Phylogenetic Analysis of Phenotypically Characterized Cryptococcus laurentii Isolates Reveals High Frequency of Cryptic Species

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Abstract

Background: Although Cryptococcus laurentii has been considered saprophytic and its taxonomy is still being described, several cases of human infections have already reported. This study aimed to evaluate molecular aspects of C. laurentii isolates from Brazil, Botswana, Canada, and the United States.

Methods: In this study, 100 phenotypically identified C. laurentii isolates were evaluated by sequencing the 18S nuclear ribosomal small subunit rRNA gene (18S-SSU), D1/D2 region of 28S nuclear ribosomal large subunit RNA gene (28S-LSU), and the internal transcribed spacer (ITS) of the ribosomal region.

Results: BLAST searches using 550-bp, 650-bp, and 550-bp sequenced amplicons obtained from the 18S-SSU, 28S-LSU, and the ITS region led to the identification of 75 C. laurentii strains that shared 99–100% identity with C. laurentii CBS 139. A total of nine isolates shared 99% identity with both Bullera sp. VY-68 and C. laurentii RY1. One isolate shared 99% identity with Cryptococcus rajasthanensis CBS 10406, and eight isolates shared 100% identity with Cryptococcus sp. APSS 862 according to the 28S-LSU and ITS regions and designated as Cryptococcus aspenensis sp. nov. (CBS 13867). While 16 isolates shared 99% identity with Cryptococcus flavescens CBS 942 according to the 18S-SSU sequence, only six were confirmed using the 28S-LSU and ITS region sequences. The remaining 10 shared 99% identity with Cryptococcus terrestris CBS 10810, which was recently described in Brazil. Through concatenated sequence analyses, seven sequence types in C. laurentii, three in C. flavescens, one in C. terrestris, and one in the C. aspenensis sp. nov. were identified.

Conclusions: Sequencing permitted the characterization of 75% of the environmental C. laurentii isolates from different geographical areas and the identification of seven haplotypes of this species. Among sequenced regions, the increased variability of the ITS region in comparison to the 18S-SSU and 28S-LSU sequences reinforces its applicability as a DNA barcode.

Introduction

The Cryptococcus genus includes more than 100 species of which most are considered non-pathogenic, with the exceptions of Cryptococcus neoformans and Cryptococcus gattii. During the last decade Cryptococcus laurentii has occasionally been described to infect severely immunocompromised hosts [1–3]. In most of these reports from which isolates were obtained, the blood and the cerebrospinal fluid (CSF) were the predominant sources [2–5].

C. laurentii was first identified from palm wine in the Congo by Kufferath in 1920 as Torula laurentii [6]. This isolate was then reclassified as Torula laurentii [7] and renamed in 1950 as Cryptococcus laurentii (CBS 139) [8]. Later in Japan, an isolate with identical phenotypic characteristics was described as Torula flavescens [9], reclassified in 1922 as Torulopsis flavescens [7], and then renamed as Cryptococcus flavescens (CBS 942) [8].

Cryptococcus aures, Cryptococcus carnescens, and Cryptococcus penneus, in addition to C. flavescens, were also considered to be synonymous of C. laurentii until phylogenetic analysis of the internal transcribed spacer (ITS) and D1/D2 region of 28S nuclear ribosomal large subunit rRNA gene (28S-LSU) demonstrated that they are different species [10,11].
Table 1. Isolate, species, source, and GenBank accession numbers of **Cryptococcus** spp. environmental isolates.

| Isolate | Species | Source     | Country | GenBank 18S-SSU | GenBank 28S-LSU | GenBank ITS | Hap |
|---------|---------|------------|---------|-----------------|-----------------|-------------|-----|
| CL01    | C. laurentii | Peri-hospital | Brazil  | JX393937       | JN626943       | JO968462   | 1   |
| CL02    | C. laurentii | Peri-hospital | Brazil  | JX393938       | JN626944       | JO968463   | 1   |
| CL03    | C. laurentii | Peri-hospital | Brazil  | JX393939       | JN626945       | JO968464   | 1   |
| CL04    | C. laurentii | Peri-hospital | Brazil  | JX393940       | JN626946       | JO968465   | 1   |
| CL05    | C. laurentii | Peri-hospital | Brazil  | JX393941       | JN626947       | JO968466   | 1   |
| CL06    | C. laurentii | Peri-hospital | Brazil  | JX393942       | JN626948       | JO968467   | 1   |
| CL07    | C. laurentii | Peri-hospital | Brazil  | JX393943       | JN626949       | JO968468   | 1   |
| CL08    | C. laurentii | Peri-hospital | Brazil  | JX393944       | JN626950       | JO968469   | 1   |
| CL09    | C. laurentii | Peri-hospital | Brazil  | JX393945       | JN626951       | JO968470   | 1   |
| CL10    | C. laurentii | Peri-hospital | Brazil  | JX393946       | JN626952       | JO968471   | 1   |
| CL11    | C. laurentii | Trees       | Brazil  | JX393947       | JN626953       | JO968472   | 1   |
| CL12    | C. laurentii | Peri-hospital | Brazil  | JX393948       | JN626954       | JO968473   | 1   |
| CL13    | C. laurentii | Peri-hospital | Brazil  | JX393949       | JN626955       | JO968474   | 1   |
| CL14    | C. laurentii | Peri-hospital | Brazil  | JX393950       | JN626956       | JO968475   | 1   |
| CL15    | C. laurentii | Peri-hospital | Brazil  | JX393951       | JN626957       | JO968476   | 1   |
| CL16    | C. laurentii | Peri-hospital | Brazil  | JX393952       | JN626958       | JO968477   | 1   |
| CL17    | C. laurentii | Peri-hospital | Brazil  | JX393953       | JN626959       | JO968478   | 1   |
| CL18    | C. laurentii | Trees       | Brazil  | JX393954       | JN627000       | JO968479   | 1   |
| CL19    | C. laurentii | Peri-hospital | Brazil  | JX393955       | JN627001       | JO968480   | 1   |
| CL20    | C. laurentii | Peri-hospital | Brazil  | JX393956       | JN627002       | JO968481   | 1   |
| CL21    | C. laurentii | Peri-hospital | Brazil  | JX393957       | JN627003       | JO968482   | 1   |
| CL22    | C. laurentii | Trees       | Brazil  | JX393958       | JN627004       | JO968483   | 1   |
| CL23    | C. laurentii | Peri-hospital | Brazil  | JX393959       | JN627005       | JO968484   | 1   |
| CL24    | C. laurentii | Peri-hospital | Brazil  | JX393960       | JN627006       | JO968485   | 1   |
| CL25    | C. laurentii | Trees       | Brazil  | JX393961       | JN627007       | JO968486   | 1   |
| CL26    | C. laurentii | Peri-hospital | Brazil  | JX393962       | JN627008       | JO968487   | 2   |
| CL27    | C. laurentii | Peri-hospital | Brazil  | JX393963       | JN627009       | JO968488   | 1   |
| CL28    | C. laurentii | Peri-hospital | Brazil  | JX393964       | JN627010       | JO968489   | 1   |
| CL29    | C. laurentii | Peri-hospital | Brazil  | JX393965       | JN627011       | JO968490   | 1   |
| CL30    | C. laurentii | Peri-hospital | Brazil  | JX393966       | JN627012       | JO968491   | 1   |
| CL31    | C. laurentii | Peri-hospital | Brazil  | JX393967       | JN627013       | JO968492   | 1   |
| CL32    | C. laurentii | Pets shops  | Brazil  | JX393968       | JN627014       | JO968493   | 1   |
| CL33    | C. laurentii | Pets shops  | Brazil  | JX393969       | JN627015       | JO968494   | 1   |
| CL34    | C. laurentii | Pets shops  | Brazil  | JX393970       | JN627016       | JO968495   | 1   |

Phylogenetic Analysis of **Cryptococcus laurentii** Strains
| Isolate | Species | Source | Country | GenBank Hap 1 | GenBank Hap 2 | GenBank Hap 3 | GenBank Hap 4 |
|---------|---------|--------|---------|---------------|---------------|---------------|---------------|
| CL36    | C. laurentii | Pets shops | Brazil | JX393971 | JX394050 | JQ968496 | 1 |
| CL37    | C. laurentii | Pets shops | Brazil | JX393972 | JX394051 | JQ968497 | 1 |
| CL38    | C. laurentii | Pets shops | Brazil | JX393973 | JX394052 | JQ968498 | 1 |
| CL39    | C. laurentii | Pets shops | Brazil | JX393974 | JX394053 | JQ968499 | 1 |
| E4      | C. laurentii | Pigeon dropping | Brazil | JX393977 | JX394054 | JQ968501 | 3 |
| E5      | C. laurentii | Pigeon dropping | Brazil | JX393978 | JX394055 | JQ968502 | 3 |
| E6      | C. laurentii | Pigeon dropping | Brazil | JX393979 | JX394056 | JQ968503 | 3 |
| E7      | C. laurentii | Pigeon dropping | Brazil | JX393980 | JX394057 | JQ968504 | 1 |
| E11     | C. laurentii | Pigeon dropping | Brazil | JX393981 | JX394058 | JQ968505 | 3 |
| E12     | C. laurentii | Pigeon dropping | Brazil | JX393982 | JX394059 | JQ968506 | 3 |
| DS288   | C. laurentii | Mopane tree | Botswana | KC469712 | KC485478 | KC469756 | 1 |
| DS386   | C. laurentii | Mopane tree | Botswana | KC469715 | KC485481 | KC469757 | 1 |
| DS388   | C. laurentii | Mopane tree | Botswana | KC469718 | KC485483 | KC469759 | 1 |
| DS390   | C. laurentii | Mopane tree | Botswana | KC469720 | KC485484 | KC469760 | 3 |
| DS391   | C. laurentii | Mopane tree | Botswana | KC469722 | KC485486 | KC469761 | 3 |
| DS392   | C. laurentii | Mopane tree | Botswana | KC469723 | KC485487 | KC469762 | 3 |
| DS400   | C. laurentii | Mopane tree | Botswana | KC469724 | KC485488 | KC469764 | 5 |
| DS402   | C. laurentii | Mopane tree | Botswana | KC469725 | KC485489 | KC469765 | 6 |
| DS403   | C. laurentii | Mopane tree | Botswana | KC469726 | KC485490 | KC469766 | 6 |
| DS444   | C. laurentii | Mopane tree | Botswana | KC469727 | KC485491 | KC469767 | 6 |
| DS447   | C. laurentii | Mopane tree | Botswana | KC469728 | KC485492 | KC469768 | 6 |
| DS448   | C. laurentii | Mopane tree | Botswana | KC469729 | KC485493 | KC469769 | 6 |
| DS455   | C. laurentii | Mopane tree | Botswana | KC469730 | KC485494 | KC469770 | 6 |
| DS529   | C. laurentii | Norway spruce | USA | KC469735 | KC485495 | KC469771 | 3 |
| DS530   | C. laurentii | Norway spruce | USA | KC469736 | KC485496 | KC469772 | 3 |
| DS531   | C. laurentii | Norway spruce | USA | KC469737 | KC485497 | KC469773 | 3 |
| DS532   | C. laurentii | Norway spruce | USA | KC469738 | KC485498 | KC469774 | 3 |
| DS533   | C. laurentii | Norway spruce | USA | KC469739 | KC485499 | KC469775 | 3 |
| DS534   | C. laurentii | Norway spruce | USA | KC469740 | KC485500 | KC469776 | 3 |
| DS535   | C. laurentii | Norway spruce | USA | KC469741 | KC485501 | KC469777 | 3 |
| DS536   | C. laurentii | Norway spruce | USA | KC469742 | KC485502 | KC469778 | 3 |
| DS537   | C. laurentii | Norway spruce | USA | KC469743 | KC485503 | KC469779 | 3 |
| DS538   | C. laurentii | Norway spruce | USA | KC469744 | KC485504 | KC469780 | 3 |
| DS539   | C. laurentii | Norway spruce | USA | KC469745 | KC485505 | KC469781 | 3 |
| DS540   | C. laurentii | Norway spruce | USA | KC469746 | KC485506 | KC469782 | 3 |
| DS744   | C. laurentii | Douglas Fir tree | Canada | KC469730 | KC485495 | KC469771 | 1 |
| DS746   | C. laurentii | Douglas Fir tree | Canada | KC469731 | KC485496 | KC469772 | 1 |
| DS748   | C. laurentii | Douglas Fir tree | Canada | KC469732 | KC485497 | KC469773 | 1 |
| DS778   | C. laurentii | Douglas Fir tree | Canada | KC469733 | KC485498 | KC469774 | 1 |
| DS782   | C. laurentii | Douglas Fir tree | Canada | KC469734 | KC485499 | KC469775 | 1 |
| DS784   | C. laurentii | Douglas Fir tree | Canada | KC469735 | KC485500 | KC469776 | 1 |
| DS786   | C. laurentii | Douglas Fir tree | Canada | KC469736 | KC485501 | KC469777 | 1 |
| DS788   | C. laurentii | Douglas Fir tree | Canada | KC469737 | KC485502 | KC469778 | 1 |
| DS790   | C. laurentii | Douglas Fir tree | Canada | KC469738 | KC485503 | KC469779 | 1 |
| DS792   | C. laurentii | Douglas Fir tree | Canada | KC469739 | KC485504 | KC469780 | 1 |
| DS794   | C. laurentii | Douglas Fir tree | Canada | KC469740 | KC485505 | KC469781 | 1 |
| DS796   | C. laurentii | Douglas Fir tree | Canada | KC469741 | KC485506 | KC469782 | 1 |
| DS798   | C. laurentii | Douglas Fir tree | Canada | KC469742 | KC485507 | KC469783 | 1 |
| DS800   | C. laurentii | Douglas Fir tree | Canada | KC469743 | KC485508 | KC469784 | 1 |
| DS802   | C. laurentii | Douglas Fir tree | Canada | KC469744 | KC485509 | KC469785 | 1 |
| DS804   | C. laurentii | Douglas Fir tree | Canada | KC469745 | KC485510 | KC469786 | 1 |
| DS806   | C. laurentii | Douglas Fir tree | Canada | KC469746 | KC485511 | KC469787 | 1 |
| DS808   | C. laurentii | Douglas Fir tree | Canada | KC469747 | KC485512 | KC469788 | 1 |

Phylogenetic Analysis of Cryptococcus laurentii Strains
### Table 1. Cont.

| Isolate | Species | Source | Country | GenBank 18S-SSU | GenBank 28S-LSU | GenBank ITS | Hap |
|---------|---------|--------|---------|-----------------|-----------------|-------------|-----|
| DS782   | C. laurentii | Tree | Canada | KC469747        | KC469749        | KC469793    | 5   |
| DS783   | C. laurentii | Tree | Canada | KC469748        | KC469750        | KC469792    | 5   |
| DS797   | C. laurentii | Tree | Canada | KC469751        | KC485515        | KC469794    | 3   |
| DS798   | C. laurentii | Tree | Canada | KC469752        | KC485517        | KC469796    | 3   |
| DS799   | C. laurentii | Tree | Canada | KC469753        | KC485518        | KC469777    | 7   |
| DS802   | C. laurentii | Tree | Canada | KC469754        | KC485519        | KC469797    | 5   |
| O242A   | C. flavescens | Tree | Brazil | JX393984        | JX394006        | JQ968509    | 10  |
| I113A   | C. flavescens | Air | Brazil | JX393985        | JX394007        | JQ968510    | 10  |
| I283A   | C. flavescens | Air | Brazil | JX393989        | JX394011        | JQ968513    | 12  |
| CBS 942T | C. flavescens | Air | Japan | AB085796        | AB035042        | AB035046    | 12  |
| I572B   | C. terrestris | Tree | Brazil | JX393991        | JX394012        | JQ968512    | 13  |
| I573B   | C. terrestris | Tree | Brazil | JX393992        | JX394014        | JQ968514    | 13  |
| 1B2011  | C. terrestris | Pigeon dropping | Brazil | JX393993        | JX394015        | JQ968517    | 13  |
| 1C2011  | C. terrestris | Pigeon dropping | Brazil | JX393994        | JX394016        | JQ968518    | 13  |
| DS233   | C. terrestris | Mopane tree | Botswana | KC469710        | KC469711        | KC469735    | 13  |
| DS234   | C. terrestris | Mopane tree | Botswana | KC469712        | KC469713        | KC469737    | 13  |
| DS290   | C. terrestris | Mopane tree | Botswana | KC469714        | KC469715        | KC469739    | 13  |
| DS291   | C. terrestris | Mopane tree | Botswana | KC469716        | KC469717        | KC469741    | 13  |
| DS401   | C. terrestris | Mopane tree | Botswana | KC469718        | KC469719        | KC469743    | 13  |
| DS402   | C. terrestris | Mopane tree | Botswana | KC469720        | KC469721        | KC469745    | 13  |
| DS403   | C. terrestris | Mopane tree | Botswana | KC469722        | KC469723        | KC469747    | 13  |

Phylogenetic Analysis of Cryptococcus laurentii Strains
In 2005, Cryptococcus rajasthanensis (CBS 10406) was described and differentiated from C. laurentii due to 1.6% and 7.5% divergence of the nucleotide identity of the 28-LSU and ITS regions, respectively [12]. More recently, Cryptococcus terrestris (CBS 10810), the cryptic species of C. flavescens, was isolated and described from soil in Brazil [13].

Currently, most C. laurentii isolates described around the world have been identified by morphological criteria, which can miss subtle differences and misidentify cryptic species [1,14,15]. Unlike C. neoformans and C. gattii, few studies have applied DNA sequencing to describe the molecular phylogeny of C. laurentii [11,16,17]. Thus, considering the potential pathogenicity of this species, this study aimed to evaluate the molecular phylogeny of clinical and environmental C. laurentii isolates through the sequencing of multiple ribosomal DNA regions.

**Methods**

**Identification and fungal strains**

We evaluated 100 environmental isolates of C. laurentii that were identified by classical mycological methods, such as India ink test, urease and phenoloxidase activity, thermotolerance at 37°C on Sabouraud dextrose agar, nitrate and carbon assimilation assays, carbohydrate fermentation, and microculture on cornmeal with Tween 80 [18,19]. Of the 56 Brazilian isolates, 26 were obtained from peri-hospital areas, 5 from unidentified trees species, 7 from captive bird droppings in Uberaba, Minas Gerais State, and 18 from various environmental sources (bird droppings, trees, and air samples) from São Paulo State. The 18 isolates from Botswana were isolated from Mopane trees (Colophospermum mopane), the 14 isolates from New York State from Norway spruce (Picea abies) and trembling aspen (Populus tremuloides), and the 12 isolates from Vancouver, Canada from Douglas fir (Pseudotsuga menziesii) and other unidentified trees (Table 1). Isolates from Canada and the United States were isolated from swab samples collected in 2010 using single-swab BD CultureSwab Liquid Amies (Becton, Dickinson and Company, Sparks, Maryland, USA). The swabs were streaked onto yeast peptone dextrose agar (YPD, Becton, Dickinson and Company, Sparks, Maryland, USA) or Niger seed (NGS) agar containing chloramphenicol (0.5 g/L, Sigma-Aldrich, St. Louis, MO, USA), and yeast colonies were selected and colony purified [20].

All isolates were stored at -20°C in 70% YPD broth with 30% glycerol in 2-mL eppendorf tubes at the Mycology Laboratory at the Triangulo Mineiro Federal University (UFTM) for further analyses.

**DNA sequencing**

Genomic DNA was extracted from yeast cells in accordance with previously described methodology [21]. The 5' end of the 18S nuclear ribosomal small subunit rRNA gene (18S-SSU) (AFTol project available at http://aftol.org/primers.php), internal transcribed spacer (ITS) region [10,22], and D1/D2 region of 28S-LSU [10,23] were amplified from genomic DNA by PCR using the primers and conditions denoted in Table 2. PCR of the 18S-SSU, 28S-LSU, and ITS regions were performed using a PTC-100 Thermocycler (MJ Research Inc., Watertown, MA, USA) in a final volume of 50 μL. Each reaction contained 20 ng of genomic DNA, 1 x PCR buffer (10 mmol L$^{-1}$ Tris-HCl pH 8.3, 50 mmol L$^{-1}$ KCl, and 1.5 mmol L$^{-1}$ MgCl$_2$), 0.25 mmol L$^{-1}$ each of dATP, dCTP, dGTP and dTTP, and 1.25 U of Taq DNA polymerase (Invitrogen, São Paulo, SP, Brazil). The amplicons were stained with 0.5 mg mL$^{-1}$ of

| Isolate    | Species          | Source     | Country       | GenBank Hap   | 18S-SSU   | 28S-LSU | ITS  |
|------------|------------------|------------|---------------|---------------|-----------|---------|------|
| DS446      | C. terrestris    | Mopane tree| Botswana      | KC646401      | KF267575  | KF557797 | NA   |
| CBS 10810T | C. terrestris    | Soil       | USA           | EF370393      | EU200782  | NA      | NA   |
| CBS 142    | C. albidus       | Air        | Japan         | AB000826      | AB000826  | NA      | NA   |

NA: Not applicable. T: Type strain.
ethidium bromide and visualized under UV light after two hours of electrophoresis at 80 V [22].

Each PCR product was independently sequenced with the forward and reverse primers of each region using the BigDye terminator v. 3.1 reagent kit (Applied Biosystems, Foster City, CA, USA) including Tag DNA polymerase (Invitrogen, São Paulo, SP, Brazil) in an automated DNA sequencer (ABI PRISM 3130xl Genetic Analyzer, Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions.

Sequencing analysis

Sequences were edited using the software Sequence Scanner V. 1.0 (Applied Biosystems, USA). Only nucleotide sequences with a Phred quality score ≥20 were included in our analysis to limit the possibility of incorporating an incorrect base to ≤1 in 100 (≥99% accuracy). Bioedit software was used to obtain consensus sequences from aligned forward and reverse sequence reads. Each consensus sequence was submitted to the Basic Local Alignment Search Tool (BLAST), and identity values ≥99% were obtained to assign species. All generated sequences were deposited in GenBank (Table 1) and The Barcode of Life Database (BOLD) (http://www.barcodinglife.org) [24].

Phylogenetic relationships

Consensus sequences from newly identified isolates and those obtained from GenBank were aligned with the Clustal W2 algorithm (https://www.ebi.ac.uk/Tools/msa/clustalw2/) [25]. The phylogenetic analysis was calculated by the neighbor-joining, unweighted pair group method with arithmetic mean (UPGMA), and maximum likelihood methods in the MEGA 6.0 software [26]. For the neighbor-joining and maximum likelihood methods, the evolutionary distances were calculated in accordance with Kimura [27], while the Tamura 3-parameters method with the variation rate among sites modeled with a gamma distribution (shape parameter = 1) was used for UPGMA [28]. Phylogenetic relationships were calculated for each of the three regions and for the concatenated sequences applying a bootstrap analysis with 1,000 random resamplings. The type strain Cryptococcus albidus CBS 142 was designated as the outgroup in all phylogenetic analyses [29,30]. Nucleotide sequences from the CBS-KNAW Fungal Biodiversity Centre (CBS) type strains were obtained from GenBank (Table 1).

To evaluate which of the three regions presented the highest variability, the intraspecific and interspecific pairwise distance was calculated by the Kimura 2-parameter model [27] in the MEGA 6.0 software [26].

Haplotype network and goeBURST analysis

Haplotype networks were generated from the three concatenated sequence regions to visualize the differences and diversity among the C. laurentii isolates. The number and diversity of each haplotype were estimated using the software DNAsp v5.10 (http://www.molekulare-evol.de/dnaasp/) [31]. Median-joining networks [32] for the concatenated dataset were obtained and graphed using the software Network 4.610 (http://fluxus-engineering.com).

To confirm the haplotypes obtained by median-joining networks the analyses were replicated in MLSTest software (available at http://mltest.codeplex.com) and graphed by goeBURST algorithm in PHILOVIZ software [33,34]. The minimum spanning tree representing the comparison between the isolates sources and their haplotype was also calculated by goeBURST.

Coalescent species analyses

In order to estimate the time divergence between species and haplotypes, the interspecific and intraspecific net nucleotides substitutions (d) and standard error of the concatenated sequences were calculated in accordance to Kimura [27] with a bootstrap (500 replicates) as a variance method in the MEGA 6.0 software [35,36]. The distance and standard error between closest species e. g. (C. laurentii x C. rapsthananensis 0.016±0.003; C. aspenensis x C. flavescens 0.071±0.007; C. terrestris x C. flavescens 0.009±0.002) were obtained and applied in the equation $d = 2kt$, where $d$ is the number of nucleotide substitutions per site between a pair of sequences, $t$ is the divergence time, and $k$ the rate of nucleotide substitution. Here, we applied the constant ($\lambda$)
10−3/bp/year previously obtained for the Eurotiumysites lineage due to the absence of a known fossil for C. laurentii species [36]. The resulting time of divergence were used as prior parameters to calibrate the tree in the coalescent analyses.

The optimal molecular evolutionary model for the concatenated sequences was determined using the corrected Akaike Information Criterion (AICc) as executed in the software jModelTest 2 [37,38]. The optimal molecular evolutionary model General Time Reversible (GTR+I+G) with the respective parameters: AC: 0.7675, AG: 2.3377, AT: 1.8759, CG: 0.4999, GT: 1.0, alpha (IG): 0.5430, and pinv (IG): 0.5130 were obtained and used as priors in the coalescence analyses.

The BEAST software version 1.8.0 [39] was used to calculate the mean time to the most common recent ancestor (TMRCA) by applying the Bayesian Markov-chain Monte Carlo (MCMC) method assuming a relaxed log-normal model of molecular rate heterogeneity. The chain lengths were 107 generations with parameters sampled every 103 generations with an initial burn-in of 10%. The posterior probability for a given clade was the frequency that the clade was present among the posterior trees which means that the probability of the lineage be considered monophyletic in the used dataset. Convergence of parameter values in the MCMC were assessed by the effective sample size (ESS) in the Tracer software version 1.6 [40].

Nucleotide diversity of C. laurentii isolates

The extent of DNA polymorphisms, such as the number of polymorphic sites (S), nucleotide diversity (π), number of haplotypes (h), haplotype diversity (Hd), and average number of nucleotide differences (k), were calculated using DNAsp v5.10 [31]. In addition, Tajima’s D, Fu & Li’s D*, Fu & Li’s F*, and Fu’s Fs neutrality were calculated. Negative or positive results in these tests suggest evidence of purifying or balancing selection, respectively.

Fluorescence-activated cell-sorting (FACS) analysis

The FACS protocol was modified from Tanaka et al. [41]. Cells were grown overnight at 25°C in YPD broth, collected by centrifugation, and washed with 1 x PBS. Cells were then fixed in 1 ml of 70% ethanol overnight at 4°C with mild agitation. Cell pellets were obtained by centrifugation and the supernatants were discarded. Cells were resuspended and washed with 1 mL of NS buffer (10 mM Tris-HCl pH 7.2, 0.25 M sucrose, 1 mM EDTA, 1 mM MgCl2, 0.1 mM CaCl2, 0.55 mM Phenylmethylsulfonyl fluoride, 0.1 mM ZnCl2, 0.049% 2-mercaptoethanol). Cells were then resuspended in 180 μL NS buffer with, 14 mL RNase A (15 mg/μL, Qiagen) and 6 μL of Propidium iodide (1.0 μg/μL, CALBIOCHEM) and incubated in the dark for 4–8 hrs at room temperature. After incubating 50 μL of the cells were mixed with 500 μL of Tris-PI mix [482 μL 1M Tris pH 7.5+18 μL Propidium Iodide (1 μg/μL)]. Flow cytometry was performed on 10,000 cells with slow laser scan, on the FL1 channel with a Becton-Dickinson FACScan.

This study was approved by the Ethical Board of Triangulo Mineiro Federal University and is registered under the protocol number 32 CBIO/UFTM.

Nomenclature

The electronic version of this article in Portable Document Format (PDF) will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic version are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide print copies. In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MycoTaxo.aspx?Link=T&Rec=MB009723. The online version of this work is archived and available from the following digital repositories: PubMed Central and LOCKSS.

Results

All isolates produced capsule and urease but not melanin and were phenotypically characterized as C. laurentii due to their ability to assimilate arabinose, α-methyl-D-glucoside, cellobiose, D-glucose, D-mannitol, D-ribose, D-trehalose, DL-lactate, dulcitol, galactose, inositol, L-rhamnose, lactose, maltose, melitizose, raffinose, sacarose, sorbose, xylose, and 2-keto-glutarate. However, the isolates were negative for fermentation of dextrose and assimilation of inulin and potassium nitrate. FACS analysis indicated that most of the isolates are haploid (Figure S1).

A 550-bp product was amplified from the 5’ end of 18S-SSU and sequenced with the primers NS-1 and NS-2, from which a 339-bp alignment was obtained. In this analysis, 75 isolates shared 99–100% identity with the C. laurentii CBS 139 (AB032640) type strain. Another 16 isolates shared 99% identity with C. flavescentis CBS 942 (AB085796). The remaining 9 shared 99% identity with both Bullera sp. VY-68 (AB106094) from Japan and with C. laurentii RY1 from India (EF063147). High bootstrap values generated by neighbor-joining, UPGMA, and maximum likelihood analyses supported the differentiation of the following clades: C. laurentii (bootstrap values of 79, 87, and 78, respectively), C. flavescentis (bootstrap values of 96, 99, and 98, respectively) and Bullera sp./C. laurentii (bootstrap values of 63, 65, and 64, respectively) (Figure 1A).

Due to the low genetic variability of the C. laurentii clade obtained at the 5’ end of the 18S-SSU gene, we sequenced two additional ribosomal loci: D1/D2 of the 28S-LSU and the ITS gene regions. The alignment and analysis of the 530-bp long amplicon of the 28S-LSU region confirmed the identification of 75 isolates as C. laurentii and showed more intraspecific variability differentiating three major groups (group Ia, Iib, and Ibc) within C. laurentii isolates. Of the 16 C. flavescentis isolates identified by the 18S-SSU sequencing, only 6 were confirmed in the C. flavescentis clade by the 28S-LSU region with high bootstrap values of 79 (neighbor-joining), 95 (UPGMA), and 96 (maximum likelihood). The C. flavescentis clade was split into two groups (Group Ia and a possible hybrid) by 28S-LSU and three groups ( Groups Ia, Ib, and a possible hybrid) by ITS and analyses of the 1,328-bp amplicon of the concatenated regions. The two possible hybrid isolates I322A and O242A from Brazil were more related to C. terrestris in the ITS and concatenated sequences analyses (Figure 1B and 2). The remaining 10 isolates shared 99% identity with C. terrestris (CBS 108110), which has been recently described in Brazil and the United States (Figure 1B).

Among the nine Bullera sp./C. laurentii isolates identified by the 18S-SSU, isolate 4P82A shared 99% identity of the 28S-LSU and ITS regions with Cryptococcus rajasthanensis CBS 10406 (AM262324) from India. The eight remaining isolates (DS569, DS570, DS572, DS573, DS712, DS713, DS715, and DS716) recovered from a trembling aspen tree (Populus tremuloides) were designated as Cryptococcus aspenensis sp. nov. because they shared 100% identity with two undescribed isolates of Cryptococcus sp. APSS-862 (FM178286) and Cryptococcus sp. APSS-823.
eight isolates exhibited a genetic distance of 3.8% and 7.1% from C. rajasthanensis and 2.3–2.7% and 6.4–7.3% from C. laurentii by 28S-LSU and ITS region analysis, respectively (Figure 1B and 2A).

Overall, the pairwise distance of the three sequenced regions showed the highest intraspecific and interspecific variability in the ITS region (genetic distance higher than 15%) when compared with the 2.5% and 5.0% obtained with 18S-SSU and 28S-LSU, respectively (Figure 3).

The haplotype diversity of the concatenated regions was assessed using DNAsp and MLSTTest software. Multiple haplotype groups were identified within C. laurentii and C. flavescens, but not the C. aspenensis sp. nov. and C. terrestris (Figure 4A and 4B). The C. laurentii isolates were represented by seven haplotypes (H1 to H7). Haplotype 1 (H1) included 44 isolates, of which 38 (86.4%) were from Brazil, followed by the H3 composed of 6 from Brazil, 6 isolates from Canada, and 6 from the United States (Figure 4C and 4D). The highest genetic distance (12 polymorphisms) in the C. laurentii haplotypes was observed between H7 (CBS 132 type strain) and H6 (DS402 and DS444 isolates). Five of the seven C. laurentii isolates were recovered from Africa despite very limited sampling; three were unique haplotypes (H4, H6, and H7) and two were only observed in Brazil (H1) or Canada (H5). H4, which was obtained from Mopane trees in Botswana, was identified as the ancestral of C. laurentii in both Network and MLSTTest analyses. H7 (C. laurentii type strain CBS 139) was restricted to the Congo and was in much closer proximity to Botswana than any other

Figure 1. Phylogenetic analysis of 100 environmental Cryptococcus spp. isolates generated by the neighbor-joining, UPGMA, and maximum likelihood methods using partial nucleotide sequences of the (A) 5’ end of 18S SSU-rDNA and (B) D1/D2 region of 28S LSU-rDNA. Numbers at each branch indicate bootstrap values >50% based on 1,000 replicates (NJ/UPGMA/ML). The analysis involved 103 and 105 nucleotide sequences for the 18S-SSU and 28S-LSU respectively. T: Type strain.

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sample region. H3 was distinct from Botswana and was comprised of isolates from North and South America (Figure 4).

Three haplotypes were identified in *C. flavescens* isolates (H10, H11, and H12), with the ancestral haplotype H11 restricted to Brazil. H10 presented the highest genetic distance (9 polymorphisms) when compared with H11 and H12 (2 polymorphisms). H10 was also positioned closer to the *C. terrestris* haplotype H13 and could be a unique species, or ancestral genotype, or recombinant hybrid isolate between *C. flavescens* and *C. terrestris*. The *C. aspenensis* sp. nov. H9 was a completely unique genotype from New York, USA (Figure 4).

Estimates of the mean time to divergence for the *C. flavescens* and *C. terrestris* isolates were 4.02–5.46 ± 0.87 million years (about 9 million years ago) with an effective sample size (ESS) of 1213.3 and 1006.8, respectively. For *C. laurentii* population, the TMRCA were 8.03–9.61 ± 1.83 million years (about 16.4 million years ago) (ESS = 6615.0) while for the new species *C. aspenensis* sp. nov. 26.7 ± 3.9 million years (about 37.9 million years ago) (ESS = 355.5). Coalescent analysis was strongly supported with >95.0 Bayesian posterior values (Figure 5). Phylogenetic and coalescent analyses agree demonstrating additional support for the recognition of additional related haplotypes and species.

The *C. laurentii* nucleotide sequences of the 18S-SSU, 28S-LSU, ITS, and the concatenated regions presented 0, 3, 11, and 14 polymorphic sites, respectively (Table 3). The highest nucleotide diversity ($\pi$) of 0.0039 was observed for ITS. Low values of haplotype (Hd = 0.604) and nucleotide diversity ($\pi$ = 0.0014) of the concatenated regions may suggest clonal reproduction in this species (Table 3).
Taxonomy

**Cryptococcus aspenensis.** Ferreira-Paim, K., Ferreira, T. B., Andrade-Silva, L., Mora, D. J., Springer, D. J., Heitman, J., Fonseca, F. M., Matos, D., Melhem, M. S. C., et Silva-Vergara, M. L. sp. nov. [urn:lsid:mycobank.org:names:MB809723].

After 3 days on YPD agar at 25°C, *Cryptococcus aspenensis* colonies are circular, cream-colored with an entire margin, smooth, mucous to butyrous, glistening, and raised. Growth (poor) at 37°C was also observed. After 3 days at 25°C in YPD broth, the cells are ellipsoid to globose (7.5–8.7 to 5–6.2 μm), and they may be single or with one attached polar bud (Figure 6). After 15 days in slide cultures on commeal agar, pseudomycelium or mycelium is not formed. Fermentation ability is negative. Arabinose, α-methyl-D-glucoside, cellobiose, D-glucose, D-mannitol, D-ribose, D-trehalose, DL-lactate, dulcitol, galactose, inositol, L-rhamnose, lactose, maltose, melitioze, raffinose, sacarose, sorbose, xylose, and 2-keto-glutarate are assimilated. Cells were haploid by FACS analysis (Figure S1).

Unambiguous identification and phylogenetic placement is based on DNA sequences of the following nuclear loci: ITS (KC469778), 18S-SSU (KC469734), D1/D2 of 28S-LSU (KC485500). The type strain DS573 was isolated from the bark of a trembling aspen (*Populus tremuloides*) in the New York, United States and has been deposited in the Centraalbureau voor Schimmelcultures, The Netherlands, as CBS 13867 and in the Westmead Millennium Institute, Australia, as WM 14.137. Other strains belonging to this species include DS570 (CBS 13868, WM 14.138), and DS715 (CBS 13869, WM 14.139) which were isolated from a single trembling aspen tree in New York.

**Etymology:** The specific epithet *aspenensis* L. adj. *aspenensis* associated with trembling aspen (*Populus tremuloides*), the tree substrate from which the type strain was isolated.

**Discussion**

Fungal identification and taxonomy has markedly improved during the last decade and as a result, several recognized species, such as *Sporothrix schenckii*, *Paracoccidioides brasiliensis*, and *Coprinopsis cinerea*, have been distinguished as cryptic species complexes [35,42,43]. In this context, the sequencing of the 18S-SSU, D1/D2 of 28S-LSU, and ITS of the ribosomal region have been useful in yeast identification for more than 10 years. However, the low variability of the 18S-SSU and 28S-LSU regions may prohibit identification of cryptic species, while the variability of the ITS region has been frequently utilized for fungal phylogenetic studies and the fungal tree of life barcoding projects [44,45].

*C. laurentii* has classically been considered a saprophytic yeast, although 24 cases of human infection have been described, suggesting that *C. laurentii* is an opportunistic pathogen with potential similarities to the distantly related pathogenic *C. neoformans* and *C. gattii* species [3,44,46–48]. Cryptococcosis due to *C. laurentii* has been associated with severely immuno-compromised patients and/or those presenting with other underlying diseases. In such cases, *C. laurentii* was most frequently
Figure 4. Median-joining haplotype network (A) of environmental *C. laurentii* isolates based on concatenated nucleotide sequences of the 5' end of 18S-SSU, D1/D2 of 28S-LSU, and ITS regions. The tree represents 103 *Cryptococcus* spp. isolates from Brazil, Botswana, Canada, Japan, India, and the United States. The seven *C. laurentii* and three *C. flavescens* haplotypes are clearly distinguished. The Botswana ancestral haplotype (H4) of *C. laurentii* is presented and highlighted in yellow. Each circle represents a unique haplotype (H), and the circumference is
isolated from the blood, but also from several other body sites such as the CSF, skin, and lungs [49,50].

In this study, we evaluated 100 phenotypically identified \textit{C. laurentii} isolates from several countries. Of these, 75 were confirmed to be \textit{C. laurentii} by phylogenetic analysis of the 18S-SSU, 28S-LSU, and ITS regions. The obtained sequences shared 99–100\% identity with sequences from Brazil, China, South Africa, and the United States, demonstrating its worldwide and overlapping geographic distribution with \textit{C. neoformans} and \textit{C. gattii}. Although, in North America, \textit{C. gattii} has been associated with clinical infection in patients from New York, Rode Island, and other states [51–53]. At present, \textit{C. gattii} has only been environmentally isolated from the Western United States [54] and Canada [55], while \textit{C. neoformans} is broadly associated with pigeon guano in many regions of the United States, including the state of New York [56]. Hence, our study suggests that in the United States, \textit{C. laurentii} appears have a much broader distribution than \textit{C. gattii} as noted from its isolation in association with grasses in the USA, and goose guano and trees in New York State [57].

Within the \textit{C. laurentii} clade, intraspecific variability of 0.2\% (1 polymorphism), 0.2–0.4\% (1–3 polymorphisms), and 0.3–2.4\% (1–11 polymorphisms) was obtained for the analysis of the 18S-SSU, 28S-LSU, and ITS regions, respectively. These features are proportional to haplotype frequency (H1: 44 isolates; H2: 1; H3: 18; H4: 2; H5: 8; H6: 2; H7: 1; H8: 1; H9: 8; H10: 2; H11: 3; H12: 2; H13: 10; H14: 1; outgroup \textit{C. albidos} CBS 142). Yellow dots represents the number of mutation sites, excluding gaps, between the haplotypes. Black dots (median vectors) are hypothetical missing intermediates. Minimum spanning trees (B) using the goeBURST algorithm confirm the haplotype relationships among \textit{C. laurentii} isolates determined by median-joining network analysis. The size of the circle corresponds to the number of isolates within that haplotype, and the numbers between haplotypes represent the genetic distance of each haplotype, excluding the gaps. Minimum spanning trees as described in B modified to show the distribution of haplotypes according to the country of origin (C) or environmental source (D).

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Figure 5. Species tree of the \textit{C. laurentii} species complex resulting from coalescent analyses of the concatenated data set. The speciation of \textit{C. aspenensis} from \textit{C. laurentii} and \textit{C. rajasthanensis} took place 37.9 million years ago. The \textit{C. laurentii} haplotype (H4) from Botswana was the first haplotype to be differentiated (6.8 million years ago). Numbers at branches represent the Bayesian posterior support values while the bold numbers represent the nodes ages (in millions of years).

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consistent with a previously published report indicating that one polymorphism exhibited in the 28S-LSU region exist up to 11 substitutions in the ITS region [58]. Through phylogenetic analysis of the 28S-LSU and ITS regions, three divergent groups were distinguishable from the 75 C. laurentii isolates. Groups Ia and II of the 28S-LSU and ITS regions contained eight isolates from Botswana and Vancouver, which differed from the remaining 67 isolates in 1–3 and 5–11 nucleotides in the 28S-LSU and ITS regions, respectively, and constituted H5 in the network and goeBURST analysis. Additional analysis of environmental and clinical samples from outside of Brazil will be valuable to determine whether H5 is distinct to Brazil. The majority of Brazilian isolates are H1 (44 isolates). The high frequency of the H1 haplotype may be related to microevolution and/or adaptation of these isolates to the environment, while the H2 haplotype may be rare.

Despite the differences in the total number of C. laurentii isolates, those from Botswana (n = 12) shared five of the seven haplotypes observed, two of them unique (H4 and H6). Interestingly, the ancestral H4 is only represented in Botswana suggesting that similar to C. neoformans var. grubii, C. laurentii may have originated from Africa [59]. The historical haplotype (H7) from palm wine is also restricted to the Congo, which is near to Botswana. Other haplotypes common in Africa are only also observed from Brazil (H1) or Canada (H5). Therefore, it is possible that C. laurentii was introduced into Brazil and Canada from Africa. To test this hypothesis, the coalescent gene analyses was performed which showed that the isolates within the haplotype 4 are the oldest in our data set (6.8 million years ago).

The remaining 25 isolates that were originally identified as C. laurentii by standard phenotypic assays were identified by ITS, 18S-SSU, and 28S-LSU analyses as C. terrestris (n = 10), the C. aspenensis sp. nov. (n = 8), C. flavescens (n = 6), and C. rajasthanensis (n = 1). C. rajasthanensis isolates are rare, and few have been previously reported in GenBank from India, Thailand, China, and Brazil. The C. rajasthanensis isolate in our study was recovered from hollow trees in São Paulo, Brazil and differed from C. laurtenii by 0.4–0.6%, 1.7–2.1%, and 4.3–4.8% in the 18S-SSU, 28S-LSU, and ITS regions, respectively. In previous studies, the C. laurentii type strain (CBS 139) differed from the known Indian C. rajasthanensis reference isolate (CBS 10406) by 1.6% in the 28S-LSU region and 7.5% in the ITS region.

Despite the genetic distance observed between C. flavescens and C. laurentii (4–5.2% in 28S-LSU and 16.8–18.9% in the ITS), the species have long been considered phenotypically indistinguishable. For example, one previously identified clinical isolate of C. laurentii was later distinguished to be C. flavescens [4,60], suggesting that opportunistic pathogen traits may have evolved more than once within this group, similar to the presence of sporadic opportunistic pathogens in K. variicola and Cryptoococcus heveanensis species groups [61].

C. flavescens has only recently been differentiated as a sibling species of C. terrestris [13] with the advancements in multi-locus sequence analysis. It is likely that the delayed recognition of C. terrestris and C. flavescens hindered the recognition of divergent phenotypic traits now recognized as important species characteristics. C. terrestris can be differentiated phenotypically from C. flavescens by delayed and/or weak assimilation of ribose and salicin [13,44]. Our analysis supports the previous reported genetic differentiation; C. flavescens diverged from C. terrestris by 1.2–1.6% (6–10 polymorphisms) and 0.5–2.5% (2–10 polymorphisms) in the 28S-LSU and ITS regions, respectively. This difference probably occurred 9.1 million years ago as demonstrated by the coalescent analyses.

| Region | Length | S | π | $k$ | $\mu$ | $\delta$ | $D_{FS}$ | $D_{FD}$ | $D_{TT}$ |
|--------|--------|---|---|----|-----|-----|--------|--------|--------|
| 18S-SSU | 530 | - | 0.039 | - | 0.015 | -0.521 | 0.0006 | 0.3291 | 0.280 |
| 28S-LSU | 281 | - | 0.0006 | - | 0.0001 | 0.028 | 4 | 0.570 | 0.737 |
| ITS | 399 | - | 0.0039 | - | 0.0014 | 0.591 | 4 | 0.560 | 1.079 |
| Concatenated | 1328 | - | 0.0014 | - | 0.0014 | 0.591 | 7 | 0.604 | 2.373 |

Table 3. DNA polymorphisms in the ribosomal loci of the 75 C. laurentii environmental isolates.

S: number of polymorphic sites; $\pi$: nucleotide diversity; $k$: average number of nucleotide differences per sequence; $\mu$: number of hapatotypes; $H$: haplotype diversity; $D$, $D_{FS}$, and $D_{FD}$: Tajima’s $D$, Fu and Li’s $D^{*}$, Fu and Li’s $F^{*}$ and $d$, Fu’s $F_{S}$, respectively.

...
The six *C. flavescens* isolates recovered from Brazil were similar to isolates from China, Egypt, Italy, Japan, South Africa, and the United States, confirming the ubiquity of this species. The intraspecific variability of 0.2%, 0.4% and 0.8–2.2% observed in the 18S-SSU, 28S-LSU, and ITS regions, respectively, and the description of one haplotype in 18S-SSU, two in 28S-LSU, and three in the ITS region and concatenated analyses for the first time is relevant in the biological context of this species. Both isolates within H10 (I332A and O242A) share higher similarity with *C. terrestris* in 18S-SSU, the ITS region, and concatenated sequence but are more similar to *C. flavescens* in 28S-LSU. H10 may be a second haplotype of *C. terrestris* or a possible hybrid haplotype between the two species, as has been observed between *C. gattii* and *C. neoformans* [62–64]. Both isolates within this unique haplotype appear haploid by FACS which suggest this isolate may be a recombinant between *C. flavescens* and *C. terrestris* or an ancestral genotype. Coalescent analysis does not support the hypothesis that the two isolates in haplotype 10 are ancestral to both species and hence it is likely this haplotype arose from a productive introgression between *C. flavescens* and *C. terrestris*. Whole genome sequencing and the development of multilocus sequence primers specific to *C. laurentii* will be needed to support these hypotheses. Furthermore, the ancestral haplotype of *C. flavescens* appears to be H11 (revealed by MLSTest but not by DNAp), which is confined to Brazil, suggesting that it may have originated in Brazil. Additional environmental and clinical isolates must be evaluated to better define the place of origin of *C. flavescens*.

A newly identified but distinct haploid group that we designated as *C. aspenensis* sp. nov. was identified consistently through phylogenetic analyses of individual and concatenated loci and confirmed by coalescent analyses. At present, this constitutes a previously unidentified species that appears to be restricted to New York, United States. All eight isolates obtained in the H9 group appear to be nearly identical/clonal and were obtained from sampling one trembling aspen tree in Long Island, New York, United States. An additional isolate was just identified from soil sample collect on July 13 in Copake, New York (personal communication D. J. Springer) and supports the recognition of a potential haplotypes within *C. aspenensis* and resolve the phylogenetic placement of the closely related species *C. rajasthanensis*, *C. flavescens*, *C. terrestris*, and the *C. aspenensis* sp. nov. described in this analysis.

### Supporting Information

**Figure S1** Representative Fluorescence-activated cell-sorting (FACS) analysis of the *Cryptococcus* spp. included in the study. All isolates except three *C. laurentii* (CL11, CL19, and E11) and one *C. flavescens* (I234A) appear haploid. Positive haploid (CBS10574) and diploid controls (XL143) were included in each FACS run.

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### Author Contributions

Conceived and designed the experiments: KFP TBF FMF MLSV. Performed the experiments: KFP TBF FMF LAS DJS. Analyzed the data: KFP LAS DJM DJS JH MSCM MLSV. Contributed reagents/materials/analysis tools: KFP FMF DJS JH DM MSCM MLSV. Wrote the paper: KFP DJS JH MLSV.
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