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Article

Talaromyces santanderensis: a New Cadmium-tolerant Fungus from Cacao Soils in Colombia

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Abstract: Inorganic pollutants in Colombian cocoa (*Theobroma cacao L.*) agrosystems cause problems in the production, quality, and exportation of this raw material worldwide. There has been an increased interest in bioprospecting studies of different fungal species focused on the biosorption of heavy metals. Furthermore, fungi constitute a valuable, profitable, ecological, and efficient natural soil resource that could be considered in the integrated management of cadmium mitigation. This study reports a new species of *Talaromyces* isolated from a cocoa soil sample collected in San Vicente de Chucurí, Colombia. The culture was evaluated on six standardized media and distinguished by characteristic colony morphology: biverticillate and monoverticillate penicilli, acerose phialides, and slightly globose smooth-walled conidia. Culture was featured by bright yellow mycelium in young culture on CYA and CYAS medium. Colonies grew faster on Malt and Oat agar, attaining 36 and 32 mm diameter after seven days at 25 ºC. High acid production on CREA medium at 25-35 ºC was observed. Phylogenetic analysis was based on the ITS region, the RPB2, CaM, and β-Tubulin genes that indicate that it is new to science and is named *Talaromyces santanderensis* sp. nov. This new species belongs to the Talaromyces section and is closely related to *T. lentulus, T. soli*, *T. tumuli,* and *T. pratensis* (inside the *T. pinophilus* species complex) in the inferred phylogeny. Mycelia growth of the fungal strains was subjected to a range of 0-400 ppm Cd and incorporated into malt extract agar (MEA) in triplicates. Fungal radial growth was recorded every three days over a 13-day incubation period and *In vitro* cadmium tolerance tests showed a high tolerance index (0,81) when the mycelium was exposed to 300 ppm of Cd. Results suggest *that T. santanderensis* showed tolerance to Cd concentrations that exceed the permissible limits for contaminated soils, and it is promising for its use in bioremediation strategies to eliminate Cd from highly contaminated agricultural soils.

Keywords: fungal systematics; *Talaromyces santanderensis*; cacao soils; cadmium; mycoremediation

1. Introduction

Heavy metal pollution has become a severe problem worldwide [1]. The cadmium (Cd) released from different sources, whether natural or anthropogenic, can lodge in the soil and therefore bioaccumulate in crops of agricultural interest such as *Theobroma cacao* L, a native tree from tropical American rainforests, where until today, it is found in its wild state, from Peru to Mexico [2]. Cocoa ranks third after sugar and coffee in the world food market, demanded mainly by American and European companies [3]. Nonetheless, the cadmium present in agricultural soils is accumulated by certain species of plants where cocoa is listed [4], representing a problem for the cacao quality, food safety, and the international market. Cd can bioaccumulate, is non-biodegradable, and has been associated as a precursor of several human diseases such as cancer and many other illnesses related to its presence in the human body that causes oxidative stress, inflammation, and tissue damage [5]. Cd is found in the earth's crust at an average concentration of 0.1 mg

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kg-1 [6]. The average level of cadmium in agricultural soil fluctuates between 0.07 and 1.1 μg g-1, with a natural base level of 0.5 μg g-1 [7].

The production of cocoa beans in Colombia has been evaluated on numerous occasions for its high levels of cadmium. A study by Echeverri & Reyes (2016) [8] showed that the cadmium concentration in chocolate with more than 30% content of Colombian cocoa yielded an average value of 4.0477 mg/Kg of cadmium. This data puts the health of local producers and consumers at risk and pose a threat to the productive chain of cocoa derivatives because it exceeds the maximum limits established by the recent EU provision (Regulation No. 488/2014 with tolerable values between 0, 1, and 0.80 μg g-1 for derived cocoa products) [9]. This situation limits the growing access to international markets for the Colombian cocoa sector, which in 2018 managed to export a total of 7,056 tons of cocoa to 23 countries, and had a FOB income of 16 Million USD [10].

The search for different and better strategies to mitigate cadmium concentration in cocoa-producing soils has aroused interest in Central and South American countries. According to a recent study by Bravo et al. (2021) [11], in Colombian cacao-growing farms, Cd concentration in the soil samples ranged from 0,01 ppm to 27 ppm, especially in the Santander region, where the highest levels of Cd were found. Thus, many genetic, chemical, and agronomic strategies have been applied and studied to counteract the Cd of agricultural soils. Nowadays, mycoremediation has an excellent acceptance in the remediation processes of contaminated soils due to their different advantages, such as the low application cost, small environmental impact, and high effectiveness. Therefore, these advantages reduce the enormous ecological stress caused by heavy metals [12].

Several metal-tolerant filamentous fungi have been isolated from multiple heavy metal contaminated sites and soils [13, 14, 15, 16, 17, 18]. They have been recognized for their effectiveness in heavy metal remediation processes due to their particular attributes such as rapid growth, ability to thrive in extreme temperature, pH, and tolerance to high concentrations of metals. In the particular case of cadmium stress alleviation in plants, *Talaromyces* has stood out as a promising endophyte [19, 20]. Moreover, for the treatment of substrates and hydrological sources, species like *Talaromyces emersonii* and *Talaromyces amestolkiae* have been used as organic phosphate sources for treating uranium-contaminated water [21, 22]. Also, *Talaromyces helicus* has been widely used to remove cadmium, cobalt, copper, and lead from industrially contaminated sediments [23, 24].

Talaromyces spp. is characterized by a cosmopolitan distribution and joint in many substrates (most commonly found on soils) [25]. Furthermore, by their capacity to produce heat-resistant ascospores [26]. This monophyletic genus belongs to the order Eurotiales and contains seven sections (*Bacillispori*, *Helici*, *Islandici*, *Purpurei*, *Subinflati*, *Talaromyces*, and *Trachyspermi)* [26]. It was recently classified at the family and genus level, considering its traditional phenotypic characters, such as its texture that can be strictly velutinous to floppy and even synnematous or funiculous. Penicilli, in general, are biverticillate, but certain species can have both biverticillate and monoverticillate with acerose or ampulliform phialides. Conidia are usually described as ellipsoidal to fusiform and globose with a lesser proportion. Its taxonomy has been improved using sequence-based approaches [27]. To date, Talaromyces contains more than 250 described species in the Mycobank database [28] and Talaromyces section (sect.). Talaromyces contains roughly 86 species [29]. Additionally, inside the Talaromyces sect. Talaromyces is located in the informal group *Talaromyces pinophilus* complex, which harbors *T. lentulus, T. mae, T. soli, T. malicola, T. adpressus, T. annesophiae,* and *T. pratensis* [29, 30]. However, this complex requires improved phylogenetic elucidation to support this subgroup species' evolutionary relationships further.

In the present study, we performed a collection of an agricultural soil sample from the Santander region in Colombia. After the isolation in multiple media and a morphological identification, the *Talaromyces* sample could not be assigned to any known species. Thus, we performed a polyphasic identification approach by adding phylogenetic analysis of partial ITS, β-tubulin (*BenA*), calmodulin (*CaM*), and RNA polymerase II second

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largest subunit (*RPB2*) gene sequences, and the use of macro-and micro-morphological data to delimitate the new species and section in this genus.

2. Materials and methods

2.1. Sample Soil Description

A soil composite sample, with high content of Cd (18 ppm), from *the Theobroma cacao* crop was randomly collected from the village of Monserrate, municipality of San Vicente de Chucurí from the Santander region in Colombia 73.4097601W decimal longitude, 6.881000N decimal latitude. The sample for this study was provided by the Colombian Cocoa Growers Federation (Fedecacao) and deposited in the Agro-environmental research laboratory of Biotechnology-LIIBAAN from the University of Santander (UDES). The physical-chemical analysis of the soil sample was carried out by a laboratory certified by the ICA (Colombian Agricultural Institute) according to standard methods (Table 1).

2.2. Isolation of Strains and Screening for Cadmium Tolerant Fungi

The fungal strain was initially isolated on Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) by serial dilution. Samples were diluted to 10^{-4} in sterile water. 0,1 mL of different dilutions were spread on Petri dishes (10 cm diameter) containing 20 mL of medium. The plates were incubated at 25°C and 35°C in dark conditions and monitored daily for up to 7 days. Each developed colony was subcultured and isolated on fresh PDA plates supplemented with 25 mg/L of Cadmium adjusted to a pH of 5.5 to 5.7. The plates were incubated under the same conditions.

2.3. Cadmium Tolerance Test

The cadmium tolerance was evaluated based on the selection of strains whose mycelium initially grew at 25 ppm of Cd, equal to the control. Tolerant strains were cultivated in higher concentrations of Cd (50, 100, 200, 300, and 400 ppm) in PDA and MEA culture media. Sterile filtered CdCl2 salts (pore size of 0,25 μm) were incorporated, supplemented with 25 mg/mL^{-1} of streptomycin, and pH was maintained at 5.7. The experiment was performed in triplicate for both the control and the concentrations. Eight-day-old 5 mm diameter mycelium discs were individually inoculated from the pure cultures. Plates were incubated at 25 and 35 ± 1 ° C for equal days, during which the radial growth of the mycelium was monitored. The cadmium tolerance potential of the fungal species in the test medium was calculated in relation to the growth control radials (Eq. 1). The tolerance to heavy metals of the fungi was scored as follows: 0,00-0,39 (very low tolerance), 0,40-0,59 (low tolerance), 0,60-0,79 (moderate tolerance), 0,80-0,99 (high tolerance) and 1,00–> 1,00 (very high tolerance) [15].

$$Tolerance\ index = \frac{Radial\ growth\ (mm)\ of\ fungus\ in\ medium\ with\ Cadmium}{Radial\ growth\ (mm)\ of\ \ fungus\ in\ medium\ without\ Cadmium} \quad (1)$$

2.4. Cultivation Conditions and Colony Morphology

Isolates were inoculated by placing 1 μL of conidia from semi-solid agar (0,2% agar + 0,05% Tween 80) in 90 mm Petri dishes with Malt Extract Agar (MEA), Oatmeal Agar (OA), Yeast Extract Sucrose Agar (YES), Creatine Sucrose Agar (CREA), Czapek Yeast Autolysate Agar (CYA), and Czapek Yeast Autolysate Agar + 5 % NaCl (CYAS). Plates were incubated for eight days in the dark at 25°C and 35°C. The Colony features were studied following Visagie et al. 2014 [31] and Yilmaz et al. 2014. [26] Colony diameters were also measured after seven days at 25°C and 35°C and photographed (Nikon, camera, model FE-220/X-785), two perpendicular diameters were measured for each colony, and the average was calculated. Phenotypic characteristics, such as obverse and reverse culture appearance, colony texture, mycelium color, sporulation, exudates, and medium changes, were also recorded. The names of colors were referenced by Ridgway [32].

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2.5. Microscopic identification

The microscopic slides were prepared from 7-day-old cultures grown on Malt Extract Agar (MEA). The conidia were suspended in 1 ml of 60% lactic acid [26]. Phenotypic characteristics of conidia (shape, ornamentation of the cell wall) and conidiophores (number of branching points between stipe and phialides, and shape/texture of phialides) were recorded. Microscopic examinations were done using a microscope Nikon (model Eclipse Ni-u). Two diameters (length and width) were measured for 100 conidia of each isolate in the Quick Photo Camera software program (Imagen Pro).

2.6. DNA Extraction and Amplification

The isolate was cultured in a Sabouraud dextrose medium for 7 days at 28 °C for DNA extraction. Approximately 100 mg of mycelium was scraped from the surface of the plate using a sterile blade and then transferred to a 1.5 mL sample tube. Total DNA extractions were performed using a phenol-chloroform method with modifications suggested by Chi et al. (2009) [33]. The mycelia were crushed in liquid nitrogen and placed in 1 mL of Lysis Buffer (40 mM Tris-HCl, 20 mM sodium acetate, 10 mM ethylenediaminetetraacetic acid, and 1% sodium dodecyl sulfate, pH 8.0) per 100 mg of powdered mycelium. The polymerase chain reaction (PCR) method was used to amplify marker genes for species; internal transcribed spacer (ITS1-4), β-tubulin (BenA), Calmodulin (CaM), and the second subunit of DNA-dependent RNA polymerase II (RPB2).

PCR amplifications were conducted with the following primer pairs: RPB2-5F (5′-3′, CCRAARTGATCWCKRTCRTC) [34] and bRPB2-7.1R (5′-3′ CCCATRGCYTGYTTMCCCATDGC) [35] for a fragment of approximately 1000 pb of RPB2 gene. CMD5 (5′-3′ CCGAGTACAAGGARGCCTTC) and CMD6 (5′-3′ CCGATRGAGGTCATRACGTGG) primer pair for a fragment of approximately 600 bp of Calmodulin gene. ITS1-F (3′-5′ TCCGTAGGTGAACCTGCGG) and ITS4-R (3′-5′ TCCTCCGCTTATTGATATGC) for a fragment of 500 bp of ITS. T1 (5′-3′, AACATGCGTGAGATTGTAAGT) and T22 (3′-5′ TCTGGATGTTGTTGGGAATCC) for a fragment of approx 1350 bp of the β-Tubulin gene [36].

The amplification programs were used with the following parameters: for ITS; 5 min at 95ºC, 35 cycles x (1 min 94 ° C, 1 min 55 °t C, 2 min 72 ° C), 5 min at 72 ° C. For B-tubulin, 5 min at 95 ° C, 35 cycles x (35 sec 94 ° C, 55 sec 55.4 ° C, 2 min 72 ° C), 5 min at 72 ° C. For Calmodulin, it was 5 min at 95 ° C, 35 cycles x (1 min 94 ° C, 55 sec 50 ° C, 2 min 72 ° C), and 5 min at 72 ° C. For RPB2, it was 5 min at 95 ° C, 35 cycles x (1 min 94 ° C, 55 sec 52,5 ° C, 2 min 72 ° C), and 5 min at 72 ° C. All reactions were performed in a C1000 Thermal Cycler (BioRad Technologies). The amplification products were visualized on a 1% agarose gel and quantified in Nanodrop (ThermoFisher) for subsequent shipment to the Sanger sequencing service at the Genecore facility of the Universidad de Los Andes, Colombia. Obtained chromatograms were assembled using Tracy v.0.5.8 [37].

2.7. Molecular characterization and phylogeny

For the phylogenetic reconstruction, a four-gene phylogeny from 80 species described for *Talaromyces* section *Talaromyces* was downloaded from NCBI. Strain codes and database accession numbers are supplied in Table 1. Phylogenetic analyses were performed with *BenA, CaM, RPB2*, and ITS sequences individually and concatenated, with *T. dendriticus* CBS 660.80 of sect. *Purpurei* as the outgroup.

Sequences were aligned with MAFFT v 7.453 [38]. For evolution model determination and Maximum Likelihood (ML) analysis, IQTREE [39] was implemented with Ultra-Fast Bootstrap. Bayesian Inference (BI) analysis was performed with BEAST2 [40] with 10.000.000 generations; the molecular clock was set as default. Substitution models and rates among sites were set as TPM2+I+G4 for *BenA*, TIM3e+I+G4 for *CaM*, TPM2+I+G4 for *RPB2*, and TIM2+F+R3 for ITS for ML and BI analyses. Trees were visualized in Figtree.

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3. Results

Analysis of soil of composite sample pH value, the content of macro and micronutrients, and other data of interest as cationic ratios are shown in Table 1.

Table 1. Physical-chemical analysis of cocoa soil.

Parameter	Value	Unit	Suitable range	Interpretation
PH	6,8			
Organic material	5,31	%		
Nitrogen (N)	0,27	%	0,19-0,35	Medium
Phosphorus(P)	51,34	ppm	15-30	High
Potassium (K)	0,12	meq/100g	0,20-0,50	Low
Magnesium (Mg)	2,27	meq/100g	1,50-3,0	Medium
Calcium (Ca)	22,30	meq/100g	3,0-60	High
Sodium(Na)	0,16	meq/100g	0,10-0,50	Low
Sulfur(S)	10,82	ppm	10-15	Low
Iron(Fe)	72,13	ppm	25-50	High
Boron(B)	0,15	ppm	0,40-0,80	Low
Copper(Cu)	2,50	ppm	1,50-3,0	Medium
Manganese(Mn)	1,25	ppm	5,0-25	Low
Zinc(Zn)	19,55	ppm	2,0-3,5	High
Cadmium (Cd)	18,83	ppm	-	High
Cationic ratios				
Ca/Mg	9,82		3,0-6,0	High
Ca/K	181,30		15-30	High
Mg/K	18,46		10-15	High
(Ca+Mg)/K	199,76		20-40	High
% Na -saturation	0,64		5-15	Low
% K -saturation	0,49		2-3	Low
% Ca -saturation	89,74		50-70	High
% Mg -saturation	9,13		10-20	Low
% saturation of bases	100,01		35-50	High
%aluminum saturation	-		10-50	-

Analytical Methods

11. interchangeable aluminum expressed in terms of acidity (acid-base titration, Yuan method-KCL). 2. PH (Potentiometric) 3. Sulfur (Turbidimetric, Ca monobasic phosphate extraction 0,008 M). 4. Boron (Colorimetric), 5. Bases of change (atomic absorption) 6. Cation exchange capacity (ammonium acetate extraction), 7. Electric conductivity (electrometric saturation extract).8. Available phosphorus (colorimetric, Bray II), 9. Micronutrients

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Atomic absorption, extraction with DTPA)10. Organic material (Walkley Black), 11. Texture(Bouyoucos).12. Cadmium (atomic absorption spectroscopy using air-acetylene flame)

3.1. Cadmium tolerance test

The presence of filamentous fungi in contaminated sites would indicate their adaptation to rough soil conditions and the development of specific mechanisms for such resistance, as shown by the new species of Talaromyces isolated in this study (Fig.1A and 1B). *Talaromyces santanderensis* demonstrates a high tolerance rating at 100-400 ppm cadmium with IC50=354.72 ppm, calculated by sigmoid function [41].

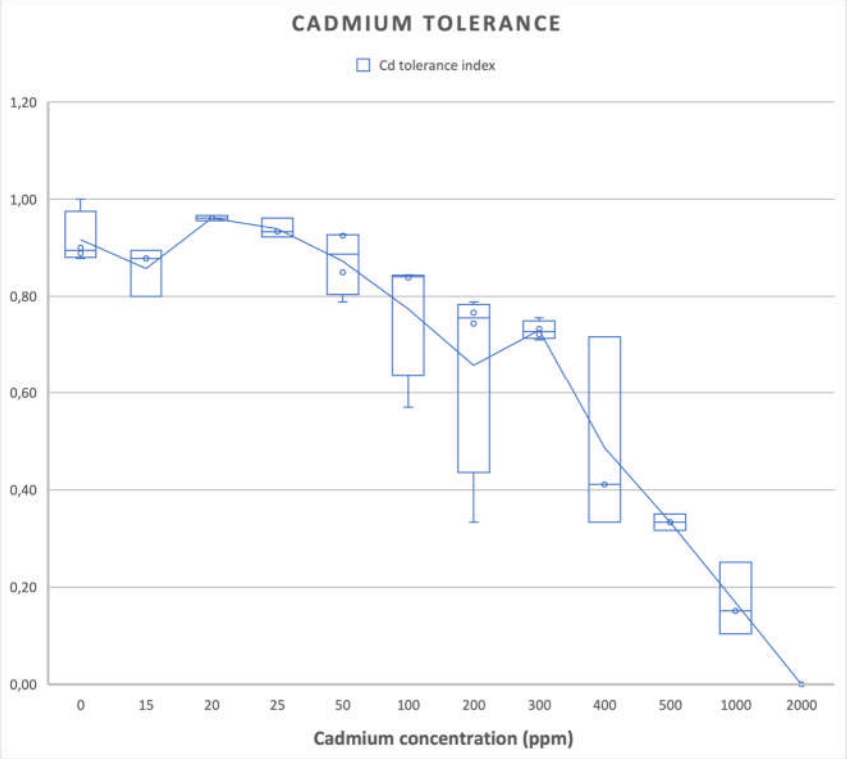

Figure 1. Effect of different concentrations of Cadmium on mycelial radial growth (Cd tolerance index of 3 replicates ± SE).

3.2. Growth in different culture media

Each isolate was inoculated on oatmeal agar (OA), creatine sucrose agar (CREA), Czapek yeast extract agar (CYA), CYA + 5 % NaCl (CYAS), yeast extract sucrose agar (YES), and malt extract agar (MEA). The isolates were incubated for seven days at 10°C, 25°C, 35°C, and 37°C in darkness. The morphological characteristics of the colonies at 25°C, 35°C, and 37°C were very similar in terms of color and texture in the different culture media tested, observing a change in the growth rate at 10°C, where a slower growth was observed, and the size of the colonies reached 5-7 mm in diameter, after seven days. Growth rates were better at 25°C and 35°C in all culture media except CREA agar, where the mycelium did not develop.

The results of the radial growth diameters and the macro morphological characteristics are presented in Figs. 1A -1B.

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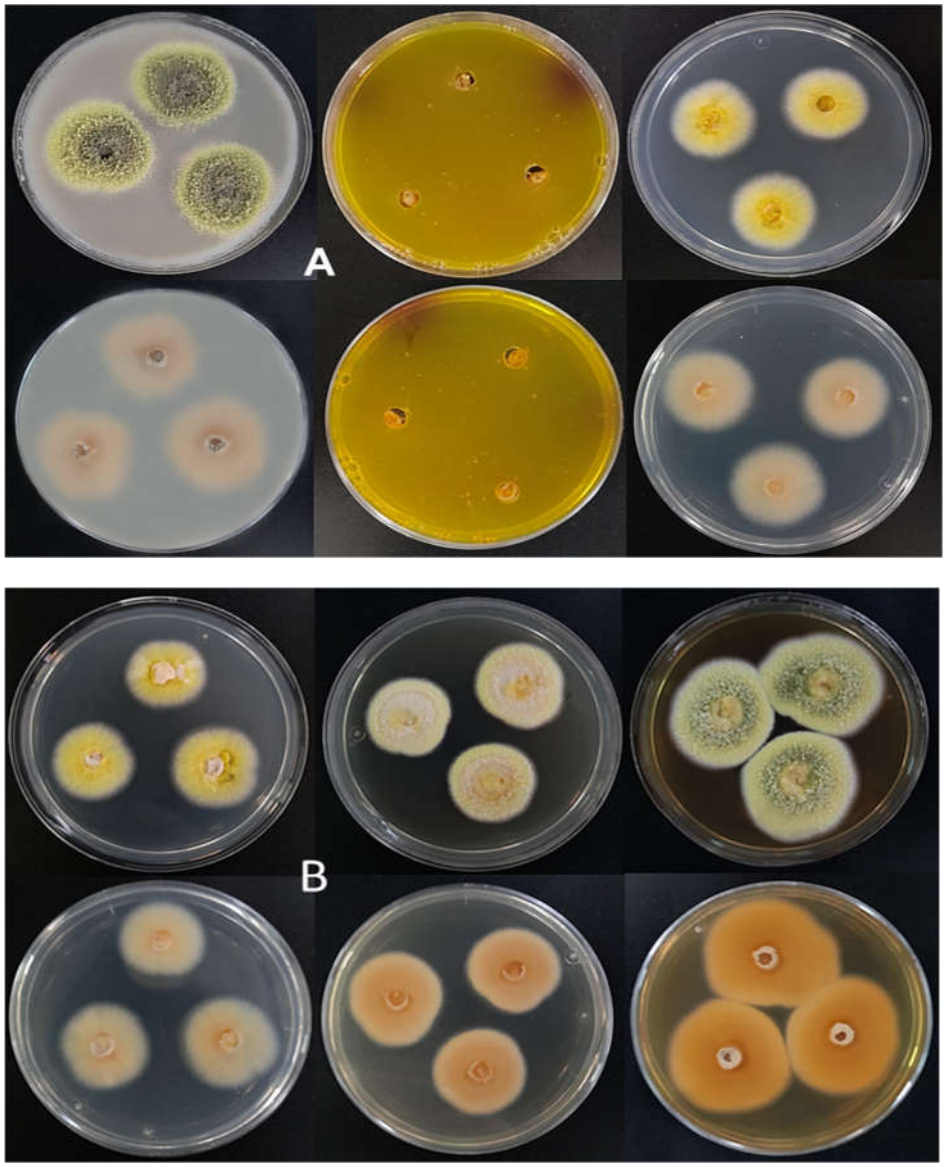

Figure 2. A: Colonies from left to right (top row) OA, CREA, CYA supplemented with 5 % NaCl (CYAS), (bottom row) OA reverse, CREA reverse, and CYA supplemented with 5 % NaCl (CYAS), B: Colonies from left to right (top row) CYA, YES, Malta (bottom row) CYA reverse, YES reverse, and Malta at 25°C after 1-wk incubation. .

The isolate had a plane-low surface with filamentous colonies and grew moderately on every culture medium. The isolate had a low growth ratio in the CREA medium, where the mycelium did not develop and produced high acidification (change of the medium color from purple to yellow) at 25°C, 35°C, 37°C. Colonies had the better growth ratios on Malt and Oat agar, attaining 36 and 32 mm diameters after seven days at 25°C. The front side in both culture mediums was greenish yellow (R.PI.V), with a wide cream margin (R.PI.XVI), 6~9 mm. In contrast, the reverse side of the colony was salmon (R.PI.XIV) on OA and Cinnamon Rufous (R.P.I. XIV) on Malt agar. Sporulation was dense, and conidia were numerous. The texture of the colony was floccose to powdery in both culture media. On YES, they attained a diameter of 29~30 mm after seven days at 25°C. The front side of the colony was cream (R.PI.XVI), which turned to pale orange-yellow over time (R.PI.III), and the reverse side was cinnamon rufous (R.P.I.XIV). The texture of the colony was floccose. Sporulation was sparse to moderately dense, and conidia were numerous. On CYA supplemented with 5 % NaCl (CYAS) and CYA, the growth of the mycelium was slow. The average growth was 26mm on CYA and 24 mm on CYAS, the colonies were lemon yellow (R.P.I.IV), and the reverse side was salmon (R.PI.XIV) (Fig. 2A and 2B). The texture

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of colonies was at the margin floccose, sporulation was dense, and conidia were numerous. Figs. 2A-2B. The isolate manifested growth at 25°C, 35°C, and 37°C. Soluble pigments were absent in all cultures. Clear exudates were present on Oat and Malta agar.

Growth size (mm) and other morphological aspects of the new *Talaromyces* species are described and compared with five *Talaromyces* species that are phylogenetically related (Table 2).

Table 2. Morphological comparisons of new *Talaromyces* species and their closely related species.

Species	CYA mm	MEA mm	YES mm	Conidio-phore	Conidia shape	Conidia Wall	Conidia Size-µm	Source	Reference
T. santan-derensis	22- 24	34-36	26-30	Mono-to biverticillate	Slightly globose	Smooth	1.8-2.2	Cocoa soil, Santander, Colombia	This study
T. pinophilus	16- 31	37- 45	12-35	Biverticillate, occasionally monoverticil-late	Subglo-bose, to ellipsoidal	Smooth to finely rough-ened	2.5-3.5 (-9) x2.5-3.0 (-5)	Mycobank MB560662	Peterson, S. W., & Jurjević, Ž. (2019).
T. adpressus	32- 33	42-43	42-43	Biverticillate	Subglo-bose to el-lipsoidal	Smooth	2.5-4.5 (-4.5) × 2–3.5	Indoor en-viron-ments in Beijing China	Chen, A. J.,et.al (2016)
T. lentulus	26- 27	43–44	37–38	Biverticillate	Globose	Smooth	2.5–3.0	Alkaline soil, Ying-kou, Shan-dong, China	Jiang, X. Z.et.al(2018)
T. pratensis	20- 22	34-36	16-17	Biverticillate, occasionally monoverticil-late	Globose to subglo-bose, oc-casionally broadly ellipsoidal	Smooth to finely rough-ened walls	2.5-3.0 (-7) x 2.5- 3.5 (-4.5)	Effluent of water treatment plant Cin-cinnati, W. B.	Peterson, S. W., & Jurjević, Ž. (2019).
T. soli	20-26	37-42	14-26	biverticillate rarely mono-verticillate	subglo-bose to broadly ellipsoidal	thick, to finely rough-ened	2.5-3.5 (-5.5) x2.5-3.5(-4.5)	Isolated from soil.	Peterson, S. W., & Jurjević, Ž. (2019).

3.3. Micromorphology analysis

The isolates showed Conidiophores arising from surface hyphae with smooth-walled stipes (250-–330 × 2.0–2.5 µm) (Figs.3A-b). Conidiophores had mono-to-biverticillate penicilli with acerose-shaped phialides and short blunt necks (Figs 3. B, C, D). Conidia are smooth-walled and slightly globose (1,8~2,2 µm in diameter). Loose conidia or chains of conidia were irregularly observed in masses of 6 to 8 conidia (Figs.3 E-F). 3–5 Metulae per stipe (9-10 × 2.5–2.8 µm) were observed; phialides (2–4 per metula) were acerose with short collula (7–9 × 1.5–2.0 µm). The stipes were smooth-walled.

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Conidia were olive green (R.PI.IV) on OA and MEA medium and pale greenish yellow (R.PI.V) on CYA, CYAS, and YES medium. For ascoma production, OA, MEA and CYA plates were incubated for up to four weeks. No structures of sexual reproduction were found in any of the observed samples for the microscopic analysis.

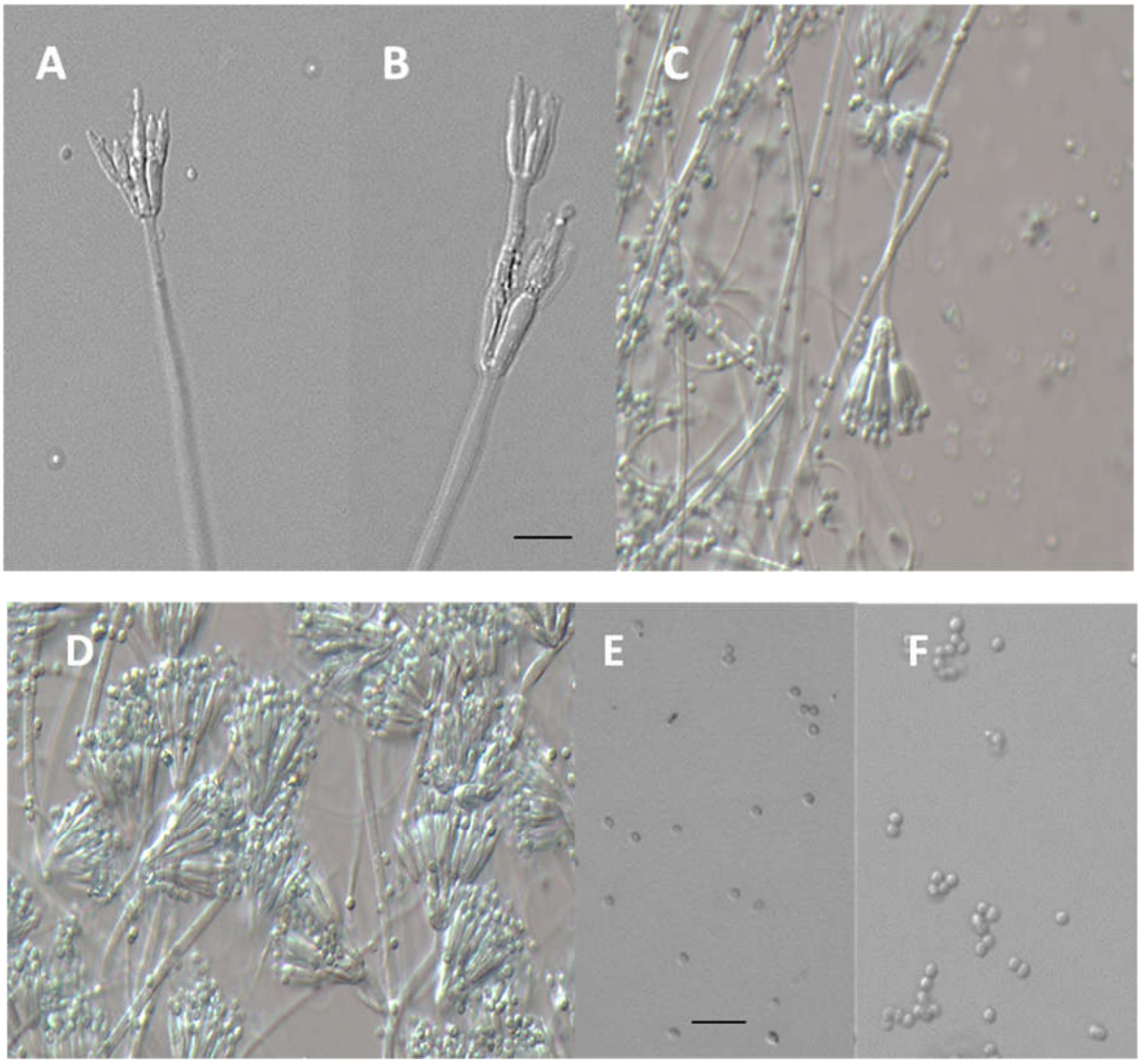

Figure 3. (A-D) Monoverticillate and Biverticillate Conidiophores. (E-F) Conidia. Scale bars= 10 µm.

3.4. Molecular identification and sequence analysis

Sequences were reported to Genbank under the code accesses: OP082331, ITS1-4; OP067657, BenA; OP067656, CaM and OP067655, RPB2. PCR amplification generated amplicons of *BenA* about 1447 bp, *CaM* about 449 bp, *Rpb2* about 1033 bp and ITS about 569 bp. The trimmed alignments of *BenA, CaM, Rpb2,* ITS and the combined *BenA-CaM-Rpb2-*ITS sequences were 520, 639, 1008, 692 and 2859 characters with gaps, respectively.

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3.5. Phylogenetic analysis

Figure 4. Topologies reconstructed with ITS, CAM, BenA and RPB2, bootstrap and posterior probability included at the nodes.

BI phylogram inferred from the concatenated BenA-CaM-Rpb2 sequences. Ultrafast-bootstrap percentages over 90% derived from 10000 replicates and posterior probabilities over 0.9 of posterior support are indicated at the nodes. T indicates ex-type strains, strains

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belonging to new species are indicated in boldface. Scale Bar: number of substitutions per nucleotide position.

Figure 5. Individual phylogeny of BenA. BI phylogram inferred from partial BenA sequences. Ultrafast-bootstrap percentages over 90% derived from 10000 replicates and posterior probabilities over 0.9 of posterior support are indicated at the nodes. T indicates ex-type strains, strains belonging to new species are indicated in boldface. Scale Bars: number of substitutions per nucleotide position.

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The phylogenetic trees generated by the concatenated BenA-CaM-Rpb2-ITS and individual loci show the isolate as a distinct species of sect. *Talaromyces*. The specific epithet refers to the region of type locality

T. santanderensis sp. nov. forms a clade with *T. lentulus* [42] with 100%/1 bootstrap/pp support in the concatenated BenA-CaM-Rpb2-ITS (Fig. 4) and individual phylogenies of BenA (Fig. 5) and CaM. RPB2 phylogeny supports this clade with 99%/1 bootstrap/pp support. Nevertheless, *T. lentulus* forms a separate lineage in ITS phylogeny. This clade is located inside the Talaromyces pinophilus complex, which includes *T. soli, T. tumuli, T. adpressus, T. pinophilus, T. pratensis, T. domesticus, T. sayulitensis, T. malicola, T. annesophieae, and T. mae* [30]. In the concatenated analysis, other clades supported inside the *T. pinophilus* complex were *T. domesticus* and *T. sayulitensis* with 100/1 bootstrap/pp support and *T. mae* with *T. pinophilus* and *T. annesophieae* with 100/1 bootstrap/pp support. In the individual BenA phylogeny, *T. domesticus* and *T. sayulitensis* formed a clade with 94/0.98 bootstrap/pp support and *T. pinophilus* with *T. annesophieae* with 100/0.98 bootstrap/pp support.

4. Discussion

This study demonstrated by phylogenetic analysis that the new species of Talaromyces is closely related to *T. lentulus* [42]; nevertheless, both species have characteristic morphological differences. *T. lentulus* has biverticillate penicilli, with acerose phialides with short collula, and develops vivid yellow mycelium in MEA, with a funiculus and floccose texture. On CYA, conidiogenesis is absent, and the mycelium shows a Pale Salmon color. Colonies with a 26–27 mm diameter at 25°C after seven days are thin with few radial sulci; velutinous with sparsely overlaid mycelium. Conidia are globose, smooth-walled, and present approximately 2.5–3.0 mm in size. In contrast, *T. santanderensis* presents conidiophores with mono to biverticillate penicilli, with acerose-shaped phialides and short blunt necks, developing greenish yellow mycelium in MEA, with a broad cream margin, and floccose to powdery texture. On CYA conidiogenesis is present, and the mycelium shows a Lemon Yellow color. Colonies with 22–24 mm diameter at 25°C after seven days, at the margin were floccose, sporulation was dense, and conidia were numerous, slightly globose conidia and are smaller than *T. lentulus* with an average size of 1.8-2.2 mm.

To evaluate the ascoma production on *T. santanderensis*, OA, MEA, and CYA plates were incubated for up to four weeks. No structures of sexual reproduction were found in any of the observed samples for the microscopic analysis. Nevertheless, other studied species of Talaromyces have shown that they need a longer incubation time for ascospore production (from 6 to 20 weeks) [26]. Sexual reproduction structures are difficult to observe in some species of fungi under laboratory conditions, for which these species have been considered "asexual" or anamorphic, representing a challenge for their study. Some species of ascomycete fungi known as "asexual" are capable of reproducing sexually, induced under laboratory-controlled conditions [43, 44]. Therefore, more studies will be required to elucidate if the new species described in this work could develop a teleomorphic state.

It is suggested that *T. santanderensis* is a new species inside *the T. pinophilus* complex using a polyphasic approach, including different phylogenetic analyses of combined and individual ITS, CaM, BenA, and RPB2 gene sequences. Nevertheless, relationships between members of the *T. pinophilus* complex remain disputed. Further barcoding or sampling efforts are needed. *T. santanderensis* is the fifth species from the *Talaromyces* sect. *Talaromyces* isolated from Colombia after *T. amazonensis*, *T. francoae*, *T. purgamentorum,* and *T. veerkampii* [29, 45]. Recently, the number of *Talaromyces* species has increased, but further studies are needed to understand the entire biodiversity of the group in Colombia.

Furthermore, a recent study by Bravo et al. 2021 [11] shows the distribution of Cd in soils of cocoa crops in different districts of Colombia, in which Santander had the broadest range of Cd concentrations (0.01-27 ppm) of all the districts analyzed, supporting Cd concentrations found in the studied soil sample. *T. santanderensis* displays a high tolerance

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index (0,81) to cadmium in vitro tests. It has been reported that this tolerance to metals by filamentous fungi is directly correlated to their isolation soil, the toxicity of the tested metal, its medium concentration, and the competence of the isolate [46]. This isolate is already adapted to the specific conditions of the soil. This ability to accumulate heavy metals by species of fungi is based on several mechanisms of adaptive genetic and physiological constituents that have been widely described [47, 48, 49]. Thus, the tolerance of *T. santanderensis* to cadmium presents a promising opportunity for bioremediation strategies focused on eliminating the stress caused by this heavy metal in the agroecosystems of *T. cacao*, reducing the bioavailability of the heavy metal and offering a competitive relationship with its niche and microbial community. More studies are needed to understand its role in the rhizospheric community of *T. cacao* and characterize its potential for bioremediation processes.

Supplementary Materials: Figure S1: BI phylogram inferred from partial CaM sequences. Ultrafast-bootstrap percentages over 90% derived from 10000 replicates and posterior probabilities over 0.9 of posterior support are indicated at the nodes. T indicates ex-type strains, and strains belonging to new species are indicated in boldface. Scale bars: number of substitutions per nucleotide position. Figure S2. BI phylogram inferred from partial RPB2 sequences. Ultrafast-bootstrap percentages over 90% derived from 10000 replicates and posterior probabilities over 0.9 of posterior support are indicated at the nodes. T indicates ex-type strains, and strains belonging to new species are indicated in boldface. Scale bars: number of substitutions per nucleotide position. Figure S3. BI phylogram inferred from partial ITS sequences. Ultrafast-bootstrap percentages over 90% derived from 10000 replicates and posterior probabilities over 0.9 of posterior support are indicated at the nodes. T indicates ex-type strains, and strains belonging to new species are indicated in boldface. Scale bars: number of substitutions per nucleotide position.

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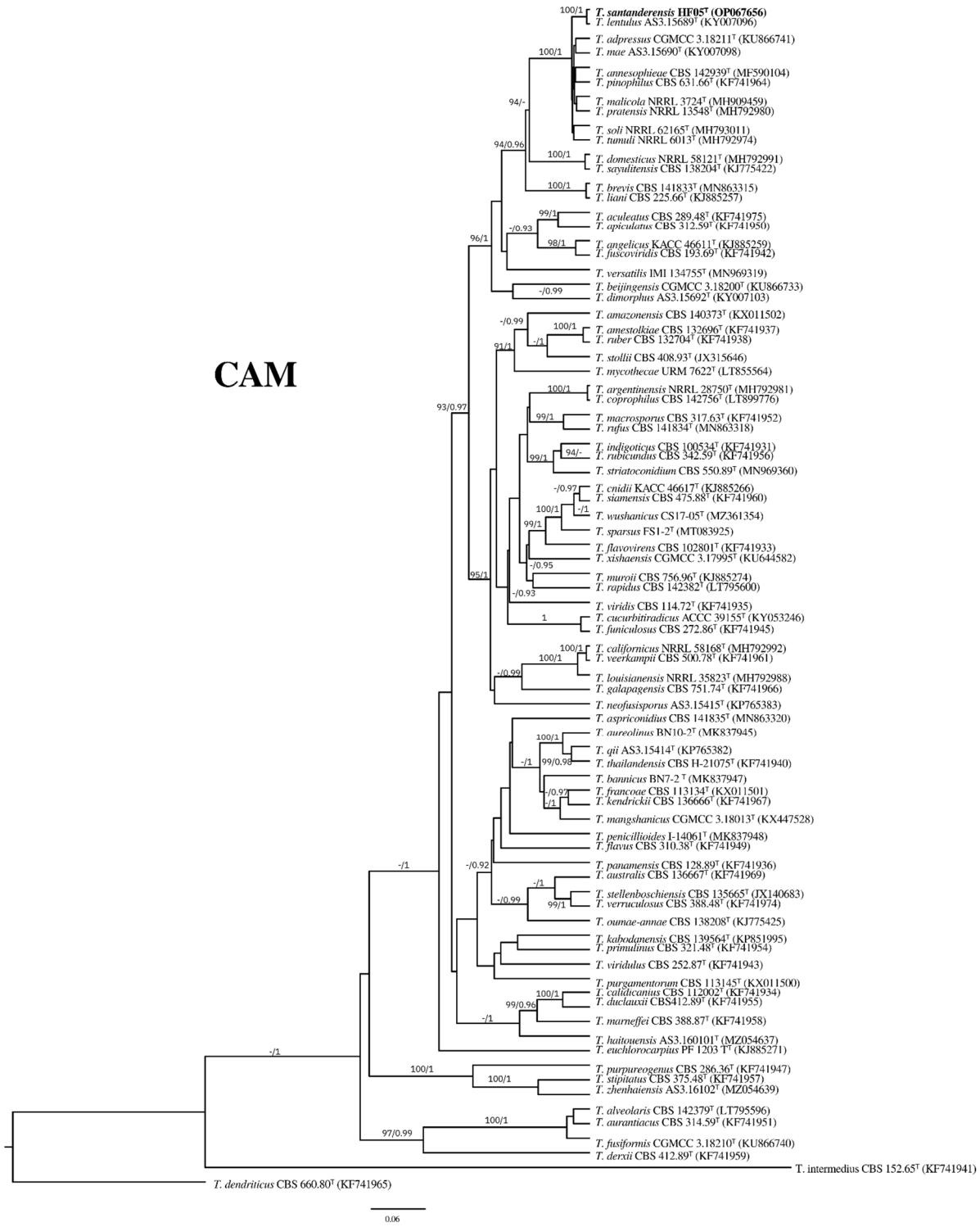

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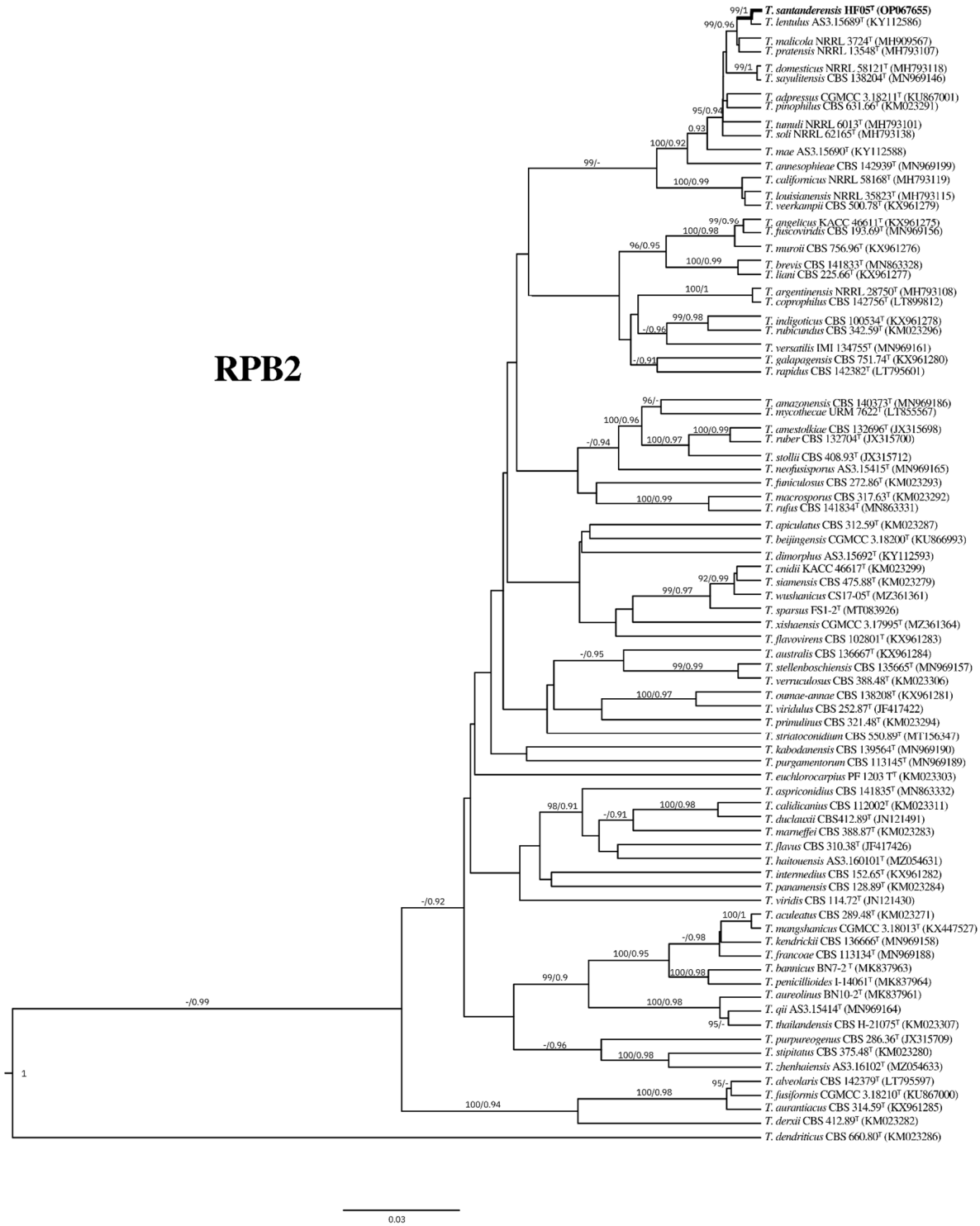

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Lgal considerations: This study was carried out following the collection permit granted by Resolution No. 01749 of December 29, 2017, to the University of Santander (UDES) with NIT number 804.001.890-1, which contributed to the procurement of a composite soil sample of approximately 10 kg, from the Monserrate Farm - municipality of San Vicente de Chucurí. The corresponding credits will be given to the National Federation of Cocoa Growers for the support provided. The strain of *T.santanderensis* was deposited in the Biological Collection of the Universidad de Santander-Colombia, under the following identification: Biological Record ID: CBUDES: UDES:3068, Record number: CNAT_2LIB_HThCd_02_03, Catalog number: CBUDES-CN-HThCd02. The number of the biological collection registered on the National Single Registry of Biological Collections with Alexander von Humboldt Biological Resources Research Institute- Colombia is RNC: 128. Additionally, the guidelines of the framework contract for access to genetic resources and their derived products No. 127, signed between the Ministry of Environment and Sustainable Development and EAFIT University, were followed. Also, Article 6 of Law 1955 of 2019, the National Development Plan 2018-2022 "Pact for Colombia, Pact for Equity," is issued.

Data Availability Statement: The sequences newly generated in this study can be found in the NCBI database.

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