Ensaios e Ciência
ISSN 1415-6938 e ISSN 2178-695X

DOI: 10.17921/1415-6938.2025v29n3p582-594

Morphological and Molecular Identification of Yeasts Isolated from Brazilian Savannah Fruits Submitted to Concomitant Stress in Sugarcane Juice

Identificação Morfológica e Molecular de Leveduras Isoladas de Frutos da Savana Brasileira Sob Estresse Concomitante em Suco de Cana-De-Açúcar

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Received on: Aug 20, 2024
Accepted on: Mar 7, 2025

Abstract

In a biotechnological system, the precise species identification is paramount for an efficient process. In industrial conditions, fermentation is conducted without aseptic, and consequently, the process is subject to constant contamination by bacteria and yeast strains, including wild species of *Saccharomyces cerevisiae*. The characterization and identification of yeast species have been based on morphology and physiological characteristics. However, these laborious techniques often leave room for doubt. Currently, molecular biology techniques allow the rapid identification of yeast species involved in the fermentation process efficiently and economically. The bioprospection of yeast more resistant to stress conditions may reveal strains with good potential for use. However, correct isolate molecular and morphological characterization is required. The present study aimed to characterize five yeasts isolated from Brazilian savannah fruit extracts on a morphological and molecular level. Morphological analysis of the yeasts L32, L34, L36, L38, and B32 considered factors such as colony size, color, surface, and edges. This characterization was complemented with molecular identification performed using the PCR and PCR/RFLP. The results show predominantly smooth and shiny colonies. Analysis of the amplification of ribosomal DNA fragments, after digestion with the enzymes *Hae III* and *Hinf I*, resulted in bands corresponding to the profile of S. cerevisiae. With these results, it was possible to conclude that the method of yeast isolation by pressure of simultaneous stresses allows the selection of the *S. cerevisiae* yeasts.

Keywords: Selective Isolation. PCR. PCR/RFLP. Ribosomal DNA. Colonies.

Resumo

Em um sistema biotecnológico, a identificação precisa das espécies é de suma importância para um processo eficiente. Em condições industriais, a fermentação é conduzida sem assepsiar e, consequentemente, o processo está sujeito a constante contaminação por cepas de bactérias e leveduras, incluindo espécies silvestres de *Saccharomyces cerevisiae*. A caracterização e identificação de espécies de leveduras tem sido baseada na morfologia e em características fisiológicas. No entanto, estas técnicas laboriosas muitas vezes deixam margem para dúvidas. Atualmente, a utilização de técnicas de biologia molecular permite a rápida identificação das espécies de leveduras envolvidas no processo fermentativo de forma eficiente e econômica. A bioprospecção de leveduras mais resistentes a condições de estresse poderá revelar cepas com bom potencial de utilização. Contudo, é necessária uma correta caracterização molecular e morfológica do isolado. O objetivo do presente estudo foi caracterizar cinco leveduras isoladas de extratos de frutos do cerrado brasileiro em nível morfológico e molecular. A análise morfológica das leveduras L32, L34, L36, L38 e B32 levou em consideração fatores como tamanho das colônias, cor, superfície e bordas. Esta caracterização foi complementada com identificação molecular realizada por PCR e PCR/RFLP. Os resultados mostram colônias predominantemente lisas e brilhantes. A análise da amplificação dos fragmentos de DNA ribossômico, após digestão com as enzimas *Hae III* e *Hinf I*, resultou em bandas correspondentes ao perfil de *S. cerevisiae*. Com estes resultados foi possível concluir que o método de isolamento de leveduras por pressão de estresses simultâneos permite selecionar as leveduras *S. cerevisiae*.

Palavras-chave: Isolamento Seletivo. PCR. PCR/RFLP. DNA ribossômico. Colônias.

1 Introduction

Yeasts are unicellular microorganisms with essential applications in biotechnological processes, particularly in ethanol production. These microorganisms are widely distributed in nature. Studies aiming to isolate and identify yeasts have been conducted in several countries to search for new species and strains that may have desirable characteristics for optimal fermentation processes (López-Arboleda *et al*., 2010; Parapouli *et al*., 2020; Voidarou *et al*., 2021).

Yeasts ferment glucose, maltose, and fructose in preference to other monosaccharides, disaccharides, and disaccharides (Parapouli *et al.*, 2020). Monosaccharides, such as glucose and fructose, are more abundant in flowers, fruits, seeds, and the juice of certain plants (Santos *et al*., 2016), making these substrates a natural habitat for yeasts (Marçal, 2005; Li *et al*., 2018; Zaman *et al*., 2008).

The isolation and correct identification of yeasts is crucial for understanding the enzymatic reactions that may occur during the early fermentation stages (Amoikon *et al.*, 2018). The fermentation process may start with the growth of various species of yeast belonging to the genera *Candida, Debaryomyces, Hanseniaspora, Hansenula, Kloeckera, Metschnikowia, Pichia, Schizosaccharomyces, Torulaspora* and *Zygosaccharomyces* (Esteve-Zarzoso *et al*., 1999).

Classical methods classify yeasts based on morphological and physiological characteristics and biochemical properties (Baleiras; Van Der Vossen; Hofstra, 1994; Barnet; Payne; Yarrow, 1990; Parapouli *et al.*, 2020). However, these methods present some drawbacks, such as using various tests to obtain an accurate identification at the species level. In addition, they are very laborious techniques that require a long process to obtain the result, as well as considerable analyst's expertise to identify varieties of the same species since they can undergo morphological changes according to the environment and development stage (Blanco *et al*., 2006; Blanco-Piñón *et al.*, 2014; Esteve-Zarzoso *et al.*, 1999; Kuthan *et al*., 2003; Sun; Gresham, 2021).

Yeast cells can be spherical, oval, elliptical, or elongated. Yeast morphology is not indicative of the species. Pleomorphism may not be considered a form of contamination. In general, yeast cells are larger than bacteria cells, exhibiting considerable variation in their dimensions.

Yeast cells may range from 1 to 5 µm in width and 5 to 30 µm in length. Each species has a characteristic shape, although even in pure cultures, there are considerable variations in size and shape depending on the cells age and the environment in which they develop (Ceccato-Antonini, 2010; Kukhtevich *et al*., 2020; Zakhartsev; Reuss, 2018).

Methods based on molecular techniques have been developed to enable quick and efficient identification of the yeast species involved in the fermentation processes (González-Alonso *et al*., 2021; Tristezza *et al.*, 2014). The following are the most commonly used methods to differentiate species and strains of yeasts: molecular karyotyping using pulsed field gel electrophoresis (PFGE), based on polymorphism of the chromosomal DNA (Schwartz; Cantor 1984; Vezinhet; Blondin; Hallet, 1990), restriction fragment length polymorphism analysis of mitochondrial DNA (RFLP-mtDNA) (Guillamón; Barrio; Huerta, 1994; 1996; López *et al.*, 2001; Querol; Barrios; Ramo´N, 1992), polymerase chain reaction (PCR), amplification of the ITS1 and ITS2 5.8S regions and digestion with restriction endonucleases (PCR-RFLP of ITS) (Bockelmann et al., 2008; Esteve-Zarzoso *et al.*, 1999;), analyzes of random amplified polymorphic DNA (RAPD) (Walczak; Czaplinska; Barszczewski, 2007), gene sequencing, amplification of repetitive sequences (SSRs) and microsatellite (Querol Barrio; Ramón 1994; Vaudano; Garcia-Moruno, 2008), real-time PCR and flow cytometry (Martorell; Querol; Fernández-Espinar, 2005; Hierro *et al.*, 2007; Andorra *et al.*, 2011).

Molecular techniques associated with phenotypic methods are powerful tools in the identification and monitoring of the yeasts population dynamics during the fermentation process (González-Alonso *et al.*, 2021). The present study aimed to characterize yeasts on a molecular level,

isolated from Brazilian savannah fruit extracts and subjected to the simultaneous stress of temperature, acidity, alcohol content, and osmotic pressure for 72 hours.

2 Material and Methods

The present study was conducted in the Microbiology and Molecular Biology laboratories of Universidade Católica Dom Bosco (UCDB), Campo Grande, Mato Grosso do Sul, Brazil. The fruit used were lobeira (*Solanum lycocarpum* St. Hil), baru (*Dipteryx alata* Vogel), bocaiúva (*Acrocomia crassiflora* Mart), inga (*Inga alba*), araticum (*Annona crassiflora* Mart) and guavira (*Campomanesia cambessedeana* Berg).

All fruits were collected in the Brazilian savannah near Campo Grande County, MS, at latitude 20º24'406' South, longitude 54°34'004'' West, and an altitude of 654 m. The fruits were collected at their maximal physiological maturity, according to their annual schedule and availability. Certain fruits at a high maturation stage were collected in the soil or on the plants. The collection periods of each fruit were as follows: araticum (March), bocaiúva (July), baru and inga (September), lobeira (October), and guavira (November).

2.1 Isolation and characterization of yeast strains

Only five yeast strains resisted all the stress conditions (pH 3, osmotic pressure 20ºB, 8% alcohol content, and temperature ranging from 32°C to 38°C) (Oliveira *et al*., 2012). Four strains were derived from the lobeira fruit (*Solanum lycocarpum*) and received L32, L34, L36, and L38 designations. One strain was obtained from the baru fruit (*Dipteryx alata)* and received the designation $B_{32.}$

2.2 Characterization of the yeast strains

The strains were grown at 30 ºC for 48 hours in a Potato Dextrose Agar (PDA) culture medium containing ampicillin (500 mg/L). With the aid of a colony counter (Phoenix CP 600), morphological characterization was performed according to the colonies size, color, surface, and edges.

2.3 Genomic DNA extraction

In addition to genomic DNA extraction from five strains of isolated yeasts, DNA extraction was performed with the yeasts PE2 (Pedra 2) and *Saccharomyces cerevis*iae (CEFAR CCCD-SS001) as a positive control.

Firstly, the samples were incubated on PDA at 30 °C for 48h. Then, they were washed twice with 200 uL of phosphate buffered (137mM NaCl, 2.7mM KCl, 4.7mM Na_2HPO_4 x $7H_2O$, 1.5mM KH_2PO_4) pH 7.2 at 4 ºC. Then, 50 µL of proteinase K (20 mg/mL), 200 µL of sodium dodecyl sulfate

20% (SDS), 800 µL of chloroform, and 400 µL of protein precipitation solution (3M $C_2H_3KO_2$, 2M CH_3COOH) were added. The samples were centrifuged at 14000 xg for 10 minutes.

The supernatants were collected and treated with 1 mL of absolute ethanol. After a new centrifugation step at 14000 xg for 5 minutes, the supernatant was discarded, and 1 mL of 70% ethanol was added to the formed pellet. The samples were centrifuged at 14000 xg for 3 minutes, and the supernatant was discarded again. The pellets were dried at room temperature, suspended in 100-150 µL of ultrapure water, and incubated at 65 ºC for 5 minutes. The samples were stored at -20 ºC until use.

2.4 Molecular identification

The molecular characterization of the yeast strains selected under concomitant stress was performed using polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP). The fragments amplification of the ITS1, ITS2 5.8S ribosomal DNA was performed as described by Esteve-Zarzoso *et al*. (1999), using the oligonucleotides ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*. 1990). Specific amplification of *S. cerevisiae* DNA was carried out using the primers SC1d (5-ACATATGAAGTATGTTTCTATATAACGGGTG-3) and SC1r (5-TGGTGCTGGTGCGGATCTA-3), based on the conserved region of the large subunit ribosomal 26S (Martorell; Querol; Fernández-Espinar, 2005).

The amplification reactions (both ITS and 26S) were performed using 0.5 µl of genomic DNA (approximately 100 ηg/µL) in 49.5 µL of PCR mix containing 0.5 µM of each oligonucleotide, 10µM dNTPs, 1X Taq buffer, 1.5 mM $MgCl_2$, and 0.5 U of Taq DNA polymerase. The reactions were performed in an automated thermal cycler (MJ Research, USA) under the following conditions: denaturation at 94 °C for 31 minutes, 35 cycles of denaturation at 94 °C for 1 minute, annealing at 52 °C for 45 seconds (ITS) and 60 °C (26S) and extension at 72°C for 1 minute, followed by a final extension step at 72 °C for 10 minutes.

The amplified PCR products were digested with the restriction endonucleases *Hae* III (Sigma) and *Hinf* I (Invitrogen), following the manufacturer's instructions. PCR products and restriction fragments were analyzed using 2% agarose gels in 1X TBE buffer (Tris borate EDTA). The gels were stained with ethidium bromide and visualized under ultraviolet light. The amplicon sizes were estimated by comparison with a base pair ladder of 50-1000 bp (Invitrogen) with the aid of Alfa DigiDOC software. The restriction patterns obtained were compared to the wild yeasts PE 2 and *Saccharomyces cerevisiae* (CEFAR CCCD-SS001), used as positive controls for the molecular

analysis.

2.5 Sequencing

Eight samples showing an amplified fragment of approximately 300 bp were selected for sequencing reactions. The amplicons were purified using the Qiaex II gel extraction kit (Qiagen), quantified by comparison with standard Low mass (Invitrogen), and sequenced using the Sanger method and the automatic sequencer ABI 3130xl (Applied Biosystems®) in the laboratory of Biotechnology at Universidade Católica de Brasília (UCB).

The obtained sequences were analyzed using PHRED, PHRAP, and Bioedit 5.0.9 software to assess the sequence quality, perform alignment, and create the consensus sequence. Homology analysis was performed using BLASTn (Basic Local Alignment Search Tool—NCBI), which sought homologous sequences in the GenBank.

The genetic fragment of the large subunit ribosomal 26S from strains L_{32}, L_{34}, L_{36}, and L_{38} (*lobeira* fruit), and B_{32} (*baru* fruit) was aligned to the sequences of the same gene in *S. cerevisiae* (GenBank accession numbers gi329136676, gbU00027, gi259146797), as well as other yeast species (*Pichia kidriaziizewii* 146) O (accession number g1422778941) and *Aspergillus oryzae* (accession number gi309754740) as an out-group. The alignment was initially performed with the aid of CLUSTAL W software and subsequently corrected manually using Bioedit 5.0.9. The size of the sequences used in the phylogenetic analysis ranged from 235 bp to 290 bp, with a total of 107 variable sites in 290 aligned positions, using only the sequences obtained in the present study.

Comentado [U1]: O que gostaria de dizer nesse trecho por favor?

Comentado [JBdS2R1]: como um grupo externo

The phylogenetic tree was inferred by the maximum likelihood method (ML), based on the Tamura-Nei model using the MEGA 5 program (Tamura *et al*. 2011). Bootstrap resampling (1000 replicates) was performed to statistically support the reliabilities of the nodes on the trees.

3 Results and Discussion

The colonies morphology of the isolated strains was characterized. All five strains showed colonies with a similar morphology (Table 1 and Figure 1). Even under the same medium and cultivation conditions, there was variation in the size of the colonies (<1mm to 3mm). However, all yeast strains exhibited a white smooth and shiny surface for the colonies and edge of the colonies.

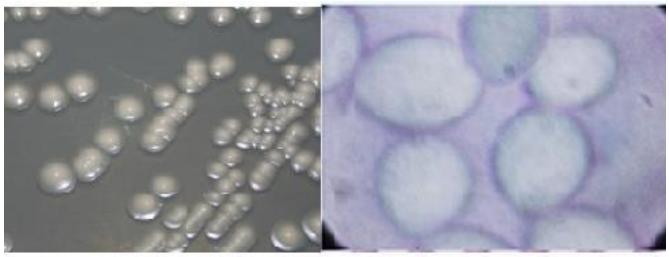
All yeast colonies were convex and had satisfactory growth in the PDA culture medium. Similar results were found by Pradeep et al. (2013) when analyzing the Influence of Culture Media on Growth and Pigment Production by Single *Fusarium* KUMBF1201 Isolated from Paddy Field Soil.

Table 1 - Morphology of yeast strains colonies isolated from samples grown in solid PDA, using 500 mg/l ampicillin

	Strains				
Description	**L_{32}**	**L_{34}**	**L_{36}**	**L_{38}**	**B_{32}**
Diameter (mm)	<1mm	1-2mm	1-2mm	< 1mm	1-3 mm
Texture	shiny	shiny	shiny	shiny	shiny
Color	white	white	white	white	white
Surface	smooth	smooth	smooth	smooth	smooth
Edge	smooth	smooth	smooth	smooth	smooth
Raise	convex	convex	convex	convex	convex

Source: research data.

Figure 1 - Morphological aspects of yeasts isolated from Potato Dextrose Agar – A) Smooth colonies; B) Yeast cells (magnification 1000x)



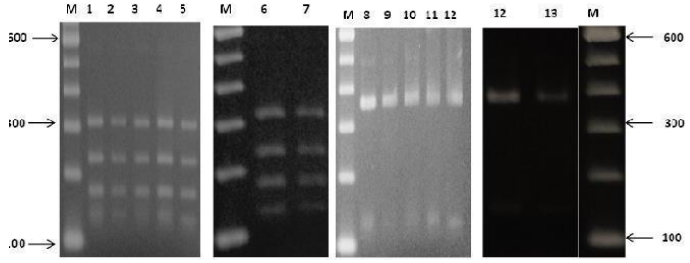
Source: research data.

All the strains isolated (L32, L34, L36, L38 and B32), including standard strains (PE 2 and *S. cerevisiae* ATCC), exhibited an amplicon of 850 bp.

The amplicon size observed is characteristic of the species *S. cerevisiae.* Thus, it was possible to confirm that all yeast isolates belonged to the genus *Saccharomyces*. The yeast contaminants, most commonly found in ethanol production processes, exhibited ITS fragments of 400 bp (*Candida intermedia* and *C. lusitaniae*), 450 bp (*C. pararugosa*), 480 bp (*C. xylopsoci*), 540 bp (*Pichia fermentans*), 650 bp (*P. caribbica* and *P. anomola*), and 850 bp (*S. cerevisiae*), making it possible to perform preliminary characterization of yeasts through this technique (Basílio *et al.*, 2008).

Enzymatic digestion was also performed with the amplified ITS fragments of the yeast strains isolated in the present study, revealing a restriction profile compatible with *S. cerevisiae* (Table 2 and Figure 2).

Figure 2 - Electrophoresis of PCR products of ITS1-ITS4 (2% agarose gel). Fragments were obtained after enzymatic digestion with *Hae* III (Lines 1-7) and *Hinf* I (Lines 8-13). Line M: Molecular weight 100 bp (Invitrogen TM), Lines 1 and 8: L32, Lines 2 and 9: L34, Lines 3 and 10: L36, Lines 4 and 11: L38, Lines 5 and 12: B32, Lines 6 and 7: PE-2, Lines 7 and 13: *S. cerevisiae* (CEFAR CCCD-SS001)



Source: research data.

Table 2 - Size of the PCR products and restriction fragments obtained from yeast strains

Strain	PCR (ITS)	*Hinf* I	*Hae* III
L_{32}	850	370+370+125	320+230+180+150
L_{34}	850	370+370+125	320+230+180+150
L_{36}	850	370+370+125	320+230+180+150
L_{38}	850	370+370+125	320+230+180+150
B_{32}	850	370+370+125	320+230+180+150
PE 2	850	370+370+125	320+230+180+150
S. cerevisiae	850	370+370+125	320+230+180+150

Source: research data.

The enzymatic digestion of amplified fragments may ensure more information when it is required to differentiate species (Combina *et al*., 2008; Granchi; Bosco; Messini, 1999; Mccullough *et al*., 1998; Redzepovic; Orlic; Sikora, 2002). Esteve-Zarzoso *et al.* (1999) successfully identified and differentiated several yeasts using PCR-RFLP/ITS analysis and the restriction enzymes *Hinf* I and *Hae* III, including species of the *Saccharomyces sensu stricto* complex.

Granchi *et al.* (1999) studied the yeast populations present in wine fermentation and reported that the RFLP-PCR/ITS technique was more efficient than other traditional methods, enabling the monitoring of changes in the yeast community throughout the fermentation process. However,

Comentado [U3]: Preciso deste trecho em português, por favor.

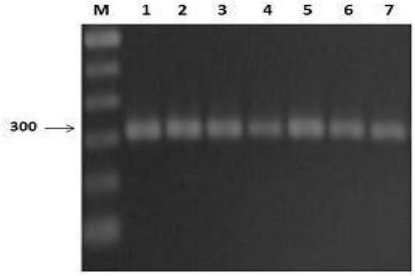
Comentado [JBdS4R3]: A digestão enzimática de fragmentos amplificados pode garantir mais informações quando necessário para diferenciar espécies (Mccullough; Clemons; Mccusker, 1998; Granchi; Bosco; Messini, 1999; Redzepovic; Orlic; Sikora, 2002; Combina et al., 2008). Esteve-Zarzoso et al. (1999) identificaram e diferenciaram com sucesso diversas leveduras usando análise de PCR-RFLP/ITS e as enzimas de restrição Hinf I e Hae III, incluindo espécies do complexo Saccharomyces sensu stricto.

Comentado [U5R3]: Está perfeito, agora entendi, faz sentido, obrigado.

Mccullough *et al*. (1998) failed to differentiate strains of *S. boulardii* from isolates of *S. cerevisiae* despite using ten different restriction enzymes.

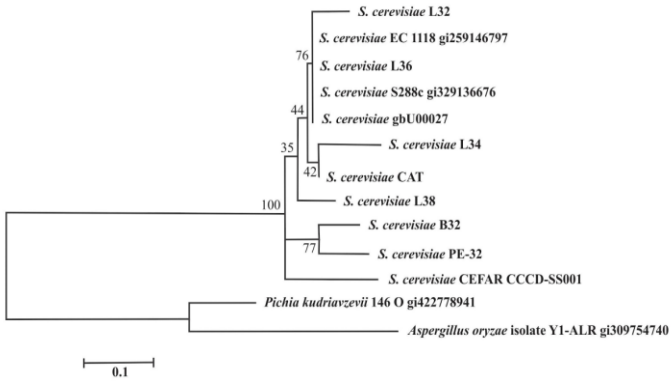
In the present study, PCR was also performed using primers that amplify a fragment of approximately 301 bp, specifically for *S. cerevisiae* (Martorell; Querol; Fernández-Espinar, 2005). This assessment showed that all the yeast strains exhibited a fragment based on the expected result (Figure 3), confirming the identification of *S. cerevisiae*.

Figure 3 - Electrophoresis of PCR products (2% agarose gel) - Line M: Molecular weight 100 bp (Invitrogen TM), Line 1: L32, Line 2: L34, Line 3: L36, Line 4: L38, Line 5: B32, Line 6: PE-2, Line 7: *S. cerevisiae*



Source: research data.

Figure 4 - Phylogeny of *Saccharomyces cerevisiae* using the 26S gene and the maximum-likelihood method with bootstrap resampling of 1000 replicates



Source: research data.

The yeast strains DNA sequencing resulted in sequences of approximately 235 bp, exhibiting 99% identity with the 26S gene of *S. cerevisiae* (best hits e-value 7e-117) and corresponding to

position 443619-443845 in the genome of *S. cerevisiae* S288c chromosome VIII (GenBank accession number 329136676). The phylogenetic tree constructed with 11 sequences of *Saccharomyces*, based on the 26S gene.

In the phylogenetic tree (Figure 4), it is possible to observe that, after separating *Pichia kidriaziizewii* 146 and *Aspergillus oryzae*, the other yeasts gathered in a single statistically defined group (100% of the topologies generated by bootstrap analysis). Although the differences in the topology did not exhibit statistical sustainability, the formation of a group with the isolates L32, L34 and the species *S. cerevisiae* was notable (GenBank access numbers gi329136676, gbU00027 and gi259146797). Concerning the remainder of the division, L34 and CAT were observed, followed by a branch with L38, and finally, the formation of a statistically sustainable group containing Baru (B32), PE-2 (70% bootstrap), and one branch with *S. cerevisiae* (CEFAR CCCD-SS001).

The phylogenetic hypothesis proposed, with the tree constructed by the maximum likelihood method analyzing the branches' size, reflects the nucleotide substitutions rate in each sequence. In other words, larger branches indicated greater numbers of nucleotide substitutions. Short branches were generated in the present study (Figure 4) and there were no significant differences among the yeast isolates, thus demonstrating that the region of the 26S gene is highly conserved.

4 Conclusion

Based on the results of the present study, it may be stated that the isolating yeast method by pressure of simultaneous stresses allows the selection of *S. cerevisiae* yeasts.

Acknowledgements
The authors would like to thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support through the granting of a scholarship (Process 551372/2010-9) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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