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Endophytic fungi from the genus *Colletotrichum* are abundant in the *Phaseolus vulgaris* and have high genetic diversity

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Abstract

Aims: To evaluate the diversity of endophytic fungi from the leaves of the common bean and the genetic diversity of endophytic fungi from the genus *Colletotrichum* using IRAP (inter-retrotransposon amplified polymorphism) and REMAP (retrotransposon-microsatellite amplified polymorphism) analyses.
Methods and Results: The fungi were isolated by tissue fragmentation and identified by analysing the morphological features and sequencing the internal transcribed spacer (ITS) regions and the rDNA large subunit (LSU). Twenty-seven different taxa were identified. *Colletotrichum* was the most commonly isolated genera from the common bean (32·69% and 24·29% of the total isolates from the Ouro Negro and Talismã varieties, respectively). The IRAP and REMAP analyses revealed a high genetic diversity in the *Colletotrichum* endophytic isolates and were able to discriminate these isolates from the phytopathogen *Colletotrichum lindemuthianum*.
Conclusions: Fungi from the genus *Colletotrichum* are abundant in the *Phaseolus vulgaris* endophytic community, and the IRAP and REMAP markers can be used to rapidly distinguish between *C. lindemuthianum* and other *Colletotrichum* members that are frequently found as endophytes.
Significance and Impact of the Study: This is the first report of the diversity of endophytic fungi present in the common bean and the use of IRAP and REMAP markers to assess the genetic diversity of endophytic fungi from the genus *Colletotrichum*.

Introduction

Endophytes are defined as organisms that colonize the internal tissue of plants without causing pathological symptoms or harm to the host (Petrini 1991) or micro-organisms isolated from the surface or interior of sterilized plant tissues that cause no apparent damage to the plant (Hallmann *et al.* 1997).

In the symbiotic relationship between plants and endophytic fungi, the plant gains competitive ability and resistance to biotic and abiotic factors due to the metabolites produced by the fungus, while these fungi benefit from the nutrients and shelter provided by the host plant (Müller and Krauss 2005). Several studies have demonstrated the relevance of endophytic fungi in the induction of host plant resistance (Dingle and McGee 2003; Kavroulakis *et al.* 2007), plant growth promotion (Hamayun *et al.* 2009b; You *et al.* 2012), increased tolerance to abiotic stresses (Redman *et al.* 2002; Khan *et al.* 2012), biological control of pests and disease (Cao *et al.* 2009; Zhang *et al.* 2009) and the production of metabolites of pharmacological interest, such as antibiotics, antioxidants and anticancer agents (Zhang *et al.* 2006; Chandra 2012; Radić and Strukelj 2012; Budhiraja *et al.* 2013).

The bean is a herbaceous plant species that belongs to the legume family (Fabaceae or Leguminosae) and includes several species of the genus *Phaseolus* (common bean) and the specie *Vigna unguiculata* (cowpea). The most commonly cultivated bean is *Phaseolus vulgaris* L., which is one of the most important agricultural products

in the world economy and has great importance for human nutrition (Broughton *et al.* 2003).

One of the main factors affecting bean production is fungal diseases such as anthracnose of the common bean, which is caused by the fungus *Colletotrichum lindemuthianum* (Barrus 1918). In addition to *C. lindemuthianum*, the genus *Colletotrichum* includes other species of phytopathogens (Bailey and Jeger 1992) such as *Colletotrichum truncatum*, which is the species most often associated with anthracnose in the soya bean (Manandhar *et al.* 1985). However, phytopathogenic species from this genus are capable of mutualistic or commensal lifestyles on plants other than those in which they cause disease (Redman *et al.* 2001). Different *Colletotrichum* species have been found in association with endophytic fungal communities in a large variety of plants from different ecosystems, such as *Vigna unguiculata* (Rodrigues and Menezes 2002), *Taxus mairei* (Wang *et al.* 2008), *Coffea arabica* (Fernandes *et al.* 2009), *Camptotheca acuminata* (Ding *et al.* 2010) and *Jatropha curcas* (Kumar and Kaushik 2013).

Molecular markers are useful tools for studying fungal ecology. IRAP (inter-retrotransposon amplified polymorphism) and REMAP (retrotransposon-microsatellite amplified polymorphism) markers are based on sequences of retrotransposons alone or retrotransposons and microsatellites, respectively (Kalendar *et al.* 1999). REMAP and IRAP markers can be used for isolate identification (Zein *et al.* 2010), the evaluation of genetic diversity (Branco *et al.* 2007; Zein *et al.* 2010; Santana *et al.* 2012) and genetic mapping (Manninen *et al.* 2000). In our laboratory, we have developed IRAP and REMAP markers to study intraspecific and interspecific diversity within the genus *Colletotrichum* (Santos *et al.* 2012). These markers are also effective for use in other fungi (Santana *et al.* 2013).

Endophytic fungi have been studied in various crops of economic interest such as cacao (Rubini *et al.* 2005), coffee (Fernandes *et al.* 2009), cowpea (Rodrigues and Menezes 2002), corn (Orole and Adejumo 2011) and soya bean (Pimentel *et al.* 2006; Hamayun *et al.* 2009a; Khan *et al.* 2011; Leite *et al.* 2013). However, to our knowledge, the endophytic fungal community of the common bean (*P. vulgaris*) has not yet been studied. Thus, the aim of this study was to determine the diversity of endophytic fungi from the leaves of *P. vulgaris* and to describe the genetic diversity of the endophytic fungi from the genus *Colletotrichum* using IRAP and REMAP markers.

Materials and methods

Place of collection and processing of the plant material

Fifteen leaves were collected from different plants of the Ouro Negro and Talismã varieties (Table 1) after 45 days

Table 1 Origin and characteristics of the varieties used in the isolation of bean leaf endophytic fungi

Variety	Origin	Characteristics
BRSMG Talismã	Brazil (2002)	Carioca (beige with light brown stripes) type grain; average weight for 100 seeds of 26–27 g; prostrate structure; medium cycle; resistant to the common mosaic and anthracnose
Ouro Negro	Honduras (1991)	Black type grain; average weight for 100 seeds of 25–27 g; prostrate structure; normal cycle; high capacity of symbiotic nitrogen fixation; resistant to rust and anthracnose; cold tolerant

of cultivation under the same edaphoclimatic conditions in the Diogo Alves de Melo experimental field at the Universidade Federal de Viçosa, in Viçosa—MG in Zona da Mata Mineira (latitude 21°45′ south and longitude 42°51′ west). The leaves were washed in running water to eliminate impurities from the leaf surface such as soil and dust residue. The leaves were then divided into 5×5 mm (0.25 cm^2) fragments using a scalpel.

Isolation of endophytic fungi

The leaf fragments were decontaminated by transferring to a solution of 70% ethanol with Tween 80 (two drops of Tween 80 per 100 ml of 70% ethanol) for 1 min, then to a solution of sodium hypochlorite (NaClO) containing 2–2·5% active chlorine for 4 min and finally to a solution of 70% ethanol with Tween 80 (two drops of Tween 80 per 100 ml of 70% ethanol) for 30 s. The surface disinfection was performed using a relationship between concentration of sodium hypochlorite (NaClO) and exposure time of the leaves to this reagent. The optimum relationship of 'concentration-time' was found by preliminary tests in which different concentrations and different exposure times were used (data not shown). After decontamination, the fragments were washed three times for 2 min in sterile deionized water to remove residual chlorine. The leaf fragments were then plated into 90 mm petri dishes containing YMC medium (10 g l^{-1} malt extract, 2 g l^{-1} yeast extract, 13 g l^{-1} agar), pH 6, supplemented with streptomycin (50 mg l^{-1}) and tetracycline (50 mg l^{-1}) to inhibit bacterial growth. The plates were incubated at 22 $\pm$ 2°C with a photoperiod of 12 h for 18 days. An aseptic control of the leaf surface disinfection was performed by printing the adaxial portion of the leaf fragments (imprinting), at previously marked locations, in YMC culture medium containing antibiotics. The absence of fungal growth in the control demonstrated that the surface disinfection technique was effective. In

addition, aliquots of the final rinse water of the leaf fragments were also plated as a complementary test of surface disinfection (Pereira 1993).

The fungal colonies isolated from the leaf fragments were purified by isolation on extinction of inoculum (yeast) and by monospore purification (to filamentous forms). The purified isolates were evaluated for morphology and separated into different morphotypes.

Extraction of total DNA

At least one representative isolate from each morphotype was streaked onto YMC medium. After 10 days of growth, a portion of the mycelium was collected for DNA extraction using the UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions.

Amplification of ITS and LSU regions

The following primers were used to amplify the internal transcribed spacer (ITS) region (approx. 600 pb): ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes and Bruns 1993) and ITS4 (5′-TCCTCCGCTTATTGATAT GC-3′) (White *et al.* 1990). The amplification conditions were as follows: initial denaturation at 95°C for 2 min, followed by 39 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 7 min. The amplification reaction was performed in a final volume of 25 μl containing 5 μl of Colorless GoTaq® Flexi Buffer (5×) (Promega, Madison, WI), 2·5 μl MgCl$_2$ (25 mmol l^{-1}) (Promega), 1 μl dNTPs (2·5 mmol l^{-1} each dNTP), 1 μl of the ITS1F primer (5 mmol l^{-1}), 1 μl of the ITS4 primer (5 mmol l^{-1}), 0·25 μl of Go Taq® DNA Polymerase (5 U l^{-1}) (Promega) and 5 μl of genomic DNA (2 ng μl^{-1}).

The rDNA large subunit (LSU) region (approx. 600 pb) was amplified using the primers LR0R (5′-AC-CCGCTGAACTTAAGC-3′) and LR16 (5′-TTCCACC-CAAACACTCG-3′) according to the protocol described by Botella and Diez (2011).

The amplified ITS and LSU fragments were electrophoresed on a 1·2% agarose gel, stained with ethidium bromide and visualized with the Eagle Eye® imaging system (Stratagene, La Jolla, CA, USA). The PCR products were sent to Macrogen Inc. (South Korea) for purification and sequencing using the same primers that were used for the amplification. The sequences of both DNA strands were grouped into contigs and manually corrected using the Sequencher ver. 4.1.4 program (Genecodes Corporation, Ann Arbor, MI). All sequences were compared with sequences deposited in GenBank using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi). The ITS

region sequences were deposited in GenBank under the accession numbers JQ753956 to JQ754047, and the LSU region sequences were deposited under the accession numbers JQ754048 to JQ754139.

Phylogeny

The ITS and the LSU DNA sequences were separately aligned using the MEGA 5 program (Tamura *et al.* 2011). Based on the alignment, a distance matrix was created for each region using Phylemon2 (http://phylemon.bioinfo.cipf.es/). The resulting matrix for each region was analysed using the program MOTHUR (Schloss *et al.* 2009) to group the sequences into OTUs (operational taxonomic units that share at least 97% sequence identity).

To construct the phylogeny for each region, one sequence was selected from each OTU, and additional sequences from GenBank were added. The sequences for each region were aligned using MEGA 5 (program) and manually edited to perform the phylogenetic analysis by Bayesian inference. This analysis was performed using the program MrBayes 3.1 (http://mrbayes.sourceforge.net/) to generate a consensus tree that contained the isolated endophytic species (Yang and Rannala 1997).

Diversity indices

The distribution of the isolates was used to calculate diversity indices using the program Past ver. 2.01 (Hammer *et al.* 2001).

IRAP and REMAP

The endophytic isolates from the *Colletotrichum* genus isolated in this study and 14 pathogenic isolates from the species *C. lindemuthianum* (9UFV, 45·3457, 64·497, 65·451, 67·491, 72·801, 73·497, 75·415, 83·501, 87·1234, 89.A2$^{2-3}$, 89·112, 95·28 and Lv101) from the culture collection of the Universidade Federal de Viçosa were used for the IRAP and REMAP analyses. For the IRAP analysis, we used the primers CIRAP2 (5′-AATAACGTC TCGGCCTTCAG-3′) and CIRAP4 (5′-CTTTTGACGAG GCCATGC-3′), and for the REMAP analysis, we used the primers CIRAP2 and MS1 (5′-GGCGGCGGCGGCGG CGGCGGCT-3′). The IRAP and REMAP reactions were performed using the amplification conditions described previously (Santos *et al.* 2012). The amplicons were subjected to gel electrophoresis on a 1·5% agarose gel containing ethidium bromide (0·2 mg ml^{-1}) in 1× TBE buffer. A 1 Kb DNA Ladder (Promega, Madison, WI, USA) molecular weight marker was used. The band pattern was analysed using the BioNumerics® ver. 6.0 software

(Applied Maths, Kortrijk, Belgium). The similarity matrix was constructed by calculating the densitometric curves using Pearson's correlation coefficient. The clustering was performed using the UPGMA algorithm (Unweighted Pair Group Method with Arithmetic Mean), and the correlation is expressed as percentage similarity. The quality of the branches was determined by calculating the cophenetic relationship. All IRAP and REMAP amplifications were repeated in three independent experiments.

Results

Isolation, phylogenetic analysis and identification

A total of 122 isolates were obtained, including 52 from the Ouro Negro variety and 70 from the Talismã variety. Initially, the isolates were divided into 58 morphotypes according to the feature exhibited on YMC medium. These 58 morphotypes were reduced to 27 taxa after molecular phylogenetic analysis. Identification of the taxa was carried out, with ≥97% similarity defined as ≥97% LSU rDNA similarity based on the results obtained from the BLASTN analysis (Table S1).

The phylogenetic trees for the ITS and LSU regions generated using the Bayesian method are shown in Figs 1 and 2. Both the Ouro Negro and the Talismã varieties showed isolates from the phyla Ascomycota and Basidiomycota. Isolates from phylum Basidiomycota accounted for 25% of all isolates from the Ouro Negro variety and 20% of all isolates from the Talismã variety, and these isolates belonged to the orders Tremellales (*Hannaella* and *Cryptococcus*) and Agaricales (*Crinipellis*). The isolates from the phylum Ascomycota belonged to the orders Capnodiales, Chaetothyriales, Diaporthales, Eurotiales, Glomerellales, Helotiales, Hypocreales, Magnaporthales, Pleosporales, Tuberculariales and Xylariales.

The three most commonly isolated genera from the Ouro Negro variety were *Colletotrichum* (32·69%), *Hannaella* (23·8%) and *Cochliobolus* (19·23%). *Colletotrichum* and *Hannaella* were also among the three most highly represented genera isolated from the Talismã variety ranking 1st and 3rd and accounting for 24·29% and 17·14% of the isolates, respectively. The 2nd most frequent genus was *Phomopsis*, which accounted for 18·57% of the isolates from the Talismã variety (Table 2).

Diversity indices

The diversity indices (Table 3) were calculated to facilitate the visualization of the diversity of isolates from each cultivar. These indices showed that the species richness and evenness were greater in the Ouro Negro variety.

IRAP and REMAP

A total of 35 endophytic isolates belonging to the genus *Colletotrichum* and 14 *C. lindemuthianum* isolates were evaluated by IRAP and REMAP, and all of the amplicons showed high reproducibility (Fig. 3). The *C. lindemuthianum* isolates formed a single group, while the endophytic isolates showed a greater genetic diversity and formed several groups (Fig. 4).

Discussion

In this study, we successfully isolated and identified endophytic fungi from the leaf tissue of two varieties (Ouro Negro and Talismã) of the common bean. Isolates from the classes Dothideomycetes and Sordariomycetes, belonging to the phylum Ascomycota, accounted for 75·41% of the total composition of the endophytic community, which is consistent with previous studies in other plant species (Arnold and Lutzoni 2007). According to these authors (2007), approx. 75% of the endophytic fungi isolated from plants from different environments belong to the classes Dothideomycetes and Sordariomycetes, and the abundance can vary relative to the other classes.

Fungi from the phylum Basidiomycota were also highly represented in the endophytic microbial community of the common bean. Unlike the phylum Ascomycota, there are few reports of the isolation of endophytic fungi from the phylum Basidiomycota in the literature. Fungi from this phylum have been isolated from orchids (Tao *et al.* 2008; Zhu *et al.* 2008), as well as some monocots and dicots (Waller *et al.* 2005; Arnold *et al.* 2007; Thomas *et al.* 2008). In our study, 22·13% of the total isolates belong to the phylum Basidiomycota, which indicates an important role for this phylum in the endophytic community of the leaves of the common bean. The fungi isolated with the highest frequency were from the order Tremellales, mainly from the genus *Hannaella*.

The most abundant genera detected in this work were *Colletotrichum*, *Hannaella*, *Cochliobolus* and *Phomopsis*. This abundance maybe due to the intrinsic characteristics of the fungi from these genera, which grow rapidly and are highly competitive in nonselective or plant-based media such as the YMC medium used for isolation in this study.

The Ouro Negro and Talismã varieties of the common bean were chosen because they are largely recommended for plantation in Minas Gerais State (the second major producer of this crop in Brazil). The anthracnose resistance feature was another reason for the selection of these two varieties. Initially, our hypothesis was that *Colletotrichum lindemuthianum* maybe colonized Talismã and Ouro Negro varieties as endophytic and cannot cause disease because of their anthracnose resistance features.
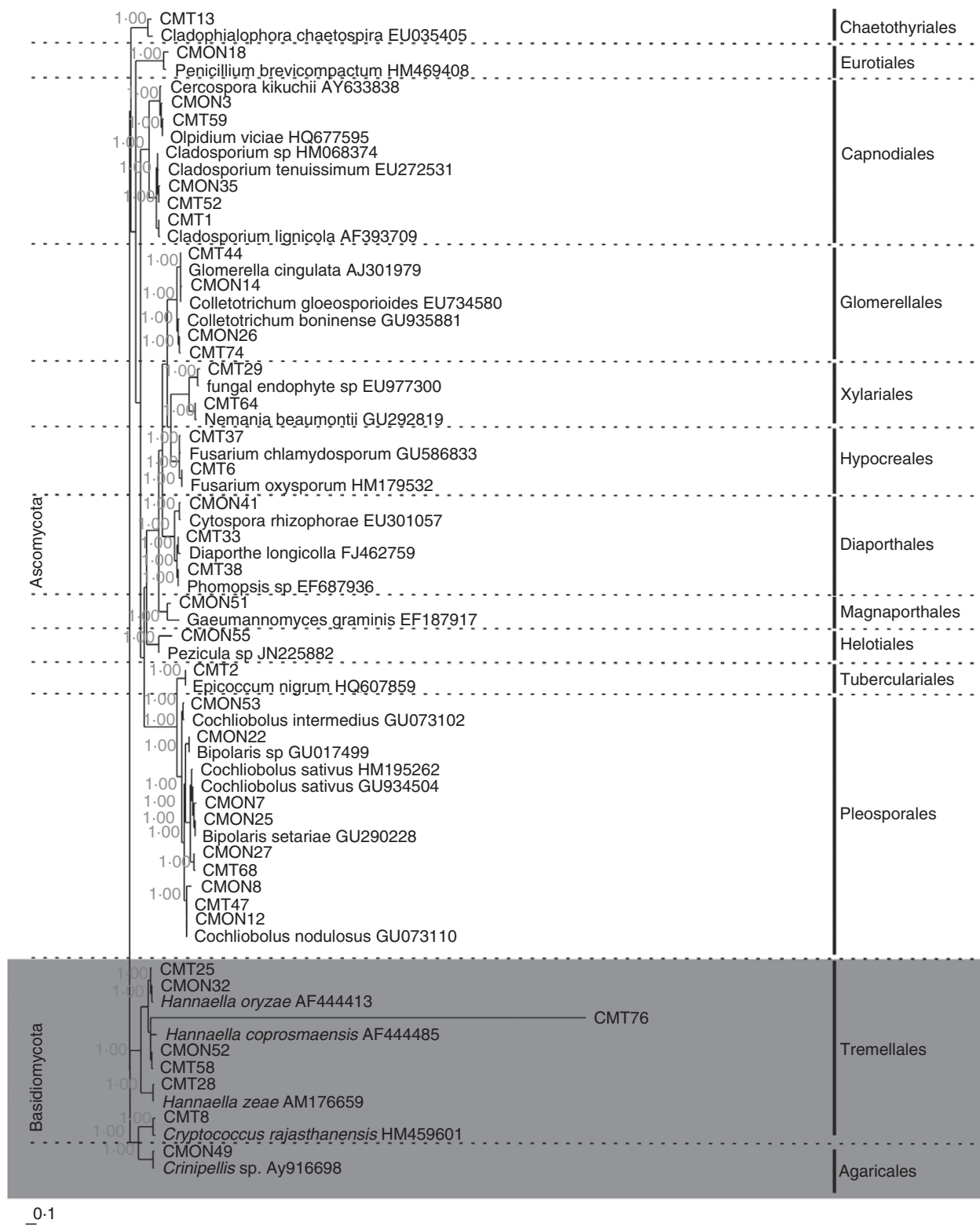
Figure 1 Phylogenetic tree obtained by Bayesian analysis using the nucleotide sequences of the ITS region of rDNA from representative isolates of endophytic fungi obtained from variety Ouro Negro and Talismã. Each ancestor node with the posterior probability of 1·00 was evidenced, and the ancestral nodes without numbers had a posterior probability of 0·50.
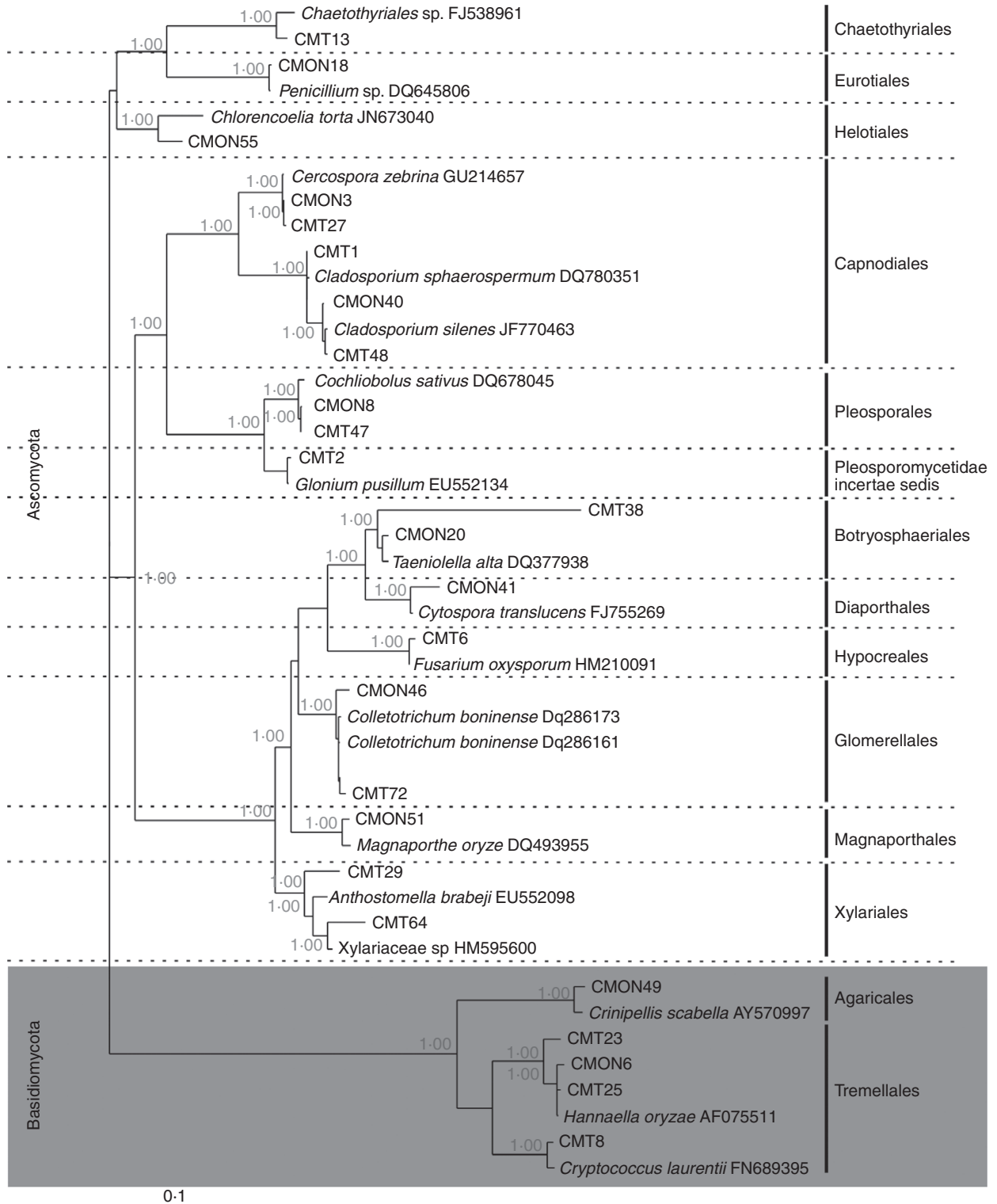
Figure 2 Phylogenetic tree obtained by Bayesian analysis using the nucleotide sequences of the LSU region of rDNA from representative isolates of endophytic fungi obtained from variety Ouro Negro and Talismã. Each ancestor node with the posterior probability of 1·00 was evidenced, and the ancestral nodes without numbers had a posterior probability of 0·50.

Table 2 Species of endophytic fungi isolated from common bean varieties Ouro Negro and Talismã and abundance for each isolate

	Taxa	Espécie	ONG	TAL	Total
Ascomycota	1	*Anthostomella* sp.	0	1	1
	2	*Bipolaris papendorfii*	1	0	1
	3	*Cercospora* sp.	3	4	7
	4	*Cercospora zebrinae*	0	2	2
	5	*Chlorencoelia* sp.	1	0	1
	6	*Cladophialophora* sp.	0	1	1
	7	*Cladosporium silenes*	0	2	2
	8	*Cladosporium* sp.	2	1	3
	9	*Cladosporium tenuissimum*	1	0	1
	10	*Cochliobolus sativus*	8	7	15
	11	*Cochliobolus* sp.	2	0	2
	12	*Colletotrichum boninense*	1	10	11
	13	*Colletotrichum gloeosporioides*	15	6	21
	14	*Colletotrichum* sp.	1	1	2
	15	*Cytospora* sp.	1	0	1
	16	*Epicoccum nigrum*	0	2	2
	17	*Fusarium oxysporum*	0	4	4
	18	*Fusarium* sp.	0	1	1
	19	*Magnaporthe grisea*	1	0	1
	20	*Nemania* sp.	0	1	1
	21	*Penicillium brevicompactum*	1	0	1
	22	*Phomopsis longicolla*	1	13	14
Basidiomycota	23	*Crinipellis* sp.	1	0	1
	24	*Cryptococcus* sp.	0	1	1
	25	*Cryptococcus zeae*	0	1	1
	26	*Hannaella oryzae*	8	12	20
	27	*Hannaella* sp.	4	0	4
		Total	52	70	122

ONG, Ouro Negro variety; TAL, Talismã variety. The grey shade discriminates the genera from the basidiomycota phylum from the Ascomycota phylum.

Table 3 Number of taxa, individuals and diversity index for each cultivar

Diversity indices/ Parameters	Formula†	Bean cultivar	
		ONG	TAL
Taxa (S)	–	18	16
Individuals (*n*)	–	70	52
Dominance (D)	$D = Sum(n_i/n)^2$	0·112	0·158
Shannon (H)	$H = Sum((n_i/n)\ln(n_i/n))$	2·451	2·235
Simpson (1-D)	$1 - D = 1 - Sum(n_i/n)^2$	0·888	0·843
Evenness (E)	$E = e^H/S$	0·645	0·584
Menhinick (db)	$Db = S/\sqrt{n}$	2·151	2·219
Margalef (Ma)	$Ma = (S-1)/\ln(n)$	4·001	3·796
Equitability (J)	$J = H/Hmax$	0·848	0·806
Fisher alpha (FA)	$S = \alpha*\ln(1 + n/\alpha)$	7·843	7·897
Berger-Parker (d)	$d = n/Nt$	0·186	0·308

†S, number of taxa; n, number of individuals; ni, number of individuals of taxon i; Nt, number of individuals in the dominant taxon; Hmax=log S, * Fisher's alpha; ONG, Ouro Negro variety; TAL, Talismã variety.

However, all fungi from the genus *Colletotrichum* isolated in this work were not from the *C. lindemuthianum* species.

Some fungi exhibit different symbiotic lifestyles depending on the host plant and/or the environmental conditions (Redman *et al.* 2001). Thus, fungi that are pathogenic for a particular plant species may colonize other species in an endophytic manner. Several genera of fungi isolated in this study are known to cause disease in plants, including the genera *Colletotrichum*, *Cercospora*, *Cladosporium*, *Cochliobolus*, *Fusarium*, *Penicillium* and *Crinipellis*. Furthermore, pathogenic fungi can often be found living as endophytic fungi in asymptomatic host plant tissues (Schulz *et al.* 1999). *Colletotrichum* was the most abundant genus isolated from both bean varieties in

our study; however, we did not isolate any *C. lindemuthianum* fungi. Out of all of the taxa from which isolates were obtained in this study, only the taxum *Fusarium oxysporum* (four isolates) belongs to a species known to be pathogenic for the common bean (Pastor-Corrales and Abawi 1987). Although this species is considered to be a pathogen of the plant we studied, this does not imply that the isolate described in this study is indeed pathogenic, as we did not test Koch's postulates for this isolate. Taxonomic evidence supports a close phylogenetic relationship between endophytic and pathogenic organisms, which can be considered sister species (Carroll 1988).

Regarding to potential biotechnological uses for endophytic fungi, *Colletotrichum gloeosporioides*, one of the *Colletotrichum* species that is abundant in the common bean endophytic community, produces various bioactive metabolites with antimicrobial (Zou *et al.* 2000; Arivudainambi *et al.* 2011), antitumor (Gangadevi and Muthumary 2008; Nithya and Muthumary 2009; Xiong *et al.* 2013), antioxidant, anti-inflammatory and anti-hyperlipidemic (Zhang *et al.* 2012) activity, among other activities of interest to the pharmaceutical industry. Thus, the *C. gloeosporioides* isolates identified in this study are strong candidates for future studies regarding potential biotechnology applications.

To compare the community structure of the two common bean varieties studied, diversity indices were calculated (Table 3). Diversity takes into account different concepts: species richness, relative abundance and community evenness. In our study, we observed 18 taxa among Ouro Negro variety isolates and 16 taxa among Talismã variety isolates. The abundance of species obtained from Talismã variety was greater than that of
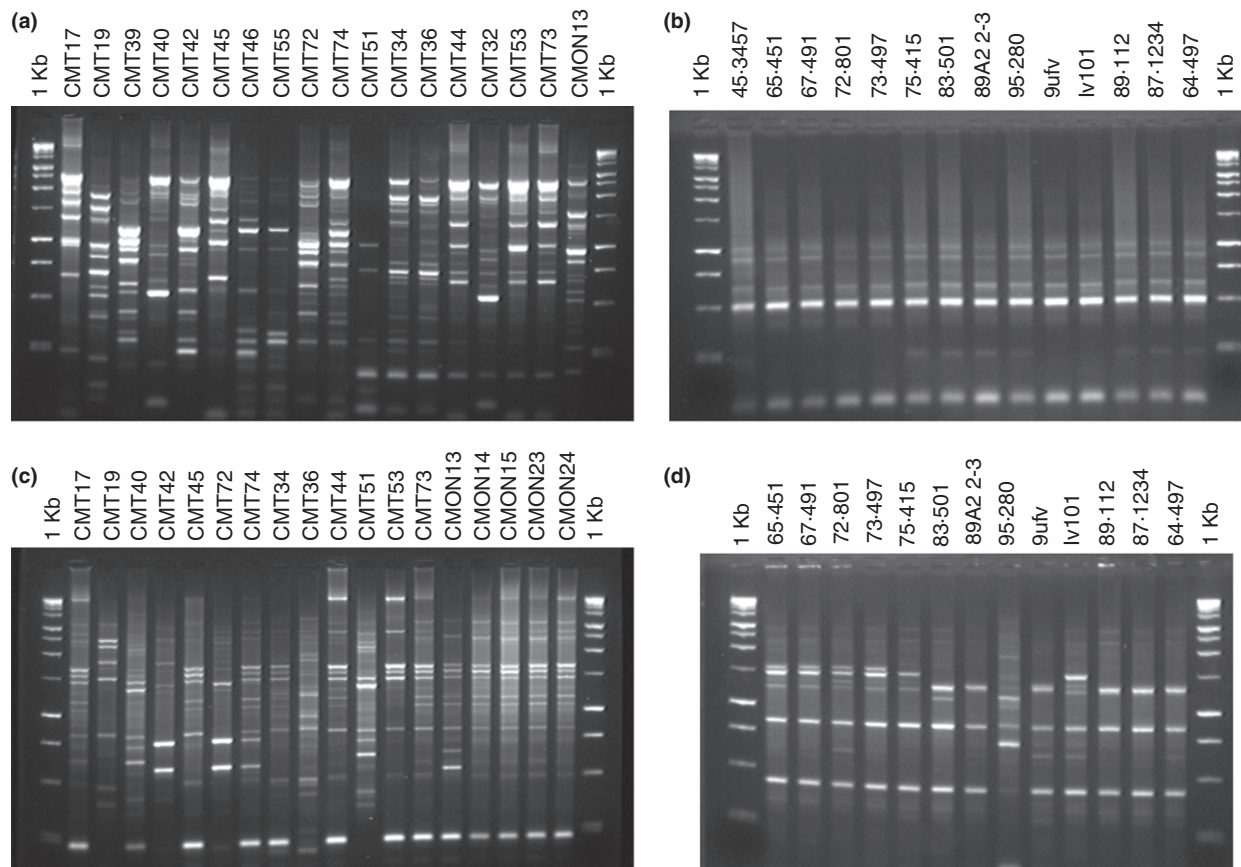
Figure 3 Electrophoretic profile of the DNA of *Colletotrichum* generated by IRAP and REMAP. a—Result of the combination of CLIRAP2 and CLI-RAP4 oligonucleotides of 18 endophytic isolates of the genus *Colletotrichum*. b—Result of the combination of CLIRAP2 and CLIRAP4 oligonucleotides of 14 isolates of *Colletotrichum lindemuthianum*. c—Result of the combination of CLIRAP2 and MS1 of 18 endophytic isolates of the genus *Colletotrichum*. d—Result of the combination of CLIRAP2 and MS1 oligonucleotides of 13 isolates of *Colletotrichum* lindemuthianum. 1 Kb DNA ladder represents the molecular marker. Letters and numbers in each well correspond to the identification of isolates.

the Ouro Negro variety as shown by Dominance and Berger-Parker indices. However, Simpson index was greater for Ouro Negro variety. Based on evenness and equitability indices, the evenness was greater in the fungal community of Ouro Negro variety. According to Schulz and Boyle (2005), the differences between host plants and their endophytic colonizers might be due to virulence of the endophyte; prevailing microhabitats, environment conditions, stress, host senescence and host defence responses. In addition, Gamboa *et al.* (2002) pointed that culture conditions, surface sterilization protocols, leaf fragment size and type of growth medium also correlate with entophytic fungi isolation. The two varieties assessed in this study were growing in the same edaphoclimatic conditions. Therefore, this suggests distinct endophytic communities between the varieties.

Santos *et al.* (2012) successfully used molecular markers (IRAP and REMAP) based on the sequence of the retrotransposon *RetroCl1* (Retroelement *Colletotrichum*

lindemuthianum 1) to characterize the significant genetic diversity of *C. lindemuthianum* and showed that these markers can be used in other species from the genus *Colletotrichum*. Some studies have reported discrepancies between dendrograms when two different molecular marker techniques were used (Herzberg *et al.* 2002; Chadha and Gopalakrishna 2007). Our results are consistent with these studies, in that some of the isolates grouped differently based on the IRAP and REMAP techniques. However, analysis with both types of markers showed that the *C. lindemuthianum* isolates were grouped separately from the endophytic isolates. Consequently, the IRAP and REMAP markers used were able to discriminate between the endophytic isolates from the genus *Colletotrichum* obtained from the leaves of *P. vulgaris* and the various *C. lindemuthianum* isolates.

Our results show that the endophytic fungi from the genus *Colletotrichum* have a high genetic diversity. The *Colletotrichum* species generate and maintain genetic
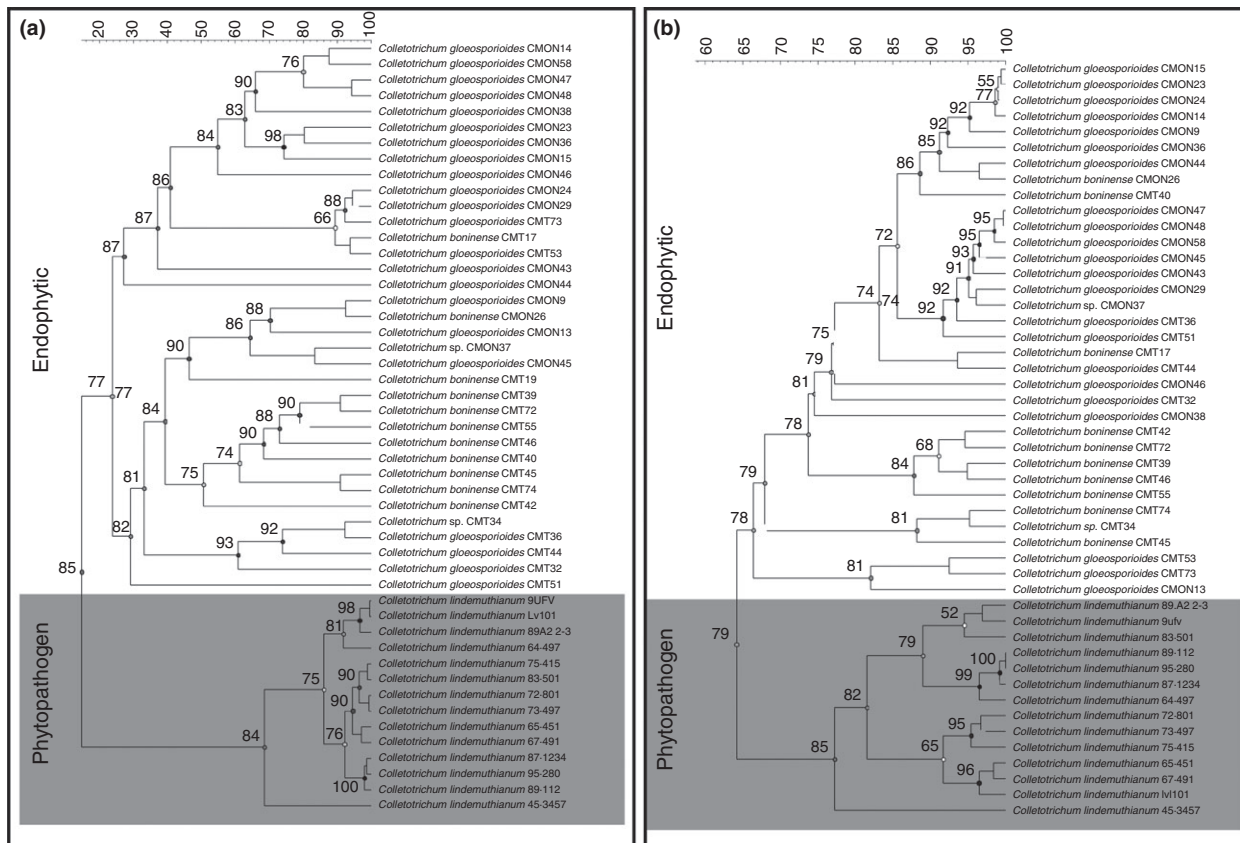
Figure 4 Dendrogram (UPGMA) based on IRAP markers (a) and REMAP markers (b). CMON = isolate from Ouro Negro Variety; CMT = isolate from Talismã Variety. The *Colletotrichum lindemuthianum* isolates are identified by its races followed by the identification number of mycological collection (exception by isolates LV101 and 9UFV that races are not defined).

variability by several means, including the sexual cycle (Mahuku and Riascos 2004), the parasexual cycle (Rodríguez-Guerra *et al.* 2003) and the exchange of genetic material by conidial anastomosis (Roca *et al.* 2004; Ishikawa *et al.* 2012). Due to the high genetic variability found both in the *Colletotrichum* endophytic fungi and in the phytopathogenic *C. lindemuthianum*, we speculate that the exchange of genetic material between endophytes and phytopathogens is possible. The mechanisms by which *C. lindemuthianum* generates and maintains high genetic variability have not yet been fully elucidated, and conidial anastomosis can occur between different species from the genus *Colletotrichum* (Roca *et al.* 2004).

The UPGMA analysis did not reveal any specific clustering based on the cultivar studied, as subgroups were formed that contained isolates from both cultivars. In addition, some isolates identified as *Colletotrichum boninense* clustered with isolates identified as *C. gloeosporioides*. The isolates were identified based on sequence identity in the ITS and LSU regions and phylogenetic analysis of these regions; however, the LSU sequences of *Colletotri-*

chum type strains are not currently available in public databases. Another obstacle to identifying fungi from the genus *Colletotrichum* by molecular methods is the possibility of incorrect annotation of sequences deposited in GenBank (Hyde *et al.* 2009). It is interesting to note that the use of the IRAP and REMAP markers revealed a genetic diversity that was not evident based on analysis of the ITS and LSU sequences. These molecular markers demonstrated the existence of genetic variability within a species, as isolates identified as being part of the same species formed several different clusters.

Thus, the IRAP and REMAP molecular markers can be used to rapidly distinguish between *C. lindemuthianum* and other *Colletotrichum* members that are frequently found as endophytes of the common bean. These markers can be used to analyse the genetic diversity of these fungi, facilitating a better understanding of the variability within this genus. To our knowledge, this is the first study to isolate endophytic fungi from leaf tissue from the common bean, thus contributing to a greater understanding of the community of endophytic fungi that colonize this important legume.

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Conflict of Interest

The authors declare no conflict of interests.

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Supporting Information

Additional Supporting Information maybe found in the online version of this article:

Table S1 Identity of the ITS and LSU sequences of the fungi isolates from *Phaseolus vulgaris* with the sequences deposited in the GenBank database.