Characterization of *Trichoderma* species from agricultural soils of Paraguay

Caracterización de especies de *Trichoderma* de suelos agrícolas de Paraguay

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ABSTRACT

There is a growing interest in the development of sustainable alternatives to the use of chemical pesticides for pest management in agricultural systems. This research aimed to isolate and characterize native strains of *Trichoderma* spp. from different soils of Paraguay using morphological and molecular criteria. We processed plant and soil samples from eight commercial farms distributed in different departments of Paraguay and isolated 14 monosporic isolates of *Trichoderma* spp., obtaining two isolates from the Department of Alto Paraná (FCQ36 and FCQ37), four isolates from Cordillera (FCQ42, FCQ43, FCQ44, and FCQ46), one isolate from Central (FCQ32), and seven isolates from Itapúa (FCQ13, FCQ16, FCQ18, FCQ19, FCQ21, FCQ23, and FCQ47). In addition, phylogenetic analyses using the ITS and *tef1α* loci were carried out. A better resolution of the *tef1a* gene than the ITS region was observed. Moreover, a third phylogenetic tree from the concatenated ITS and *tef1α* sequences matrix was generated, obtaining the same topology with higher bootstrap support values. Through this approach, we reported for the first time the presence of *Trichoderma koningiopsis* (FCQ19, FCQ36, and FCQ37), *Trichoderma neokoningii* (FCQ13), and *Trichoderma asperellum* (FCQ42, FCQ43, FCQ44, and FCQ46), *Trichoderma brevicompactum* (FCQ18 and FCQ21), and *Trichoderma longibrachiatum* (FCQ 47) in Paraguay. The *Trichoderma* species identified in this study can be used to develop effective biocontrol products for agricultural and industrial purposes in Paraguay.

Key words: biological control, phylogenetics, fungi, taxonomy.

RESUMEN

Existe un creciente interés en el desarrollo de alternativas sostenibles al uso de plaguicidas químicos para el manejo de plagas en los sistemas agrícolas. El objetivo de este trabajo fue aislar y caracterizar por criterios morfológicos y moleculares cepas nativas de *Trichoderma* spp. de diferentes suelos de Paraguay. Se procesaron muestras de plantas y suelo de ocho fincas comerciales distribuidas en diferentes departamentos de Paraguay para el aislamiento de 14 aislados monospóricos de *Trichoderma* spp., obteniendo dos aislados del Departamento de Alto Paraná (FCQ36 y FCQ37), cuatro aislados de Cordillera (FCQ42, FCQ43, FCQ44 y FCQ46), un aislado de Central (FCQ32) y siete aislados de Itapúa (FCQ13, FCQ16, FCQ18, FCQ19, FCQ21, FCQ23 y FCQ47). Además, se realizaron análisis filogenéticos utilizando los loci ITS y *tef1α*. Se observó una mejor resolución del gen *tef1α* en comparación con la región ITS. Además, se generó un tercer árbol filogenético a partir de la matriz concatenada de ambas secuencias ITS y *tef1α* obteniendo la misma topología con mayores valores de soporte bootstrap. A través de este enfoque, se reporta por primera vez la presencia de *Trichoderma koningiopsis* (FCQ19, FCQ36 y FCQ37), *Trichoderma neokoningii* (FCQ13) y *Trichoderma asperellum* (FCQ42, FCQ43, FCQ44 y FCQ46), *Trichoderma brevicompactum* (FCQ18 y FCQ21) y *Trichoderma longibrachiatum* (FCQ 47) en Paraguay. Las especies de *Trichoderma* identificadas en este trabajo pueden ser utilizadas en el desarrollo de productos de control biológico eficaces para fines agrícolas e industriales en Paraguay.

Palabras clave: control biológico, filogenética, hongos, taxonomía.

Introduction

The genus *Trichoderma* comprises more than 375 species of free-living fungi that are present mainly in the organic matter and as endophytes in plant roots colonizing their rhizosphere (Samuels, 2006; Pappu, 2018; Cai & Druzhinina, 2021). *Trichoderma* species are cosmopolitan and widely diverse in the tropics (Rivera-Méndez *et al*., 2020). Several studies have demonstrated that these fungi benefit plants due to their protection against plant pathogens and as plant growth promoters (Howell, 2003; López-Quintero *et al*., 2013). Different species of *Trichoderma* produce different compounds that activate systemic plant defenses and regulate pathogen infections, significantly altering plant physiology (Li *et al.,* 2019; Patel *et al.,* 2019). Because of this, *Trichoderma* has been applied to crops as a plant growth

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promoter and inductor of resistance to abiotic stress and plant pathogens (Alkooranee *et al.,* 2019; Mayo-Prieto *et al.,* 2020; Moreno-Ruiz *et al.,* 2020).

Currently, commercial products of the fungus *Trichoderma* spp. are formulated for its application in agricultural systems (Fraceto *et al*., 2018). These products do not always have the same efficiency because they depend on environmental conditions (Altintas & Bal, 2008; Nieto-Jacobo *et al*., 2017; Di Lelio *et al*., 2021). The biocontrol activities of *Trichoderma* species are significantly affected by the host plant and soil type as well as other microbial populations present in the same ecological niche (Lombardi *et al*., 2018; Naseby *et al*., 2000; Morán-Diez *et al*., 2020). Therefore, *Trichoderma* species can produce highly specialized metabolites to interact with specific plant hosts and phytopathogens (Silva *et al*., 2014; Contreras-Cornejo *et al*., 2016; Kubicek *et al*., 2019). Consequently, isolation and identification of effective native strains that are well adapted to a location´s edaphoclimatic conditions in which they are to be deployed are necessary for effective biological control (Consolo *et al*., 2012; Yendyo *et al*., 2017; Ferreira *et al*., 2020).

Numerous studies have been conducted in Latin America to identify *Trichoderma* isolates from different countries. These studies employ a polyphasic characterization approach that combines traditional phenotypic and physiological methods with modern molecular biology. Endophytic biological control agents such as *Trichoderma ovalisporum* have been isolated from the Peruvian Amazon's ecozones to manage cocoa diseases. These isolates are described morphologically, physiologically, and molecularly (Holmes *et al*., 2004). Similarly, in Argentina research focused on studying the genetic diversity of *Trichoderma* species and their biocontrol mechanisms allow the registration of commercial products based on *Trichoderma,* which are used as biological control agents and plant growth promoters (Consolo *et al*., 2012; Amerio *et al*., 2020). Research on the use and application of *Trichoderma* in southern Brazil began in 1989, with the production and distribution of native strains performed by Empresa Brasileira de Pesquisa Agropecuária (Embrapa) (Bettiol & Morandi, 2009). *Trichoderma* species in Brazil, such as *Trichoderma harzianum*, *Trichoderma tomentosum*, *Trichoderma asperellum*, *Trichoderma ghanense, Trichoderma azevedoi*, *Trichoderma peberdyi*, *Trichoderma reesei,* and *Trichoderma atroviride*, are used for biocontrol of plant pathogens (Lopes *et al*., 2012; Inglis *et al*., 2020), enzyme production (Horta *et al*., 2018), and secondary metabolite isolation (Brito *et al*., 2014). In Paraguay, the isolation and description of *Trichoderma* species has recently started compared to other countries in the region (Stauffer Bonzon, 1999; Ortellado Franco & Orrego Fuente, 2013; Sanabria Velázquez, 2020). Therefore, Paraguay remains an unexplored source of new *Trichoderma* species with potential biotechnological applications.

The first description of the Paraguayan species of *Trichoderma* at the molecular level was in 2017 when seven *Trichoderma* species from sesame soils were isolated from northeastern Paraguay. These *Trichoderma* spp. are characterized using only the Internal transcribed spacer (ITS) region as a DNA marker (Fernández Gamarra *et al*., 2017). Despite this DNA marker being widely accepted as a tool for the preliminary taxonomic identification of fungi, it does not have enough resolution to differentiate between species of *Trichoderma* because of a high level of homoplasy (Druzhinina *et al.,* 2005; Schoch *et al.,* 2012). Therefore, employing more genes with good resolution to describe these species is critical. A commonly used marker is the translation-elongation factor 1α (*tef1*α) since it is highly polymorphic and helps as an additional DNA marker for species delimitation of *Trichoderma* (Hermosa *et al*., 2004; Chaverri *et al*., 2015; Stielow *et al*., 2015; Rivera-Méndez *et al*., 2020).

The appropriate characterization of native species of *Trichoderma* is critical for developing commercial biocontrol products and to deploy these appropriately in the field (Chaverri *et al*., 2015). Moreover, *Trichoderma* isolates can produce novel molecules of interest for biotechnological industries that require the correct identification and preservation of these selected isolates to register novel commercial products (Woo *et al*., 2014; Błaszczyk *et al*., 2016). Previous studies could not characterize *Trichoderma* fungi in Paraguay to the species level nor preserve them appropriately. Therefore, this research aimed to identify and describe the morphological, physiological, and molecular characteristics of the *Trichoderma* isolates. This information can be valuable for understanding the diversity and distribution of *Trichoderma* in the agricultural soils of Paraguay. To achieve this goal, we isolated native strains of *Trichoderma* spp. from different agricultural fields of Paraguay and characterized them morphologically using macro and microscopic morphometric observations and molecularly using ITS 1-4 and *tef1α* gene regions. Since these isolates were obtained from commercial farms distributed in different parts of Paraguay, they have the potential to be used as biocontrol agents or for other biotechnological applications.

Materials and methods

Sampling and isolation

We collected soil, rhizosphere, and plant samples from different Paraguay crop production areas to isolate *Trichoderma* spp. Soil samples were collected using a soil sampler to a depth of approximately 20 cm. We labeled each sample and georeferenced them through a global positioning system (GPS) and transported them to the laboratory for analysis (Tab. 1). We prepared serial dilution of each sample following previously described methods (Samuels & Hebbar, 2015). Two hundred µl of soil suspension was poured on the surface of a PDA (Papa-Dextrose-Agar, Liofilchem®, Teramo, Italy) culture medium with oxytetracycline (TerramicinaLA®, Zoetis, AR).

In order to isolate potential endophytic *Trichoderma* spp. from the plants, we cut the tissues into pieces of approximately 0.5 cm, washed them with 70% ethanol for 30 s, then washed them with sodium hypochlorite 3% solution for another 30 s, and rinsed the material three times with sterile distilled water. We dried the disinfested samples and placed four pieces on the surface of the PDA medium with oxytetracycline. All plates were incubated at 28 ± 2°C for 5 d in complete darkness. Colonies with macroscopic characteristics of *Trichoderma* spp. were selected, and microscopic structures, conidiophores, and we analyzed conidiospores with appropriate identification keys (Barnett & Hunter, 1998). We transferred colonies identified as *Trichoderma* spp. to PDA medium to obtain a pure culture. All plates were incubated at 28 ± 2°C for 5 d. Monosporic isolates of *Trichoderma* spp. were obtained to ensure genetic homogeneity.

We prepared serial dilutions of the suspension from the pure culture and placed 200 µl on the surface PDA medium and incubated at 28 ± 2°C for 24 or 48 h. We transferred the first germinated spores to new PDA media. We identified cultures using sequential alphanumeric codes and stored them for posterior analyses in test tubes with PDA culture medium at 4°C and Eppendorf tubes with sterile glycerol at -20°C.

Morphology of the *Trichoderma* isolates

We grew fourteen Paraguayan monosporic isolates of *Trichoderma* spp. on PDA medium for 3 d at 28 ± 2°C and measured colony growth and color. To describe colony colors, we used the RGB color model available at ArtyClick Colors (ArtyClick Pty Ltd, Sydney, Australia, https://colors.artyclick.com/color-name-finder/). We measured the width and length of conidiophores, phialides, and conidia using a microscope with a digital camera (3MP, AMScope, USA), employing the software AMScope Version 4.7. We reported descriptive analyses of the measurements using Infostat (version 2017, Córdoba, AR), presenting means followed by standard deviation values. We used three biological replicates per isolate for macroscopic measurements, using 30 replicates per isolate for microscopic structural measurements.

TABLE 1. Description of the *Trichoderma* isolates obtained from different locations and crops from different production areas in Paraguay and their GenBank accession numbers.

Sample type	Crop	Isolate code	Specie	Genbank accession N°	
				ITS	*tef1*α
Soil	Tomato (*Solanum lycopersicum*)	FCQ13	*T. neokoningii*	MZ339274	MZ442668
Soil	Tomato (*S. lycopersicum*)	FCQ16	*T. harzianum*	MZ339256	MZ442661
Soil	Tomato (*S. lycopersicum*)	FCQ18	*T. brevicompactum*	MZ339234	MZ442659
Soil	Tomato (*S. lycopersicum*)	FCQ19	*T. koningiopsis*	MZ339263	MZ442663
Soil	Tomato (*S. lycopersicum*)	FCQ21	*T. brevicompactum*	MZ339235	MZ442660
Rhizosphere	Pepper (*Capsicum annuum*)	FCQ23	*T. harzianum*	MZ339258	MZ442662
Soil	Pepper (*C. annuum*)	FCQ32	*T. harzianum*	MZ339273	MZ442666
Soil	Corn (*Zea mays*)	FCQ36	*T. koningiopsis*	MZ339264	MZ442664
Soil	Soybean (*Glycine max*)	FCQ37	*T. koningiopsis*	MZ339265	MZ442665
Soil	Pepper (*C. annuum*)	FCQ42	*T. asperellum*	MZ339228	MZ442655
Soil	Pepper (*C. annuum*)	FCQ43	*T. asperellum*	MZ339229	MZ442656
Soil	Stevia (*Stevia rebaudiana*)	FCQ44	*T. asperellum*	MZ339230	MZ442657
Parasite of *Colletotrichum* spp.	Ornamentals (*Tagetes* spp.)	FCQ46	*T. asperellum*	MZ339231	MZ442658
Cortex	Lemon verbena (*Aloysia citrodora*)	FCQ47	*T. longibrachiatum*	MZ339223	MZ442667

DNA extraction and amplification

Fourteen monosporic isolates of *Trichoderma* spp. were grown in PDB medium (Potato-Dextrose-Broth, Liofilchem®, Teramo, IT) for 5 d at 25±2°C. We filtered the mycelium using a Büchner funnel, washed with sterile distilled water, and frozen until further processing. The modified hexadecyltrimethylammonium bromide (CTAB) method was used to extract the DNA (Murray & Thompson, 1980). Each frozen sample was briefly macerated with liquid nitrogen and incubated with 2×CTAB buffer at 65°C for 40 min. An equal volume of chloroform was added, and the phases were separated by centrifugation. The DNA in the supernatant was precipitated with isopropanol, washed with 70% ethanol, and resuspended in ultrapure water (Invitrogen, Carlsbad, CA). The ITS region was amplified by PCR using ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) and *tef1*α locus using EF1-728F (CATCGAGAAGTTCGAGAAGG) and TEF1REV (GCCATCCTTGGAGATACCAGC) primers (Hermosa *et al*., 2004; Samuels & Hebbar, 2015). In both cases, 25 µl of mix containing 1 µl of a 1:10 dilution of the DNA sample, 2.5 µl of Buffer 10X TopTaq, 0.5 µl of 10 µM dNTPs, 1 µl of each primer 10 µM, and 0.25 µl of TopTaq polymerase (Qiagen, Mississauga, Ontario, Canada) were used. Amplifications were carried out in a thermal cycler (SimpliAmp™, ThermoFisher) with the following conditions: an initial denaturation cycle at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min for ITS regions or 59°C during 1 min for the (*tef1*α) gene, 72°C for 1 min with a final extension of 72°C for 10 min. The PCR products were resolved through a 1% electrophoresis agarose gel and purified using PureLink™ Quick Gel Extraction Kit (Invitrogen, Carlsbad, USA). The PCR products were sequenced by Macrogen (Seoul, Korea).

Phylogenetic analysis

The sequencing files were first analyzed for quality and trimmed with BioEdit 7.0.5.3. We identified the sequences of the ITS and *tef1*α regions with TrichoMARK2020 (https://www.trichokey.com/index.php/trichomark) that was formerly available on the homepage of the International Commission on *Trichoderma* (ICTT). We also performed a BLAST search to find related sequences in the NCBI database. We carried out sequence alignment using AliView 1.26 (Larsson 2014) with the MAFFT v. 7.450 binary (Katoh *et al*., 2019), using local alignment parameters. The ICTT reference dataset ITS56 was first used to generate a local database (Cai & Druzhinina, 2021) (Tab. S1). After further primer removal, we analyzed the sequences to determine the best evolutionary model. We selected the K2+G model (10.1007/BF01731581) according to MEGA X Software 10.0.5 (Kumar *et al*., 2018), and the Neighbor-Joining trees were assembled with 1000 bootstrap replications. We assembled the phylogenetic trees using previously reported *tef1*α gene sequences (Tab. S1) to generate a local database. We removed the primer portions and aligned the sequences using the same parameters as previously described. We then joined together the processed ITS and tef1α sequences for the concatenated tree.

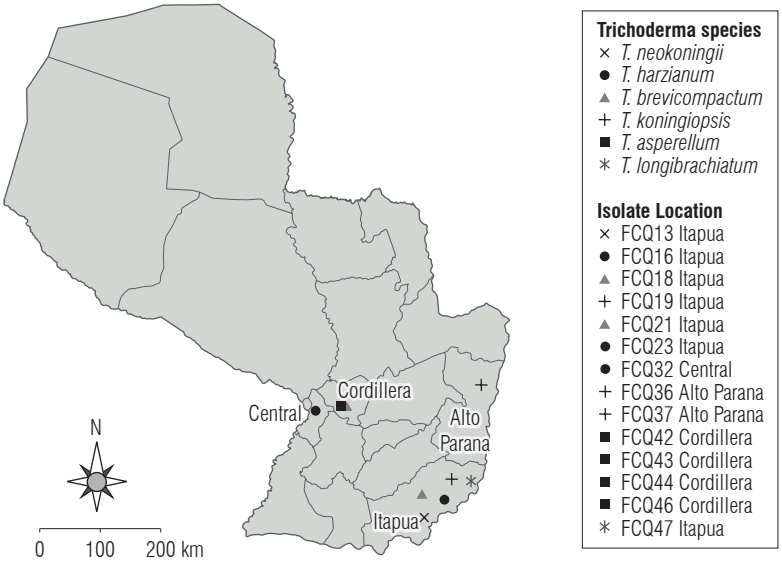

FIGURE 1. Map of Paraguay indicating sampled locations of *Trichoderma* spp. isolates obtained in 2016.

Results

Isolation and morphological characterization

We processed plant and soil samples from eight commercial farms distributed in different departments of Paraguay (Fig. 1) to isolate fourteen monosporic isolates of *Trichoderma* spp.

We obtained two isolates from departments of Alto Paraná (FCQ36 and FCQ37), four from Cordillera (FCQ42, FCQ43, FCQ44, and FCQ46), one isolate from Central (FCQ32), and seven isolates from Itapúa (FCQ13, FCQ16, FCQ18, FCQ19, FCQ21, FCQ23, and FCQ47). All isolates showed morphological characteristics of the genus *Trichoderma* with septate hyphae, branched conidiophores, and small conidia scattered or grouped (Fig. 2). Also, all isolates were able to cover completely the surface of the PDA medium on the 90 mm Petri plate after 3 d of incubation at 28 ± 2°C, except for the isolate FCQ13 that presented the slowest growth with a rate of 13.60 ± 1.10 mm d^{-1} (Tab. 2).

The colonies of isolate FCQ13 were often white at first, forming greenish-grey conidiophores after one week of incubation, with cylindrical phialides of 7.11 ± 2.79 × 2.59 ± 0.37 µm of length and width. Conidia were ellipsoidal and measured 3.36 ± 0.93 × 2.47 ± 0.51 µm on average (Tab. 2, Fig. 2A). Isolate FCQ16 produced light green and yellowish conidiophores that grew scattered throughout the plate with yellow pigment secreted into the agar (Fig. 2D). The 3-d-old monosporic cultures had ampulliform phialides of average length and width of 7.47 ± 1.25 × 3.46 ± 0.29 µm, and conidia were subglobose with measurements averaging 2.94 ± 0.8 × 2.64 ± 0.45 µm.

The isolate FCQ18 sporulated after 72 h, producing greenish-grey colonies with scant aerial mycelia, compact tufts, and without pigmentation on the reverse side of the plate. Microscopic examination of conidiophores revealed ampulliform to lageniform phialides of 6.67 ± 1.61 × 3.26 ± 0.78 µm in length and width (Fig. 2C). The conidia were subglobose with a length and width of 2.69 ± 0.47 × 2.56 ± 0.39 µm. Although isolated from a similar soil and host (Tab. 1), the colonies of FCQ19 were slightly different from FCQ18, with a glade green color, and grew uniformly on PDA medium. Conidiophores of FCQ19 supported lageniform phialides of 8.22 ± 1.9 × 4.12 ± 0.9 µm of length and

TABLE 2. Morphological characterization of 14 *Trichoderma* strains isolated from different locations and crops from different production areas in Paraguay.

Isolate	Colony			Conidia			Phialides		
	Growth[a]	Color	RGB color code	Length[b]	Width[b]	Characteristics	Length[b]	Width[b]	Characteristics
FCQ13	13.60±1.10	Greenish Grey	#99AA99	3.36±0.93	2.47±0.51	Ellipsoidal	7.11±2.79	2.59±0.37	Cylindrical
FCQ16	28.47±0.83	Greenish Yellow	#99A540	2.94±0.8	2.64±0.45	Subglobose	7.47±1.25	3.46±0.29	Ampulliform
FCQ18	27.88±1.10	Greenish Grey	#99AA99	2.69±0.47	2.56±0.39	Subglobose	6.67±1.61	3.26±0.78	Ampulliform to lageniform
FCQ19	27.04±1.91	Glade Green	#668066	3.86±0.9	2.64±0.35	Ellipsoidal	8.22±1.9	4.12±0.9	Lageniform
FCQ21	27.57±0.91	Green Smoke	#99AA66	2.88±0.71	2.73±0.42	Subglobose to ovoidal	6.33±2.44	3.71±0.7	Ampulliform to lageniform
FCQ23	27.80±1.29	Green Smoke	#99AA66	2.84±0.67	2.78±0.45	Subglobose	7.01±1.56	3.77±0.52	Ampulliform
FCQ32	28.26±0.70	Glade Green	#668066	2.45±0.44	2.4±0.48	Subglobose	6.33±1.34	3.5±0.24	Ampulliform
FCQ36	28.36±1.21	Greenish Grey	#99AA99	3.61±0.75	2.56±0.92	Ellipsoidal	8.55±1.53	3.79±1.01	Lageniform
FCQ37	28.07±1.46	Glade Green	#698366	3.94±0.98	2.96±0.55	Ellipsoidal	7.9±1.56	3.42±0.9	Lageniform
FCQ44	28.15±0.87	Glade Green	#698366	4.01±0.75	3.66±0.82	Subglobose to ovoidal	8.48±1.73	3.65±0.87	Lageniform
FCQ42	27.76±1.37	Glade Green	#698366	3.62±0.48	2.98±0.46	Subglobose to ovoidal	8.46±0.82	2.81±0.72	Lageniform
FCQ43	28.35±0.90	Glade Green	#698366	3.56±0.55	2.88±0.75	Subglobose to ovoidal	8.4±1.58	3.55±0.24	Lageniform
FCQ46	27.18±1.36	Green Pine	#365536	3.79±0.84	3.08±1.18	Subglobose to ovoidal	9.82±0.76	3.75±0.97	Lageniform
FCQ47	28.54±1.20	Glade Green	#668066	3.51±0.6	3.03±0.58	Ellipsoidal	9.83±2.12	3.18±0.84	Lageniform

[a] Measured in mm d^{-1} . Values are means ± standard deviation of three replicates per isolate.

[b] Measured in μm. Values are means ± standard deviation of 30 replicates.

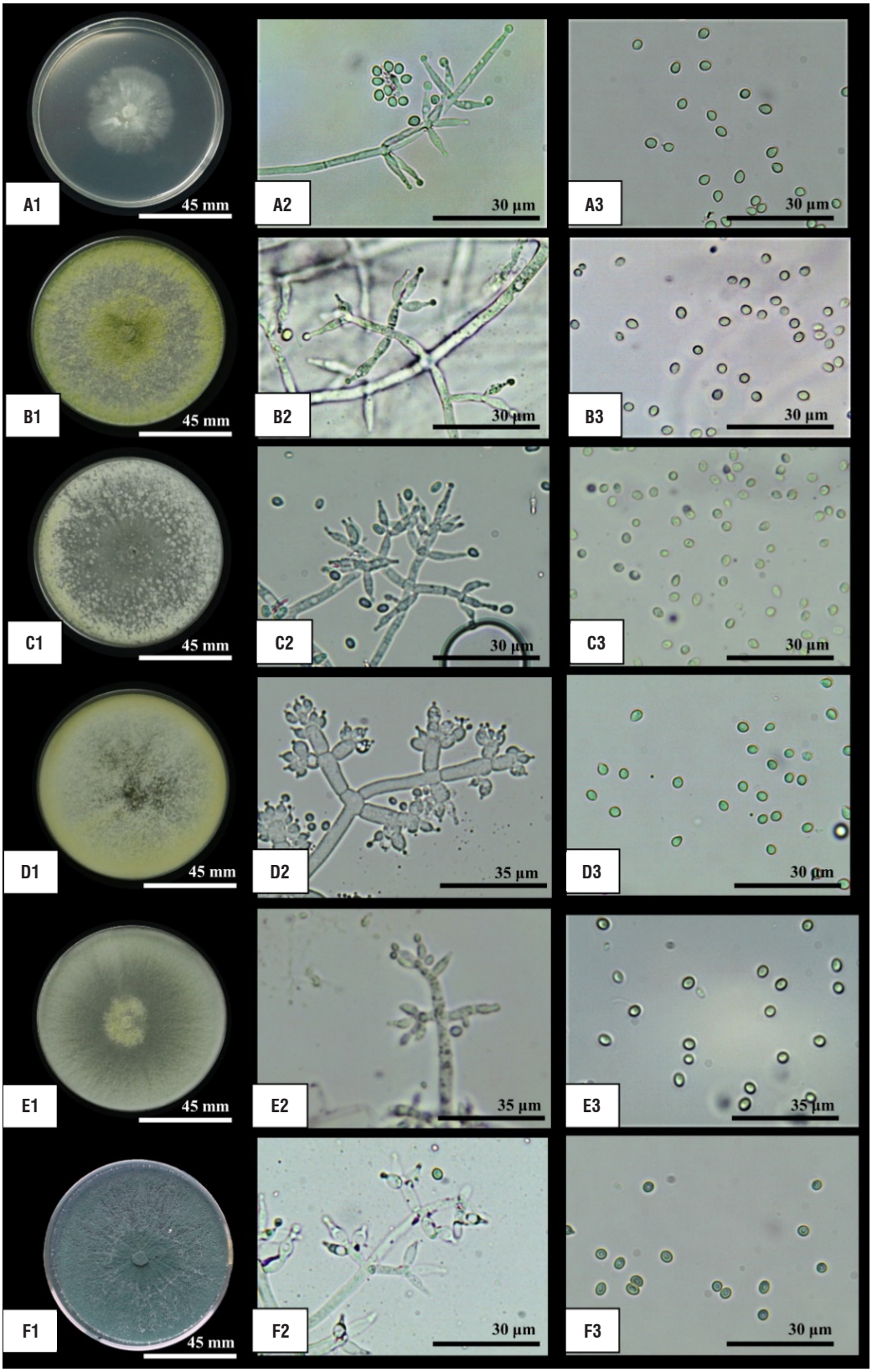

FIGURE 2. Representative cultures of each *Trichoderma* species: A) *T. neokoningii*, FCQ13; B) *T. asperellum*, FCQ42, FCQ43, FCQ44; FCQ46. C) *T. brevicompactum*, FCQ18, and FCQ21; D) *T. harzianum*, FCQ16, FCQ23, and FCQ32; E) *T. koningiopsis*, FCQ19, FCQ36, and FCQ37; and F) *T. longibrachiatum*, FCQ47. Each column represents the following: 1) potato-dextrose-agar (PDA) cultures after 3 d of incubation at 28°C; 2) Conidiophores; and 3) conidia of each *Trichoderma* species observed under a light microscope with 400 × magnification.

width with terminal ellipsoidal conidia measuring an average of 3.86 ± 0.9 × 2.64 ± 0.35 µm (Tab. 2, Fig. 2E). The isolate FCQ21 produced green smoke colonies without pigmentation on the reverse side of the plate. All colonies sporulated on PDA with conidiophores that terminated in ampulliform to lageniform phialides of 6.33 ± 2.44 × 3.71 ± 0.7 µm (Fig. 2C).

We obtained the isolates FCQ23 and FCQ32 from similar soil and host (Tab. 1) that yielded morphologically similar colonies. Monosporic colonies of FCQ23 had abundant mycelia, loose tufts, and filiform margins. The conidiophores had ampulliform phialides of 7.01 ± 1.56 × 3.77 ± 0.52 µm in length and width. Conidia were subglobose and averaged a length and width of 2.84 ± 0.67 × 2.78 ± 0.45 µm (Fig. 2D). Similarly, the isolate FCQ32 showed green colonies with loose tufts that were not uniformly distributed on the PDA medium. The conidiophores observed under the microscope presented opposite branches with shorter ampulliform phialides of 6.33 ± 1.34 × 3.5 ± 0.24 µm. The conidia were subglobose and yellowish-green in color with an average size of 2.45 ± 0.44 × 2.4 ± 0.48 µm (Fig. 2D).

The isolates FCQ36 and FCQ37 were obtained from similar agricultural fields but different crops (Tab. 1). Still, the colonies of both isolates were morphologically similar and had greenish-grey color colonies that grew uniformly on PDA. For isolate FCQ36, conidiophores were long with lageniform phialides of 8.55 ± 1.53 × 3.79 ± 1.01 µm, while for isolate FCQ37 these were 7.9 ± 1.56 × 3.42 ± 0.9 µm. Likewise, the conidia of both isolates were ellipsoidal with FCQ36 measuring an average of 3.61 ± 0.75 × 2.56 ± 0.92 µm and FCQ37 and measuring 3.94 ± 0.98 × 2.96 ± 0.55 µm (Fig. 2E).

The isolates FCQ42, FCQ43, and FCQ44 were obtained from different soils and fields (Tab. 1). Colonies were morphologically similar, with a glade green color, except for FCQ46 that had a darker green color (Tab. 2, Fig. 2B).

Closer examination revealed that conidiophores in these isolates were symmetrical, ending in three or more lageniform phialides and with subglobose or ovoid conidia (Fig. 2B). The isolate FCQ47 obtained from the plant tissue cortex (Tab. 1) was morphologically different from the previous isolates. Colonies were glade green with abundant mycelia, and loose tufts were uniformly distributed (Fig. 2F). Closer examination of microscopic structures confirmed septate hyphae and branched conidiophores with longer main branches and terminal lageniform ellipsoidal phialides 9.83 ± 2.12 × 3.18 ± 0.84 µm. Conidia were green and ellipsoid with an average of 3.51 ± 0.6 × 3.03 ± 0.58 µm of length and width (Tab. 2).

Phylogenetic analysis

Fourteen sequences of the ITS region of Paraguayan isolates of *Trichoderma* were obtained, with an amplification product size ranging from 540 to 599 base pairs. The pairwise similarity of the obtained sequences was compared phylogenetically with ITS reference sequences and assigned to the *Trichoderma* genus (Tab. S1). Paraguayan isolates clustered in different sections with three isolates grouped within section *Harzianum/Virens*: Isolates FCQ16, FCQ23, and FCQ32, grouping with AY605713 (*T. harzianum*) and NR144868 (*Trichoderma lentiforme*) (Fig. 3). Two isolates, FCQ18 and FCQ21, were grouped within the *Brevicompactum* clade alongside EU330941 (*T. brevicompactum*) with 100% bootstrap support. Moreover, eight isolates clustered within section *Trichoderma*: FCQ43, FCQ44, and FCQ46 grouped with MH021852 (*T. asperellum*) with 75% bootstrap support with FCQ42 clustering nearby this branch with 68% support. The isolate FCQ47 grouped within the unresolved section *Longibrachiatum,* close to *T. longibrachiatum*, *T. reesei*, and *Trichoderma parareesei*, and had 59% bootstrap support with NR120298 (*T. longibrachiatum*) (Fig. 3, Tab. S2).

Due to the difficulty of resolving the complex of *T. harzianum* and divide *Trichoderma* using only ITS region sequences, we obtained *tef1*α sequences for each isolate by PCR, with amplicons ranging from 566 to 645 base pairs. Was observe longer distances for species grouping within the *Harzianum* clade, confirming a better resolution of the *tef1*α gene than the ITS region (Fig. 4, Tab. S3).

Isolate FCQ32 grouped within the *Harzianum* clade, alongside FJ463310 (*T. lentiforme*), EU279992 (*T. harzianum),* and HM142375 (*T. amazonicum*) with 56% bootstrap support. Notably, despite FCQ16 and FCQ23 also clustering within the *Harzianum* clade with 83% bootstrap support, no *Trichoderma* reference sequence grouped near these two sequences (Fig. 4). As both isolates fell within the *T. harzianum* complex species, they either belong to *T. harzianum strictu senso* or another species within this complex.

In the section *Trichoderma*, isolates FCQ43, FCQ44, and FCQ46 were grouped in the same clade as EU279961 (*T. asperellum*) with 55% bootstrap support and FCQ42 nearby this same clade with 99% support. In addition, isolates FCQ19, FCQ37, and FCQ36 grouped alongside EU279998 (*T. koningiopsis*) with 91% bootstrap support. The isolate FCQ13 clustered next to KJ665620.1 (*T. neokoningii*) with

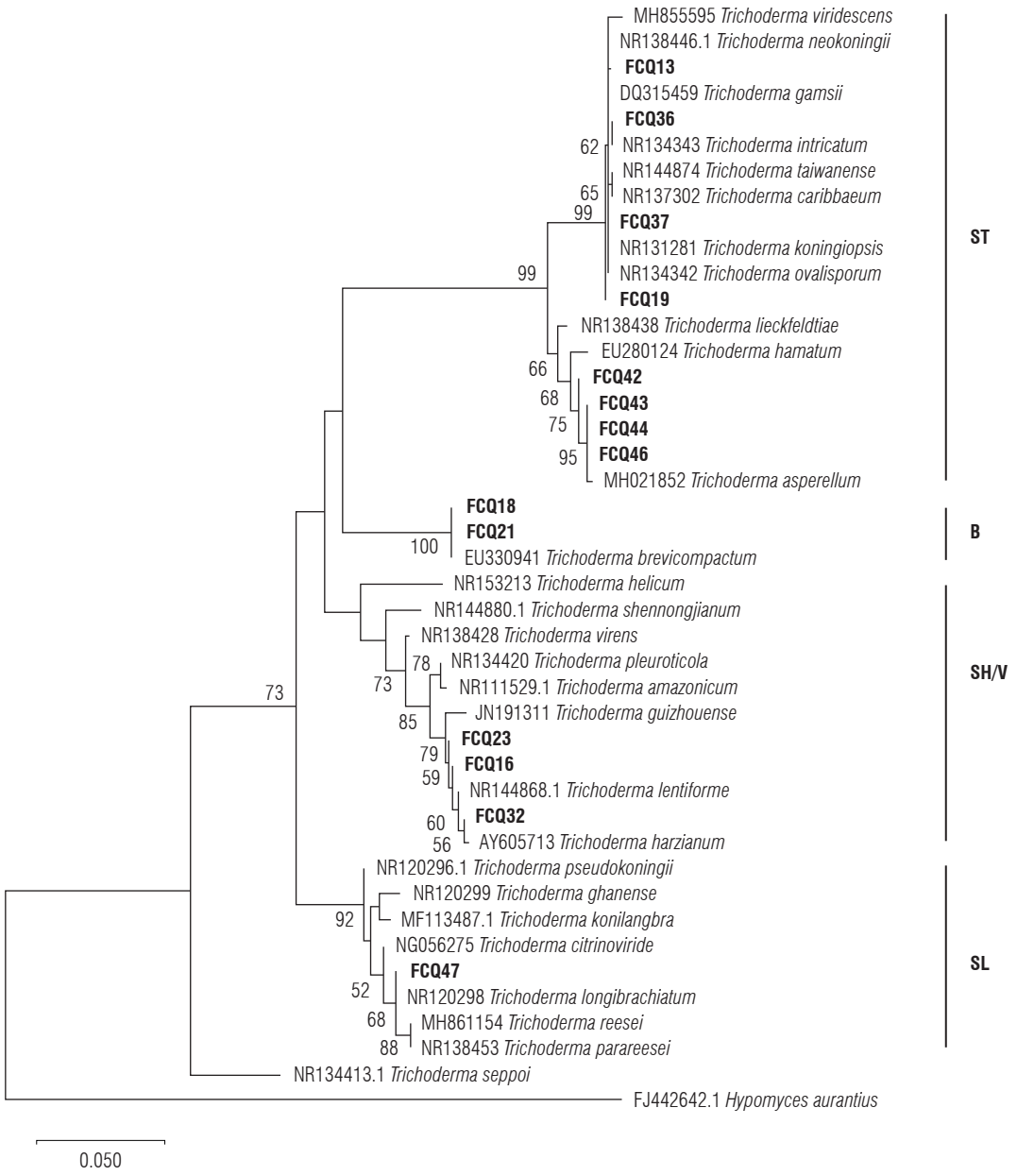

FIGURE 3. Phylogenetic tree of the Internal transcribed spacer (ITS) sequences from 14 *Trichoderma* isolates with *Hypomyces aurantius* as the outgroup. The scale bar corresponds to the number of substitutions per site. ST - section *Trichoderma*, SH/V - section *Harzianum/Virens*, SL - section *Longibrachiatum*, and B - clade *Brevicompactum*. The phylogenetic tree was obtained using DNA distance-based and neighbor-joining analysis. Bootstrap percentages higher than 50% (1000 bootstraps) are indicated above the branches.

99% support (Fig. 4). Isolates FCQ18 and FCQ21 were grouped with EU280061 (*T. brevicompactum*) within the *Brevicompactum* clade with 99% bootstrap support. Finally, the isolate FCQ47 was grouped within section *Longibrachiatum*, alongside AY8656408 (*T. longibrachiatum*) with 99% bootstrap support (Fig. 4).

A third phylogenetic tree was generated from the concatenated matrix of both ITS and *tef1*α sequences (Fig. 5). Overall, we saw the same topology of the *tef1*α tree with no changes in the clustering of Paraguayan *Trichoderma* isolates (Figs. 3-4). The differences between the concatenated ITS-*tef1*α tree were mainly related to each node's branch length and bootstrap support values. For example, FCQ32 and *T. lentiforme* grouped with a bootstrap value of 76%, compared to 56% from the *tef1*α gene alone (Fig. 4), while the value for FCQ16 and FCQ23 went from 83% support from the *tef1*α tree (Fig. 4) to 95%. At the same

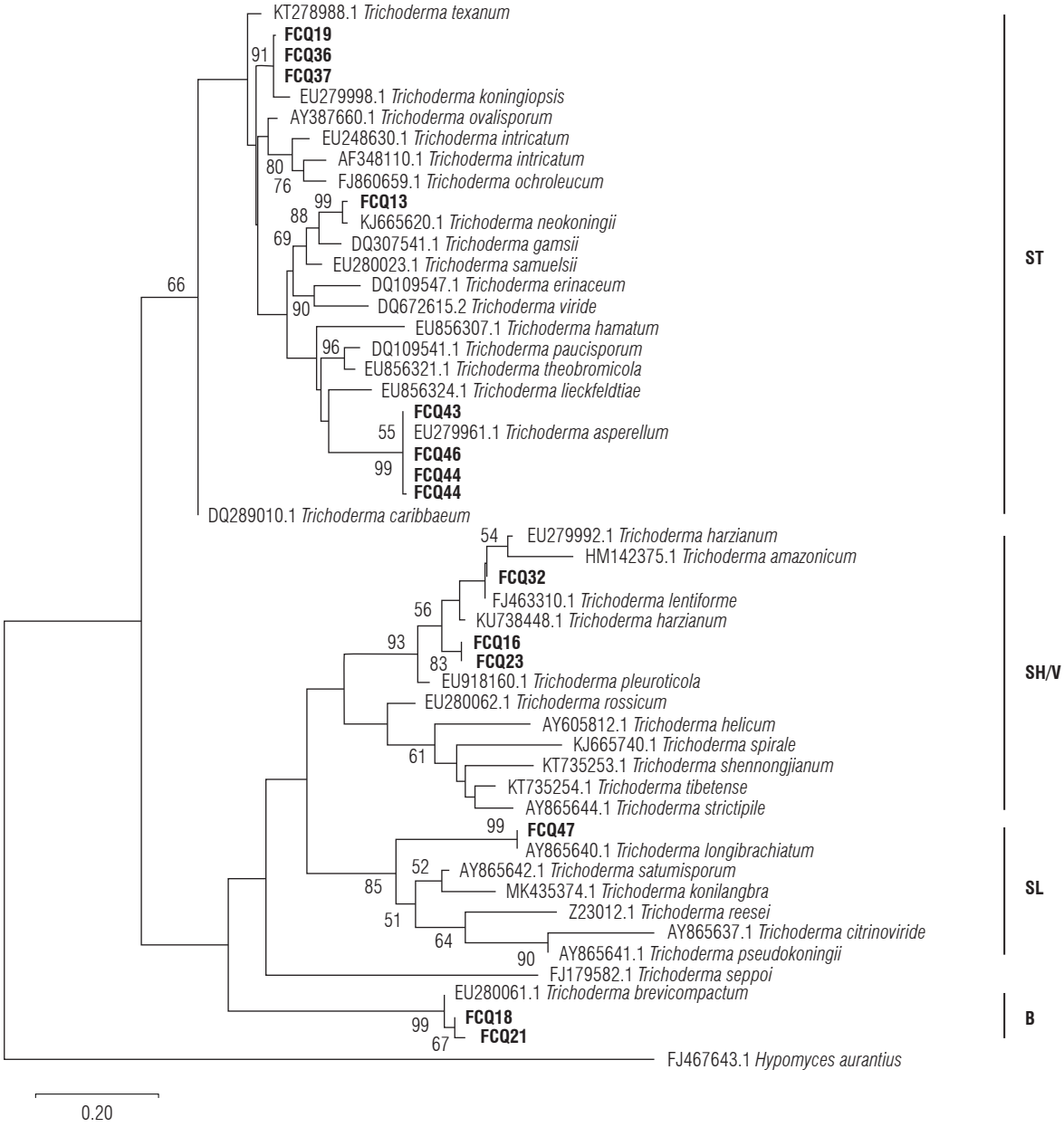

FIGURE 4. Phylogenetic tree of elongation factor gene 1 alpha (*tef1*α) sequences from 14 *Trichoderma* isolates with *Hypomyces aurantius* as the out-group. The scale bar corresponds to the number of substitutions per site. ST - section *Trichoderma*, SH/V - section *Harzianum/Virens*, SL - section *Longibrachiatum*, and B - clade *Brevicompactum*. The phylogenetic tree was obtained using DNA distance-based and neighbor-joining analysis. Bootstrap percentages higher than 50% (1000 bootstraps) are indicated above the branches.

time, the bootstrap value of the *T. harzianum* complex that includes the species cited so far, went from 93% (Fig. 4) to 98% (Fig. 5).

In section *Trichoderma*, the bootstrap support value of the clade with isolates FCQ19, FCQ36, FCQ37, and *T. koningiopsis* increased slightly to 98% (Fig. 5) from 91% in the *tef1α* tree (Fig. 4). Bootstrap support of the isolates FCQ43, FCQ44, FCQ46, and *T. asperellum* increased from 55% (Fig. 4) to 76% (Fig. 5). But, the bootstrap support for FCQ42 was slightly reduced from 99% in the previous *tef1*α tree to 95% bootstrap support in the concatenated phylogram (Fig. 5). Similarly, there was a slight reduction of support for FCQ13 and *T. neokoningii* from 99% support to 96% in the concatenated tree. The bootstrap values of the clades containing isolates FCQ18 and FCQ21 increased slightly from 99% support on the *tef1α* tree to 100% on the concatenated tree, similar to the clade

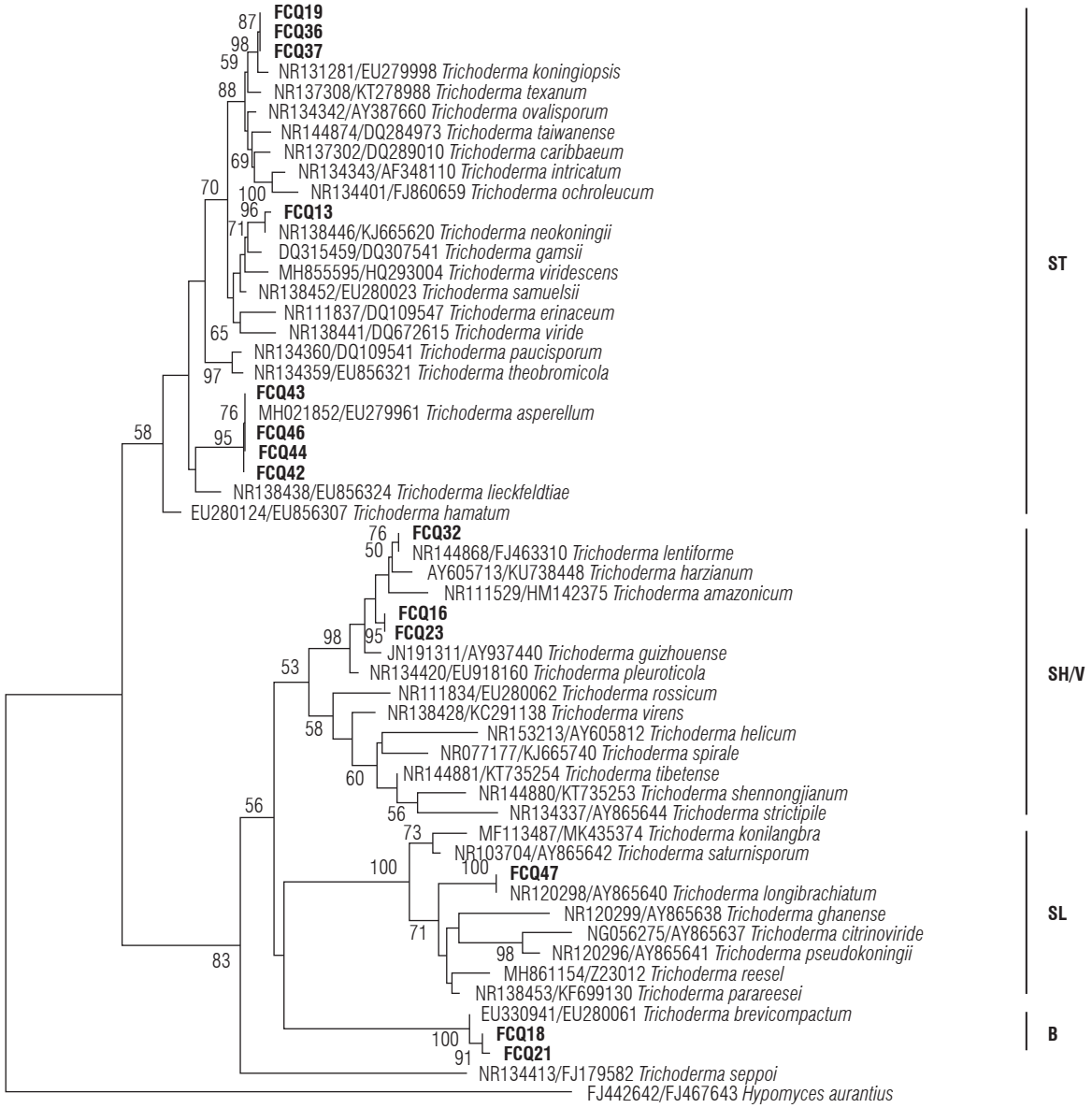

FIGURE 5. Phylogenetic tree of the concatenated ITS (left accession code) and *tef1*α (right accession code) sequences from 14 *Trichoderma* isolates with *Hypomyces aurantius* as the out-group. The scale bar corresponds to the number of substitutions per site. ST - section *Trichoderma*, SH/V - section *Harzianum/Virens*, SL - section *Longibrachiatum*, and B - clade *Brevicompactum*. The phylogenetic tree was obtained using DNA distance-based and neighbor-joining analysis. Bootstrap percentages higher than 50% (1000 bootstraps) are indicated above the branches.

containing isolate FCQ47 and the reference sequences for *T. longibrachiatum* (Fig. 5).

Discussion

Species of *Trichoderma* naturally inhabit agricultural soils, making them an interesting source of beneficial strains (Inglis *et al*., 2020). In this research, a total of 14 isolates of *Trichoderma* were obtained from various agricultural production areas of Paraguay. In addition, *Trichoderma* isolates were characterized morphologically and molecularly using ITS and *tef1*α markers, with species identified for the first time in Paraguay.

The morphology of colonies and conidiophores agreed with those commonly observed in *Trichoderma* spp. Morphological features between isolates were diverse, allowing their separation based on dissimilarities; however, these differences were not consistent given that, in some cases, isolates of the same species had different morphologies. For example, colonies FCQ18 and FCQ21 (*T. brevicompactum*) had slightly different morphologies; the conidia of the latter

tended to be ovoid, while the former were subglobose. In addition, it was difficult to differentiate between conidia of different species; this was the case for FCQ21 (*T. brevicompactum*) that was morphologically similar to FCQ23 (*T. harzianum*). Morphological characteristics can vary significantly depending on the incubation parameters, media, and even brands of reagents (Jaklitsch, 2011). Optimal incubation temperatures for *Trichoderma* range between 25-30°C in darkness with differences in growth rate between species not greater than 10 mm (Chaverri & Samuels, 2003; Samuels & Hebbar, 2015), which is supported by our results. The recommended media to conduct morphological characterization of *Trichoderma* species is PDA Difco (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), as described in the reference work of Samuels and Hebbar (2015). Other commonly used media are Spezieller Nahrstoffarmer agar (SNA), Cornmeal agar (CMA), and Malt Extract Agar (MEA) that allow better micromorphology characterization (Chaverri *et al*., 2015; Samuels & Hebbar, 2015). However, in this work, we used only the PDA medium from Liofilchem®, which could have influenced the morphological characteristic of the isolates.

Initial characterization of *Trichoderma* isolates in Paraguay only used the morphological and antagonistic activity against plant pathogens as a method of characterization of *Trichoderma*. The first attempts to investigate the potential of native strains of *Trichoderma* as biological control agents were conducted by Stauffer (1999). Afterward, more studies focused on the isolation and description of native *Trichoderma* at the morphological level to develop alternative management products for sesame and soybean seed treatment against *Macrophomina phaseolina* (Garcete & Orrego, 2011; Ortellado Franco & Orrego Fuente, 2013). These studies encouraged the interest in the application of *Trichoderma* in other pathosystems aiming to protect other specialty crops such as macadamia (Sanabria Velazquez & Grabowski, 2016), stevia (Britos & Jongdae, 2016), and chia (Albrecht *et al*., 2017). However, none of these previous studies employed molecular tools for species identification and were named only *Trichoderma* spp. Since phenotypic traits do not allow for species-level differentiation, they are complementary to the molecular characterization (Cai & Druzhinina, 2021).

The ITS region is considered the molecular barcode for fungal identification and is the most commonly used marker for diagnosing fungal species (Raja *et al*., 2017). However, the ITS region does not have enough resolution to resolve the species complex of *T. harzianum* and divide groups of *Trichoderma*. This lack of resolution was confirmed by multiple authors (Druzhinina & Kubicek, 2005; Samuels *et al*., 2006; Chaverri *et al*., 2015; Cai & Druzhinina, 2021). The ITS region proved useful for exploring the genetic diversity of *Trichoderma* in Brazil, resulting in more than 33% of the isolates being related to *T. harzianum* in the *Harzianum* complex. However, this region's analysis alone was insufficient to distinguish between species belonging to the same complex (Feitosa *et al.,* 2019).

The *tef1*α sequences were analyzed to determine the species complex of *T. harzianum* and the section *Trichoderma*. This analysis showed better resolution of the *tef1*α gene than the ITS gene. Two isolates, FCQ16 and FCQ23, clustered together and within the section *Harzianum*/*Virens*, yet these could not be properly identified as there was no close match for a reference sequence. Nonetheless, both isolates fell within the *T. harzianum* complex of species. *Trichoderma harzianum* is considered a complex of polyphyletic species that are morphologically indistinguishable. Similarly reported in previous works (Chaverri *et al.,* 2015), the analysis of *tef1α* sequences provided a better-defined section *Trichoderma*. The intricate nature of the *T. harzianum* species complex has been previously reported by Druzhinina *et al.* (2010) and Kubicek *et al.* (2019).

The concatenation of ITS and *tef1α* sequences improved statistical support for species identification. Similar to our results, previous recent reports using the combination of ITS and *tef1*α have successfully characterized *Trichoderma* species (Lisboa *et al*., 2017; Haouhach *et al*., 2020). Other genes used for this purpose were *rpb2*, *ech42*, *cal1*, *act*, *acl1*,18S rRNA, and 28S rRNA. However, as not all *Trichoderma* species were sequenced using these genes, for example old sequences from public databases, they have reduced utility for molecular identification at the species level. Cai and Druzhinina (2021) argue that using *tef1*α and *rpb2* regions allows the correct identification of *Trichoderma* species based on the sequence similarities between the query strain and the reference strains. More complete evidence of different species within the complex could be achieved by considering sequence similarities and phylogenetic concordance.

We found the species *T. brevicompactum* (FCQ18 and FCQ21), which have been reported to produce high concentrations of trichodermin, an antifungal metabolite that inhibits protein synthesis and is deadly to living cells (Degenkolb *et al*., 2008; Malmierca *et al*., 2012; Barúa *et al*., 2019). This compound has been reported to inhibit seed germination and reduce plant growth (Tijerino *et al*., 2011). Also, when tomato seedlings were treated with *T.*

brevicompactum, the root length and plant size were significantly reduced. In addition, there were more necrotic lesions when treated with *T. brevicompactum* and inoculated with the plant pathogen *Botrytis cinerea*. For these reasons, the isolates FCQ18 and FCQ21 may potentially produce trichodermin in high concentrations, thus limiting their use as biological control agents. However, this capacity is an attribute of strains that cannot be attributed to the production of a specific metabolite or their endophytic capacity.

T. asperellum was frequently found in this study (FCQ42, FCQ43, FCQ44, and FCQ46). Similarly, isolates of *T. asperellum* were reported from agricultural soils of northern Paraguay but based only on morphological and ITS region analysis (Fernández Gamarra *et al*., 2017). The species *T. asperellum* has been studied extensively because of its potential as a biological control agent for plant pathogens (Wu *et al*., 2017). In the neighboring country, Brazil, *T. asperellum* application in soybean seeds significantly increased the yield (Chagas *et al*., 2017) and suppressed the growth of the pathogen *Sclerotinia sclerotiorum* isolate to varying degrees (Macena *et al*., 2020).

We reported one isolate of *T. longibrachiatum*, FCQ47, obtained from the cortex of lemon verbena (*Aloysia citrodora*) with the potential to act as an endophytic strain, though this was not confirmed in this study. Previous research reported the potential of endophytic strains of *T. longibrachiatum* isolated from *Dendrobium nobile* stem segments to increase plant metabolites and inhibit bacterial pathogens (Sarsaiya *et al*., 2020), suggesting that this species has the potential to be employed as a biocontrol agent. This hypothesis was confirmed by Zhang *et al.* (2018), who reported that the strain of *T. longibrachiatum* T6 was effective against *Valsa mali,* the causal agent of apple tree valsa canker disease, and the primary mechanism of control of the pathogen, the production of secondary metabolites. However, some characteristics might limit the potential of *T. longibrachiatum* employed as a commercial biocontrol agent. This fungus produced trichokonin VI (TK VI), a peptaibol compound that significantly reduced the root growth of *Arabidopsis* (Shi *et al*., 2016). Moreover, *T. longibrachiatum* was described as an opportunistic pathogen of immunocompromised humans in several publications (Kuhls *et al*., 1999; Richter *et al*., 1999; Akagi *et al*., 2017).

The results of various studies show that the species composition of *Trichoderma* in agricultural soils in Latin America is diverse (Holmes *et al*., 2004; Consolo *et al*., 2012; Amerio *et al*., 2020; Inglis *et al*., 2020). This can be contrasted with the findings in Paraguay, considering that the sample of isolates is relatively small; and, thus, the results should be interpreted with caution.

A large number of fungal species can act as biological control agents of agricultural pests (McSpadden Gardener & Fravel, 2002), whose antagonistic properties are based on the activation of multiple mechanisms such as competition for space and nutrients (Sempere Ferre & Santamarina, 2010), mycoparasitism (Atanasova *et al*., 2013), and antibiosis with the production of enzymes (Wu *et al*., 2017) and secondary metabolites such as antibiotics, mycotoxins, and phytotoxins that participate in the control of the pathogen (Tijerino *et al*., 2011; Barúa *et al*., 2019; Cubilla-Ríos *et al*., 2019). Although many researchers focused their attention on the potential that these metabolites have for the biological control of phytopathogens, there is a diverse group of secondary metabolites that differ in chemical structure, and that exhibit multiple biological functions for applications in agriculture (McSpadden Gardener & Fravel, 2002), pharmacy (Daniel & Rodrigues Filho, 2007), and food processing (Galante *et al*., 1998) that cannot be ignored. Therefore, this work of characterization of native *Trichoderma* isolates can help to raise interest in producing Paraguayan biotechnological products based on *Trichoderma* species as well as providing documentation of the biogeographic distribution of this economically significant group of microscopic fungi in this region of the world. In summary, we report *T. asperellum, T. brevicompactum, T. longibrachiatum, T. koningiopsis, and T. neokoningii* for the first time in Paraguay, based on morphological and molecular data from the ITS and *tef1*α loci.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

ADSV curated the data, conducted formal analysis, and wrote the original draft. MMFP conducted investigations, performed formal analysis, and contributed to the original

draft writing. LIA also conducted the experiments. MEFG conceptualized the project, wrote and edited reviews, and contributed to the writing. MCRR provided supervision, writing, and visualization. PHS developed the methodology, validated the findings, and reviewed and edited the project. JEBC conceptualized the project, provided resources, and edited the work. All authors reviewed the final version of the manuscript.

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SUPPLEMENTARY TABLE 1. *Trichoderma* spp. sequences used for the phylogenetic analyses.

Species	Genbank accession N°	
	ITS	*tef1*α
Hypomyces aurantius	FJ442642	FJ467643
Trichoderma amazonicum	NR111529	HM142375
Trichoderma asperellum	MH021852	EU279961
Trichoderma brevicompactum	EU330941	EU280061
Trichoderma caribbaeum	NR137302	DQ289010
Trichoderma citrinoviride	NG056275	AY865637
Trichoderma erinaceum	NR111837	DQ109547
Trichoderma gamsii	DQ315459	DQ307541
Trichoderma ghanense	NR120299	AY865638
Trichoderma guizhouense	JN191311	AY937440
Trichoderma hamatum	EU280124	EU856307
Trichoderma harzianum	AY605713	EU279992, KU738448
Trichoderma helicum	NR153213	AY605812
Trichoderma intricatum	NR134343	AF348110, EU248630
Trichoderma konilangbra	MF113487	MK435374
Trichoderma koningiopsis	NR131281	EU279998
Trichoderma lentiforme	NR144868.	FJ463310
Trichoderma lieckfeldtiae	NR138438	EU856324
Trichoderma longibrachiatum	NR120298	AY865640
Trichoderma neokoningii	NR138446	KJ665620
Trichoderma ochroleucum	NR134401	FJ860659
Trichoderma ovalisporum	NR134342	AY387660
Trichoderma parareesei	NR138453	KF699130
Trichoderma paucisporum	NR134360	DQ109541
Trichoderma pleuroticola	NR134420	EU918160
Trichoderma pseudokoningii	NR120296	AY865641
Trichoderma reesei	MH861154	Z23012
Trichoderma rossicum	NR111834	EU280062
Trichoderma samuelsii	NR138452	EU280023
Trichoderma saturnisporum	NR103704	AY865642
Trichoderma seppoi	NR134413	FJ179582
Trichoderma shennongjianum	NR144880	KT735253
Trichoderma spirale	NR077177	KJ665740
Trichoderma strictipile	NR134337	AY865644
Trichoderma taiwanenense	NR144874	DQ284973
Trichoderma texanum	NR137308	KT278988
Trichoderma theobromicola	NR134359	EU856321
Trichoderma tibetense	NR144881	KT735254
Trichoderma virens	NR138428	KC291138
Trichoderma viride	NR138441	DQ672615
Trichoderma viridescens	MH855595	HQ293004

SUPPLEMENTARY TABLE 2. BLAST results for the ITS sequences of each *Trichoderma* species isolated in the study.

Isolate	Species	Identity (%)	Accession length	Matched accession
FCQ13	*T. koningiopsis*	100.00	613	MK791649.1
	T. viride	100.00	576	KC576682.1
	T. sp.	99.81	578	MF136557.1
FCQ16	*T. harzianum*	100.00	604	MN262484.1
	T. rifaii	100.00	585	OL757372.1
	T. afarasin	100.00	604	FJ442665.1
	T. inhamatum	99.81	545	MH861135.1
FCQ18	*T. turrialbense*	100.00	627	MT530012.1
	T. sp.	100.00	585	MN602854.1
	T. brevicompactum	100.00	579	MK253291.1
FCQ19	*T. sp.*	100.00	580	MW765133.1
	T. sulphureum	99.81	624	MT530250.1
	T. koningiopsis	99.81	565	MT520626.1
	T. afroharzianum	99.81	1131	MN644679.1
FCQ21	*T. turrialbense*	100.00	627	MT530012.1
	T. sp.	100.00	585	MN602854.1
	T. brevicompactum	100.00	579	MK253291.1
FCQ23	*T. harzianum*	100.00	619	MK738150.1
	T. azevedoi	100.00	603	MK714903.1
	T. sp.	100.00	582	MK808887.1
	T. simmonsii	100.00	560	MF078647.1
	T. lixii	100.00	592	OL741785.1
	T. lentiforme	99.81	603	FJ442251.1
FCQ32	*T. lentiforme*	100.00	600	MN262489.3
	T. harzianum	100.00	570	MK751758.1
	T. breve	100.00	640	MN400089.1
	T. lixii	100.00	653	KY315574.1
	T. simmonsii	100.00	598	MZ835628.1
	Sordariomycetes sp.	100.00	1106	MW529552.1
	T. harzianum	99.81	599	KX379172.1
FCQ36	*T. sulphureum*	99.81	624	MT530250.1
	T. sp.	99.81	565	MT520637.1
	T. koningiopsis	99.81	565	MT520626.1
	T. afroharzianum	99.81	1131	MN644679.1
FCQ37	*T. sulphureum*	100.00	624	MT530250.1
	T. koningiopsis	100.00	565	MT520626.1
	T. afroharzianum	100.00	1131	MN644679.1
	T. atroviride	100.00	602	MN341303.1
	T. caribbaeum var. caribbaeum	100.00	658	NR _ 166015.1
	T. neokoningii	100.00	581	MW269083.1
	T. ovalisporum	100.00	588	MW268857.1
	T. ghanense	100.00	862	MT892811.1

to be continued

Isolate	Species	Identity (%)	Accession length	Matched accession
FCQ42	*T. asperellum*	100.00	588	MT367901.1
	T. sp.	100.00	599	MT150599.1
	T. hamatum	100.00	898	MT111894.1
	T. yunnanense	100.00	597	MT102857.1
	T. viride	100.00	577	MT007532.1
	Phytophthora cinnamomi	100.00	603	MZ771300.1
	T. pubescens	100.00	548	MW280120.1
	T. koningii	100.00	548	MW265008.1
	Fusarium oxysporum f. sp. Ricini	100.00	609	MW074252.1
	T. harzianum	100.00	583	MT995126.1
FCQ43	*T. hamatum*	100.00	634	MT355443.1
	T. asperellum	100.00	595	MT341772.1
	T. sp.	100.00	557	MT133836.1
	T. harzianum	100.00	543	MW965792.1
FCQ44	*T. hamatum*	100.00	634	MT355443.1
	T. asperellum	100.00	595	MT341772.1
	T. sp.	100.00	557	MT133836.1
FCQ46	*T. hamatum*	100.00	634	MT355443.1
	T. asperellum	100.00	595	MT341772.1
	T. sp.	100.00	557	MT133836.1
	T. harzianum	100.00	543	MW965792.1
FCQ47	*T. longibrachiatum*	100.00	599	MT520646.1
	T. sp.	100.00	598	MT520642.1
	Fusarium oxysporum	100.00	604	MW775868.1

SUPPLEMENTARY TABLE 3. BLAST results for the *tef1*α sequences of each *Trichoderma* species isolated in the study.

Isolate	Species	Identity (%)	Accession length	Matched accession
FCQ13	*T. neokoningii*	98.43	818	KJ871265.1
	T. neokoningii	97.50	601	DQ841718.1
	T. sp. vd1	91.68	611	DQ841711.1
	T. gamsii	91.13	644	KR051477.1
	T. paraviridescens	91.12	629	MW791206.1
FCQ16	*T. harzianum*	100.00	516	KP890327.1
	T. sp. VB-2019a	100.00	531	MH352423.1
	T. camerunense	100.00	524	MG822709.1
	T. azevedoi	100.00	485	MN585285.1
	T. rifaii	98.00	555	MK644113.1
FCQ18	*T. brevicompactum*	98.72	631	MT058876.1
	T. sp. VNB-2019b	97.87	420	MT300493.1
	T. turrialbense	93.49	540	EU338282.1
	T. arundinaceum	89.63	623	MT058877.1
FCQ19	*T. koningiopsis*	99.22	959	EU279995.2
	T. arenarium	97.67	520	MT242306.3
FCQ21	*T. brevicompactum*	99.63	877	AB558910.1
	T. arundinaceum	90.00	623	MT058877.1
	T. turrialbense	93.70	540	EU338282.1
	T. sp. VNB-2019b	96.68	420	MT300493.1
FCQ23	*T. harzianum*	100.00	516	KP890327.1
	T. sp. VB-2019a	100.00	531	MH352423.1
	T. camerunense	100.00	524	MG822709.1
	T. azevedoi	100.00	485	MN585285.1
	T. rifaii	97.95	555	MK644113.1
	T. pollinicola	97.95	973	MF939619.1
	T. lixii	100.00	610	KJ855089.1
FCQ32	*T. lentiforme*	96.72	546	MK644114.1
	T. harzianum	96.32	888	AY605768.1
	T. lixii	95.88	601	JQ040449.1
	T. guizhouense	96.19	522	FJ463289.1
FCQ36	*T. koningiopsis*	100.00	959	EU279995.2
	T. arenarium	97.67	520	MT242306.3
FCQ37	*T. koningiopsis*	100.00	959	EU279995.2
	T. arenarium	97.67	520	MT242306.3
FCQ42	*T. asperellum*	99.62	535	GU198232.1
FCQ43	*T. asperellum*	99.81	892	MN307415.1
FCQ44	*T. asperellum*	100.00	892	MN307415.1
FCQ46	*T. asperellum*	100.00	892	MN307415.1
FCQ47	*T. longibrachiatum*	100.00	871	MH208265.1
	T. bissettii	100.00	817	HG931271.1