussi (1950), Woolfit et al, in 2007 selected the CBS 2499 strain for genomic analysis because it is the largest representative of the *D. bruxellensis* clade, because its genome size is estimated at 19.4 Mb, because it is easy to manipulate in the laboratory and especially because it produces -petite‖ mutants, allowing sequencing for the construction of clones without contamination of mitochondrial DNA, although among the contigs generated some of them show similarity to mitochondrial genes when compared by BlastX. From this sequencing, 14,860 random reading frames were obtained from this genomic library which are found within 5,407 contigs, totaling approximately 7.6 Mb of sequence data, giving an estimate of the size of the genome, thus indicating that only 40% of it has been sequenced with an estimated gene density in the order of 7,430 protein-coding genes (Woolfit et al., 2007). 2,606 gene sequences with partial or total homology to *S. cereviseae* and 277 orthologous genes to non-S. *cereviseae* species were identified.

S. cereviseae which, however, belonged to the *Saccharomycetales* family, showing thus, the high rate of horizontal transfer from these species to *D. bruxellensis* . Repeats of the 18S, 5.8S, 25S and 5S ribosomal DNA locus were observed, as well as at least 24 tRNA genes.

To try to pinpoint the phylogenetic position within the hemiascomycetes, Woolfit *et al* (2007) constructed 366 model trees based on the protein sequences of genes orthologous *to D. bruxellensis* and nine from other fungal species. A cluster analysis was carried out, in which successive exclusions of the species furthest from *D. bruxellensis* were made from these 366 trees in order to verify the most accepted topology among these 366 possible trees. Three topologies were created and accepted as the explanation of *Dekkera*'s position within the hemiascomycetes and how it diverged. These topologies contrasted in how this species actually positioned itself among the three topologies generated. To clarify this issue, a Shimodaira-Hasegawa statistical test was carried out which rejected topology 3, which placed *D. bruxellensis* in a clade isolated from the other species of hemiascomycetes, and topology 1, in which the most significantly accepted was topology 2, which explains that *D. bruxellensis* diverged at some point from its sister group composed of *C. albicans* and *D. hansenii*.

In order to determine whether there really was any relationship between *C. albicans* and *S. cereviseae* and *D. bruxellensis*, an analysis was carried out comparing the amino acid sequences of the latter with syntenic species, thus verifying the identity between each of them and *D. bruxellensis.* This comparison showed that *D. bruxellensis* had undergone a trichotomy of the other species mentioned, giving an idea that the rate of evolution of *D. bruxellensis* was much higher than the others. This perspective was only proven when the GC content of these species was compared, verifying that, expressively, *D. bruxellensis* contained a high GC content when compared to the other two species in question (Woolfit *et al*, 2007).

In order to verify the rate of rearrangements between the predicted trichotomy and to confirm whether this in *D. bruxellensis* was generated by a chromosomal duplication event, a comparison of gene order was made. The result was elucidated where the rate of conserved genes between *S. cereviseae* and *D. bruxellensis* is higher than in *C. albicans and* where the rate of rearranged genes is higher in *C. albicans* than in *S. cereviseae.* Also in this study, it was observed that there were gaps

between the coding genes which were not similar to any other gene in the entire database using BlastX; these regions were supposedly identified as intergenic regions. A comparison was made in which it was found that these spaces contain introns that are orthologous *to S. cereviseae* and others that are specific to *D. bruxellensis*, evidencing kinship and the hypothesis that it is so close to *S. cereviseae*. As this species is a high value-added yeast, as it participates in fermentation processes, this characteristic is an excellent indication that *D. bruxellensis* can be used as a fermenting yeast (Woolfit *et al*, 2007).

Mitochondrial DNA

The biogenesis of the ATP synthesis machinery requires input from two physically separate genomes: one in the nucleus and the other in the mitochondria. The mitochondria supply much of the cell's energy processes, producing ATP by phosphorylating the oxidative apparatus of the outer mitochondrial membrane. In humans, more than 80 proteins participate directly in oxidative phosphorylation, of which 13 are encoded by mitochondrial DNA. The distribution of the components of oxidative phosphorylation that are encoded by the nuclear and mitochondrial genomes varies between species. However, in all species the essential contribution of the mitochondrial genome requires that this mtDNA be inherited with fidelity to ensure that respiratory function is maintained during growth and development (Chen and Butow, 2005).

Since the discovery that mitochondria have their own genome, independent of the nuclear genome, the question has been raised as to whether they are subject to the same repair mechanisms that occur in the nuclear genome. However, for a long time it was believed that DNA repair was not present in the genome of this organelle. In this sense, repair would not be necessary due to the redundancy of the genetic information present in the various copies of the organelles' DNA. This view was reinforced by the observations that the genome of this organelle does not have any genes that encode enzymes responsible for DNA repair (Britt, 1996). The mtDNA has a higher mutation rate when compared to the nuclear DNA (Richter et al., 1988), and from this fact, some lesions with UV irradiation, producing pyrimidine dimers, are not efficiently removed in the mtDNA (LeDoux et al., 1992).

The control region is responsible for regulating the replication and transcription of the entire mtDNA. Replication begins in this region and is carried out by the displacement of one strand in relation to the other, forming a loop, known as the D-loop. In the control region, mtDNA polymorphisms are checked (Upholt and Dawid, 1977).

These organelles have a double membrane and are present in the cytoplasm, being responsible for many crucial metabolic processes such as oxidative phosphorylation. For this reason, mitochondria are known as the energy plants of cells. It is believed that mitochondria are evolutionarily derived from an ancestral bacterium, which would have formed an intracellular symbiotic relationship with the first eukaryotic cells, which explains the fact that it has its own genome (Gray, 1992). Over hundreds of millions of years, this ancestor lost the ability to function as an independent organism, so its genome became very attenuated. In fact, most of the mitochondria's functional proteins are encoded by genes in the nucleus (Lang *et al*. 1997). What remained in the mitochondria *of Saccharomyces* was a circular genome of 42,889 base pairs (bp) that contains 43 genes, of which 27 encode transporter RNAs, two encode ribosomal RNAs and 19 encode proteins/enzymes involved in the electron transport chain of oxidative phosphorylation and ATP production (Wallace 1992).

Mitochondrial DNA evolves around five times faster than nuclear DNA (Cann and Wilson 1983). This variation is primarily due to the fact that mitochondria are major generators of free radicals, providing a favorable environment for DNA mutations. Another cause is the absence of histones, which play a protective role in nuclear DNA (Yakes and Van Houten, 1997). In addition, the mitochondrial DNA polymerase enzyme has low corrective activity when compared to nuclear DNA polymerase (Kunkel and Loeb, 1981) and nucleotide excision-dependent DNA repair is not present in mitochondria (Croteau *et al*. 1999). In relation to the coding region, some portions of the control region are highly variable between individuals, evolving five times faster than the rest of the molecule (Greenberg *et al*. 1983), presumably due to the weak selection exerted on the non-coding region of the DNA. It should also be considered that the typical *D-loop* structure, where there is momentary formation of single strands, can influence the point mutation pattern (Reyes *et al*. 1998), since the depurination rate of single stranded DNA is four times higher than that of double stranded DNA (Lindahl and Nyberg, 1972). For these reasons, phylogenetic identification tests focus on sequence variation within the variable regions of genes (Holland *et al*. 1993; Wilson *et al*. 1993; Parson *et al*. 1998).

ANALYSIS PHYLOGENETIC

Phylogenetics is the field of biology that seeks to identify and understand the evolutionary relationships between the different forms of life on Earth. The first objective criteria for phylogenetic reconstruction were based on morphological data. With recent access to the structure of macromolecules (DNA, RNA and proteins), phylogenetic analysis has advanced dramatically. In order to work with phylogenetic reconstruction based on molecular data, it is necessary to know some properties of the sequences to be compared.

When reconstructing any type of phylogeny, a systematist's primary concern should be homology (Hennig, 1966; Philips et al, 2000), i.e. we should always compare homologous characters in different species. The term homology was coined in the 19th century by Owen, an English anatomist who, although a contemporary of Darwin, did not believe in evolution (Lewin, 1997).

Homology is currently understood as a property relating to entities that have a common origin. More simply put, two characters are homologous if their identical or similar parts have a common origin (Patterson, 1988; Titus and Frost, 1996; Lewin, 1997; Graur and Li, 2000). Homology is therefore a qualitative term, not a quantitative one. Even so, many authors still mistakenly use the concept of homology as a synonym for similarity.

There are three types of homology: orthology, when the sequences have a single common ancestor; paralogy, when they originate from a gene duplication; and xenology, when they originate from lateral (or horizontal) incursion. Only orthologous sequences can provide phylogenetic information on the history of organisms.

Once the sequences have been obtained, they must be correctly aligned. To do this, although there are many computer programs that perform this task, the sequences must be aligned manually. However, automatic alignments can be a first step. This stage is extremely important, as a wrong alignment will compromise the entire analysis. For a coherent alignment, the different types of mutations and nucleotide substitutions must be known. Mutations occur randomly anywhere in the genome and can be transitions, transversions, deletions/insertions and inversions. Substitutions refer to coding regions and can be synonymous (when there is no change in

the coded amino acid), non-synonymous (when there is a change) and meaningless (when a stop codon is generated).

The aim of the alignment is to ensure that the position of each base being compared between the sequences considered is homologous. Because of the problem of stretches being lost or gained, it is necessary to insert gaps in the sequences of species that have lost stretches or that have not gained them (Phillips *et* al, 2000). Nowadays, there are several computer programs for aligning amino acid and nucleotide sequences (ClustalX, ESEE, Macaw, Pileup, TreeAlign, etc.) These programs are efficient and recommendable.

In the ClustalW program (Thompson ET AL, 1994) and in most programs, alignment is done in two stages. In the first, all the sequences are compared pair by pair and a maximum similarity measure between each two sequences is calculated. To calculate this similarity measure, a points is constructed where its two axes represent the two sequences being compared. For each identity between the bases, a point is placed on the graph. In the case of two identical sequences, a row of dots will be found on every diagonal of the graph, regardless of nucleotide composition. Based on this matrix of dots, the program will maximize the similarity between the sequences, using what are called penalties. Two types of penalties are used in this case: the interval penalty, which is the number of identical bases we must add to insert an interval, and the substitution penalty. The final similarity value between each two sequences is calculated as the total number of identical bases minus the number of substitutions, minus the number of gaps multiplied by the gap penalty.

From the pairwise similarity data, a dendrogram is built and the final alignment is made from the innermost nodes. In other words, the multiple alignment program starts by aligning the most similar sequences first, then those that connect to them, and so on, until all the sequences are aligned. The final result of the alignment is actually an excellent indicator of how suitable your gene is for the specific phylogenetic problem.

In all molecular phylogenetic methods, each position occupied in the sequence (nucleotide or amino acid) is considered to be a multi-state character (it can be one of the four nucleotides or one of the twenty amino acids) and each character is considered to be independent of the others. The variation in character states will provide phylogenetic information. Phylogenetic methods are essentially statistical and can be classified into two main groups according to their criteria: quantitative (distance methods); 2) qualitative (parsimony and likelihood methods). In distance methods, the differences between two sequences are reduced to a single variable (number of differences) and their evolutionary relationships are not taken into account. In this method, the distance is first calculated and then the phylogenetic tree is reconstructed using a specific algorithm. There are different models that can be used to construct a distance matrix (NEI *et al*, 2000; MATIOLI, 2001).

The tree will be reconstructed from this matrix using a given algorithm, the most commonly used being UPGMA and Neighbor-Joining. In the qualitative criteria, the differences between the molecules are considered as a series of variables discontinuous. The most commonly used methods, although they are not the only ones, are Maximum Parsimony (MP) and Maximum Likelihood (MV). The principle of MP is that the simplest hypothesis, i.e. the one in which the tree with the fewest steps (=fewest changes in character state) is chosen to explain a given set of data. Thus, there is no explicit evolutionary model underpinning this methodology (Lesk et al., 2008).

Operational problems can only be avoided

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by observing a very large number of equally more parsimonious trees or the excessive influence of homoplasies. However, the most critical question is philosophical: Is Nature parsimonious? MV is based on explicit evolutionary models of nucleotide substitution. These models are evaluated on their likelihood of explaining a set of data in a way that reflects the most credible evolutionary history. The model with the best likelihood value, which for operational reasons is given in logarithmic form, will be chosen as the basis for reconstructing the tree. For both MP and MV, there are two types of algorithm for finding the best tree: 1) Exhaustive Search, in which all possible topologies are checked; 2) Heuristic Search, in which only a subset of probabilistically most likely trees is examined. The latter, in practical terms, significantly reduces computational processing time which, in some cases, depending on the number of sequences compared, can last days or even months (NEI *et al*, 2000; MATIOLI, 2001).

OBJECTIVES

GENERAL OBJECTIVE

- Analyze the COX2 mitochondrial gene of the different industrial strains

SPECIFIC OBJECTIVES

- Analyze the evolutionary characteristics of the mitochondrial gene and its sequences of the different strains *of D. bruxellensis*.
- *Determine* whether polymorphisms occur between the sequences of *D. bruxellensis* in industrial sugarcane juice.
- Investigate whether *Dekkera* strains belong phylogenetically to the hemiascomycetes group.
- Construct primers for mitochondrial structural genes.

MATERIALS AND METHODS

LINEAGES AND MEDIA

Cell lines

The *D. bruxellensis* strains GDB237, GDB239, GDB240, GDB242, GDB248 and GDB251 were sourced from different alcohol distilleries in the north-east of Brazil. The strains CBS60, CBS739, CBS5512, CBS9919 CBS79, CBS74 were kindly provided by Prof. Desmond Clark-Walker of the Australian National University, Canberra, Australia.

Cell culture media

The cells were grown in YPD medium (2% glucose, 2% peptone, 1% yeast extract) for cell growth or in YPA (2% potassium acetate, 2% peptone, 1% yeast extract) to induce sporulation. This medium was used to increase the cell's mitochondrial DNA content, as it induces cellular respiration. The cells were pre-cultured in 20 ml of YPA medium for 24 h and these cultures were used to inoculate a further 180 ml of the same medium. The cultures were incubated for a further 24 h or until they reached 3 g of biomass.

GENETIC ANALYSIS

DNA extraction with mtDNA-enriched medium

The cells were inoculated in YPA medium and cultured for 48 hours at 30º C. After collection by centrifugation, the cells were incubated with 3 ml of protoplastization solution (20 mg. ml^{-1} of the lytic enzyme glucanex; 10 mg. ml^{-1} of BSA; 0.8 M KCl; 20 mM sodium citrate) for 3 hours at 30º C. The protoplasts were washed with solution 01 (1 M sorbitol, 50 mM citrate buffer pH 5.8) and resuspended in 2 ml of solution 02 (200 mM sucrose; 65 mM KCl; 20 mM EGTA; 10 mM $MgCl_2$; 50

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mM HEPES-KOH pH 7.2). Then 3 ml of solution 03 (20 mM sucrose; 65 mM KCl; 20 mM EDTA; 50 mM HEPES-KOH pH 7.2) and 1 ml of 10 U.ml DNAse A solution^{-1} were added to hydrolyze most of the nuclear DNA. After incubation for 3 hours at 75° C, 3 ml of solution 04 (10mM EDTA; 50mM Tris-HCl pH 8.2; 50µg.ml^{-1} proteinase K; 0.3% SDS) for lysis of the mitochondrial membrane, followed by extraction and purification of mitochondrial DNA using phenolic extraction with phenol:-chloroform:isoamyl alcohol (25:24:1). The DNA was precipitated with absolute ethanol at 4ºC. The precipitate was resuspended three times with 70% ethanol and resuspended in 30 µl TE buffer (10mM Tris-HCl ph 8.0, 1mM EDTA). The extracted DNA was analyzed by 1% agarose gel electrophoresis and its concentration was estimated by spectrophotometry and agarose gel with Hindλ marker where the DNA concentration was measured at 60 ng/µl.

Quantification and assessment of total DNA integrity

The DNA samples were quantified using a spectrophotometer. Quantification was carried out using the following conversion: one unit of absorbance at 260 nm represents 50 µg/ml of DNA. The samples were diluted (1:100) and the OD measured. To determine concentration and purity, the absorbances at 260 nm and 280 nm were observed. The ideal Abs260/Abs280 ratio should be close to two for pure DNA, with values between 1.9 and 2.1 being acceptable. To determine the integrity of the DNA samples, they were subjected to electrophoresis in a 1% agarose gel, using the appropriate running buffer. For the run, 4 µl of DNA and 1 µl of sample buffer were used. The gel was stained with ethidium bromide and visualized in a UV transilluminator. The efficiency of the method was tested using semi-quantitative PCR assays using the nuclear primer Db and the mitochondrial primer DbCOX2 designed using the Primer3 program (http://fokker.wi.mit.edu/primer3/input.htm). The amplification products were separated on a 0.8% agarose gel and visualized after staining with ethidium bromide.

***COX2* gene sequence analysis**

The sequences homologous to the *COX2* gene of *S. cerevisiae* were taken from GenBank. The sequences were aligned using CLUSTALX and consensus regions were identified where the primers were designed based on the consensus. The *primers* were designed using *Primer 3 software* (http://fokker.wi.mit.edu/primer3/input.htm). The following criteria were used to construct the *primers*: amplicon size of around 607pb, oligo size of between 20 and 25 nucleotides, GC content of between 20 and 80% and average Tm of 60° C. The reaction took place in 100µL tubes containing Taq DNA buffer polymerase (20mM Tris-HCl pH 8.0, 50mMKCl), 1.5mM MgCl2, 200µM of each dNTP, 10pmol of each primer oligonuceotide, 500ng of extracted DNA and 2.5 units of Taq DNA polymerase (Invitrogen). The reagents were mixed and heated at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C per 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds. The amplification was visualized by electrophoresis on a 1% agarose gel using a 1 kb Plus molecular weight standard (Invitrogen) for comparison (Sambrook and Russell, 2001). The amplification product was then purified using the PCR purification kit (High Pure PCR purification Kit - PROMEGA) and quantified by 1% agarose gel electrophoresis using the λ Hind III molecular weight marker. From the amplification of the GDB237 strain, the industrial strains GDB237, GDB239, GDB240, GDB242, GDB248 and GDB249 and the strains provided by Prof. Clark walker CBS60, CBS739, CBS5512, CBS9919 CBS79, CBS74 were extracted, purified and sequenced and subjected to sequence analysis.

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Sequencing reaction

The initial work was carried out using 2µl of Big dye in a total reaction volume of 10µl. A PCR reaction was then set up in the following proportions: 2µl of DNA, 3µl of milliQ water, 2µl of buffer, 1µl of 3.2 mM COX2 () forward primer and 2µl of BigDye. After the polymerase chain reaction, the plates (PCR-Optical 96 well reaction plate number N-801-0560) are removed from the thermal cycler and given 65% isopropanol. They are sealed with alcohol-resistant adhesive (Adhesive PCR Film N- 21950), mixed by inversion three times, then left to stand in the dark at room temperature for 15 min. They are then centrifuged for 45 min at 3400 rpm. The excess isopropanol is discarded and the plates inverted onto paper towels. 60 % ethanol is added and centrifuged at the same speed for 10 min. The excess ethanol is discarded again and the plates are inverted on the paper towel. To dry, the plates are spun at 300 rpm with the plates inverted on the paper towel and then sent to the oven at 37 ºC for 15 minutes. After drying, the plates receive 10 mL of formamide and are sent to the thermal cycler for the denaturation reaction. After this stage, the plates were sent to the ABI 3700 Prism Analyser automatic DNA sequencer.

Phylogenetic analysis of the *COX2* gene sequences of the different isolates.

The nucleotide sequences of the *COX2* gene were realigned using the ClustalW program. The pairwise sequence alignment distances were derived using the method described by Feng et al. (1985) and Feng and Doolitle (1990) who correct the composition of partial bases using the following relationship: $D = -\ln(S_{real} - S_{aoacaso}) / (_{Sidentic} - S_{aoacaso})*100$, where S_{real} is the score of the original alignments, $S_{aoacaso}$ is the score of random sequences of the same size and base composition and $_{Sidentic}$ is the average of the two sequences, each aligned with itself. The phylogenetic tree was built using the -neighbor-joining‖ method of Saito and Nei (1987).

Collection of gene sequences, construction of degenerate *primers* and amplification conditions

To build the degenerate primers, various topologies were analyzed based on the work of Woofit et al (2007), which lists the clades referring to the genera and species closest to *Dekkera bruxellensis* based on the analysis of nuclear genes. This made it possible to infer a physical map of the mitochondrial genome of this clade. Six species showing nuclear genomic similarity to *D. bruxellensis* were subjected to multiple alignments using the Mega v. 4.0 computer program (http://www.megasoftware.net) based on the following gene order in the *S. cereviseae* mitochondrial DNA genome: COX2, COX3, COX1, ATPase8, ATPase6, CYTb, ATPase9 and VAR1. The programs CODEHOP (http://bioinformatics.weizmann.ac.il) and Codon Usage (*http://www.bioinformatics.org/ sms2/codon_usage.html*) were used to refine the design of degenerate primers in order to amplify the orthologous genes of *D. bruxellensis*.

RESULTS

LINE SELECTION

All the strains showed good cell growth in YPA medium, in which acetate was the only carbon source. This means that *D. bruxellensis* can develop on a purely respiratory metabolism. However, strain GDB237 was selected for the continuation of the trials due to the better growth observed.

EVALUATION OF THE EXTRACTION AND AMPLIFICATION OF THE COX2 GENE

The protocol developed for extracting mitochondrial DNA showed a high yield due to the large amount of material obtained at the end of the process (Figure 1). The efficiency of the process was assessed using semi-quantitative PCR experiments, which showed that the extracted material was most amplified using the DbCOX2 primers, which amplify a 650 bp fragment related to the mitochondrial *COX2* gene (Figure 1), and least amplified using the Db primers, which amplify the nuclear gene that codes for 26S ribosomal RNA.

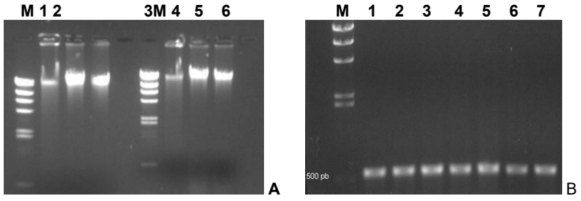


Figure 1. Optimization of the *D. bruxellensis* mitochondrial DNA extraction protocol. **A.** Agarose gel of the mitochondrial DNA samples respectively (GDB237, GDB239, GDB240, GDB242, GDB248, GDB249). **B.** Amplification of the mitochondrial *COX2* gene from mitochondrial DNA samples (GDB237). Line M: *λ-HindIII* marker.

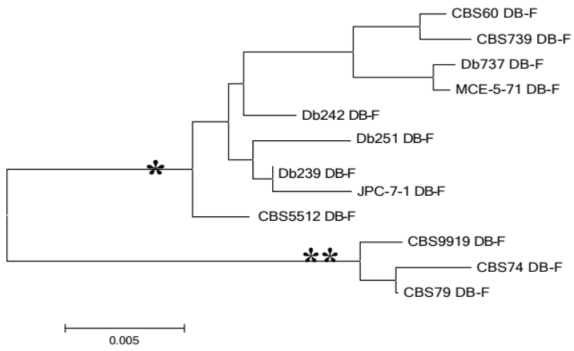
EVALUATION OF SEQUENCES

These isolates are interesting from the point of view of the evolution of the mitochondrial genome in *D. bruxellensis*, since previous studies by the group showed that they were consistently identified as *D. bruxellensis*, but had unique molecular markers in the mitochondrial genome due to intraspecific variation. These modifications translated into punctual changes in the amino acid sequence from Ile to Met, or vice versa (Figure 3). In addition to these unique changes, two unique changes in the use of codons can be seen in the DNA sequence (Figure 2): the first change at the 31st amino acid is evident in the sequenced industrial strains where an ACA-type codon differs from the *D. bruxellensis* and B custersii strains from Genbank in that it presents ACT ,, the second is found at the 65th amino acid where TTT is evident for the sequenced industrial strains and TTC in *D. bruxellensis* and *B. custersii.*

Of the total number of alterations, 18 were transitions and 30 were transversions. This result is satisfactory when compared to the mutability of mammalian mitochondrial DNA, in which the rate of transversions exceeds the rate of transitions by a ratio of 2:1.

PHYLOGENETIC ANALYSIS

When subjected to a clustering analysis, the *COX2* gene sequences of the industrial strains *of D. bruxellensis* grouped together in the *B. custersii* yeast clade (strain type CBS 5512) (Figure 5).



Phylogenetic analysis by the Neighbor Joining clustering method of the nucleotide sequences of the *COX2* gene of the industrial strains of *D. bruxellensis* compared with the collection strains of the *B. custersii* clade (*) and the *D. bruxellensis* clade (**).place booststrap and number of aligned base pairs

However, there is great genetic variability within this group. This suggests that the *COX2* gene is under great selective pressure where, in most cases, the substitution of Met residues (present in the type strain of *D. bruxellensis* CBS 74) for Ile residues are characteristic of *B. custersii*

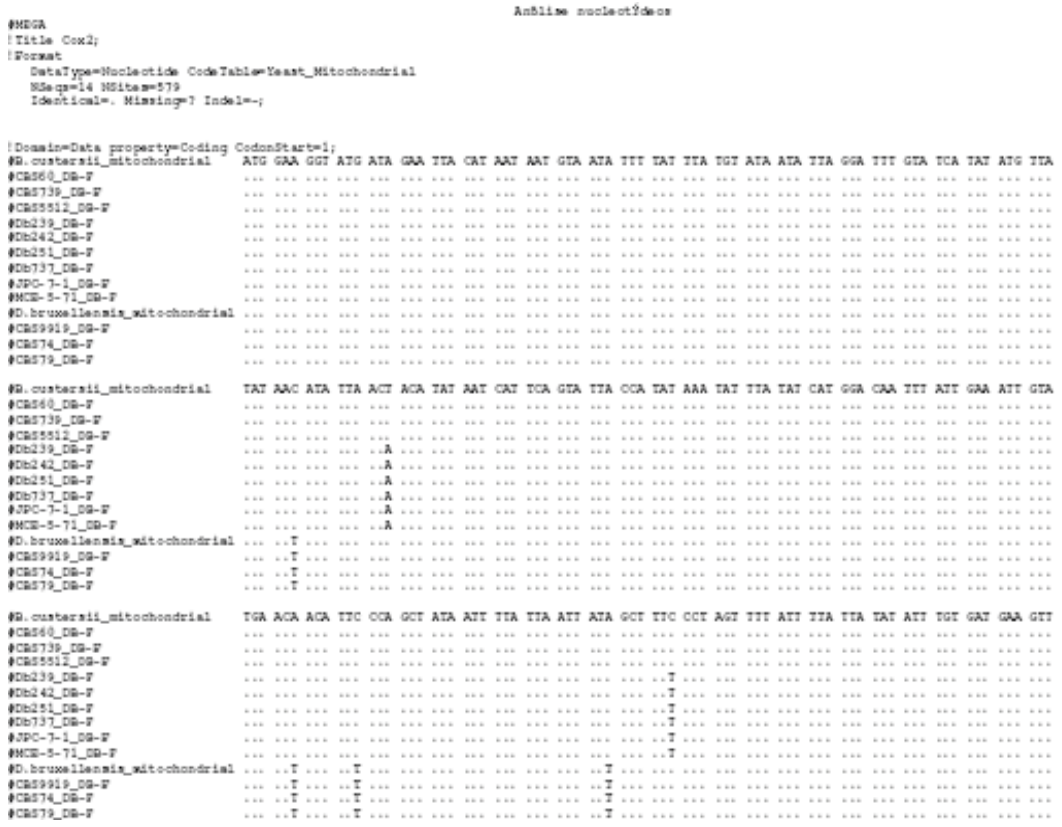


Figure 2. Multiple nucleotide sequence alignment of the *COX2* gene of industrial strains of *D. bruxellensis* and collection strains of *D. bruxellensis* and *B. custersii*.

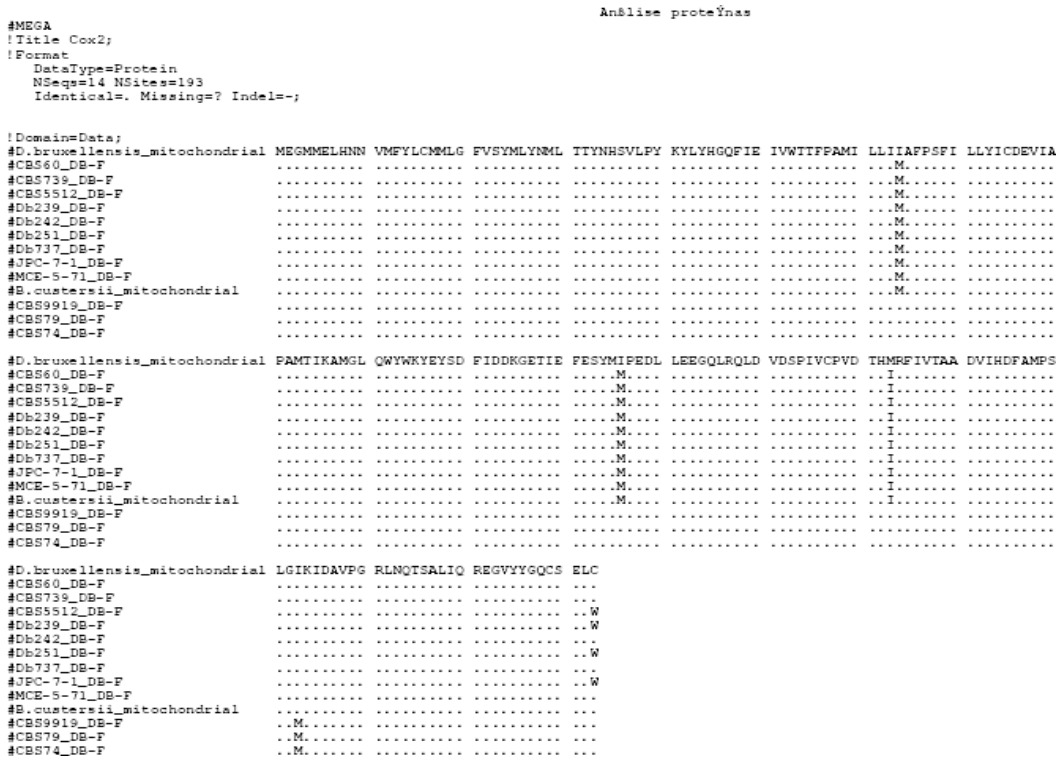


Figure 3. Multiple alignment of the amino acid sequence of the *COX2* gene of industrial strains of *D. bruxellensis* and collection strains of *D. bruxellensis* and *B. custersii*.

IN SILICO ANALYSIS OF THE MITOCHONDRIAL GENOME

Since the mitochondrial genome *of D. bruxellensis* has not been sequenced, the construction of primers for the amplification and cloning of its structural genes was carried out using computational tools through the MEGA4 program, using sequences deposited in the NCBI and Génolevures banks. The following genes were considered: *COX1*, *COX3*, *ATP6*, *ATP8*, *ATP9*, *CYTb* and *VAR1*, according to the discrimination for *S. cerevisiae*. The first analysis was carried out in order to assess the phylogenetic relationship between the mitochondrial genomes of the available ascomycetes. This made it possible to construct a physical map of the mitochondrial genome of this clade (Figures 6 to 12).

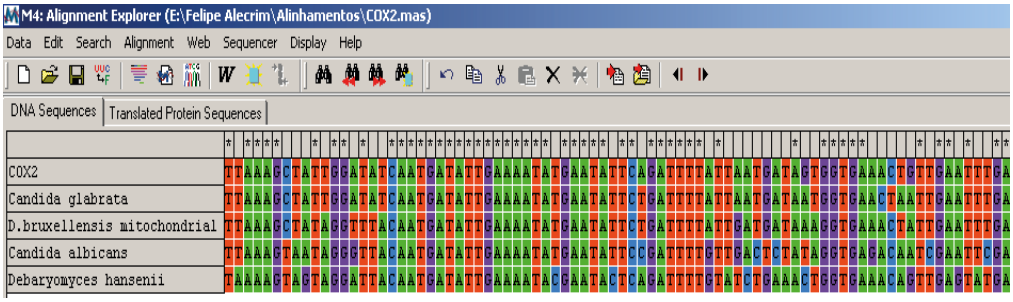


Figure 6. Multiple alignment of the *COX2* gene for ascomycetous yeasts.

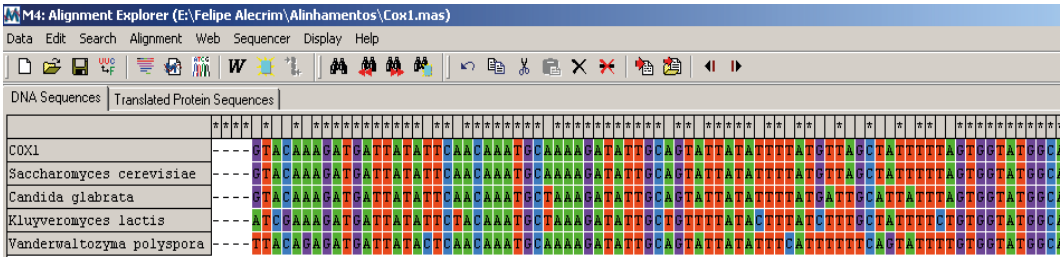


Figure 7. Multiple alignment of the *COX1* gene for ascomycetous yeasts.

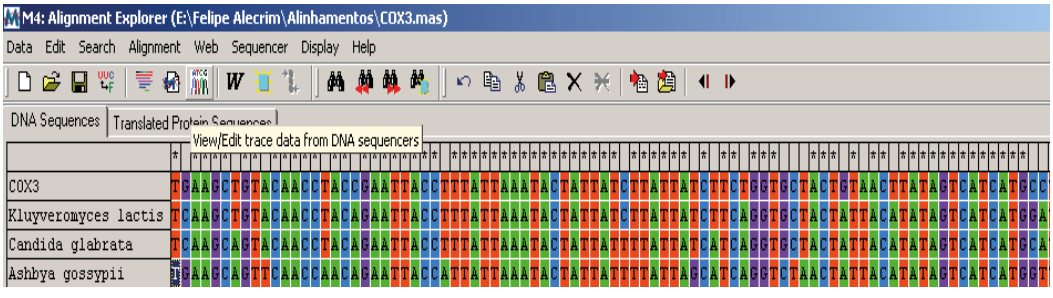


Figure 8. Multiple alignment of the *COX3* gene for ascomycetous yeasts.

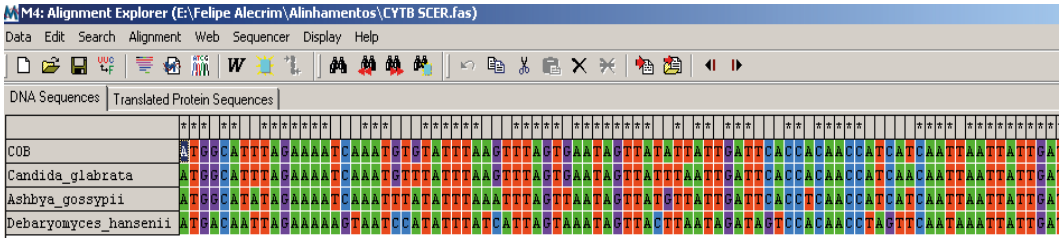


Figure 9. Multiple alignment of the *CYTb* gene for ascomycetous yeasts

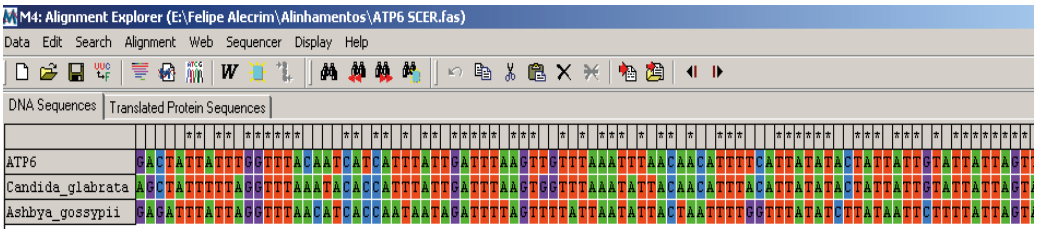


Figure 10. Multiple alignment of the *ATP6* gene for ascomycetous yeasts.

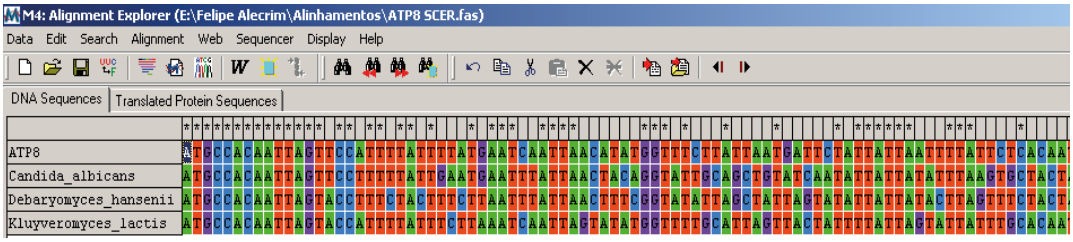


Figure 11. Multiple alignment of the *ATP8* gene for ascomycetous yeasts.

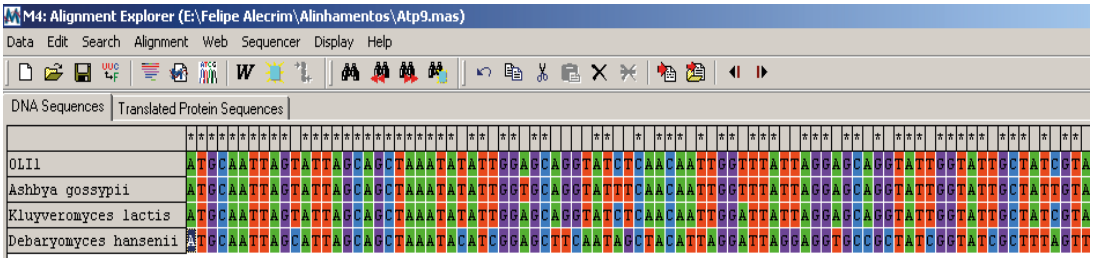


Figure 12. Multiple alignment of the *ATP9* gene for ascomycetous yeasts.

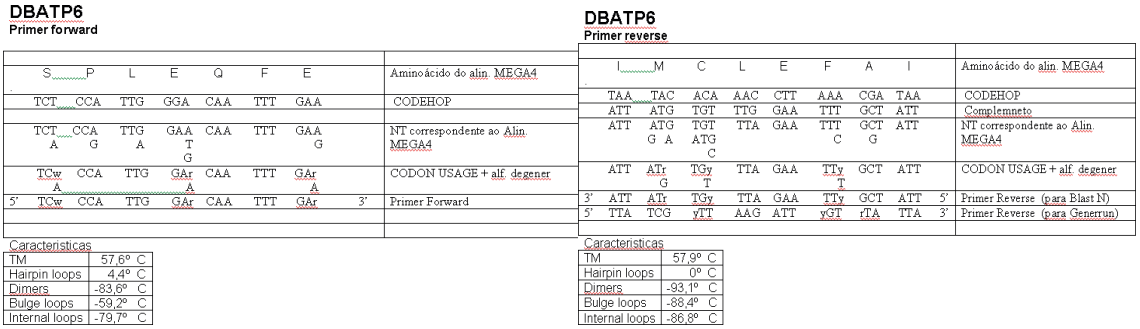


Figure 13. Analysis of the consensus sequences for the mitochondrial genes of ascomycetes with a view to constructing degenerate primers for amplification and sequencing of the orthologs in *D. bruxellensis* (example *ATP6* gene).

Primer name	Sequence (5'-3')		
cons-Cox3-FW	CAACAACATCCWTTYCATATRG	cons-ND1-RV	TAATTCWGATTCAGCTTCAAC
cons-Cox3-RV	ATRTATAARAATAATCARATWAC	cons-ND4-FW	CATACWTGATTACCWGTWGTWCA
cons-Atp6-FW	TCWCCATTAGARCAATTTG	cons-ND4-RV	TAAWCCTTGATAATAWAT
cons-Atp6-RV	AATAGCRAATTCTAACAWYATAAT	cons-CytB-FW	TTATTAGGWTTATGTTTAGTWA
cons-Cox1-FW	AGATGATTATAYTCWACAAATGC	cons-CytB-RV	TAAYACTTTRAAWGTATTWCCTCT
cons-Cox1-RV	CWGCWGGWGGWSWWGTTAA	cons-ND5-FW	GCTACWCGTAAAAGTGCWCAA
cons-ND1-FW	TTAATGGCTATWGCTGATG	cons-ND5-RV	CCWTATAATGATTCDATAATRATATCTTT

Table 1. Degenerate primer sequences for amplification and sequencing of orthologs in *D. bruxellensis*.

The results showed that the yeast *Debaryomyces hansenii* did not group with the ascomycetes studied, but with the sequences that, in principle, would serve as outgroups.

The CODEHOP and Codon Usage programs were used to refine the design of degenerate primers for amplifying the orthologous genes of *D. bruxellensis* (Figure 13). The alignments proved to be representative for the construction of primers, since good conservation was observed between the syntenic gene sequences of the aforementioned structural genes. These data provide a basis for future analysis of the genetics and evolution of population of *D. bruxellensis, which will serve as a* basis for establishing correlations between genetic variability and the physiological capacities of different strains. The primers were designed (Table 1) and ordered. The experiments to amplify the *D. bruxellensis* genes with the degenerate primers were partially successful, with 50% of the primers tested amplifying and being submitted for sequencing. Initially, tests are being carried out on the suitability of the PCR reaction, both in terms of the concentrations of the reaction reagents and the amplification program. So far, the *COX1* and *ATP6* genes have been amplified. These fragments will be cloned and sequenced.

DISCUSSION

The cell growth experiments revealed that glucose and sucrose are the most suitable carbon sources to support the growth of the yeast *D. bruxellensis*. Conterno *et al.* (2006) carried out physiological characterization of various strains of this yeast and found that these same carbon sources are more readily used by *D. bruxellensis*. However, Basílio *et al.* (2008) showed that sucrose consumption by *D. bruxellensis* cells is lower than that observed for *S. cerevisiae* cells. Therefore, this factor should not contribute to the better adaptation of *D. bruxellensis* to sugarcane juice must. On the other hand, cell cultivation experiments showed that *D. bruxellensis* grows better in a medium with potassium acetate as an alternative carbon source, providing a better growth rate than media containing glucose, glycerol and calcium acetate. As the YPA medium favors respiration, there are indications that this yeast, which has a positive Crabtree metabolism, is using the oxidative pathway and its genes to maintain itself in the vats even when sucrose is completely depleted by *Saccharomyces cerevisiae*.

From the extracted DNA, it was possible to amplify the *COX2* gene from all the samples. These fragments were subjected to nucleotide sequencing and the results showed intense variation with twelve changes identified. According to Hoeben *et al* (1993) these changes are in line with the high rate of evolution of the *COX2* gene in these species. Mitochondrial DNA evolves around five times faster than nuclear DNA (Cann and Wilson 1983). This intraspecific variation is due, firstly, to the fact that mitochondria are major generators of free radicals, providing a favorable environment for DNA mutations. Another cause is the absence of histones, which play a protective role in nuclear DNA (Yakes and Van Houten, 1997). In addition, the mitochondrial DNA polymerase enzyme has low corrective activity when compared to nuclear DNA polymerase (Kunkel and Loeb, 1981) and nucleotide excision-dependent DNA repair is not present in mitochondria (Croteau *et al.* 1999). Based on this fact, of the twelve changes shown in the DNA sequences, only the second and sixth changes deserve attention. There are base splits in the industrial strains that are unique and cannot be compared to either *D. bruxellensis* or *B. custersii*. The ACA and TTT splits in the industrial strains are differ from ACT and TTC of the *D. bruxellensis* and *B. curstesii* strains. Of the total number of alterations, 18 were transitions and 30 were transversions.

In comparison, the amino acid sequence, translated with the universal code using the Mega 4.0 program, showed that the industrial strains identify more with *B. curstesii* than with *D. bruxellensis* as the predominant yeast in sugarcane juice distilleries. Liberal *et al*. (2005) described these industrial yeasts as *D. bruxellensis using specific primers*, but their study shows that changes in the amino acid sequence of the *COX2* gene show that they are more similar to *B. curstesii*. According to Hoeben *et al* (1993) *Dekkera bruxellensis* encodes ATA for isoleucine in the universal code, but the industrial strains encode ATT methionine, showing the same codon in the *B. custersis* species and vice versa. Clark-Walker *et al* (1992) state that there is a great similarity between *D.bruxellensis* and *B.curstesii,* which is emphasized by the identity of the amino acid sequences despite the DNA sequences in which only 13 punctual alterations were located. However, these changes always occur in the third position, which supports the theory seen in the work by Jupeng *et al* (1996) that separates these two species into different clades, demonstrating that they are phylogenetically different species, which leads us to believe that the industrial strains are *B. custersii* and not *D. bruxellensis*

This type of identification using DNA and amino acid sequences has already proved to be useful. Gerdini et al (2000) typified pathogenic *Candida glabrata* species by comparing the DNA and amino acid sequences of different species and strains, thus demonstrating the efficacy of the method using the cytochrome oxidase two gene.

The phylogenetic analysis of the *COX2* gene was accurate in terms of the suspicion that the industrial strains are grouped close to *B. custersii* and not *Dekkera bruxellensis*, however, as described by Hoeben et al (1986), this gene is under great selective pressure and the mutation rate is high, generating controversy over the certainty of the classification.

Despite its importance, *D. bruxellensis* is still poorly characterized genetically. In fact, Woolfit *et al.* (2007) pioneered the genetic study of this yeast, providing much of the data used for current research into the genetic basis of its physiological capabilities. These researchers carried out a partial sequencing that covered approximately 40% of the *D. bruxellensis* genome, identifying around 3000 genes. Among of these, it is interesting to note the presence of genes involved only with nuclear genomes, since *petite* strains were used by Professor Piskur's group. In order to elucidate this issue, degenerate *primers* were synthesized to perform a phylogeny by concatenating structural genes. This analysis was carried out -in silico‖, generating seven pairs of primers

In contrast to the work of Woolfit et al, 2007, in which only mutants without mtDNA were used, this work is relevant to the present work in that it provides guidance in determining the consensus sequences for the different oxidative metabolism genes. Based on the *Saccharomyces cereviseae* genome, the gene order was defined as L-rRNA COII COIII S-rRNA COI ATPase 8 ATPase 6 Cyt b ATPase 9 Var 1, using data from Genbankaromyces *cereviseae*. The programs CODEHOP *(*http://bioinformatics.weizmann.ac.il) and Codon Usage *(*http://www.bioinformatics.org/sms2/codon_usage.html) were used to refine the design of degenerate primers for amplifying the orthologous genes of *D. bruxellensis*. Kurtzein et al, (2007) concatenated genes through -in silico‖ analysis for the group of hemiascomycetes, defining *Dekkera bruxellensis* as one of the hemiascomycetes. Of the primers tested, 50% amplified with the number of base pairs predicted by the CAP3 program, but they were not sequenced or cloned, and it is hoped that these primers will be used in future research to elucidate the mitochondrial genome of this yeast.

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From studies of these concatenated genes it will be possible to have precise information on the phylogenetic position of the industrial species, *although in the study carried out the degree of identity with B custerssii is much greater than in D. bruxellensis.* The only two specific changes in the industrial strains, which are in the third base of the 31st amino acid and the third base of the 62nd amino acid, identify a certain polymorphism in this gene. However, the rate of identical bases that align to a degree of similarity means that the predicted industrial strains are classified as B. custersii.

CONCLUSION

- The mtDNA extraction protocol process favored respiration and, consequently, obtaining the COXII gene sequence.
- The analysis of the DNA sequences was significant in terms of identifying new cracks from the industrial strains.
- Amino acid analysis confirms that the industrial strains resemble *B. custersii.*
- The synthesis of primers is beneficial in terms of serving as a basis for future studies.
- Therefore, analysis of the COXII gene showed that the industrial strains known as *D.bruxellensis* in sugarcane juice distilleries are more similar to *B. custersii.*

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