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ORIGINAL ARTICLE

Development of a taxon-discriminating molecular marker to trace and quantify a mycorrhizal inoculum in roots and soils of agroecosystems

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Abstract

Crop inoculation with *Glomus cubense* isolate (INCAM-4, DAOM-241198) promotes yield in banana, cassava, forages, and others. Yield improvements range from 20 to 80% depending on crops, nutrient supply, and edaphoclimatic conditions. However, it is difficult to connect yield effects with *G. cubense* abundance in roots due to the lack of an adequate methodology to trace this taxon in the field. It is necessary to establish an accurate evaluation framework of its contribution to root colonization separated from native arbuscular mycorrhizal fungi (AMF). A taxon-discriminating primer set was designed based on the ITS nrDNA marker and two molecular approaches were optimized and validated (endpoint PCR and quantitative real-time PCR) to trace and quantify the *G. cubense* isolate in root and soil samples under greenhouse and environmental conditions. The detection limit and specificity assays were performed by both approaches. Different 18 AMF taxa were used for endpoint PCR specificity assay, showing that primers specifically amplified the INCAM-4 isolate yielding a 370 bp-PCR product. In the greenhouse, *Urochloa brizantha* plants inoculated with three isolates (*Rhizophagus irregularis*, *R. clarus*, and *G. cubense*) and environmental root and soil samples were successfully traced and quantified by qPCR. The AMF root colonization reached 41–70% and the spore number 4–128 per g of soil. This study demonstrates for the first time the feasibility to trace and quantify the *G. cubense* isolate using a taxon-discriminating ITS marker in roots and soils. The validated approaches reveal their potential to be used for the quality control of other mycorrhizal inoculants and their relative quantification in agroecosystems.

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Introduction

Arbuscular mycorrhizal fungi (AMF) play an important role in natural ecosystems as well as in agroecosystems (Brundrett 2009). These soil fungi form symbiotic associations with the majority of plant species existing in nature (Smith and Read 2008), leading to several benefits to plants and soils, such as increasing nutrient and water absorption by plants and improving some physical and chemical soil properties. This results in higher crop yields and better plant adaptation to biotic and abiotic stress. Moreover, these fungi can improve plant resistance/tolerance (foliar and root) to pathogens and contribute to crop tolerance against diseases (Plenchette et al. 2005; Hamel and Strullu 2006; Barea et al. 2013; Verbruggen et al. 2013; Yang et al. 2014; Priyadharsini and Muthukumar 2015). Consequently, AMF are an essential and active component of sustainable agroecosystems.

Positive results from AMF inoculation under field conditions were previously reported (Farmer et al. 2007; Bayrami et al. 2012; Pellegrino et al. 2012, 2015; Hijri 2016; Schütz et al. 2018). Particularly, the *G. cubense* isolate (INCAM-4, DAOM-241198) inoculation have improved productivity under the tropical field conditions of Cuba, using different soil types and many economically important crops (Rivera and Fernández 2006; Martín et al. 2010, 2017; González et al. 2015, 2016a, 2016b; Joao et al. 2017; Morejón et al. 2017; Rosales et al. 2017).

In both natural and agricultural ecosystems, inoculated AMF have to compete with native AMF communities (Verbruggen et al. 2013), and root systems have been shown to be simultaneously colonized by more than one AMF taxon (van Tuinen et al. 1998a; Wang et al. 2011; Sánchez-Castro et al. 2012; Johansen et al. 2015). The interaction between inoculated and resident AMF is influenced by numerous factors including crop identity, agricultural management, and edaphoclimatic conditions (Gosling et al. 2006; Rivera et al. 2007; Herrera-Peraza et al. 2011; Brito et al. 2012, 2018; Köhl et al. 2014, 2015).

To distinguish different AMF species or isolates based just on fungal structures within roots through microscopy is difficult, despite efforts made by scientists (Abbott 1982; Abbott and Gazey 1994). Molecular tools allow to differentiate AMF at family and species levels, as well as to trace and detect specific AMF species inoculated in soils and roots. The methods of AMF-specific amplification by nested PCR (van Tuinen et al.1998a; Redecker 2000; Farmer et al. 2007; Krüger et al. 2009), cloning and sequencing (Gollotte et al. 2004; Thiéry et al. 2016), or a combination of them (Börstler et al. 2010; Pellegrino et al. 2012; Sánchez-Castro et al. 2012) were initially used. Later, the quantitative real-time PCR (qPCR) becomes a useful tool for the specific quantification of inoculated isolate in agroecosystems, despite the presence of resident AMF species and soil biota (Pivato et al. 2007; Köhl et al. 2015; Binet et al. 2017; Buysens et al. 2017; Voříšková et al. 2017). Other alternative methods to analyze AMF communities and genotypes, with the potential to trace inoculants, have been recently reported: NGS-based approach using Illumina MiSeq platform (Berruti et al. 2017; Morgan et al. 2017) and proteomic-based approach using MALDI-TOF–MS (Crossay et al. 2017).

Most studies using qPCR and mtLSU markers were performed to trace and quantify *Rhizophagus irregularis* (syn. *Glomus irregulare* and *Rhizoglomus irregulare*), a model fungus among AMF, in greenhouse (Krak et al. 2012; Janoušková et al. 2017; Voříšková et al. 2017) and field experiments (Buysens et al. 2017), or even in a spore suspension or inoculants (Badri et al. 2016). But there is hardly any variability in the mtLSU region for many other taxa different from *Rhizophagus*. Recently, Thioye et al. (2019) used the largest subunit of RNA polymerase II gene (*rpb1*) to trace and quantify a *R. irregularis* isolate in the field. The authors suggested this marker is appropriate for the *Rhizophagus* genus as well. Other AMF-taxa were traced and quantified using nuclear large ribosomal subunit (nrLSU) region (Pivato et al. 2007; Thonar et al. 2012; Janoušková et al. 2017; Voříšková et al. 2017). The nuclear ribosomal genes (nrDNA) show more suitable marker regions for the whole-group of AMF (Voříšková et al. 2017).

Among nrDNA, the present study used a marker targeting the ITS region that provided a sufficient resolution to be proposed as a universal DNA barcode marker for fungi. The ITS region was successfully used for identification of other mycorrhizal fungi (Millner et al. 2001; Manter and Vivanco 2007; Jacquemyn et al. 2012; Schoch et al. 2012). Schmidt et al. (2013) reported ITS superior to LSU in species discrimination. The authors compared the overall probability of accurate species identification using ITS with the success of the two-marker plant barcode system. Therefore, this region probably is more suitable for an AMF taxon-discriminating primer design. Moreover, the nrDNA regions rely on solid and extensive sequence databases (Begerow et al. 2010; Krüger et al. 2012; Öpik and Davison 2016).

The *G. cubense* isolate is compatible with a wide range of host plants and soil types, considered as generalist, which constitutes an important characteristic for commercial application (Verbruggen et al. 2013; Köhl et al. 2015). However, for a massive release to the field and further evaluation of ecological impact of such inoculum, it is crucial to have a molecular genetic tool to determine the effectiveness of host plant colonization and persistence under field conditions. Thus, the aims of the present study were (1) to design and optimize a taxon-discriminating ITS molecular marker for *G. cubense* isolate (INCAM-4, DAOM-241198), (2) to validate the ITS molecular marker in roots and soils under greenhouse and field conditions.

Materials and methods

Design and optimization of ITS molecular marker to trace *G. cubense* isolate

Primer design

The AMF isolate *Glomus cubense* (INCAM-4, DAOM-241198) from the collection of the Instituto Nacional de Ciencias Agrícolas (INCA) of Cuba was the focus of the study. A primer pair targeted to the ITS rDNA region was designed for *G. cubense* (Table 1), using the NCBI accession numbers for the amplified GLO2A-GLO2R sequences (JF692724 to JF692726, Rodríguez et al. 2011). The primer set was designed by the sequences aligning with Mega7 software (Kumar et al. 2016). A consensus sequence was

Table 1 Description of *G. cubense*-discriminating primers designed and initial parameters of the PCR

Primers (forward/reverse	Primer sequence (5′–3′)	Primer conc. (µM)	Annealing temp. (°C)	Amplicon size (bp)
Gcb-ITS-F	CAACGGATCTCTTGGCTCT	0.2/0.2	60	370
Gcb-ITS-R	ATTTGATCGTATACGAATGAAC			

used with Primer/Blast in order to get a list of potential primer sets. The absence of significant similarity of designed primers with other known DNA sequences in the NCBI GenBank public database (www.ncbi.nlm.nih.gov/tools/primer-blast/) was checked in silico. Predicted specificity of primers and dimer formation were tested by simulation with the program AmplifX_1.7.0_ WIN_EN (Bill Engels, University of Wisconsin, Madison, USA). The initial PCR conditions for further standardization were established using Oligo7 software.

DNA isolation from spores

The spores of *G. cubense* were obtained from cultures with *Sorghum bicolor* L. as a host plant by wet-sieving (300–38 µm mesh sieves), decanting and centrifugation in a sucrose gradient (Gerdemann and Nicolson 1963). Spores were collected under a dissecting microscope (Carl Zeiss, Stemi 2000-C, Gottingen, Germany) by micropipetting, rinsed with mH_2O (ultrapure water), cleaned by ultrasonication for 10 min and air-dried. DNA extraction was performed with the PrepMan Ultra (PMU) reagent (Applied Biosystems) following the manufacturer's instructions. The spores were crushed using liquid nitrogen and sterile plastic micropestle. The concentration of genomic DNA (gDNA) was quantified using nanodrop spectrophotometer 2000/2000 C (Thermo Fisher). The supernatant was diluted with mH_2O to 5 ng/µL.

The gDNA (10 ng) was amplified in a semi-nested PCR using the fungal-specific primers set ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990), in the first PCR (2 min at 95 °C; 35 cycles of 45 s at 95 °C, 45 s at 45 °C and 45 s at 72 °C; a final extension of 5 min at 72 °C). The second PCR was performed using 1 µL of PCR product from the first PCR amplification as template and the fungal-specific primer pair ITS1 (White et al. 1990) and ITS4, under similar PCR parameters, but the annealing temperature was 49 °C. The PCR product from the second PCR amplification was diluted 1:100 and used as positive control for *G. cubense* after verifying the presence of a strong and unique band on gel. The PCR reaction mixtures (25 µL volume) contained 1 × GoTaq® Flexi buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer, 1.25 units of GoTaq® Flexi polymerase (Promega, Madison, USA), and template DNA.

Amplifications were performed on a thermocycler Agilent Technologies SureCycler 8800 (Malaysia) and repeated at least three times. PCR products were loaded on 1% agarose gels with 1× TAE buffer (40 mM Tris, pH 7.8, 20 mM acetic acid, 2 mM EDTA) and visualized after staining with ethidium bromide (Sambrook et al. 1989).

Optimization of endpoint PCR from spores

Cycling parameters were optimized by gradient PCRs using reaction mixtures as described above and 1 µL of PCR product from the second PCR, identified as positive control for *G. cubense*, as template. The PCR parameters were as follows: 2 min at 95 °C; 30 cycles of 30 s at 95 °C, 45 s at 50 to 65 °C and 30 s at 72 °C; and a final extension of 5 min at 72 °C. Afterwards, a direct PCR following the optimized conditions was performed using 10 ng of gDNA extracted from *G. cubense* spores (2 µL of 5 ng/µL) as template to verify that (1) the optimized technique can amplify directly the sample (not nested PCR needed) and (2) the accuracy of designed primers.

Detection limit assay To estimate the detection efficiency of *G. cubense* isolate, the sensitivity of endpoint PCR was determined using 2 µL of serial fivefold dilutions of spores gDNA from 50 ng/µL (having 100, 10, 1, 0.1, and 0.01 ng as template) and the optimized PCR conditions above described. The PCRs were repeated three times.

Specificity assay For the specificity test through endpoint PCR, 18 isolates of various AMF taxa were used. Out of them, 16 were supplied by the Biological Resource Center Johanna Döbereiner (CRB-JD) at Embrapa Agrobiology, RJ, Brazil (Table 2). The spores were produced in pot cultures on *Urochloa decumbens* (Stapf) D. Webster (brachiaria grass) plants grown under greenhouse conditions. Two *Rhizophagus* isolates from Cuban collections were also included, which were propagated on *S. bicolor* plants under greenhouse conditions. The selection of AMF isolates contained members of all main clades of *Glomeromycotina* (Spatafora et al. 2016), that allowed to check for cross-specificity among both closely and distantly related AMF isolates.

The spores were extracted from substrates whereas the gDNA was extracted from spores and their concentrations measured as described above for *G. cubense*. The

Table 2 Arbuscular mycorrhizal fungal strains of various taxa used in the specificity test and its origin

AMF isolate	Family/genera	Code	Collection/origin
Rhizophagus irregularis	*Glomeraceae/Rhizophagus*	INCAM-11, DAOM-711363	INCA/Cuba
Rhizophagus intraradices	*Glomeraceae/Rhizophagus*	A31, IES-9, BRM 049199	IES/Cuba
Rhizophagus clarus	*Glomeraceae/Rhizophagus*	A5, CNPAB 005, BRM 033301	Embrapa Agrobiology/Brazil
Rhizophagus diaphanus	*Glomeraceae/Rhizophagus*	A84, CNPAB 042, BRM 049304	Embrapa Agrobiology/Brazil
Rhizophagus manihotis	*Glomeraceae/Rhizophagus*	A83, CNPAB 041, BRM 049303	Embrapa Agrobiology/Brazil
Glomus formosanum	*Glomeraceae/ Glomus*	A20, CNPAB 020, BRM 033691	Embrapa Agrobiology/Brazil
Glomus sp1	*Glomeraceae/Glomus*	A51, Inoculum 151, BRM 049224	Embrapa Agrobiology/Brazil
Glomus sp2	*Glomeraceae/Glomus*	A100, CNPAB 056, BRM 050039	Embrapa Agrobiology/Brazil
Glomus sp3	*Glomeraceae/Glomus*	A90, CNPAB 046, BRM 049310	Embrapa Agrobiology/Brazil
Glomus sp4	*Glomeraceae/Glomus*	A91, CNPAB 047, BRM 049311	Embrapa Agrobiology/Brazil
Claroideoglomus etunicatum	*Claroideoglomeraceae/Claroideoglomus*	A44, Inoculum 51, BRM 049217	Embrapa Agrobiology/Brazil
Paraglomus brasilianum	*Paraglomeraceae/Paraglomus*	A85, CPAC LBRS, BRM 049305	Embrapa Agrobiology/Brazil
Gigaspora gigantea	*Gigasporaceae/Gigaspora*	A8, CNPAB 008, BRM 033425	Embrapa Agrobiology/Brazil
Gigaspora margarita	*Gigasporaceae/Gigaspora*	A1, CNPAB 001, BRM 033261	Embrapa Agrobiology/Brazil
Dentiscutata heterogama	*Dentiscutataceae/Dentiscutata*	A2, CNPAB 002, BRM 033298	Embrapa Agrobiology/Brazil
Scutelospora calospora	*Scutellosporaceae/Scutelospora*	A80, CNPAB 038, BRM 049299	Embrapa Agrobiology/Brazil
Cetraspora pellucida	*Racocetraceae/Cetraspora*	A70, CNPAB 029, BRM 049244	Embrapa Agrobiology/Brazil
Acaulospora morrowiae	*Acaulosporaceae/Acaulospora*	A78, CNPAB 036, BRM 049296	Embrapa Agrobiology/Brazil

INCA Instituto Nacional de Ciencias Agrícolas, *IES* Instituto de Ecología y Sistemática

numbers of spores for DNA extraction ranged from 100 to 300, depending on the spore size of the different AMF taxa. The obtained supernatants were diluted with mH_2O to final concentrations of about 5 to 10 ng/µL. Subsequently, all spore gDNAs extracted (about 10 to 20 ng) were amplified following the PCR optimized conditions.

Amplification of fungi

Fungal presence was verified in spore gDNAs using a nested PCR approach targeting the highly conserved nuclear ribosomal gene *ITS*. The first PCR step was performed using the eukaryote-specific primer pair NS31 (Simon et al. 1992) and NDL22 (van Tuinen et al. 1998b) at the following conditions: 2 min at 95 °C; 30 cycles of 45 s at 95 °C, 45 s at 52 °C and 2 min at 72 °C; a final extension of 5 min at 72 °C. The PCR reaction mixture (25 µL volume) contained 1× Green GoTaq® Flexi buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer, 1.25 units of GoTaq® Flexi polymerase, and 10 ng of template DNA. The second PCR step was performed using 1 µL of the first PCR product as template and the primer pair ITS1 and ITS4 under the conditions described above with the same reaction mixture.

Optimization of qPCR assay from spores

To prepare standards for the qPCR assays, gDNA extracted from *G. cubense* spores grown in vivo was used. The DNA concentration was measured with Qubit® dsDNA HS Assay kit (Invitrogen) using Qubit™ 3.0 fluorometer (life technology, Thermo Fisher Scientific) and diluted to 100 pg gDNA. The qPCR using PowerUp™ SYBR™ Green Master Mix kit (Applied Biosystems) was performed in 10 µL reaction mixtures on the C1000 Touch™ Thermal Cycler CFX96™ Real Time System (BIO-RAD, Singapore). The optimized reaction mixtures contained 1 × PowerUp™ SYBR™ Green Master Mix, 0.2 µM of each designed primer and 1 µL the standard (10^{-1}–10^{-3} ng/µL), using three technical replicates. The cycling conditions followed the manufacturer's instruction kit.

Detection limit assay The limit of detection (LOD) of the qPCR assay was calculated using the method proposed by Shrivastava and Gupta (2011) and applied by Badri et al. (2016), which was used to quantify a *R. irregularis* isolate. For a linear regression, LOD can be expressed as $LOD = 3S_a/b$, where S_a is the standard deviation of *y*-intercepts of regression lines and *b* is the slope of the calibration curve. LOD was calculated from standard calibration curves based on threefold dilutions of gDNA standards (10^{-1}–10^{-3} ng/µL), which were performed in triplicate. The IBM SPSS 22 software was used for the linear regression with $P \leq 0.05$.

Specificity assay For the specificity test of qPCR, nine isolates of various AMF taxa were selected (Table 2: *R. irregularis*, *R. clarus*, *G. formosanum*, *R. diaphanus*, *P. brasilianum*, *A. morrowiae*, *Glomus* sp1, *Glomus*

sp2, and *Glomus* sp3). The qPCR reactions were developed according to previously optimized conditions ("Optimization of qPCR assay from spores"). gDNAs extracted from spores were diluted about 0.2–1 ng, measured with the Qubit® fluorometer mentioned. The absolute quantification of the target sequences was performed based on the standard calibration curves using the BIO-RAD CFX96™ software (Singapore).

Validation of ITS molecular marker to trace and quantify *G. cubense* (INCAM-4) in roots and soils under greenhouse and field conditions

Biological material

Urochloa brizantha (A. Rich.) R.D. Webster (syn. *Brachiaria brizantha*) was used in this study as host plant. Seeds were disinfected with sodium hypochlorite (10%) before pre-germination on sterilized substrate composed by sand:vermiculite (2:1, v/v). Seedlings were transplanted 5 days after germination to plastic pots (4 kg) containing sterilized soil (121 °C for 1 h during three consecutive days), five plants per pot. At this moment, 1 g of inoculum (*G. cubense*, *R. irregularis*, or *R. clarus*) was deposited per pot below seedling roots. Therefore, four treatments were studied: control plants (no myc-inoculated) and inoculated plants with each of the three mentioned isolates, having five pots per treatment set up as a randomized design. All inoculants contained about 100 spores per g and other propagules.

Plants were grown under greenhouse conditions (natural photoperiod—November to February 2018, Seropédica, Rio de Janeiro, Brazil, temperature of 32/20 °C day/night) and irrigated with filtered water. The main chemical soil properties were: pH (H_2O) 6.93, C 0.29%, N 0.04%, P 51.60 mg/L, K^+ 66.74 $cmol_c/dm^3$, Ca^{2+} 0.85 $cmol_c/dm^3$, Mg^{2+} 0.24 $cmol_c/dm^3$. Plants were harvested 105 days after transplanting (Supplementary material Fig. 1).

In addition, environmental non-sterilized soils from Cuba (three trap cultures) and Brazil (two soils with corn and soybean roots) were tested. The Brazilian soil cultured with corn (*Zea mays* L.) mostly contained *Gigaspora* sp., *Acaulospora* sp., *G. macrocarpum*, *G. tortuosum*, *G. glomerulatum*, *Glomus* sp., and *Sclerocystis clavisporum*, which was the soil employed in the greenhouse experiment (see below) before sterilization. The Brazilian soil cultured with soybean (*Glycine max* L.) was mostly characterized by the presence of *Gigaspora* sp., *Acaulospora* sp., *A. mellea*, *Ambispora leptoticha*, *C. pellucida*, *Cl. etunicatum*, *Glomus* sp., *G. glomerulatum*, and *S. clavisporum* (Supplementary material Fig. 2). Cuban soils (pH between 7.12 and 8.02) were collected from three provinces (Pinar del Río, Mayabeque and Holguín), which were representative of the country. AMF spores were propagated using *S. bicolor* as trap plant and morphologically characterized. The soil from Pinar del Río was mostly characterized by the presence of *R. intraradices*, *Acaulospora* sp., *G. viscosum, G. cubense* and some different *Glomus* sp. Mayabeque soil mostly contained spores of *R. intraradices*, *Funneliformis mosseae*, *F. constrictum*, *G. viscosum*, *G. macrocarpum*, *G. microaggregatum*, *Glomus* sp., *Sclerosystis* sp., *Acaulospora* sp., *P. brasilianum* and *Racocetra fulgida*. Holguin soil was mostly characterized by the presence of *R. intraradices*, *Rhizophagus* sp., *F. constrictum*, *G. viscosum*, *Glomus* sp., *G. cubense*, and *Diversispora* sp.

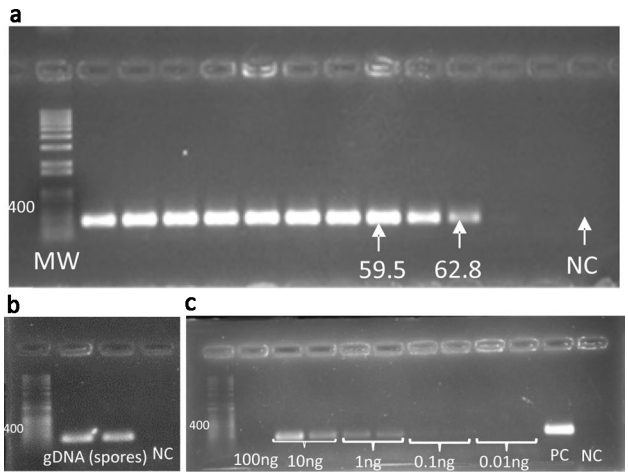

Fig. 1 Images of gels (1% agarose) stained with ethidium bromide from **a** gradient PCR using designed primers (Gcb-ITS-F/Gcb-ITS-R) and *Glomus cubense* positive control as template. Legend: well 1—molecular weight marker (MW: 1 kb plus Ladder, Invitrogen), with the position of 400 bp indicated; wells 2 to 13—temperature gradient (°C): 50.3, 50.7, 52.0, 53.8, 55.3, 56.7, 58.3, 59.5, 61.1, 62.8, 64.5, 65.0; well 14—negative control (mH2O). **b** Optimized PCR using gDNA from spores. Legend: well 1—MW; wells 2 and 3—replicated sample of *G. cubense* gDNA (spores); well 4—negative control (mH2O). **c** Detection limit assay of specific endpoint PCR, using fivefold DNA dilutions (*G. cubense* spores). Legend: well 1—MW; well 2—100 ng; wells 3 and 4—10 ng; wells 5 and 6- 1 ng; wells 7 and 8—0.1 ng; wells 9 and 10- 0.01 ng; well 11—*G. cubense* positive control; well 12—negative control (mH_2O)

Sample preparation

Roots were washed and tap roots removed. The thinnest roots were ground with a mortar and pestle in liquid nitrogen. One gram of each sample was kept at − 80 °C for further use. DNA extraction from roots (50 mg) was achieved with the PowerPlant® Pro DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions. Soil samples were lyophilized overnight first and DNA extractions from soil (200 mg)

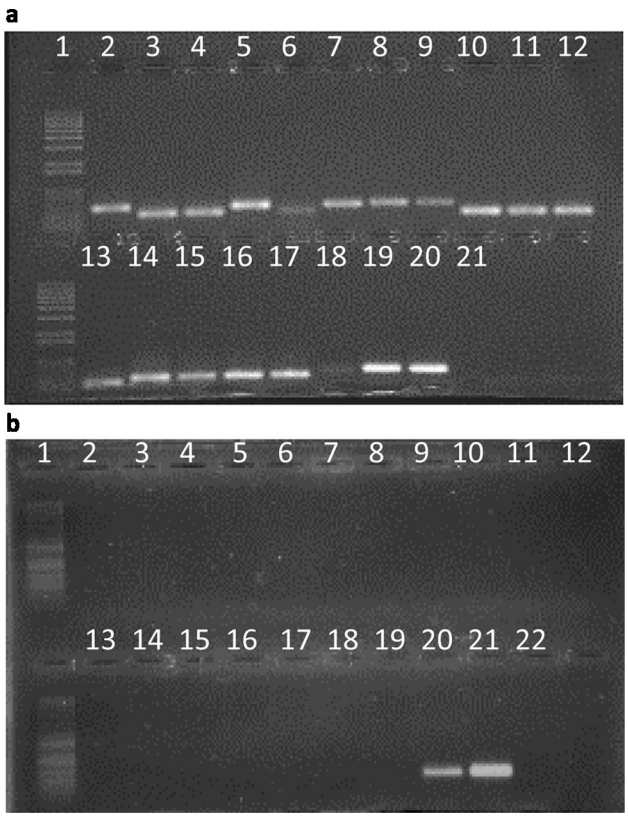

Fig. 2 Images of gels (1% agarose) from specificity assay of endpoint PCR using 18 AMF taxa and *Glomus cubense* (spores). **a** Fungal amplification by nested PCR (I: NS31/NDL22; II: ITS1/ITS4) and **b** with designed *G. cubense*-discriminating primers. Staining with ethidium bromide. Legend: well 1—molecular weight marker (1 kb plus Ladder, Invitrogen); wells 2 to 20—spores of *Gigaspora gigantea*, *Claroideoglomus etunicatum*, *Rhizophagus manihotis*, *Glomus* sp3, *Acaulospora morrowiae*, *R. diaphanus*, *Glomus* sp2, *R. clarus*, *Cetraspora pellucida*, *Scutelospora calospora*, *Dentiscutata heterogama*, *Gi. margarita*, *Glomus* sp1, *Glomus* sp4, *Paraglomus brasilianum*, *R. intraradices*, *G. formosanum*, *R. irregularis*, *G. cubense*; well 21—**a** negative control (mH2O), **b** *G. cubense* positive control; well 22—**b** negative control (mH_2O)

were accomplished using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions. In both cases, samples were incubated at 70 °C for 10 min (Thermomixer 5436, Eppendorf, Hamburg, Germany) before homogenization using the Mini-bead beater™ (Biospec Products) for 1 min at 3000 rpm. gDNA concentrations were quantified using nanodrop spectrophotometer 2000/2000 C (Thermo Fisher). A half of each extract was diluted with mH_2O to get about 1–4 ng/µL to be used in endpoint PCR. The extraction of each sample was performed in triplicate. The method used to verify fungal amplification by endpoint PCR in all samples was the same described above ("Amplification of fungi").

Estimation of root colonization by AMF

Assessment of root colonization by AM fungi was evaluated in five plants per treatment. Root systems were cleared in 2.5% KOH, then rinsed with abundant water and soaked in HCl 1%; finally, roots were stained with methylene blue (0.05% in acidic glycerol 50%) (Grace and Stribley 1991). Percentages of root fragments colonized by the fungus (F%) were evaluated by gridline intersection method according to Giovannetti and Mosee (1980) in a stereomicroscope (Carl Zeiss, Stemi 2000-C, Gottingen, Germany) using the notation scale described by Trouvelot et al. (1986) and freely available (http://www.dijon.inra.fr/Mychintec/Mycocalc-prg/download.html). Colonized root fragments were mounted on microscope slides in PVLG to take pictures (Axiovision System of Carl Zeiss (Gottingen, Germany) Camera AxioCam ERc5s).

Determination of AMF spore number

The spores obtained or present in 50 g of soils were isolated as described above, according to Gerdemann and Nicolson (1963) in three repetitions per sample. Thus, spores were counted under a stereomicroscope (Carl Zeiss, Stemi 2000-C, Gottingen, Germany) using Doncaster dishes.

Detection by endpoint PCR from soil and root samples

For endpoint PCR in soil and root samples, different cycling conditions (data not shown) were tested to optimize PCR conditions. For root samples, optimized cycling conditions were as follows: 2 min at 95 °C; 35 cycles of 30 s at 95 °C, 45 s at 60 °C and 30 s at 72 °C; and a final extension of 5 min at 72 °C. For soil samples, optimized cycling conditions were similar to root samples, with a higher cycle number (40 instead of 35). PCR reaction mixtures were as described above using 2–8 ng of gDNA for soil and root samples as template.

Quantification of *G. cubense* by qPCR with the nuclear ITS marker

All gDNAs from non-diluted soil and root samples (environmental and greenhouse) were used as template DNA for qPCR, with about 15 and 30 ng of them in each reaction, respectively. gDNA concentrations were measured with the Qubit® fluorometer mentioned. The cycling conditions and reaction mixtures were previously described ("Optimization of qPCR assay from spores"). Standard curves based on dilutions of genomic standard (DNA extract from *G. cubense* spores) were included in each run. The resulting concentrations of evaluated samples were expressed as ng of *G. cubense* DNA/ng total DNA (ng/ng DNA).

Data analysis and statistics

Spore number and root colonization percentages were analyzed after arcsine transformation for percentage values, by one-way ANOVA analyses, whereas differences between means using Tukey's test with $P \leq 0.05$ (IBM SPSS Statistics version 22).

Results

Design and optimization of ITS molecular marker to trace *G. cubense* isolate

Specificity in silico test of designed primers

The blast check of NCBI showed that only the reverse primer designed (Gcb-ITS-R) is specific, matching only the *G. cubense*-specific sequences published (JF692724 to JF692726) with 100% of homology and coverage. Moreover, the in silico study with several available sequences from analyzed AMF isolates (*G. formosanum*, *R. irregularis*, *Dentiscutata heterogama*, *Scutelospora calospora*, and *Cetraspora pellucida*) also showed low similarity with the reverse primer. These results indicate that the reverse primer (Gcb-ITS-R) could be considered as taxon-discriminating for the *G. cubense* isolate INCAM-4, DAOM-241198.

Optimization of endpoint PCR from spores

The endpoint PCR with the designed primers and initial pre-established conditions yielded a unique and strong band (Fig. 1a). The temperature gradient showed 60 °C as optimum annealing temperature. The amplicon length was about 370 bp, as it was expected. In addition, a positive and effective amplification was observed using directly 10 ng of gDNA extracted from spores as template (Fig. 1b). Therefore, the optimized setting was as follows: 2 min at 95 °C; 35 cycles of 30 s at 95 °C, 45 s at 60 °C and 30 s at 72 °C; and a final extension of 5 min at 72 °C.

Detection limit assay No band was observed when a high gDNA concentration or quantity (50 ng/µL~100 ng) was used as template in the endpoint PCR (Fig. 1c, well 2). The limit of detection was 0.1 ng (0.05 ng/µL) of *G. cubense* spore gDNA, distinguishing a very faint band with the right size (Fig. 1c, wells 7 and 8).

Specificity assay No band was visible on gel after the first PCR (data not shown). Nevertheless, in the second PCR, fungal presence was confirmed in all 18 different AMF spore samples (Fig. 2a). The unique band amplified using ITS primers by sample ranged about 500–600 bp. The specificity assay using the designed *G. cubense*-discriminating primers showed a robust band only for the *G. cubense* sample (gDNA from spores), similar to positive control, both with the expected size (Fig. 2b, well 20).

Detection limit and specificity assays of qPCR from spores

Standard curve and qPCR amplifications for the designed primer pair (*G. cubense*-discriminating) during qPCR optimization are illustrated in the supplementary material (Fig. 3). The LOD for this method was 40 Cq with $P \leq 0.05$ (Fig. 3), which means that samples with Cq < 40 contain *G. cubense* DNA, while samples with Cq ≥ 40 do not have this isolate.

Specificity results of the designed primers tested by qPCR showed no amplification for any of the nine AMF taxa evaluated, except *G. cubense*. This result agrees with the in silico analysis and the specificity assay developed by endpoint PCR using the same AMF isolates. All this corroborates that the designed primer set could be considered taxon-discriminating for *G. cubense* isolate INCAM-4, DAOM-241198.

Validation of ITS molecular marker to trace and quantify *G. cubense* (INCAM-4) in roots and soils under greenhouse and field conditions

AMF colonization and spore number

In general, the root colonization by AMF was high under experimental conditions (Table 3). *U. brizantha* roots inoculated with *R. clarus* A5, CNPAB 005 were more colonized (70%) compared to other treatments (Supplementary material Fig. 5a). No AMF presence was observed in the control treatment (no myc-inoculated). The other treatments showed non-significant statistical differences between them, reaching from 41 to 60% root colonization

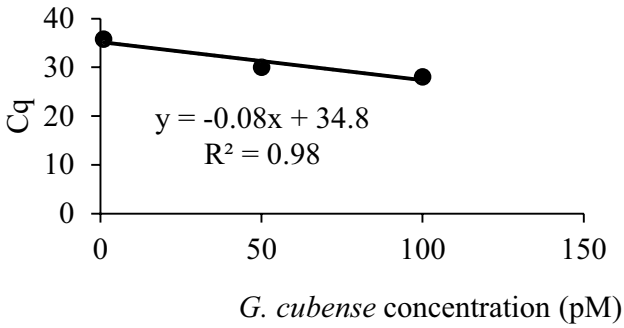

Fig. 3 Determination of limit of detection (LOD) from standard calibration curves based on threefold dilutions ($n = 3$, $P \leq 0.05$)

Table 3 Root colonization, spore number, and quantification of nrDNA ITS of *G. cubense* isolate (INCAM-4, DAOM-241198) in root and soil samples of *Urochloa brizantha* plants grown under greenhouse conditions and environmental samples

Samples	Cq	SD ±	Concentration ± SD (ng/ng DNA)		Root colonization (%)	Spore number per g of soil
Glomus cubense inoculated						
U. brizantha roots	29.8	0.12	3.43E−02 ± 4.45E−03		41.67 b	
U. brizantha roots	29.2	0.11	5.32E−02 ± 2.44E−03		59.73 b	
U. brizantha roots	28.5	0.54	8.89E−02 ± 3.13E−02		52.91 b	
U. brizantha soil	32.6	0.17	16.0E−04 ± 1.15E−04			8 d
U. brizantha soil	33.4	0.51	10.1E−04 ± 9.84E−04			11 d
U. brizantha soil	34.1	0.04	6.22E−04 ± 1.16E−05			4 d
Rhizophagus clarus inoculated						
U. brizantha root	N/A				70.00 a	
U. brizantha soil	N/A					18 c
Rhizophagus irregularis inoculated						
U. brizantha root	N/A				58.47 b	
U. brizantha soil	N/A					33 b
Control (no myc-inoculated)						
U. brizantha root	N/A			0 c		
U. brizantha soil	N/A					0 e
Environmental non sterilized						
Corn root (Brazil)	38.8	0.50	4.21E−05 ± 2.26E−06	43.50 b		
Soybean root (Brazil)	N/A			51.40 b		
Corn soil (Brazil)	39.8	0.08	1.56E−05 ± 1.22E−06			9d
Soybean soil (Brazil)	N/A					7 d
Holguín soil (Cuba)	31.4	0.04	3.16E−03 ± 5.86E−05			128 a
Mayabeque soil (Cuba)	40.3	0.21	1.09E−05 ± 1.41E−06			44 b
Pinar del Río soil (Cuba)	36.9	0.36	1.72E−04 ± 1.50E−05			22 c

Data of concentration are means ± SD ($n = 3$). Tukey's test compares means ($P \leq 0.05$) of root colonization and spore number ($n = 3$)

SD standard deviation, *N/A* non-amplification

(Table 3). The AMF spore number ranged from 4 to 33 spores per g of soil for greenhouse experiment (Supplementary material Fig. 4). In soils collected from Brazilian and Cuban fields ranged from 7 to 128 spores per g of soil (Table 3).

Detection by endpoint PCR from soil and root samples in greenhouse

For soil and root samples, no band corresponding to fungal rDNA was detected after the first PCR (Fig. 4 a and b). The specific fungal amplification products were detected after the second amplification (Fig. 4 c and d). However, the designed *G. cubense*-discriminating primers amplified the *G. cubense*-specific fragment (Fig. 4 e and f) directly from soil and root samples only in treatments inoculated with this isolate.

Quantification of *G. cubense* by qPCR with the nuclear ITS marker

Cq values of *U. brizantha* roots and soils in the greenhouse experiment ranged from 28 to 34 and fit for the standard curves (Table 3), but it was only for *G. cubense* inoculated treatment. Non-amplification (N/A) was detected in the other treatments. The *G. cubense* DNA concentration was higher in root fragments ($3.4\ 10^{-2}$–$8.9\ 10^{-2}$ ng/ng DNA) than in soil samples ($6.2\ 10^{-4}$–$16\ 10^{-4}$ ng/ng DNA). For samples from Brazilian field soils and roots, just the corn sample showed a late amplification (Cq ~ 39, about $1.6\ 10^{-5}$ and $4\ 10^{-5}$ ng/ng DNA for soil and root, respectively), which indicates *G. cubense* presence in low amount (Table 3). For trap cultures using Cuban soils, the *G. cubense* isolate was detected in Holguín and Pinar del Río, showing different and high concentrations compared to *G. cubense*-inoculated soil from the greenhouse experiment. Though, the trap culture from Mayabeque soil noticed a Cq of 40.3 (Table 3), which is superior to the LOD calculated for this

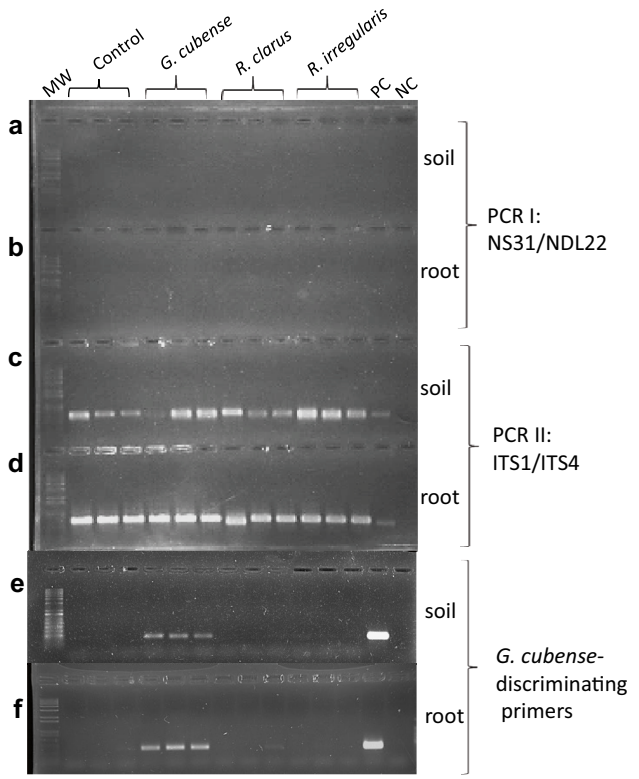

Fig. 4 Images of gels (1% agarose) from soil **a**, **c** and **e** and root **b**, **d** and **f** samples of *Urochloa brizantha* inoculated with three AMF isolates grown under greenhouse conditions. Staining with ethidium bromide. Legend: MW, molecular weight marker (1 kb plus Ladder, Invitrogen); Control, non-inoculated plants; *G. cubense*, *G. cubense*-inoculated plants; *R. clarus*, *R. clarus*- inoculated plants; *R. irregularis*, *R. irregularis*-inoculated plants; PC, positive control (*G. formosanum* spores: **a**, **b**, **c**, **d**), (*G. cubense spores*: **e**, **f**); NC, negative control (mH_2O)

system. Then, it is considered that *G. cubense* isolate (INCAM-4, DAOM-241198) was not present in this sample.

Discussion

Two specific molecular approaches involving detection of *G. cubense* isolate (INCAM-4, DAOM-241198) were validated in root and soil from greenhouse experiment and field. Well-developed and validated methods for the long-term tracing and/or quantification of inoculated AMF strains can contribute considerably to a breakthrough of the commercial application of mycorrhizal technology. At the same time, these molecular approaches can be used in ecological studies to explore effects of *G. cubense* inoculation on soil, which could contribute to avoid negative environmental consequences of agricultural management practices in agroecosystems.

This is one of the few studies that by endpoint PCR enables to detect direct and specifically an AMF taxon in root and soil samples. Previous studies used nested PCR (Tuinen et al. 1998a; Turnau et al. 2001; Farmer et al. 2007; Gamper and Leuchtmann 2007; Cesaro et al. 2008, Demir et al. 2011; Sýkorová et al. 2012). Most of these studies used nrLSU as marker and one of them used mtLSU (Sýkorová et al. 2012). The nested PCR approach involving two sets of primers in two amplification steps has been commonly used in AMF research to overcome PCR inhibition and to increase sensitivity for rare DNA templates in the presence of an overwhelming background of no target DNA (van Tuinen et al. 1998b). However, this method with two or three successive amplifications has disadvantages: (i) the intensity of amplification product cannot be linked to the fungus amount present in the sample, only the presence/absence of a fungus could be estimated (Farmer et al. 2007) and (ii) the risk of contamination by the amplified product from the first reaction. Thus, obtaining a specific product in a single PCR eliminates the contamination and artifact risk; therefore, the fungus sum existing in samples may be assessed through the band intensity. A recent study using a single PCR amplification coupled to next-generation sequencing was applied to analyze the AMF community in root samples (Řezáčová et al. 2016).

The absence of band using a high gDNA concentration or quantity (50 ng/µL~100 ng) as template in the detection limit assay by endpoint PCR (Fig. 1c, well 2) could be the consequence of PCR inhibition due to high concentrations of contaminants as a result of DNA extraction. In such cases, the best option is to use a convenient dilution of the sample. That is what often happens when AMF samples are used.

The benefits of qPCR in relation to endpoint PCR include speed, sensitivity, reproducibility and the ability to deliver quantitative results. The present study is the first, to our knowledge, that allows AMF-taxon quantification in root and soil by qPCR using an ITS marker, despite disadvantages related to the sequence polymorphism of this region and to the low ability to discriminate among closely related species or intraspecific isolates of AMF (Stockinger et al. 2010). The primer pair designed here based on ITS region showed specificity and sensitivity for the *G. cubense* isolate (INCAM-4, DAOM-241198). In particular, it was possible due to the following: (i) the ITS rDNA sequence heterogeneity within this isolate was low (from Mega7 alignment), (ii) the existence of only one known isolate representative of the species, (iii) the lack of closely related sequences from NCBI GenBank public database blast (only the sequence AF185683 of *R. intraradices*, which have 92% homology and 83% coverage).

The qPCR results showed that *G. cubense* isolate was significantly more abundant in root tissues than in soils. In correspondence, lower quantification cycle (Cq) values and higher *G. cubense* DNA concentrations were observed in root samples compared to those in soil samples (Table 3). Similar results were reported by Pivato et al. (2007) in field samples colonized by resident AMF using nrLSU marker. This finding is in agreement with the low saprophytic ability of AMF (Smith and Read 2008).

The concentration values of *G. cubense* in inoculated roots were high (ranging from 3 to 9 10^{-2} ng/ng DNA) with root colonization percentages between 41 and 59 (Table 3). Buysens et al. (2017) detected, using a mtLSU marker, the *R. irregularis* isolate MUCL41833 in potato roots under greenhouse conditions, recording concentration values of 7.4 10^{-3} ng/ng DNA (with 47% root colonization) and 3.0 10^{-4} ng/ng DNA (with 33% root colonization). Under field conditions, the authors found lower values of the inoculated AMF isolate: 2 10^{-5} ng/ng DNA with 31% root colonization for Bintje potato cultivar and 1.5 10^{-5} ng/ng DNA for Charlotte cultivar, with 46% of colonization. Results presented here also showed lower concentration values of *G. cubense* in environmental corn roots (non-sterilized soil and non-inoculated root): 4.2 10^{-5} ng/ng DNA vs. 3–9 10^{-2} ng/ng DNA in greenhouse inoculated-roots (Table 3). However, concentration value differences between greenhouse and field samples were higher in the present study with regard to Buysens et al. (2017) (10^3 vs. 10–10^2 times lower). This is consistent with the fact that field roots from Buysens et al. (2017) were inoculated with the target AMF, while our environmental roots were not inoculated with *G. cubense*. Therefore, a low concentration of *G. cubense* in corn root and soil (Table 3) seems to be the consequence of the presence of this taxon in such Brazilian soil among resident AMF (Supplementary material Fig. 2a). Despite this, its contribution to root colonization was low compared to other AMF that could colonize corn radical system, among them several producing intraradical spores (Supplementary material Fig. 5b). In this sense, we could mention AMF *Glomus brohultii*, whose presence has been reported in Cuban and Brazilian soils (Herrera-Peraza et al. 2003). Similarly, *Acaulospora mellea* described from Brazilian soil and root (Schenck et al. 1984) was lately found in Pinar del Río, Cuba (Furrazola et al. 2015). Besides, in agreement with most of the AMF present in Brazilian soils studied, they have also been observed in different Cuban ecosystems (Torres-Arias et al. 2015, 2017; Furrazola et al. 2016). In summary, this result corroborates the cosmopolitan distribution pattern of AM fungal taxa across continents and their very possible low endemism (Davison et al. 2015).

For trap cultures, the analysis is different due to the nature of these samples (propagated AMF spores using root system). The trap culture soil from Holguin reached inferior Cq and superior *G. cubense* concentration, while the trap culture soil from Pinar del Río showed higher Cq and lower *G. cubense* concentration values, compared to *U. brizantha* soils inoculated with *G. cubense*. The trap culture soil from Mayabeque exhibited a Cq superior to the LOD (Cq = 40); therefore, *G. cubense* was considered absent in this sample. However, further studies are suggested to absolutely confirm the presence of *G. cubense* in environmental samples (e.g. amplicon sequencing). AMF diversity studies based on spore morphology have shown the presence of common and different taxa, genera and families in various Cuban regions (Furrazola et al. 2015, 2016; Torres-Arias et al. 2015, 2017). The qPCR using ITS rDNA specific marker can be useful not only to determine the presence/abundance of this isolate in any soil, but also to characterize a solid inoculum based on *G. cubense*. The results expressed as ng/ng total DNA with high probability can be used to the quality control of manufactured products, such as biofertilizers.

Cross-specificity of designed primer pair was tested with both endpoint PCRs coupled with agarose gel electrophoresis and by qPCR. Through endpoint PCR, 18 AMF species were explored representing five different families (10 *Glomeraceae* mainly *Rhizophagus* (5), five *Gigasporaceae*, one *Claroideoglomeraceae*, one *Paraglomeraceae*, and one *Acaulosporaceae*) (Table 2). Nine AMF species were tested by qPCR, largely represented for *Glomeraceae* family with seven taxa, one *Paraglomeraceae* and one *Acaulosporaceae*. The AMF selection for qPCR specificity assay was firstly based on the absence of cross amplification by endpoint PCR, and secondly, the taxonomic relationship (same family most of them). Lastly, selection was based on the morphological similarity of AMF spores with *G. cubense* (small and hyaline to slight colored). As these results showed no cross amplification with any of the Glomales spores tested by two approaches, the primer set was considered taxon-discriminating for *G. cubense*, INCAM-4, DAOM-241198. Similar studies obtained cross amplification using endpoint PCR (Farmer et al. 2007; Cesaro et al. 2008) or qPCR (Thonar et al. 2012; Buysens et al. 2017) approaches; nevertheless, authors reported primers as specific being used in field experiments. This suggests the importance to establish the LOD of each approach in order to get a reliable and accurate methodology. Thonar et al. (2012) considered a primer pair as specific when a high difference in Cq values between target and no target AMF isolates was observed using nrLSU marker, with the lowest difference of 4 (*Gi. margarita vs. Gi. rosea*) and the greatest of 11 (*F. mosseae* vs. *R. intraradices*). However, same authors found that *Claroideoglomus claroideum* and *Cl. etunicatum* isolates showed Cq between 17 and 22 (Cq difference of 6 within same family) using the same primer pair, while the Cq difference between them and no target amplifications (different families) was inferior, equal to 5 (Cq ~ 27 for *R. intraradices*, *F. mosseae*, and *F. geosporum*). In addition, differences between mtDNA and nrDNA copy numbers (mt/nr) determined by qPCR were previously reported for two *Rhizophagus* sp. isolates (Krak et al. 2012), as well as between other AMF taxonomic lineages (Voříšková et al. 2017). The highest mt/nr ratio was found in *Gi. margarita*,

which is a slow root colonizer, followed by *F. mosseae*. The lowest ratios were observed in *Cl. claroideum* and *R. irregularis*, which were shown to rapidly decline in the vitality of intraradical structures (Voříšková et al. 2017). Thus, these latest authors pointed that isolate-specific features significantly influence the mt/nr ratio, which may be linked to distinct AMF root colonization patterns.

With respect to DNA region selected for specific-marker development, Krak et al. (2012) indicated that mtDNA would provide any additional information to nrDNA on the vitality of intraradical structures in established root colonization. However, Voříšková et al. (2017) suggested that nrDNA is a more suitable marker region than mtDNA, due to the possibility of quantifying different AMF taxa. This is particularly interesting if roots are colonized by multiple taxa, because the nrDNA copy numbers are better related to fungal biomass across taxa. The low variability of mtLSU region for most glomeromycetes, except to *Rhizophagus* sp., makes this marker unsuitable to study other AMF taxa. Results of the present investigation demonstrate the potential usefulness of the ITS rDNA region as biomarkers for AMF. Particularly, this region is validated as suitable to detect/trace and quantify a *G. cubense* isolate in roots and soils of agroecosystems.

All of this corroborates that the success of the specific primer set depends not only on the marker region selected, but also largely on the specific features of AMF taxa/taxon tested. Furthermore, specificity and sensitivity of primers is crucial to develop a suitable and accurate molecular approach to trace/quantify AMF in roots and soils of agroecosystems. This study validates for the first time the feasibility to trace directly and specifically a *G. cubense* taxon (INCAM-4, DAOM-241198) using an ITS nrDNA marker in roots and soils by two approaches: single endpoint PCR and qPCR. Despite advantages and disadvantages of each approach, both of them were successfully validated under greenhouse conditions. This research allows the use of the designed primer set to verify *G. cubense* establishment and survival under field conditions. This important isolate has been successfully used in commercial inoculants in Cuba. The assay could be suitable for the quality control of commercial products using *G. cubense* isolate. Moreover, the proposed approaches could be useful to elucidate the action mechanism of *G. cubense* and its interactions with soil microbiota. Finally, this study makes evident that ITS rDNA marker has potential to be used with the same purpose for other AMF isolates and provides approaches that could be applied to other AMF taxa. The methods developed here are useful to evaluate the quality control of AMF-based products by research institutions, governmental and industrial laboratories.

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