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Determination of a Minimal DNA Sequence of the Internal Transcribed Spacer Region for the *in silico* Identification of *Botryosphaeria* sp.

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Abstract: *Botryosphaeria* species are plurivorous phytopathogenic fungi colonizing a wide range of host plants of agricultural, forestry, ecological and economic importance. Species identification in the *Botryosphaeria* genus is based mainly on morphological characteristics of the anamorphs in combination with sequence analysis from the Internal Transcribed Spacer (ITS) region. The purpose of this study was to determine a minimal DNA sequence of the ITS region which could be applied for the identification of *Botryosphaeria* species. A total of 23 entries from the ITS sequences of 13 *Botryosphaeria* species and several fragment lengths obtained from these entries, were compared with those already deposited in public databases using the BLASTn search tool. The secure identification of 11 of the 13 studied species was possible from the start of the ITS1 and the end of the ITS2 region using fragments up to 200 bp. These results were similar to those obtained with the complete ITS1/5.8S/ITS2 region. Phylogenetic trees based upon the neighbor-joining method showed similar topology when generated by complete ITS sequence and compared with those obtained with 200 bp sequences. The results in this study showed that for identification of the greater number of the *Botryosphaeria* species, a simple sequence read with 200 bp obtained from the beginning of the ITS1 region, or from the end of the ITS2 region should be sufficient. This procedure makes the identification process more rapid and easier and reduces the assembly efforts.

Key words: *Botryosphaeria*, species identification, internal transcribed spacer, fungi, *in silico*

INTRODUCTION

Fungi belonging to the genus *Botryosphaeria* Ces. and De Not (Pleosporales, Loculoascomycetes) are plurivorous phytopathogens colonizing a wide range of host plants of agricultural, forestry, ecological and economic importance causing diseases (cankers, gummosis, sudden wilting, root rot and branch and whole tree dieback), as well as rots in pre-harvested fruits. These fungi are distributed worldwide in temperate and tropical climate regions (Jacobs and Rehner, 1998; Alves *et al*., 2005).

A species of *Botryosphaeria* described as ligninolytic and characterized to the species level as *B. rhodina* has been recognized as having commercial applications for the bioremediation of

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polyaromatic hydrocarbons and xenobiotic compounds (Barbosa *et al*., 1996; Dekker *at al*., 2002). This species also produced an exopolysaccharide (botryosphaeran) when grown on basal medium (Barbosa *et al*., 2003) that has potential applications as an immuno-potentiator.

Several aspects complicate species identification in the *Botryosphaeria* genus. Morphological diversity among the teleomorphs is often insufficient to allow a clear identification of the species. Thus, species identification is based mainly on morphological characteristics of the anamorphs; the most common form of *Botryosphaeria* found in nature (Jacobs and Rehner, 1998; Denman *et al*., 2000). These characteristics include shape, pigmentation and size of pycnidia. In addition, colony morphology, conditions for pycnidia formation, chromogenicity and temperature effects on mycelial growth rate have also seen used for species recognition (Jacobs and Rehner, 1998).

The Polymerase Chain Reaction (PCR), have been used extensively in inter-, as well as intra-, specific comparisons of several fungi (Guarro *et al*., 1999; Crous *et al*., 2001; Rodrigues *et al*., 2004; Magnani *et al*., 2005). Ribosomal DNA (rDNA) sequences are universal and contain conserved and variable regions allowing discrimination of fungi of different taxonomic levels (Guarro *et al*., 1999). Non-coding regions, such as the Internal Transcribed Spacers (ITS), evolve more rapidly and are consequently more variable than coding regions. These regions are easy to access using universal PCR primers ITS1 and ITS4 (White *et al*., 1990).

Sequence analysis of the ITS region alone (Jacobs and Rehner, 1998; Denman *et al*., 2000; Zhou and Stanosz, 2001a; Alves *et al*., 2004), or in combination with sequences from others regions (Slippers *et al*., 2004a; Phillips *et al*., 2005), have made a significant contribution to resolving taxonomic problems in the genus *Botryosphaeria* and have been used to study relationships among species and to distinguish closely-related *Botryosphaeria* sp. (Smith *et al*., 2001; Denman *et al*., 2003; Alves *et al*., 2004).

No data has yet been reported for the least-length minimum size of the ITS sequence capable of identifying fungal species with confidence. The uncertainty in the minimum size often necessitates having to sequence the complete ITS region. This exercise is labor intensive and costly involving the utilization of bioinformatic tools for processing and contig assembly before performing comparisons with sequences deposited in public databases.

The objective of this study was to determine a minimal DNA sequence length of the ITS region for the identification of *Botryosphaeria* species by a method that is rapid and economical, making the technology already available more accessible.

MATERIALS AND METHODS

Download and Selection of ITS Sequences from Genbank Database

The NCBI (National Center for Biotechnology and Information) GenBank database via the Entrez nucleotide portal was accessed to search for entries under the terms *Botryosphaeria* and Internal Transcribed Spacer. The entries found were downloaded in FASTA format and the DNA sequences processed to remove the genes surrounding the ITS fragment (18S rDNA and 28S rDNA). Thus, the following analyses were done with the remaining fragments, which included only the sequences of the first Internal Transcribed Spacer (ITS1), the complete 5.8S rRNA gene and the second Internal Transcribed Spacer (ITS2).

Sequences selected were subdivided into separate files for each species belonging to the genus *Botryosphaeria* and the incomplete sequences ITS1-5.8S-ITS2 discarded. Sequence alignments were performed using BioEdit software (Hall, 1999) and manual adjustments were made where necessary. Alignments were converted into genetic distances by the Kimura two-parameter model and phylogenetic trees constructed using the Neighbor-Joining (NJ) method (Saitou and Nei, 1987) within the Phylogenic Inference Package (PHYLIP version 3.57; Felsenstein, 1993). Two sequences of each

Botryosphaeria species were selected for the analysis, with the exception of *B. corticis* and *B. eucalyptorum*. The selection was based upon the distribution of sequences at the tree examining, in particular, those sequences that were more divergent.

Sequence Processing

For each species the selected sequences were processed to obtain fragments of different sizes to allow the construction of databases with 50, 100, 150, 200, 250, 300, 350, 400 and 450 bp as well as the complete ITS region (~500 bp). This procedure was undertaken from both sequence extremities, forwards from the 5′ to 3′ end of the ITS1 region, increasing the size of the fragment up to 500 bp. The same procedure was taken in the reverse direction from the 3 to 5 end of the ITS2 region increasing by 50 bp fragments in direction of ITS1(Fig. 1).

Species Recognition

For each species, both selected sequences as well as the DNA fragments with different sizes were compared with those already deposited in the GenBank database using BLASTn search tool in default conditions (Altschul *et al*., 1990). Species Recognition was Based on Two Criteria (I) Comparison between score average obtained with a match of the same species (identification zone) with the best score obtained with the second species matched; (ii) Recognition of the greater number of GenBank deposited sequences of the query's same species inside the identification zone. Thus, species identification was considered positive when the average of the score at the same species was higher than the best score showed by the second species. Sequences deposited without identification were not considered.

Phylogenetic Relationships among the Species by ITS Fragments

Sequence alignments of the complete ITS1-5.8S-ITS2 region, as well as the fragments with different sizes were used for genetic distance analyses and grouping using PHYLIP software package, version 3.5c (Felsenstein, 1993) as described above. Tree robustness was estimated by 1000 bootstrap re-sampling using the program SEQBOOT (Felsenstein, 1985).

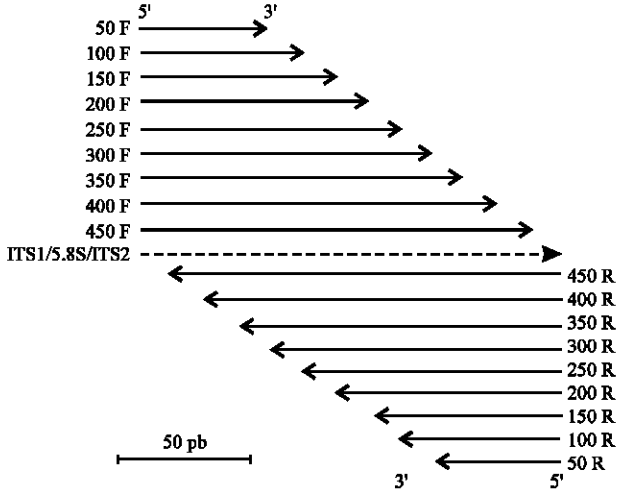

Fig. 1: Schematic representation of the model developed for the attainment of the ITS fragments used in *Botryoshaeria* species discrimination

RESULTS

From the GenBank database and following the criteria as described in the materials and methods 23 entries representing 13 *Botryosphaeria* species were selected (Table 1). Sequence alignments with these entries were performed using the BioEdit software. Length of the complete ITS1-5.8S rRNA gene-ITS2 sequences ranged from 454 bp (*B. rhodina*) (AY343483) to 504 bp (*B. stevensii*) (AY236955). The sequences were processed to obtain fragments of different sizes and compared with those in the GenBank database by BLASTn tool. The results obtained with the comparison of the different size fragments set from the 23 selected entries with those already deposited at the GenBank are shown in Table 1. The data revealed that the use of fragments from the 50 first nucleotides of the ITS1 region (50F) of *B. corticis*, *B. corticola*, *B. rhodina*, *B. stevensii* and *B. tsugae*, made possible the identification of these species, discriminating them from other *Botryosphaeria* sp.

An increase in the size of the fragments to 100 bp (100F) made an increment in the number of recognized species, allowing the identification of eight species (Table 1). An increment in the number of recognized species (11 species) could be observed with fragments of 150 and 200 bp (150F and 200F), but based on the score values, only fragments from 200 bp or more demonstrated enough sequence complexity for a meaningful identification.

The use of fragments corresponding to the last 50 nucleotides of the ITS2 region (50R) of *B. australis*, *B. corticis* and *B. corticola* allowed the identification of these species separating them from the other *Botryosphaeria* (Table 1). An increase in the fragment size to 100 bp (100R) made an increment in the number of recognized species, allowing the identification of eight species (Table 1). A total of 10 species could be identified with fragments of 150 bp (150R) and an increase of the size to 200 bp allowed the discrimination between *B. obtusa* and *Sphaeropsis sapinea*; other species belonging to the Botryosphaeriaceae family.

Species identification from the DNA fragments with different sizes was performed considering the data obtained from the best score, in spite of the sequence sense (starting from the beginning of

Table 1: Discrimination of *Botryosphaeria* species from different sized ITS region sequence fragments based on comparison with sequences deposited in the GenBank database using BLASTn search tool

	Sense and size of the fragments																		
Species/ GenBankEntries	50F	50R	100F	100R	150F	150R	200F	200R	250F	250R	300F	300R	350F	350R	400F	400R	450F	450R	Complete sequence
B. corticis/AF243397	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. corticola/AY268420	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. corticola/AY259110	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. tsugae/AF243405	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. rhodina/AY343483	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. rhodina/AY612337	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. stevensii/AY236955	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. stevensii/AF243406	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. obtusa/AY343449	+	-	+	-	+	-a	+	+	+	+	+	+	+	+	+	+	+	+	+
B. obtusa/AY343446	-a	-	-a	-	+	-a	+	+	+	+	+	+	+	+	+	+	+	+	+
B. australis/AY343386	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. australis/AY343398	-	+	-b	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. dothidea/AF027746	-c	-c	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. dothidea/AY236948	-c	-c	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. eucalyptorum/ AY339256	-d	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. lutea/AY259091	-e	-	-e	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. lutea/AY339258	-e	-	-e	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. mamane/AF246930	-d	-d	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. mamane/AF246929	-d	-d	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. parva/AY236938	-	-	-f	-f	-f	-f	-f	-f	-f	-f	-f	-f	-f	-f	-f	-f	-f	-f	-f
B. parva/AY194470	-	-	-f	-f	-f	-f	-f	-f	-f	-f	-f	-f	-f	-f	-f	-f	-f	-f	-f
B. ribis/AF243394	-	-	-g	-g	-g	-g	-g	-g	-g	-g	-g	-g	-g	-g	-g	-g	-g	-g	-g
B. ribis/AF452521	-	-	-g	-g	-g	-g	-g	-g	-g	-g	-g	-g	-g	-g	-g	-g	-g	-g	-g

(+) Positive species discrimination; (-) Negative species discrimination, (a) Positive identification related to Botryosphaeriaceae family, with the exception of *Sphaeropsis sapinea*, (b-g) Positive identification related to the *Botryosphaeria* genus, with exception of, (b) *B. lutea*; ©*B. mamane*; (d) *B. dothidea*; (e) *B. australis*; (f) *B. ribis*; (g) *B. parva*

ITS1 or from the end of ITS2). Thus, the ability of identification of one fragment only was considered when the best scores were obtained with the same species studied and when the average of these score values were higher than that observed for other species, recognizing the greater number of GenBank deposited sequences.

Thus, in taking the data obtained with the use of fragments with 200 bp (200F and 200R) of both *B. rhodina* entries (AY343483 and AY612337), the best score obtained was 396 (Table 2). The average of the scores obtained with the *B. rhodina* entries (27 to 200F and 28 to 200R) was 381 and 375 for the 200F fragments and 391 for 200R. At the same time, those scores were higher than those observed with the second species (200 and 357 for 200F and 200R, respectively). Thus, we consider positively the capacity of both 200 bp (200F and 200R) ITS fragments to recognize and discriminate *B. rhodina* from the other species deposited at the public nucleotide database. Similar results can be observed with the other species with the exception of *B. parva* and *B. ribis*, that could be separated from the other species, but without separation between themselves.

Considering these results, both ITS fragments with 200 bp (200F and 200R) were capable of identifying *Botryosphaeria* species in comparison with the data deposited at the GenBank. The results with the 200 bp fragments were the same as those observed with the complete ITS region (Table 1).

The 23 entries were aligned and used to construct a phylogenetic tree based on neighbor-joining method considering the complete ITS region. The analyses of datasets show two groups with a strongly supported (bootstrap 1000) separation. The first group was divided into two subgroups, one (bootstrap 999) containing *B. parva* and *B. ribis* (which were indistinguishable); *B. australis*, *B. lutea* and *B. eucalyptorum* and another with *B. corticis*, *B. dothidea* and *B. mamane* (bootstrap 1000). The second group (bootstrap 985) was composed of *B. corticola*, *B. obtusa*, *B. stevensii*, *B. tsugae* and *B. rhodina* with all species distinguishable.

Neighbor-joining trees were also constructed with 200 bp fragments (200F and 200R) (Fig. 2a-c). The trees topologies were very similar to that obtained with the complete ITS region. The same

Table 2: Results (best score, average score and next species score) obtained with the comparison of 200 bp fragments (F and R) from 13 *Botryosphaeria* species from the GenBank database using BLASTn search tool

		Recognized entries								
	Total				Best score		Average score		Next species score	
	deposited	Complete								
Species/GenBank	entries	sequence	200F	200R	200F	200R	200F	200R	200F	200R
B. corticis/AF243397	01	01	01	01	358	385	358	385	317	367
B. corticola/AY268420	17	16	16	16	385	385	385	385	246	379
B. corticola/AY259110	17	17	17	16	385	385	379	385	241	379
B. tsugae/AF243405	01	01	01	01	385	385	385	385	246	379
B. rhodina/AY343483	29	28	27	28	396	396	375	391	230	357
B. rhodina/AY612337	29	29	27	28	396	396	381	391	230	357
B. stevensii/AY236955	14	08	06	03	385	385	354	377	296	367
B. stevensii/AF243406	14	08	05	05	385	385	378	383	317	373
B. obtusa/AY343449	68	61	36	61	358	385	358	384	341	373
B. obtusa/AY343446	68	61	25	61	358	385	358	384	341	373
B. australis/AY343386	40	40	38	39	385	385	385	384	379	373
B. australis/AY343398	40	40	38	39	385	385	385	384	379	373
B. dothidea/AF027746	70	30	26	26	354	385	338	379	300	362
B. dothidea/AY236948	70	30	27	27	385	385	374	385	341	367
B. eucalyptorum/AY339256	03	03	03	03	385	385	385	385	352	377
B. lutea/AY259091	16	16	08	11	389	385	389	383	377	373
B. lutea/AY339258	16	16	08	16	389	385	389	383	377	373
B. mamane/AF246930	02	02	02	02	354	385	354	385	298	331
B. mamane/AF246929	02	02	02	02	354	385	354	385	298	331
B. parva/AY236938	48	ND	ND	ND	396	389	396	389	396	389
B. parva/AY194470	48	ND	ND	ND	396	396	396	396	396	396
B. ribis/AF243394	19	ND	ND	ND	394	400	394	400	394	400
B. ribis/AF452521	19	ND	ND	ND	396	396	396	396	396	396

Last verification at GenBank in 07/27/05, ND: Not Determined

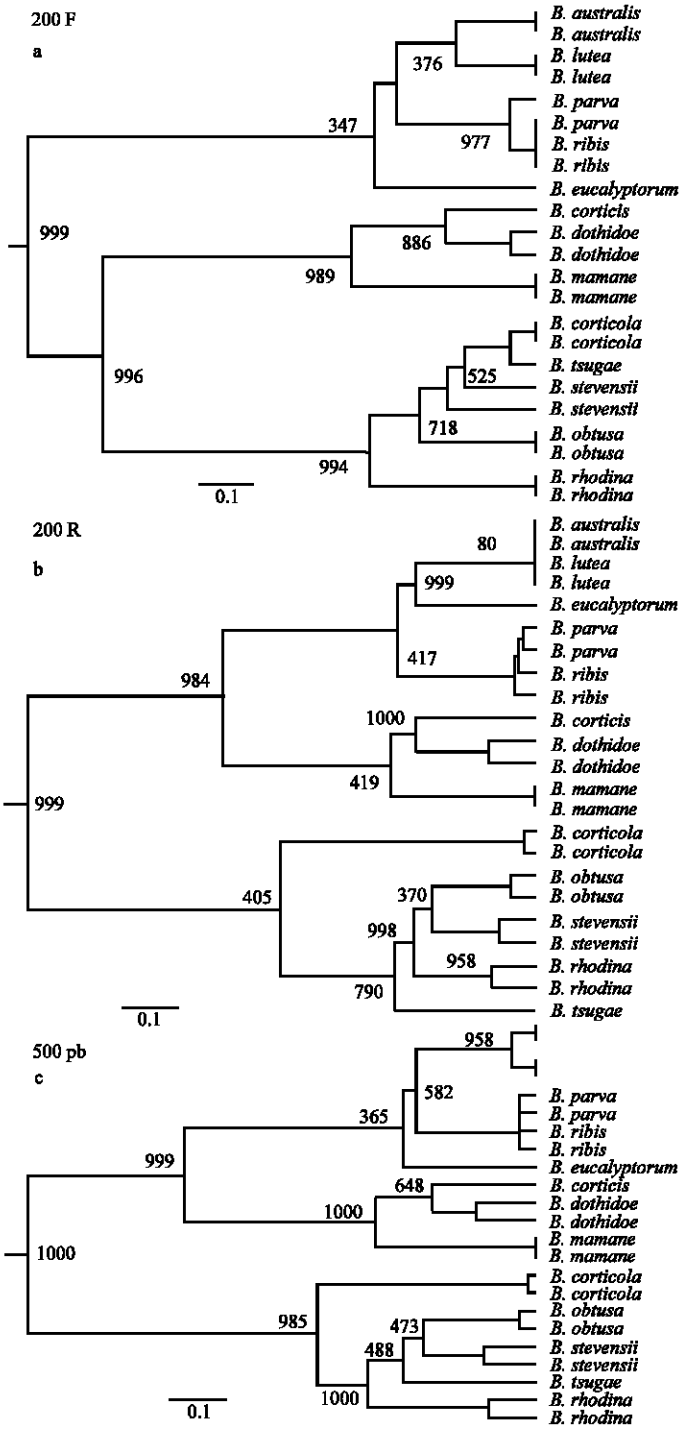

Fig. 2: Phylogenetic relationships among *Botryosphaeria* species based on neighbor-joining analyses of the complete ITS region DNA sequence data (a), 200 bp F fragments (b) and 200 bp R fragments (c). Bootstrap values (1000 replicates) are indicated at (below) the nodes. The bars represent the nucleotide changes

groups can be identified with some changes at the positioning of *B. obtusa*, *B. tsugae* and *B. stevensii* at the tree constructed with 200F and *B. eucalyptorum* and *B. tsugae* at the tree constructed with the 200R.

DISCUSSION

The correct identification of *Botryosphaeria* species is of practical importance in plant pathology, biotechnology and environmental studies. Formerly, the classification systems adopted were based on the phenotypic approach (Guarro *et al*., 1999) that, in *Botryosphaeria* species, were based mainly on morphological criteria. However, the phenotypic approach has been largely criticized for its lack of consistency, standardization, stable terminology and for its high subjectivity, still using laborious and time-consuming methods. In addition to the classical methods, the DNA approach has been extensively used in the identification of fungal species, including the *Botryosphaeria* genus (Smith *et al*., 2001; Slippers *et al*., 2004 a, b; van Niekerk *et al*., 2004).

The group of genes usually used in identification of *Botryosphaeria* species were those that codified rDNA, mainly the ITS region. This region has been used alone (Jacobs and Rehner, 1998; Denman *et al*., 2000; Zhou and Stanosz, 2001a; Alves *et al*., 2004), or in combination with other regions, such as β-tubulin and the EF1-α genes (Slippers *et al*., 2004a, b; van Niekerk *et al*., 2004; Phillips *et al*., 2005).

In this study, we tested the potential of different sized ITS rDNA sequences to recognize and discriminate the many species from the *Botryosphaeria* genus, aiming to determine the minimal DNA sequence of the ITS region useful for species identification.

Table 1 shows that ITS sequences sized with at least 200 bp (F and R) made possible the identification and the recognition of the majority of the sequences from *Botryosphaeria* already deposited in the database search. These results were similar when the complete ITS region was used with the same objective. The identification was supported by the BLASTn alignment score values between the fragments and the sequences already deposited at the GenBank. Thus, with the exception at *B. ribis* and *B. parva*, all the other fragments with at least 200 bp always showed preferential alignments with entries that corresponded to the same species of the query. These alignments showed that intra-specific score values were always higher than the inter-specific ones. The differences between score values show that the sequence complexity in this region was enough to discriminate the *Botryosphaeria* species (Table 2).

Based on the second criteria stated for each query, the greater number of GenBank available sequences corresponding to the same species were recognized. Fragments with 200 bp (F and R) were able to recognize almost the same number of entries than the complete sequence (Table 2). Entries were considered recognized when they were inside the identification zone. An exception was observed only for *B. stevensii* and *B. dothidea* sequences that recognize almost 50% of the total number of GenBank deposited sequences. However, the complete ITS region sequence recognizes the same number. The large number of partial ITS sequences deposited in the database can explain this phenomenon. Once the score was calculated not only by the quality of alignment, but also by the size of sequence aligned, partial sequences showed lower scores, which excluded them from the identification zone. This does not mean that those entries were not valid as the same species, but they did not satisfy the established criteria. Classification errors before sequence submission to the database should also be considered to explain these exceptions.

The robustness of the recognition capacity was tested by phylogenetic tree analyses. The phylogenetic relationships between the 13 species tested with the complete sequence of the ITS region were similar to those previously published with the same dataset (Slippers *et al*., 2004a;

van Niekerk *et al*., 2004). Almost the same tree topologies were observed when the 200 bp (F and R) fragment dataset was used to construct the trees. Minor changes were observed at the positioning of some species, but the major original groups were maintained.

Based on the ITS rDNA analyses, *B. parva* and *B. ribis* could not be distinguished (Table 1 and 2). This result was in agreement with that reported for the ITS region, EF1-α or the mt SSU rDNA sequences (Alves *et al*., 2004; van Niekerk *et al*., 2004; Slippers *et al*., 2004a; Zhou and Stanosz, 2001b). However, those species can be securely separated from other *Botryosphaeria* species using fragments from 100 bp (Table 1). Considering that *B. ribis* and *B. parva* are distinct species, but closely related and probably recently derived on an evolutionary scale, the discrimination of those only should be performed by the use of the multiple-gene genealogy approach, combining the sequence datasets of the ITS rDNA region, β-tubulin, EF1-α genes (Slippers *et al*., 2004a).

In almost all of the studied species, fragments with 200 bp contained the ITS1 (ranging from 134-185 bp) or the ITS2 (ranging from 154-172 bp) complete region. This observation leads to the conclusion that both ITS regions are sufficient for conclusive identification of *Botryosphaeria* species. In this way, we can recommend for sequencing the choice of one of the universal ITS1 or ITS4 primers (White *et al*., 1990), as these primers are anchored at the 18S rDNA and 28S rDNA genes, respectively, both flanking ITS1 and ITS2 regions.

The score differences observed between the identification zone and the second best match with the ITS1 region corresponding fragments (200F) were higher than those observed with the corresponding ITS2 region fragments (200R). Thus, we can recommend the preferential use of the ITS1 region for *Botryosphaeria* species identification due to its higher sequence complexity when compared with the ITS2 region.

The knowledge that for identification of *Botryosphaeria* species, a 200 bp simple sequence read is sufficient, which makes the identification faster and easier and greatly reduces costs of DNA sequencing and contig assembly programs. The major advantages of this procedure are its simplicity, the universal availability of the PCR primers, its reproducibility and its amenability to computer database analysis.

Despite all of the advantages cited above, this procedure should be treated with caution when phylogenetic closely-related species are being examined. These problems are often detected when the identification zone is not clearly demarcated showing different species with the same score. In these cases, other genomic regions, classical characters (such as morphology and biochemical), relative pathogenicity, distribution and ecology should be accessed.

Fungi identification and detection in environmental samples are stressed in the literature (González-Lamothe *et al*., 2002; Magnani *et al*., 2005). The results presented here suggest that the application of this procedure for the identification of other genera should be useful for identifying fungi species from different sources as the metagenomic approach. Thus, a knowledge of the minimal DNA sequence from the ITS region will make the *in-silico* identification routine an easier, simpler and dynamic procedure.

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