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Communication

First Description of *Simplicillium lanosoniveum*, a Potential Antagonist of the Coffee Leaf Rust from Cuba

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Abstract: (1) The fungal genus *Simplicillium* (Cordycipitaceae: Hypocreales) has an extensive distribution and a broad spectrum of hosts and substrates. The species *Simplicillium lanosoniveum* is a mycoparasite with potential for biological control of coffee leaf rust, *Hemileia vastatrix*. Morphologically, *Simplicillium* closely resembles mycoparasitic and entomopathogenic *Lecanicillium* fungi, often resulting in misidentification. A fungal isolate was obtained from leaf-rust-infested coffee plants from Cienfuegos Province, Cuba. (2) Combined analyses of morphology and molecular markers (ITS, LSU, EF-1alpha) were used for fungal identification. (3) In the NJ, ML, and BI phylogenies which were reconstructed, the isolate LBSim-01 was located in the *Simplicillium lanosoniveum* clade. This species-level identification was supported by morphological features. (4) The isolate LBSim-01 was assigned to the species *Simplicillium lanosoniveum*. This is the first description of a *Simplicillium* fungus associated with coffee leaf rust in Cuba. The presented results hold implications for the biological control of this economically relevant plant disease.

Keywords: *Simplicillium*; mycoparasitic fungi; *Hemileia vastatrix*; phylogeny; molecular taxonomy; internal transcribed spacer; LSU; translation elongation factor 1 alpha

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1. Introduction

Coffee (*Coffea* spp.) is one of the main agricultural crops and the most consumed beverages of the world [1]. The coffee sector had an annual growth rate of at least 4.28% from 2021 to 2026. The coffee species *C. arabica* and *C. canephora* dominate global markets, with production of 58% and 42%, respectively, in 2020. Since 2016, the highest producer and largest exporter countries have been Brazil, Vietnam, and Colombia, having exported 33, 29, and 14 million 60 kg bags, respectively, in 2021/2022 [2].

In Cuba, the coffee plant was introduced in 1748 and is one of the most important crops in Cuban agriculture. Cuban coffee production is a significant source of currency and meets a considerable internal demand. Currently, 85% of harvested Cuban coffee is produced in the mountainous zones of eight provinces [3].

One of the major diseases affecting coffee production is coffee leaf rust (CLR) caused by the basidiomycete fungus *Hemileia vastatrix*. CLR was first reported in Cuba in 1984, causing serious defoliation and economically relevant yield losses of up to 50% and above. During the past decade, favorable climatic conditions (median temperature of 24.7 °C,

average relative humidity of 76%, and annual precipitations of 1363.1 mm) for *H. vastratix* have been recorded in the coffee cultivation areas of Cienfuegos Province, and surveys have shown that the disease has continued to severely reduce yields of *C. arabica* [4].

Several studies have documented natural enemies of *H. vastratix*, such as larvae of gall midges of the genus *Mycodiplosis* (family: Cecidomyiidae), which feed on rust pustules. Fungi reported to display activity against CLR have been identified as *Digitopodium hemileiae* (formerly *Cladosporium hemileiae)*; *Lecanicillium* spp. from Brazil, Colombia, Indonesia, Mexico, and South Africa [5,6]; as well as *Simplicillium* spp. from Costa Rica [7].

The genus *Simplicillium* has a wide host range and a cosmopolitan distribution. Several species are associated with plant pathogenic rust fungi and are considered to have high potential for biological control [8,9]. The genus is phylogenetically related to *Cordyceps*, and its morphological characteristics are similar to those of related *Lecanicillium* or *Akanthomyces* fungi, resulting in common misclassification. Nevertheless, using a combination of classical morphological determination and phylogenetic analyses, *Simplicillium* fungi can be clearly distinguished from related fungal genera [10]. Traditionally, *Simplicillium* fungi were organized as part of the genus *Verticillium* sect. *Prostrata.* The taxonomic genus *Simplicillium* was originally introduced with *S. lanosoniveum* as the type species to designate a presumably monophyletic group of four taxa in the family Clavicipitaceae [11–13]. However, based on multi-gene phylogenetic analyses, *Simplicillium* was shown to belong to the family Cordycipitaceae (Hypocreales, Hypocreomycetidae, Sordariomycetes) [14–16]. Within the genus *Simplicillium*, species have been delineated mainly by means of ribosomal RNA operon sequence analyses [17,18].

It was the objective of the present study to identify the fungal isolate LBSim-01, isolated from pustules of *H. vastratix* in a coffee plantation in Cienfuegos Province, Cuba, based on a combination of morphological and molecular taxonomic approaches.

2. Materials and Methods

2.1. Fungal Isolation and Morphological Identification

Leaves of *Coffea arabica* (Caturra variety) with rust symptoms and the presence of white fungal mycelia in CLR colonies (Figure 1) were collected in November 2022 from a single location (coordinates 21°57′02.6″ N, 80°04′38.9″ W) in Cumanayagua, Cienfuegos, Cuba. For fungal isolation, material from the white mycelia growing in rust pustules was transferred to potato dextrose agar (PDA) plates. The fungus was isolated in pure culture via serial monosporic sub-cultivation on PDA at 26 ± 1 °C in darkness for 15 days. The pure culture of one out of several supposedly redundant isolates was maintained on PDA slants at 4 °C and deposited at the Microbial Culture Collection of the Instituto de Investigaciones de Sanidad Vegetal (INISAV), Havana, Cuba, under the strain designation LBSim-01.

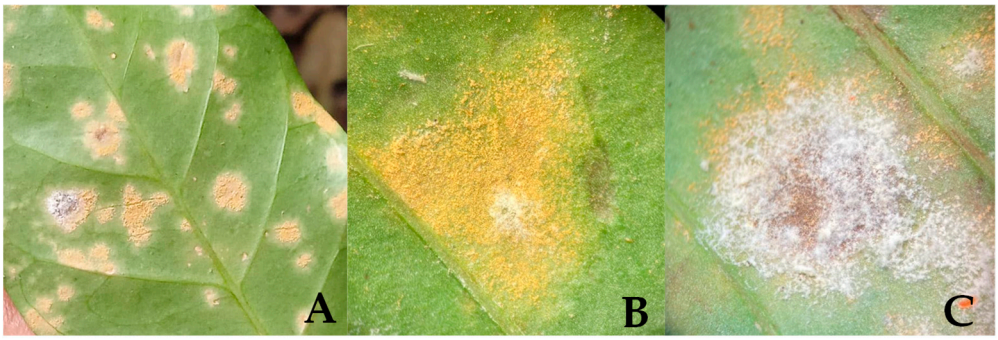

Figure 1. Coffee leaf rust colonies (pigmented orange) with mycoparasitic fungus (white) on coffee plants in the field in Cienfuegos Province: lower side of coffee leaf spotted with fungal colonies (**A**); colonies in early (**B**) and late (**C**) stages of mycoparasitism.

2.2. Morphological and Cultural Characterization

The isolate LBSim-01 was first characterized based on microscopic and cultural features. Growth was observed from 72 h to 15 days on PDA at 26 ± 1 °C. Cultural characteristics of fungal growth such as colony color (surface and reverse), diameter, border, and texture were noted. Colony color was evaluated according to the mycological color chart [19].

For microscopic characterization, the fungus was grown in a synthetic low-nutrient agar (SNA) culture medium (K_2HPO_4 1 g/L, KNO_3 1 g/L, $MgSO_4.7H_2O$ 0.5 g/L, KCL 0.5 g/L, glucose 0.2 g/L, sucrose 0.2 g/L, agar 20 g/L) at 26 ± 1 °C in darkness for 5 days. Fungal mycelium was mounted in a glass slide with 90% lactate acid solution and 0.01% lactophenol cotton blue and observed with an optical phase-contrast microscope model (Nikon Eclipse 80i (Tokyo, Japan)). The conidial morphology was described, and the conidial dimensions (length × width) were determined. Fungal structures were photographed with a Nikon DS-Fi1 Camera (Tokyo, Japan).

The taxonomic descriptions of Humber [20] and Zare and Gams [12] were referred to for fungal identification.

2.3. DNA Extraction, PCR Amplifications and Sequencing

For DNA extraction, the LBSim-01 strain was grown on YPG agar (yeast extract 2 g/L, peptone 10 g/L, glucose 20 g/L) containing 25 µg/mL tetracycline at 25 °C. Approximately 100 mg of mycelial mass was transferred to a screw-capped 2 mL microcentrifuge tube containing Lysing Matrix C (MP Biomedicals, Santa Ana, CA, USA). The sample was frozen in liquid nitrogen and processed for 30–60 s at intermediate speed in a Minilys homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). DNA from the homogenized sample was further extracted using the DNeasy Plant kit (Qiagen, Hilden, Germany) according to the standard protocol provided by the manufacturer. Purified DNA was finally eluted in 100 µL elution buffer (10 mM Tris–Cl, pH 8.5).

Three genetic markers were amplified from extracted genomic DNA on a T-One thermocycler (Biometra, Goettingen, Germany) using Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA) with PCR primers and amplicon-specific parameters, as indicated in Table 1. The internal transcribed spacer (ITS) of the ribosomal RNA operon and partial sequences of the genes encoding the 28S ribosomal RNA in the large ribosome subunit (LSU), as well as the translation elongation factor 1 alpha (EF-1alpha), were utilized. The generalized PCR protocol employed for marker amplification consisted of one initial denaturation step of 95 °C for 2 min, 35 cycles of 45 s at 95 °C, 45 s at the primer specific annealing temperature, and a 68 °C elongation step of amplicon specific time, followed by a 5 min final elongation step at 68 °C. PCR product sizes were checked by gel electrophoresis using 1% agarose gels stained with 5 µL/100 mL of Roti Gelstain (Carl Roth, Karlsruhe, Germany). PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) according to the standard protocol provided. Sanger sequencing of PCR products was performed externally using StarSEQ (Mainz, Germany), with respective PCR primers and additional internal sequencing primers, as indicated in Table 1. Raw sequence data were combined into a single consensus sequence for each marker using the MEGA v.11 software package [21] and MrBayes v. 3.2.6 [22], respectively.

Table 1. Oligonucleotide primers and reaction-specific PCR parameters used in this study.

Primer Designation	Primer Sequence (5′→3′)	Annealing Temperature (°C)	Elongation Time (sec)	Reference
LR0R	GTACCCGCTGAACTTAAGC	58	90	[23]
LR5	ATCCTGAGGGAAACTTC			[23]
ITS4	TCCTCCGCTTATTGATATGC	52	60	[24]
ITS5	GGAAGTAAAAGTCGTAACAAGG			[24]

Table 1. *Cont.*

Primer Designation	Primer Sequence (5′→3′)	Annealing Temperature (°C)	Elongation Time (sec)	Reference
EF1A-983F	GCYCCYGGHCAYCGTGAYTTYAT	52	90	[25]
EF1A-2318R	ATGACACCRACRGCRACRGTYTG			[25]
EF1A-1567R	ACHGTRCCRATACCACCSATCTT	sequencing primer		[25]
EF1A-1577F	CARGAYGTBTACAAGATYGGTGG	sequencing primer		[26]

2.4. Phylogenetic Analyses

The reference sequences of ITS, LSU, and EF-1alpha used in this study were mainly based on those used by Chen et al. [17] (Supplementary Table S1). For the reconstruction of the NJ phylogeny, nucleotide sequences were aligned using the CLUSTAL W function [27] as implemented in the MEGA v.11 software package [20]. For comprehensive analysis, an alignment of concatenated marker sequences (ITS-LSU-EF-1alpha) was generated. Phylogenies were reconstructed from unfiltered nucleotide sequence data under pairwise deletion of alignment gaps and missing data using a p-distance matrix-based neighbor joining (NJ) method, as implemented in MEGA v.11. Tree topology confidence limits were explored in non-parametric bootstrap analyses over 1000 pseudo-replicates.

For the reconstruction of ML and BI phylogenies, marker alignments were first performed with the MAFFT v.7 online server (https://mafft.cbrc.jp/alignment/server/) (accessed on 3 June 2023) [28] and subsequently adjusted manually in MEGA v.11. The sequences of the three loci were concatenated. Maximum likelihood (ML) and Bayesian inference (BI) were run in MEGA v.11 and MrBayes v. 3.2.6 [22], respectively. For ML analyses, the default parameters were used, and bootstrap support (BS) was carried out using the rapid bootstrapping algorithm with the automatic halt option. Bayesian analyses included two parallel runs of 2,000,000 generations, with the stop rule option and a sampling frequency set to each 1000 generations. The 50% majority rule consensus trees and posterior probability (PP) values were calculated after discarding the first 25% of the samples as burn-in.

3. Results

3.1. Phylogeny

Consistent consensus sequences were obtained from the LBSim-01 strain for the markers ITS (505 bp), LSU (830 bp), and EF-1alpha (906 bp), then submitted to the GenBank database under accession numbers OR592663, OR592664, and OR602870, respectively. When used one by one as query sequences for unfiltered BlastN searches across the database, the three markers revealed the greatest similarities with entries assigned to the species *Simplicillium lanosoniveum*. Importantly, the ITS and LSU marker sequences displayed 100% identity at 100% sequence coverage with orthologs from *S. lanosoniveum* (Supplementary Table S2).

Comparisons with concatenated reference sequences gave rise to an ITS–LSU-EF-1alpha alignment 2301 bp in length. In the NJ phylogeny reconstructed from the concatenated marker sequences (Figure 2), the isolate LBSim-01 was firmly located in the *Simplicillium* clade and formed a sub-clade with both *Simplicillium lanosoniveum* reference sequences. However, the *S. lanosoniveum* sub-clade received only 37% bootstrap support. In both ML and Bayesian analyses (Supplementary Figures S1 and S2), the isolate LBSim-01 was similarly located in the *Simplicillium lanosoniveum* clade.

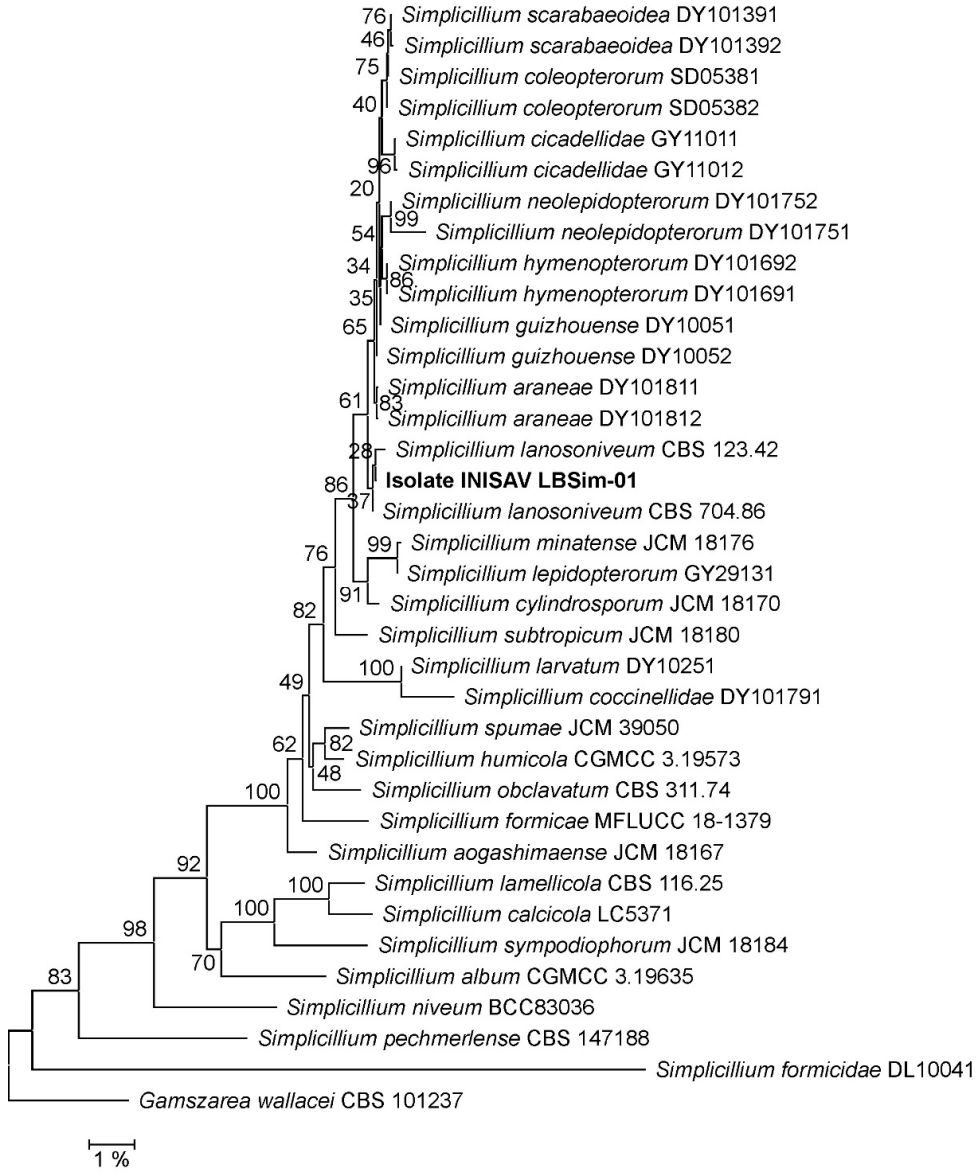

Figure 2. Neighbor joining (NJ) phylogeny of *Simplicillium* fungi as reconstructed from concatenated ITS, LSU, and EF-1alpha nucleotide sequences. Terminal branches are labeled with genus, species, and strain designations. The Cuban isolate under study is displayed in bold type. Numbers on branches indicate bootstrap support values. The size bar corresponds to 1% sequence divergence along phylogram branches. A concatenation of orthologous sequences from the related fungus *Gamszarea wallacei* was used as the outgroup.

3.2. Taxonomy

Simplicillium lanosoniveum (J.F.H. Beyma) Zare and W. Gams, Nova Hedwigia 73(1-2): 39 (2001). Figure 3 ≡ *Cephalosporium lanosoniveum* J.F.H. Beyma, Antonie van Leeuwenhoek 8: 121 (1942).

In SNA medium, the colonies were cottony at the center and slightly funiculose toward the margin. The mycelium was superficial and immersed, composed of branched, septate, smooth-walled, hyaline hyphae 1–2 µm in diameter. The conidiophores were macronematous, mononematous, cylindrical–subulate, hyaline-unbranched, 0–1(–2)-septate, and smooth-walled, and were frequently reduced to conidiogenous cells up to 30 µm long and 1.5–2.0 µm wide. Conidiogenous cells were monophialidic, cylindrical–subulate or subulate, slender, terminal, determinate, smooth-walled, and hyaline, up to 27 µm long and 1.2–1.8 µm wide. Conidia were solitary seriate, acrogenous, oval, ellipsoidal, subcylindrical

to suboblong, unicellular, smooth-walled, and hyaline, measuring 2.5–3.7 × 1–1.6 µm and accumulating in globose masses.

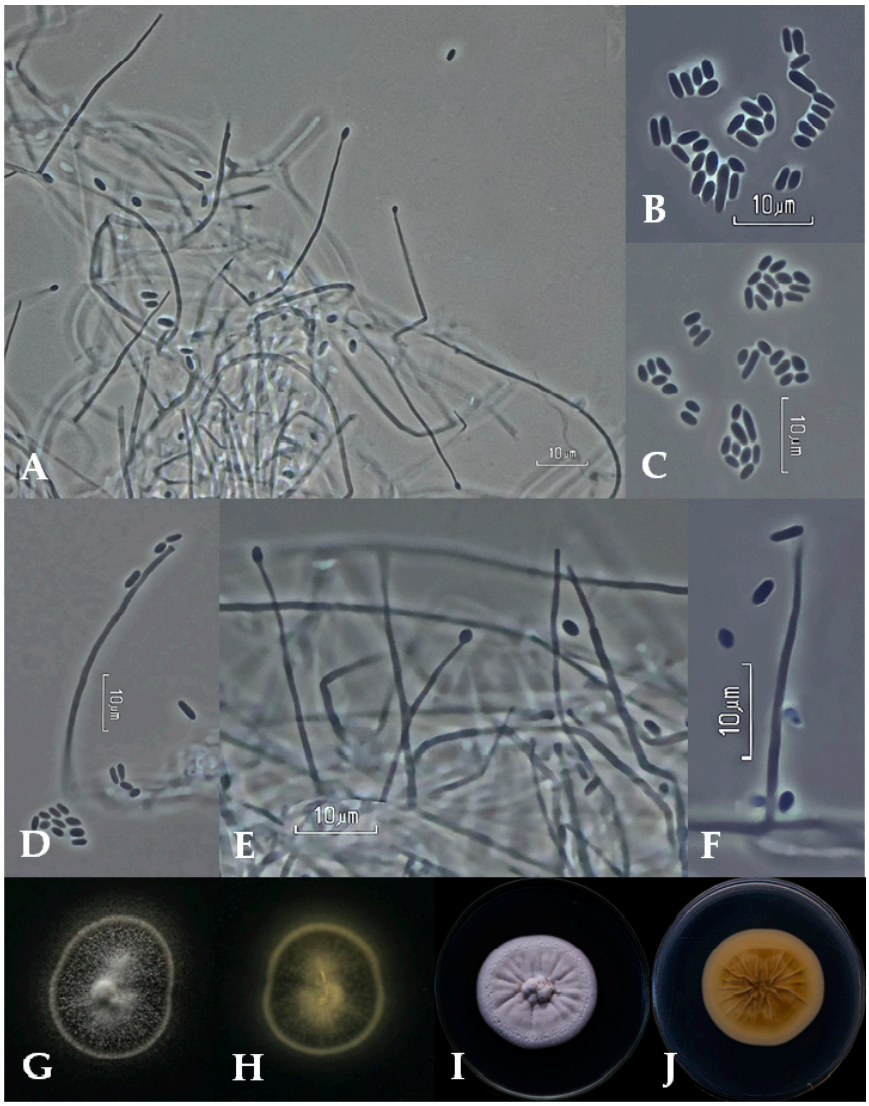

Figure 3. *Simplicillium lanosoniveum* (culture example: LBSim-01 on SNA). (**A**,**D**–**F**) Conidiogenous cells and conidia; (**B**,**C**) conidia. (**G**,**H**) Colony and reverse on SNA (**I**,**J**) on PDA.

Culture characteristics: On SNA medium, colonies reached 25–27 mm (X = 26 mm, *n* = 5) after 5 days. They were slightly cottony at the center, funiculose toward the regular margin, white, and reverse white to pale luteous. On PDA medium, colonies attained 40–45 mm in diameter (X = 42.5 mm, *n* = 5) after 10 days, and were cottony or fluffy with entire margins, as well as radially sulcate, white, and reverse pale luteous to luteous (Figure 3).

Specimen examined: Cuba, Cienfuegos Province, Cuatro Vientos region, Cumanayagua (coordinates 21°57′02.6″ N, 80°04′38.9″ W), *Coffea arabica* L. (coffee tree) plantation, on pustules of *Hemileia vastatrix*. Isel Gonzaléz-Marrero, 14 November 2022. Living culture: LBSim-01.

4. Discussion

In this study, the fungal isolate LBSim-01, obtained from CLR-infested leaves of *C. arabica* from Cienfuegos Province, Cuba, was identified morphologically through molecular taxonomy as *Simplicillium lanosoniveum*. The isolate, with a cotton-like appearance, had

previously been identified as *Lecanicillium* sp. However, more detailed morphological descriptions of LBSim-01 better matched the species *S. lanosoniveum*, as described by Zare and Gams [12]. Generally, fungi of the genus *Simplicillium* resemble the *Lecanicillium lecanii* (now: *Akanthomyces lecanii*) and *Lecanicillium Muscarium* (now: *Akanthomyces muscarius*) fungi in appearance but differ mainly in their smaller conidia and solitary phialides. Molecular phylogenetic analyses confirmed the results of morphological examinations and led to the conclusion that the isolate LBSim-01 was *Simplicillium lanosoniveum*. The genera *Lecanicillium*, *Akanthomyces*, and *Simplicillium* belong to the family Cordycipitaceae, which prominently comprises entomopathogenic and mycoparasitic ascomycetes.

Since 2020, the natural occurrence of a white-mycelia-forming fungus in association with *H. vastratix* colonies has been observed in the coffee plantations of Cumanayagua. This study constitutes the first report of the genus *Simplicillium* (Cordycipitaceae: Hypocreales) as a parasite of CLR in Cuba.

Previously, numerous authors have reported *S. lanosoniveum* as a mycoparasite of rust fungi on coffee leaves, soybeans, wheat stems, and further plant species worldwide [8,14,29,30]. The results of surveys have suggested that the fungus has high potential to inhibit the growth and spread of rust diseases, indicating this species as an ideal candidate for biological control. Several *Simplicillium* fungi with mycoparasitic action are in use for the biological control of fungal plant diseases, such as gray mold caused by *Botrytis cinerea* and powdery mildew caused by *Blumeria graminis* f. sp. *tritici* [31].

The molecular-taxonomically defined genus *Simplicillium* comprises species with a wide spectrum of host associations. Several studies have described the entomopathogenic action of *Simplicillium* species [10], and numerous *Simplicillium* species have been defined as being associated with arthropods, both insects and spiders [17,32]. Moreover, several species parasitize plant parasitic nematodes [33–35]. Importantly, *S. lanosoniveum* has been demonstrated to display dual activities: (i) as a mycoparasite on rust fungi and (ii) as entomopathogen against aphids [36–38], i.e., a specific combination of host–parasite and host–pathogen relationships reflected in phylogenetically related, but systematically distinct, *Akanthomyces uredinophilus* (formerly *Lecanicillium uredinophilum*) fungi [6]. These findings highlight the significance of *Simplicillium lanosoniveum* for both (i) research and development of dual target biocontrol of agricultural pests and plant diseases and (ii) fundamental research on the molecular biology and evolution of host–parasite and host–pathogen interactions.

5. Conclusions

The molecular taxonomic analyses and morphological study of the isolate LBSim-01 indicate that this fungus belongs to the taxonomic species *Simplicillium lanosoniveum.* This is the first report of a *Simplicillium* fungus related to coffee crops in Cuba. Its presence as a natural source of microbial agents against *H. vastratix* suggests its high potential for disease suppression as part of integrated pest management (IPM) by the Biological Control Program in Cuba. Future works will focus on the nature of interactions of LBSim-01 and *H. vastratix*, how they are related to disease development and control, their possible entomopathogenic effects against insects in coffee plantations, and the possibility of developing an efficient and sustainable biopesticide.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol4010018/s1, Figure S1: Maximum likelihood (ML) phylogeny of *Simplicillium* fungi; Figure S2: Bayesian inference (BI) phylogeny of *Simplicillium* fungi; Table S1: Reference data sets of fungal strains and marker sequences used for phylogenetic analysis; Table S2: Best hit sequence entries identified by the respective LBSim-01 query in the GenBank database.

Author Contributions: Conceptualization, Y.B.R. and A.L.; methodology, I.G.M., M.E.L.N. and C.S.; investigation, Y.B.R., I.G.M., M.E.L.N., R.F.C.R., A.P.d.l.C., H.B.G., R.G.M. and A.L.; validation, R.F.C.R. and D.-W.L.; data curation, R.F.C.R. and A.L.; writing—original draft preparation, Y.B.R. and

A.L.; writing—review and editing, Y.B.R., I.G.M., M.E.L.N., R.F.C.R., D.-W.L., A.P.d.l.C., C.S., R.G.M., H.B.G. and A.L.; project administration, Y.B.R. and A.L.; funding acquisition, Y.B.R. and A.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Sequence data analyzed in this study are publicly available from the GenBank database (https://www.ncbi.nlm.nih.gov) (accessed on 20 January 2024) under nucleotide sequence accession numbers OR592663, OR592664, and OR602870. And other data are contained within the article and supplementary materials.

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Conflicts of Interest: The authors declare no conflicts of interest.

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