Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

High genetic variability in endophytic fungi from the genus *Diaporthe* isolated from common bean (*Phaseolus vulgaris* L.) in Brazil

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Keywords
Diaporthe, DNA fingerprinting, endophytic fungus, genetic variability, IRAP, MLST, REMAP.

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2015/0775: received 20 April 2015, revised 26 August 2015 and accepted 7 September 2015

doi:10.1111/jam.12985

Abstract

Aims: The goals of the present study were to identify, to analyse the phylogenetic relations and to evaluate the genetic variability in *Diaporthe* endophytic isolates from common bean.
Methods and Results: *Diaporthe* sp., *D. infecunda* and *D. phaseolorum* strains were identified using multilocus phylogeny (rDNA ITS region; EF1-α, β-tubulin, and calmodulin genes). IRAP (Inter-Retrotransposon Amplified Polymorphism) and REMAP (Retrotransposon-Microsatellite Amplified Polymorphism) molecular markers reveal the existence of high genetic variability, especially among *D. infecunda* isolates.
Conclusions: It was concluded that the multilocus phylogenetic approach was more effective than individual analysis of ITS sequences, in identifying the isolates to species level, and that IRAP and REMAP markers can be used for studying the genetic variability in the genus *Diaporthe* particularly at the intraspecific level.
Significance and Impact of the Study: The combined use of molecular tools such as multilocus phylogenetic approach and molecular markers, as performed in this study, is the best way to distinguish endophytic strains of *Diaporthe* isolated from common bean (*Phaseolus vulgaris* L.).

Introduction

The genus *Diaporthe* (anamorph: *Phomopsis*) is distributed worldwide and has a great variety of hosts. Species of this genus may be phytopathogens, saprophytes or endophytic symbionts (Uecker 1988; Rossman *et al.* 2007; Udayanga *et al.* 2011; Gomes *et al.* 2013), i.e. living inside plant tissues for at least a part of their life cycle without causing any apparent damage to their plant host (Petrini 1991; Cabral *et al.* 1993).

Endophytic fungi from the genus *Diaporthe* have been isolated from a wide variety of hosts, from both temperate and tropical climates (Pereira *et al.* 1999; Gamboa-Gaitán *et al.* 2005; Botella and Diez 2011; González and Tello 2011; Rocha *et al.* 2011), including from agricultural crops with high economic value, such as cocoa (Rubini *et al.* 2005), coffee (Vega *et al.* 2010), soybean (Leite *et al.* 2013) and common bean (Gonzaga *et al.* 2014).

Species identification in the genus *Diaporthe* has classically been based on morphology, i.e. observation of the culture characteristics and of the vegetative and reproductive structures and, foremost, by affiliation with the host plant (Rehner and Uecker 1994). Through evidence that *Phomopsis* species may have more than one host (Wehmeyer 1933; van Niekerk *et al.* 2005), the taxonomy of this fungal genus was changed, and several studies, including those based on the rDNA ITS region, partial sequences of genes including translation elongation factor 1-α, and mating-type genes, were developed to improve the accuracy of species identification (Zhang *et al.* 1997,

1999; van Niekerk *et al.* 2005; Santos *et al.* 2010, 2011). The identification of isolates to species level in this genus is currently considered possible only with the use of molecular techniques (Santos and Phillips 2009; Santos *et al.* 2010; Udayanga *et al.* 2012).

The genealogical concordance phylogenetic species recognition method (*GCPSR)* provides higher species resolution than species identification based on morphology or reproductive compatibility criteria (Taylor *et al.* 2000; Dettman *et al.* 2003; Cai *et al.* 2011; Udayanga *et al.* 2012). Using multilocus sequence analysis, in which the same sequences of two or more genes are analysed concomitantly, the resulting genealogy concordance can provide a better resolution at the species level. This analysis is useful when the identification based on classical morphotaxonomic methods is not possible due to the absence of morphological characteristics (Rokas *et al.* 2003; Lumbsch *et al.* 2005; van der Merwe *et al.* 2010; Cai *et al.* 2011; Udayanga *et al.* 2012).

Several molecular strategies have been reported for the evaluation of genetic variability within and between species of fungi, and each approach has advantages and disadvantages. The types of markers used in these strategies include RFLP (Restriction Fragment Length Polymorphism) (Hulbert *et al.* 1988; Flood *et al.* 1992), RAPD (Random Amplified Polymorphic DNA) (Arnau *et al.* 1994; Talamini *et al.* 2006), AFLP (Amplified Fragment Length Polymorphism) (Majer *et al.* 1996; Ansari *et al.* 2004) and markers based on short repetitive intergene sequences dispersed throughout the genome (Repetitive Extragenic Palindromic – REP; Enterobacterial Repetitive Intergenic Consensus – ERIC and BOX elements) (Edel *et al.* 1995; Arora *et al.* 1996; Ash *et al.* 2010).

Among the most recently described molecular markers for assessing genetic variability in fungi are those which make use of transposable element sequences: IRAP (Inter-Retrotransposon Amplified Polymorphism) (Kalendar *et al.* 1999), which detects polymorphisms through the amplification of regions between two neighbouring retrotransposons, and REMAP (Retrotransposon-Microsatellite Amplified Polymorphism) (Kalendar *et al.* 1999), which detects polymorphisms by amplifying regions between retrotransposons and microsatellites. Polymorphisms generated by the presence or absence of retrotransposons at a given locus can be used as fingerprinting markers (Grzebelus 2006). IRAP and REMAP markers have been used for the evaluation of genetic variability in fungi (Branco *et al.* 2007; Zein *et al.* 2010; Santana *et al.* 2012, 2013; Santos *et al.* 2012), and they can also be used for gene mapping (Manninen *et al.* 2000) and for the identification of isolates (Zein *et al.* 2010).

Santos *et al.* (2012) developed and applied IRAP and REMAP markers for the evaluation of the intra- and interspecific genetic diversity of species from the genus *Colletotrichum*. In the following year, Santana *et al.* (2013) used the same markers in 27 fungal species from 11 different orders, showing that the IRAP and REMAP markers used could also be applied to analyse the genetic variability in other fungal genera, even without a sequenced genome, as is the case for the genus *Diaporthe*, subject of this study.

Diaporthe is one of the most abundant genera among the endophytic fungi isolated from Common Bean (*Phaseolus vulgaris* L.), corresponding to 11·5% of the total isolates recorded by Gonzaga *et al.* (2014). This fungal genus warrants further study for several reasons: (i) this genus does not possess any morphological markers that would allow characterization at the species level through classical taxonomy; (ii) species of this genus have been commonly found to be phytopathogens or endophytes on a wide variety of plant species (van Niekerk *et al.* 2005; Udayanga *et al.* 2012; Gomes *et al.* 2013); and (iii) fungi from the genus *Phomopsis* have been reported to produce various compounds of biotechnological interest (Horn *et al.* 1995; Wagenaar and Clardy 2001; Dai *et al.* 2005; Silva *et al.* 2005; Pornpakakul *et al.* 2007; Rukachaisirikul *et al.* 2008). Studies aiming to analyse and better characterize the genetic variability in this fungal genus are therefore of interest.

The goals of this study were to identify and to analyse the selected isolates by phylogenetic analysis using a multilocus approach; further, to evaluate the genetic variability in *Diaporthe* endophytic isolates from common bean using molecular markers based on transposable elements (IRAP and REMAP).

Materials and methods

Micro-organisms

Nine endophytic isolates of the genus *Diaporthe,* obtained from healthy leaves from Common Bean (Ouro Negro and Talismã varieties) by Gonzaga *et al.* (2014), were used in this study. Isolate CMON 20 originated from cultivar Ouro Negro, and CMT 20, CMT 38, CMT 41, CMT 43, CMT 50, CMT 60, CMT 70 and CMT 71 originated from cultivar Talismã.

Evaluation of colony morphology and mycelial growth from fungi from genus *Diaporthe*

Colony morphology of the nine *Diaporthe* isolates was evaluated following 7 days of growth on three different culture media: (i) PDA (Potato Dextrose Agar), prepared according to the manufacturer's instructions (HiMedia®, Maharashtra, India); (ii) SDA (Sabourad Dextrose Agar),

prepared according to the manufacturer's instructions (HiMedia®); and (iii) YMC (composition: 10·0 g of yeast extract (HiMedia®), 2·0 g of malt extract (HiMedia®), 15·0 g of agar (HiMedia®) and distilled water, in a 1 l total volume)). Three replicates were established for each culture medium. The plates were incubated at 22 ± 2°C with a photoperiod of 12 h.

Colony diameter, measured (mm) only in the PDA culture medium, was carried out in four different directions, using a pachymeter and the average value of the three replicates was calculated. Analysis of variance (ANOVA) was performed, followed by Tukey's test, at $P < 0{\cdot}05$, using R 3.0.0 software.

DNA extraction, amplification and sequencing

The nine tested isolates of the genus *Diaporthe* were grown in PDA culture medium. The plates were incubated at 22 ± 2°C with a photoperiod of 12 h. A maximum of 200 mg of mycelium was collected following 5–7 days of growth and used for DNA extraction using an Ultra Clean™ Microbial DNA Isolation Kit from MoBio Laboratories (Carlsbad, CA), according to the manufacturer's recommendations.

The oligonucleotide primers used for the amplification of the different genes or loci of the endophytic fungi from the genus *Diaporthe*, along with the PCR protocols, are presented in Table 1. After amplification, the PCR products were electrophoresed on a 1·2% (w/v) agarose gel (Sigma-Aldrich®, St. Louis, MO) stained with ethidium bromide (0·2 μg ml^{-1}) (Sigma-Aldrich®) in 1× TBE buffer (2 mmol l^{-1} EDTA, 0·1 mol l^{-1} Tris-HCl, and 0·1 mol l^{-1} boric acid (pH 8·0)) (Sambrook and Russell 2001) and visualized with the Eagle Eye imaging system (Stratagene, La Jolla, CA). The 1 Kb DNA Ladder (Promega, Madison, WI) was used as a molecular weight marker.

The PCR products of approx. 300–650 bp were purified using a Kit Wizard® SV Gel and PCR Clean-Up System (Promega) and sequenced according to the *dideoxy chain-termination method* (Sanger *et al.* 1977) using a BIGDYE® TERMINATOR ver. 3.1 Cycle Sequencing kit. (Applied Biosystems, Foster City, CA) Sequencing was performed at Macrogen, located in South Korea (http://dna.macrogen.com).

The sequencing products of both DNA strands were contiguously grouped, aligned and automatically corrected, using the DNA BASER SEQUENCE ASSEMBLER ver. 3.x 2012 software (Heracle BioSoft SRL Romania, http://www.DnaBaser.com). A comparative identity search of the nucleotide sequences obtained from the endophytic isolates tested was performed in GenBank Database using a local alignment algorithm for nucleotide sequences (BLASTN) (Altschul *et al.* 1990).

Phylogenetic analysis

A partition homogeneity test (PHT) was applied, as implemented in PAUP 4b10 (Swofford *et al.* 2001), to evaluate the possibility of concatenation of the data set: ITS rDNA region; EF 1-α, TUB and CAL genes. A total of five data matrices were analysed, and only the data matrix for the ITS, and the matrix for the four concatenated genes/loci were compared.

The individual phylogenetic analysis of the rDNA ITS region and the concatenated analysis of rDNA ITS region and EF 1-α, TUB and CAL partial genes, i.e. the multilocus analysis, were performed through Bayesian Inference using MRBAYES 3.2.1 software (Huelsenbeck and Ronquist 2001), after selection of the best evolutionary model for

Table 1 Genes/loci used in the multilocus analysis of the genus *Diaporthe* fungi with their respective oligonucleotide primers and PCR amplification conditions

Gene/loci	PCR primers	Sequence (5′ → 3′)	Amplification conditions	References for primers used
ITS	ITS1F	CTTGGTCATTTAGAGGAAGTAA	95°C: 2 min; (95°C 1 min, 51°C, 1 min, 72°C, 1 min) × 36 cycles; 72°C, 7 min, and final hold at 4°C	Gardes and Bruns (1993)
	ITS4	TCCTCCGCTTATTGATATGC		White *et al.* (1990)
EF 1-α	EF1-728F	CATCGAGAAGTTCGAGAAGG	95°C: 2 min; (95°C 1 min, 55°C, 1 min, 72°C, 1 min) × 36 cycles; 72°C, 7 min, and final hold at 4°C	Carbone and Kohn (1999)
	EF1-986R	TACTTGAAGGAACCCTTACC		Carbone and Kohn (1999)
TUB	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	95°C: 2 min; (95°C 1 min, 65°C, 1 min, 72°C, 1 min) × 36 cycles; 72°C, 7 min, and final hold at 4°C	Glass and Donaldson (1995)
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC		Glass and Donaldson (1995)
CAL	CAL228F	GAGTCCAAGGAGGCCTTCTCCC	95°C: 2 min; (95°C 1 min, 55°C, 1 min, 72°C, 1 min) × 36 cycles; 72°C, 7 min, and final hold at 4°C	Carbone and Kohn (1999)
	CAL737R	CATCTTTCTGGCCATCATGG		Carbone and Kohn (1999)

ITS, rDNA internal transcribed spacer region; EF 1-α, partial sequence of the gene coding for translation elongation factor 1-α; TUB, partial sequence of the gene coding for β-tubulin and CAL, partial sequence of the gene coding for calmodulin.

each partition (gene or locus) using the MRMODELTEST ver. 2.3 software (Nylander 2004). Two independent Markov Chain Monte Carlo (MCMC) runs with 10 million generations each were sampled every 1000 generations. The first 1 million generations were discarded in each run as burn-in, and the remaining sampled trees were summarized to generate a majority-rule consensus tree.

Tree branches with bootstrap values and/or *a posteriori* probability lower than 80% were omitted because that threshold indicates data with low reliability and low statistical support (Harada *et al.* 1995).

The DNA sequences of the ITS region; EF 1-α, TUB and CAL genes from the nine *Diaporthe* isolates were deposited into GenBank (http://www.ncbi.nlm.nih.gov/) (Table 2). Additional DNA sequences from ex-type cultures were obtained from GenBank (Table 2). The ex-type DNA sequences were identified and chosen from recent articles (Udayanga *et al.* 2012 and Gomes *et al.* 2013) regarding identification and/or delimitation of new species from the genus *Phomopsis*/*Diaporthe*. Representative fungal isolates were selected for which sequences, in all four loci used in the presented study, could be found in GenBank: ITS region; EF-1 α, TUB and CAL genes were present. All of the aforementioned sequences were included in the phylogenetic analysis. *Phomopsis vaccinii* (CBS 160.32) and *Diaporthe vaccinii* (CBS 122114) sequences obtained from GenBank were used as outgroup.

For the Bayesian Inference, the alignment of concatenated and manually adjusted sequences of genes/loci (ITS, EF 1-α, TUB and CAL) included 22 DNA sequences from fungal isolates, of which nine were from the isolates included in this study and 13 were from isolates obtained from GenBank. The number of characters used for the phylogenetic analysis was 1601 for the concatenated analysis and 473 for the individual analysis of the rDNA ITS region. The alignment files and phylogenetic trees were deposited into TREEBASE (www.treebase.org) and the accession number is 16711.

DNA fingerprinting

The combinations of oligonucleotide primers used in the IRAP and REMAP analysis are presented in Table 3. Amplification reactions were performed according to Santos *et al.* (2012) for the nine isolates tested: in a reaction volume of 25 μl containing 1× thermophilic DNA poly buffer (Promega), 2·0 mmol l^{-1} $MgCl_2$ (Promega), 100 μmol l^{-1} of each dNTP, 0·2 μmol l^{-1} of each oligonucleotide, 40 ng of DNA and one unit of Taq DNA polymerase (Promega). Negative controls were carried out (DNA was substituted by sterile distilled water). The amplification parameters were six cycles of 30 s at 94°C, 30 s at 50°C and 2 min at 72°C. Twenty-four extra cycles were added to these six initial cycles, and after every sixth cycle, 30 s was added to the extension time. The final extension step was 10 min at 72°C. The amplicons were separated by electrophoresis in a 1·5% agarose gel (w/v) stained with ethidium bromide (0·2 μg ml^{-1}), in TBE 1× buffer (2 mmol l^{-1} EDTA, 0·1 mol l^{-1} Tris-HCl and 0·1 mol l^{-1} boric acid (pH 8·0)) (Sambrook and Russell 2001). The 1 Kb DNA Ladder (Promega) was used as a molecular weight marker. Negative controls are not shown in the electrophoresis images, but they were performed.

The presence or absence (1 = presence; 0 = absence) of bands for each oligonucleotide primer combination used in the amplification was scored for the various isolates. The reproducibility of band profiles was tested by repeating the PCRs three times for all isolates and primers tested. Only reproducible bands were used for the analysis. The number of amplified loci and the percentage of polymorphisms were calculated using POPGENE 1.32 software (Yeh *et al.* 1999). A dendrogram (with 1000 bootstrap replicates) was built according to the unweighted pair group method (UPGMA) using *pvclust* from the R 3.0.0 software (R development Core Team 2007).

Results

The rDNA ITS region sequencing indicated that the nine isolates tested belonged to the same genus (*Diaporthe*) and that more than half, CMON 20, CMT 20, CMT 43, CMT 50 and CMT 60, belonged to the same species (*Diaporthe infecunda*) (Fig. 3).

Despite the similarities in the nucleotide sequences of the ITS rDNA region, the morphological characterization revealed differences in mycelial growth between the different isolates (Tukey test, at $P < 0{\cdot}05$) (Fig. 1), with some isolates growing faster than others, even under the same temperature, atmosphere and nutrient availability conditions. In addition, differences in colony morphology were also observed (Fig. 2).

The sequencing of partial genes coding for translation elongation factor 1-α (EF 1-α), β-tubulin (TUB) and calmodulin (CAL) of each of the nine isolates tested allowed for the study of their phylogenetic relationships using multilocus phylogenetic analysis. Figure 3 shows the phylogenetic relationship of the isolates included in this study with ex-type strains obtained from GenBank based only on the rDNA ITS region. Figure 4 shows the phylogenetic relationships based on the rDNA ITS region; EF 1-α, TUB and CAL genes/loci. In each case the majority-consensus tree resulting from Bayesian Inference was shown.

Table 2 Fungi from the genera *Phomopsis/Diaporthe* used in this study for phylogenetic analysis

Collection code	Identity	Host	Country of origin	Collector	GenBank accession numbers			
					ITS	EF 1-α	TUB	CAL
CMON 20	*Diaporthe infecunda*	*Phaseolus vulgaris*	Brazil	L Gonzaga	KP182388	KP182379	KP182397	KP182370
CMT 20	*Diaporthe infecunda*	*Phaseolus vulgaris*	Brazil	L Gonzaga	KP182389	KP182380	KP182398	KP182371
CMT 38	*Diaporthe phaseolorum*	*Phaseolus vulgaris*	Brazil	L Gonzaga	KP182390	KP182381	KP182399	KP182372
CMT 41	*Diaporthe* sp.	*Phaseolus vulgaris*	Brazil	L Gonzaga	KP182391	KP182382	KP182405	KP182373
CMT 43	*Diaporthe infecunda*	*Phaseolus vulgaris*	Brazil	L Gonzaga	KP182392	KP182387	KP182400	KP182374
CMT 50	*Diaporthe infecunda*	*Phaseolus vulgaris*	Brazil	L Gonzaga	KP182393	KP182383	KP182401	KP182375
CMT 60	*Diaporthe infecunda*	*Phaseolus vulgaris*	Brazil	L Gonzaga	KP182394	KP182384	KP182402	KP182376
CMT 70	*Diaporthe phaseolorum*	*Phaseolus vulgaris*	Brazil	L Gonzaga	KP182395	KP182385	KP182403	KP182377
CMT 71	*Diaporthe* sp.	*Phaseolus vulgaris*	Brazil	L Gonzaga	KP182396	KP182386	KP182404	KP182378
CBS 592.81	*Diaporthe helianthi*	*Helianthus annuus*	Serbia	M Muntanola Cvetkovic	AY705842	GQ250308	JX275465	JX197454
CBS 344.94	*Diaporthe helianthi*	*Helianthus annuus,* seed	n.d.	n.d.	KC343114	KC343840	KC344082	KC343356
CBS 435.87	*Diaporthe melonis*	*Glycine soja*	Indonesia	H Vermeulen	KC343141	KC343867	KC344109	KC343383
CBS 507.78	*Diaporthe melonis*	*Cucumis melo*	USA	L Berha	FJ889447	GQ250314	JX275423	JX197417
CBS 116020	*Diaporthe phaseolorum*	*Aster exilis*	USA: Mississippi	A Mengistu	KC343176	KC343902	KC344144	KC343418
CBS 116019	*Diaporthe phaseolorum*	*Caperonia palustris*	USA: Mississippi	A Mengistu	KC343175	KC343901	KC344143	KC343417
MFLUCC 10-0608	*Diaporthe phaseolorum*	*Hylocerus undatus*	Thailand	D Udayanga	JQ619875	JX275389	JX275424	JX197418
LGMF908	*Diaporthe infecunda,* sp. nov.	*Schinus terebinthifolius,* endophytic in leaf	Brazil	J Lima	KC343127	KC343853	KC344095	KC343369
LGMF912	*Diaporthe infecunda, sp. nov.*	*Schinus terebinthifolius,* endophytic in leaf	Brazil	J Lima	KC343128	KC343854	KC344096	KC343370
MFLUCC 10-0582	*Diaporthe sp.*	*Aeschynanthus radicans*	Thailand	SC Karunarathna	JQ619885	JX275399	JX275433	JX197426
MFLUCC 10-0570	*Diaporthe sp.*	Dead wood-unknown	Thailand	D Udayanga	JQ619877	JX275391	JX275428	JX197421
CBS 122114	*Diaporthe vaccinii*	*Vaccinium corymbosum*	USA: Michigan	D C Ramsdell	KC343225	KC343951	KC344193	KC343467
CBS 160.32	*Phomopsis vaccinii*	*Oxycoccus macrocarpus*	USA	HF Bain	AF317578	GQ250326	JX275436	n.d.

Abbreviations used in the isolate codes: CBS, CBS Fungal Biodiversity Centre, Utrecht, The Netherlands; LGMF, Culture Collection of Laboratory of Genetics of Microorganisms, Federal University of Paraná, Curitiba, Brazil; MFLUCC, Mae Fah Luang University Culture Collection; CMON, pure culture from cultivar Ouro Negro; CMT, pure culture from cultivar Talismã. Abbreviations used for the amplified genes: ITS, rDNA internal transcribed spacer region; EF 1-α, partial sequence of the gene coding for the translation elongation factor 1-α; TUB, partial sequence of the gene coding for β-tubulin; CAL, partial sequence of the gene coding for calmodulin.

Table 3 Oligonucleotide primers used in IRAP and REMAP analysis of endophytic fungi from the genus *Diaporthe*

Technique	Oligonucleotide	Sequence (5′ → 3′)	References
IRAP (I)	CLIRAP1	CGTACGGAACACGCTACAGA	Santos *et al.* (2012)
	CLIRAP4	CTTTTGACGAGGCCATGC	Santos *et al.* (2012)
IRAP (II)	CLIRAP2	AATAACGTCTCGGCCTTCAG	Santana *et al.* (2013)
	CLIRAP4	CTTTTGACGAGGCCATGC	Santos *et al.* (2012)
REMAP	CLIRAP4	CTTTTGACGAGGCCATGC	Santos *et al.* (2012)
	MS1	GGCGGCGGCGGCGGCGGCGGCT	Santana *et al.* (2012)

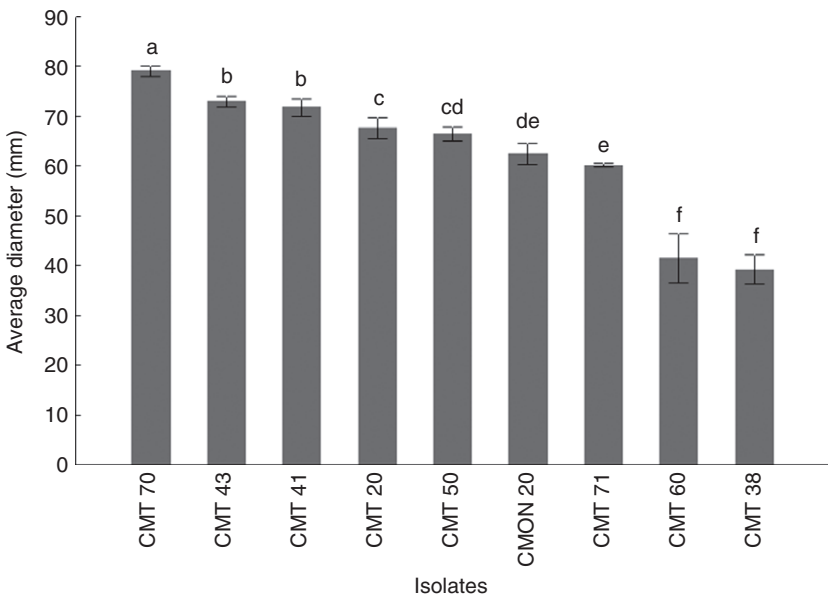

Figure 1 Mycelial growth of *Diaporthe* endophytic isolates, after 7 days of growth on PDA growth medium. Averages followed by the same letter are not statistically significantly different according to Tukey's test, at $P < 0{\cdot}05$.

The use of the rDNA ITS region alone was not able to identify the species from the genus *Diaporthe* (Fig. 3) when compared to the multilocus phylogenetic analysis (Fig. 4), which resulted in a more robust identification of the isolates tested, at both the species and genus levels, compared to isolates of ex-type strains from GenBank.

In general, the ancestor nodes of the rDNA ITS tree are not strongly supported by the *a posteriori* probability values. One example is the clade including isolates CMON 20, CMT 50, CMT 60, CMT 20, CMT 43, LGMF 908 and LGMF 912, which is common to both phylogenetic trees. In this clade, the multilocus tree is more robust than rDNA ITS tree, explained by the *a posteriori* probability values (multilocus tree, posteriori valour is: 1·00 and rDNA ITS tree, posteriori valour is: 0·82). Thus, the multilocus tree, enable us to classify the isolates as belonging to the species *D. infecunda*.

Isolates CMT 70 and CMT 38 were robustly grouped with *Diaporthe phaseolorum* isolates in the multilocus tree (CMT 70, *posteriori* valour is 1·00; CMT 38, *posteriori* valour is 1·00), whereas only CMT 70 was robustly grouped with *D. phaseolorum* in the rDNA ITS tree (bootstrap valour >97%). In addition, isolates CMT 41 and CMT 71 were dispersed in the rDNA ITS tree (bootstrap valour <80%), but were grouped with *Diaporthe helianthi/Diaporthe melonis* and *Diaporthe* sp. isolates, respectively, in the multilocus tree (CMT41, *posteriori* valour is 0·98/0·96; CMT 71, *posteriori* valour is 0·91).

For the genetic variability analysis performed using the IRAP and REMAP markers, the nine tested isolates presented amplicons with high degrees of reproducibility for all oligonucleotide combinations. In total, 74 loci were amplified and all loci were polymorphic. This means that there were no loci common to all isolates (100% polymorphic), but there are some common loci among isolates, indicating the genetic variability at the genus level. The oligonucleotide primer pair CLIRAP1 and CLIRAP4 amplified 30 loci, CLIRAP2 and CLIRAP4 amplified 26 loci, and CLIRAP4 and MS1 generated 18 loci (Fig. 5).

The formation of three main groups was observed in the grouping analysis (Fig. 6). The first group was composed of isolates CMT 41 and CMT 71, the second was

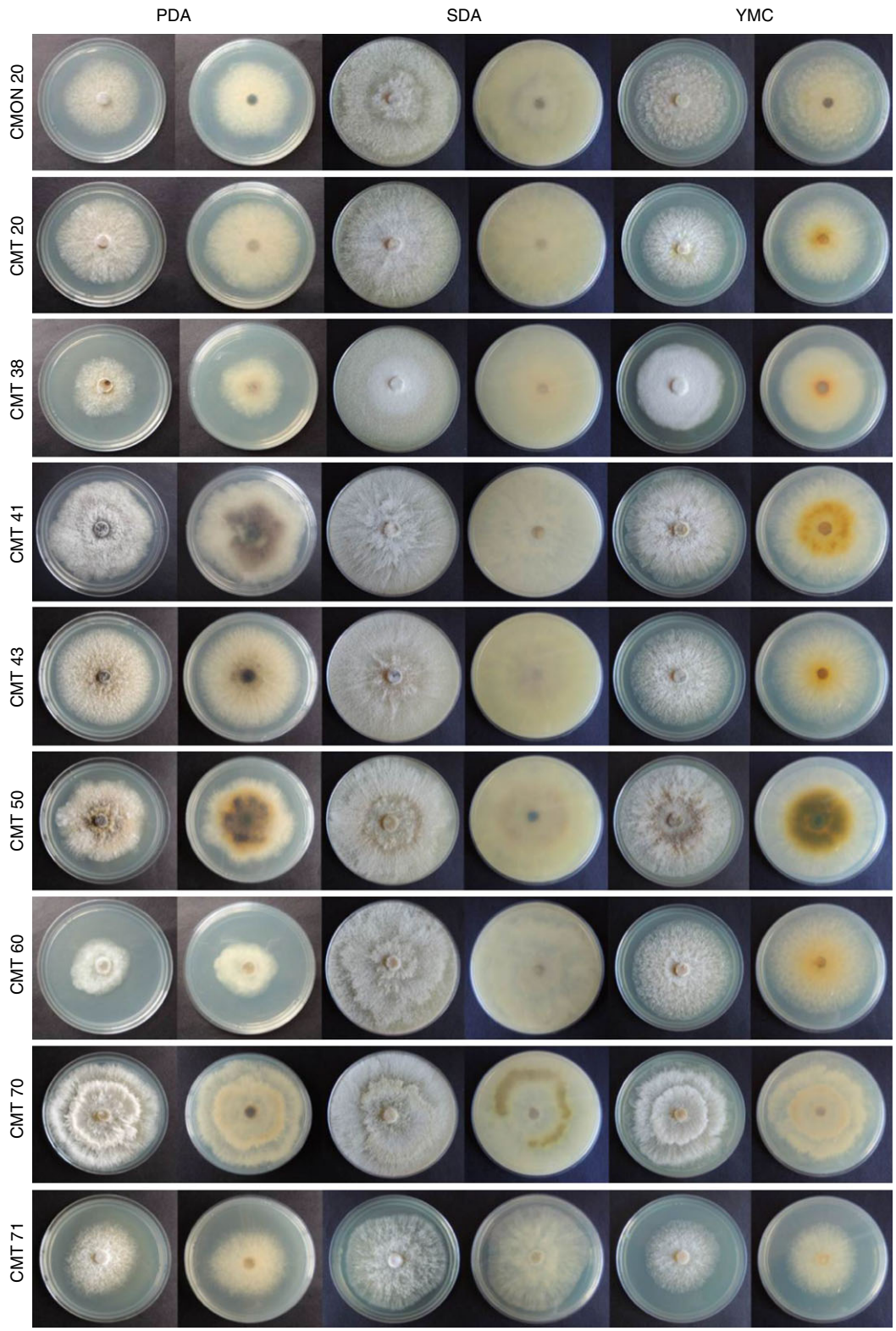

Figure 2 Morphology of colonies from endophytic isolates of the genus *Diaporthe* (CMON 20, CMT 20, CMT 38, CMT 41, CMT 43, CMT 50, CMT 60, CMT 70 and CMT 71) grown in three different culture media (PDA, Sabourad Dextrose Agar – SDA and YMC) after 7 days of growth at 25°C and under 12 h light/12 h dark photoperiod.

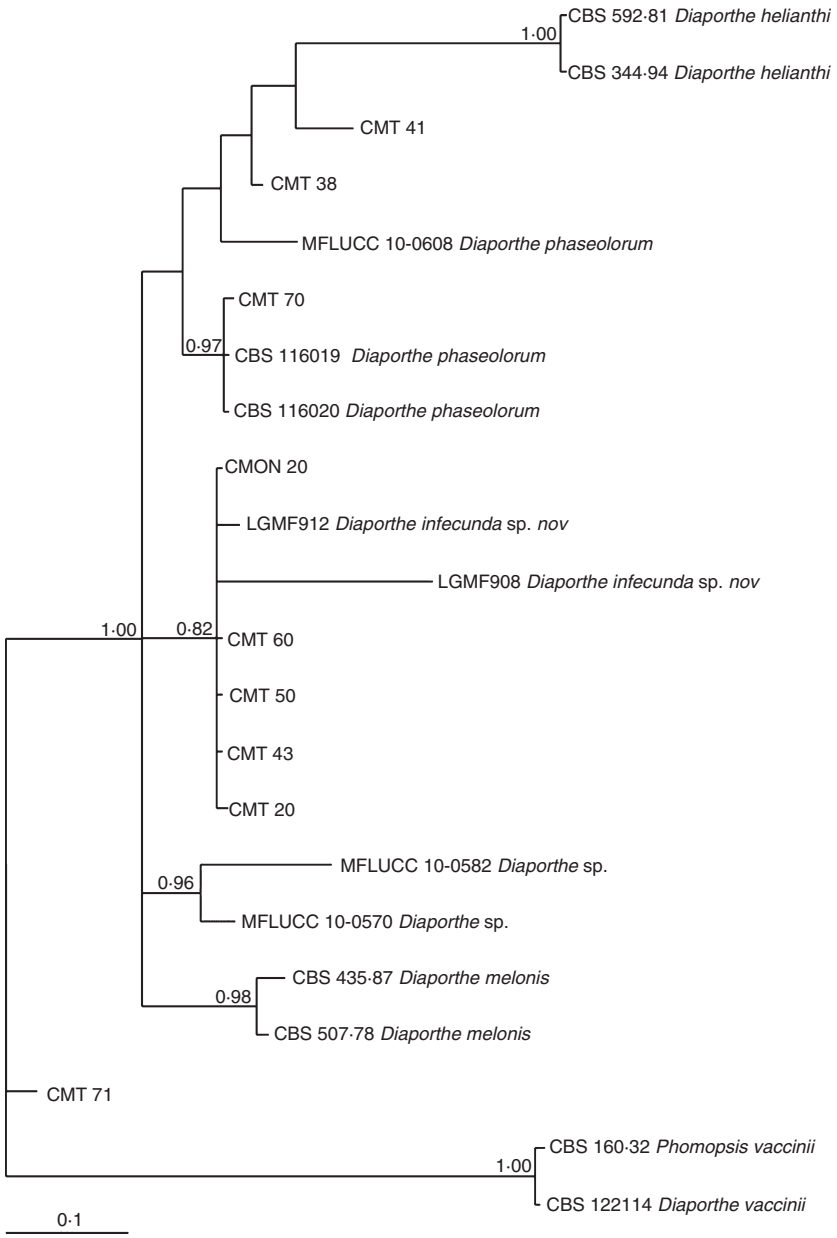

Figure 3 Phylogenetic tree obtained through Bayesian Inference (BI) using the rDNA ITS region nucleotide sequence for the nine isolates included in this study and for the 13 isolates from GenBank. Beside each ancestor node is shown, from left to right, the *posteriori* probability value. Probability values equal to or below 0·8 were omitted from the tree. The bar represents the number of changes in the nucleotide sequence of each 10 bp. TreeBase accession number: 16711.

composed of isolates CMON 20, CMT 20, CMT 43, CMT 50 and CMT 60, and the third was composed of isolates CMT 38 and CMT 70.

Interestingly, all isolates confirmed by the multilocus phylogeny analysis to belong to *D. infecunda* were clustered together in the second group. Isolate CMON 20, isolated from a different cultivar of common bean (Ouro Negro), was grouped together with isolates from cultivar Talismã. Although these five isolates belonged to the same species and were not grouped with isolates from other species in this analysis, it is still possible to observe the formation of two subgroups, one composed of isolates CMT 20 and CMT 43 and another composed of isolates CMT 50 and CMT 60.

Discussion

In this study, we investigated the genetic variability in *Diaporthe* endophytic fungi isolates from Common Bean. Our motivation for this study, primarily came from the realization that this genus was one of the most abundant in the endophytic fungal community of bean (Gonzaga *et al.* 2014). There was also interest in understanding the phylogenetic relationships of endophytic isolates from

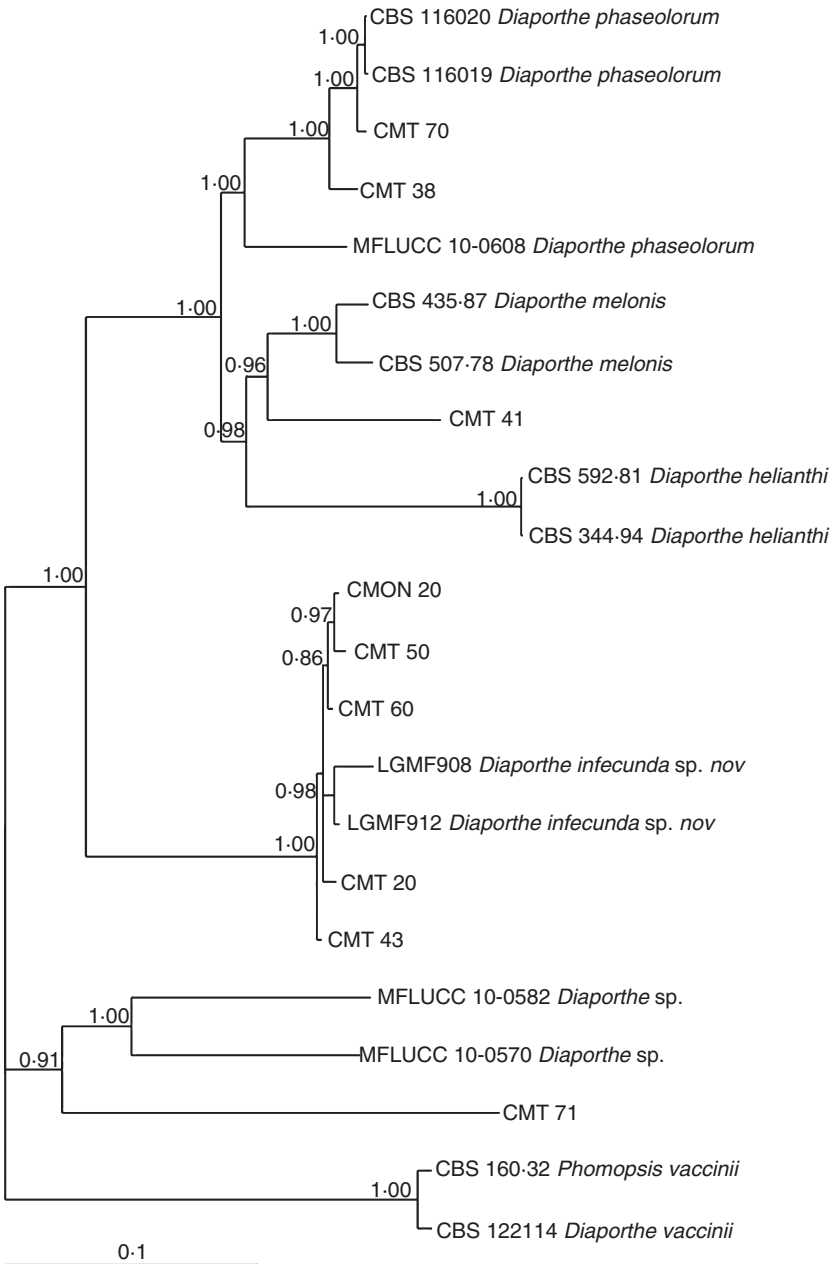

Figure 4 Multilocus phylogeny tree obtained through Bayesian Inference (BI) using the concatenated nucleotide sequence of the rDNA ITS region; EF 1-α, TUB and CAL genes, for the nine isolates included in this study and for the 13 isolates from GenBank. Beside each ancestor node is shown, from left to right, the *posteriori* probability value. Probability values equal to or below 0·8 were omitted from the tree. The bar represents the number of changes in the nucleotide sequence of each 10 bp. TreeBase accession number: 16711.

bean (*P. vulgaris*) because of the variety of interactions between species from this genus and their hosts.

Since the emergence of molecular tools, the sequencing of genes and/or intergene regions has been used to identify species or to add weight to taxonomical evaluations or re-evaluations of fungal species (Ash *et al.* 2010). Different genes and intergene regions have been described as barcode sequences for the identification of fungi, with an emphasis on the rDNA ITS region, which has been used extensively (Gardes and Bruns 1993; Schoch *et al.* 2012). Following the sequencing of this region, isolates for which pairwise comparisons (BLASTN) of nucleotide sequences present ITS region identity higher than 95% are considered to belong to a given genus and/or species (Arnold and Lutzoni 2007). The isolates tested in this study were initially identified based on sequencing of the rDNA ITS region because it was not possible to observe their conidia, from which they were considered *Mycelia sterilia* (Naik 2009).

Although the isolates studied presented significantly different mycelial growth patterns and colony morphologies, upon sequencing of the rDNA ITS region, they were

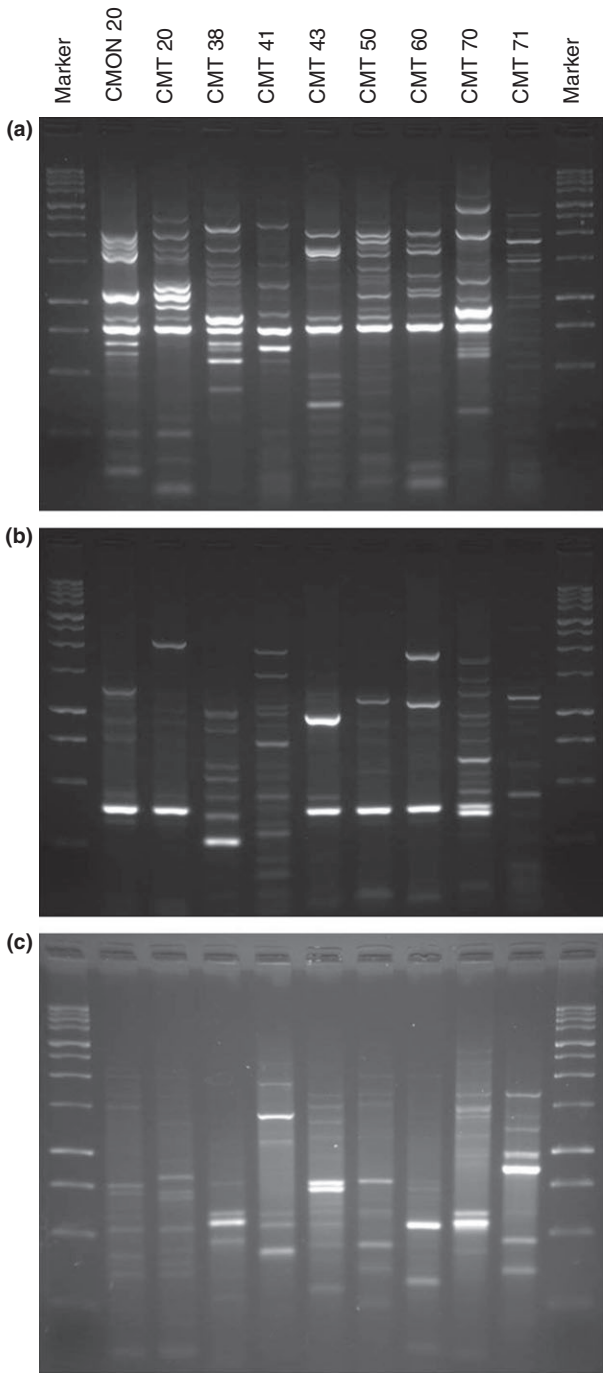

Figure 5 DNA amplification electrophoresis profile for the nine *Diaporthe* sp. isolates obtained through IRAP and REMAP. (a) Banding pattern generated by the combination of the CLIRAP1 and CLIRAP4 primers; (b) Banding pattern generated by the combination of the CLIRAP2 and CLIRAP4 primers; (c) Banding pattern generated by the combination of the CLIRAP4 and MS1 primers; 'Marker' represents the 1 kb DNA Ladder molecular marker; the isolates are represented by their codes in the mycological collection (from left to right: CMON 20, CMT 20, CMT 38, CMT 41, CMT 43, CMT 50, CMT 60, CMT 70, CMT 71); negative control is not shown.

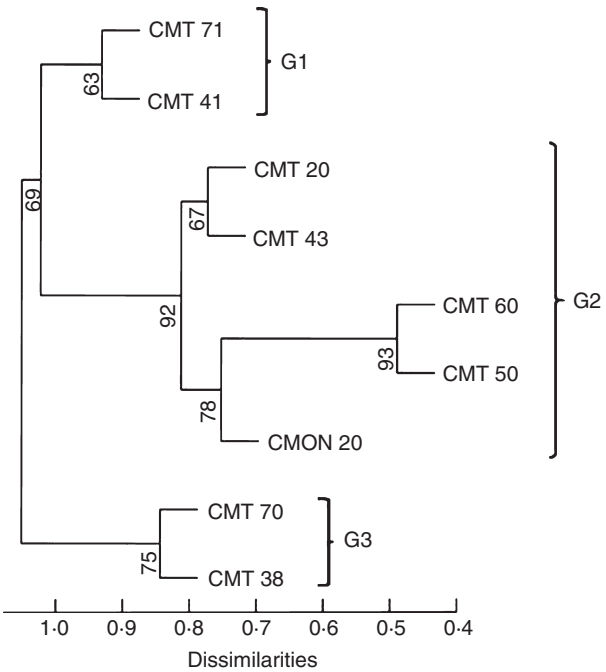

Figure 6 Genetic distance dendrogram for nine isolates of the genus *Diaporthe*. Genetic distance was based on loci obtained through IRAP and REMAP. Bootstrap values were obtained from 1000 replicates; only values higher than 63 are shown. The three main groups are represented for G1, G2 and G3. The dendrogram was built using UPGMA.

all identified as belonging to the genus *Diaporthe*, and five of them were identified as belonging to the same species, *D. infecunda*. In this way, there are differences in colony morphology, but these differences are not fully correlated with the molecular results.

The analysis using ITS sequences concatenated with partial sequences from the genes EF 1-α, TUB and CAL genes, i.e. the multilocus analysis (Fig. 4), was observed to be distinctly preferable for determining species from the genus *Diaporthe* than the phylogenetic analysis based only on rDNA ITS sequences (Fig. 3), similarly to the results found in the studies from Udayanga *et al.* (2012) and Gomes *et al.* (2013).This difference occurs because, in general, the ancestor nodes of the rDNA ITS phylogenetic tree (Fig. 3) are not strongly supported by the bootstrap and/or *a posteriori* probability values compared to multilocus phylogenetic tree (Fig. 4). In this way, multilocus analysis resulted in a more robust identification of the isolates tested, at species level.

The multilocus phylogeny analysis confirmed that the majority of the studied fungal isolates belonged to the species *D. infecunda*. This species was first described by Gomes *et al.* (2013) in a study that tried to clarify the taxonomy and phylogeny of a collection of *Diaporthe*, with isolates obtained from several hosts, including

pathogens, saprophytes and endophytes. *Diaporthe infecunda* was specifically isolated as an endophyte from leaves of *Schinus terebinthifolius*, and it presented genealogical concordance with five of our isolates (CMON 20, CMT 20, CMT 43, CMT 50 and CMT 60) as well as with leaf endophytic isolates.

Two of our isolates were identified as *D. phaseolorum*. This species has long been known as the causal agent of pod and stem blight and stem canker in soybean, and it can also cause seed rot (Hobbs and Phillips 1985; Pioli *et al.* 2003). *Diaporthe phaseolorum* was first reported to be an endophyte in *Kandelia candel* by Cheng *et al.* (2008).

Isolate CMT 41 identified as *Diaporthe* sp. did not present any clear phylogenetic relationships in the tree built only using rDNA ITS sequences but was related as *D. helianthi*/*D. melonis* by multilocus analysis. *Diaporthe helianthi* has been isolated as a common endophyte, but is mainly characterized as phytopathogen (Udayanga *et al.* 2012 and Gomes *et al.* 2013). However, to our knowledge, *D. melonis* has not yet been found, as endophyte, so far.

The IRAP and REMAP markers used in our study, originally developed to evaluate the genetic variability in fungi from genus *Colletotrichum* (Santos *et al.* 2012), were found to also be efficient for the analysis of genetic variability in the genus *Diaporthe*. Reproducible amplicons were generated for all studied isolates and for all tested oligonucleotide primer pairs. This result occurred because these markers anneal in LTR regions, which are highly conserved among the retrotransposons. In addition, retrotransposons present high dispersion, abundance and ubiquity in eukaryote genomes (Kalendar *et al.* 1999).

The results obtained with these markers for fungi from the genus *Diaporthe* showed that although they were isolated as endophytes at the same plant organ (leaf) from the same plant species (*P. vulgaris*) at the same time and geographical location (Atlantic Forest Area), they present high genetic variability. This genetic variability, revealed by IRAP and REMAP markers, cannot be accessed solely through the sequencing of the rDNA ITS region or other barcode sequence, such as the rDNA from the large ribosomal subunit (LSU), which is also used for the identification of fungal isolates (Botella and Diez 2011). This reinforces the importance of using molecular markers in studies of intraspecific fungal genetic variability.

The grouping analysis performed with data from the IRAP and REMAP markers in combination revealed that the isolates studied presented high genetic variability and formed several clusters. This result is consistent with the phylogenetic relationships indicated by the multilocus analysis. Three groups were identified using molecular markers, and two of these were formed by individuals that the multilocus analysis had confirmed as belonging to single species. This was the case of the second group, formed exclusively by *D. infecunda* isolates, and the third, formed by *D. phaseolorum* isolates.

Of the isolates included in our study, CMT 71 was the only strain which could not be identified beyond genus level, even using the multilocus analysis. CMT 41 and CMT 71, although they presented different genealogical concordances, formed a single group when analysed using IRAP and REMAP markers. However, the grouping of the CMT 41 and CMT 71 according to IRAP and REMAP is very little supported (bootstrap is equal to 63%) (Fig. 6). In the multilocus genealogical approach (Fig. 4), CMT 41 and CMT 71 were robustly grouped with *D. helianthi*/*D. melonis* and *Diaporthe* sp. respectively. The distribution pattern of molecular markers based on transposable elements does not always resemble the pattern found in phylogenetic analyses (multilocus phylogeny), which make use of molecular evolutionary clocks. Such evolutionary clocks are commonly used in phylogenetic studies, while DNA fingerprinting markers, like IRAP and REMAP are used for genetic variability purposes.

IRAP and REMAP, molecular markers based on transposable elements, were used for the first time in species of the genus *Diaporthe* to study the genetic variability. This type of molecular marker is suitable for detecting large changes in the genome (Kalendar and Schulman 2006) related to transposition events because they can detect polymorphisms generated by transposition events (Grzebelus 2006).

The activity of transposable elements can induce a variety of changes in the fungal genome, including mutations, breaks and chromosomal rearrangements, and transposition is one of the processes known to generate genetic variability in fungi (Ikeda *et al.* 2001; Daboussi and Capy 2003). For isolates composing the second group of the dendrogram, IRAP and REMAP revealed a genetic variability that cannot be accessed even using multilocus sequence analysis. The knowledge of the existence of this genetic variability, permitted by the use of molecular markers, such as the ones used in this study, is very interesting for the selection of isolates for biotechnology applications, among other uses. The use of molecular tools such as the retrotransposon-based markers used in this study is therefore suggested as an additional step for screening fungi isolates, before selection of only one isolate for biotechnological purpose, which saves time and costs.

Acknowledgements

We thank the Brazilian institutions CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e

Tecnológico) and FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais) for the financial support.

Conflict of Interest

The authors declare no conflict of interests.

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