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Fingerprinting techniques as tools towards molecular quality control of *Pseudozyma flocculosa*

Sophie J. CARON[1], Tyler J. AVIS[1], Teun BOEKHOUT[2], Richard C. HAMELIN[3] and Richard R. BÉLANGER[1]*

[1] *Département de Phytologie, Faculté des Sciences de l'Agriculture et de l'Alimentation, Université Laval, Québec G1K 7P4, Canada.*
[2] *Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT Utrecht, P.O. Box 85167, 3508 AD Utrecht, The Netherlands.*
[3] *Laurentian Forestry Centre, Natural Resources Canada, 1055 du PEPS, P.O. Box 3800, Sainte-Foy, Quebec G1V 4C7, Canada.*
E-mail: *richard.belanger@plg.ulaval.ca*

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In an effort to meet the stringent requirements towards registration of microorganisms as biopesticides, several molecular techniques were tested as part of a strategy to develop a quality control system for *Pseudozyma flocculosa*, the active ingredient of Sporodex, a biofungicide used for the control of powdery mildew fungi. In the first approach, multiplex PCR fingerprints generated by three sets of primers allowed differentiation of several isolates of *P. flocculosa* from closely related species, or genera such as *Tilletiopsis*. The same set of primers was used in quality control experiments and revealed fungal contamination that was otherwise not observed by standard culture and microscopy techniques. In addition, the use of random amplified microsatellites generated by $(GT)_n$ and $(CCA)_n$ primers was applied as a measure of possible genetic variation over 65 repeated subcultures of *P. flocculosa*. Finally, a novel technique was developed and named reverse intron PCR (RIP), based on the presence of an intron revealed by partial sequencing of mtLSU of *P. flocculosa*. RIP allowed not only differentiation of *P. flocculosa* from other species but also separated the isolates of different origins within *P. flocculosa*. This new technique could find useful applications in phylogenetic studies of closely related fungal species and isolates.

INTRODUCTION

The identification and commercial development of a fungal antagonist as a biocontrol agent is a multi-step process that includes discovery of the antagonist, small- and large-scale efficacy trials, mass production, and registration (Woodhead *et al.* 1990, Avis, Hamelin & Bélanger 2001b). There is an increasing awareness that appropriate tools must be developed to monitor the fungal antagonist at crucial and strategic moments throughout this developmental process by means of a quality control system. Quality control tools are essential in ensuring the efficacy, consistency, and reliability of the biopesticide. An effective quality control system should therefore provide means to detect contamination (Jenkins *et al.* 1998, Jenkins & Grzywacz 2000) and monitor genetic stability of the organism (Stacey, Bolton & Doyle 1991). In addition, quality control measures are essential in following guidelines imposed by legislative entities throughout the registration procedure of a biopesticide (Fravel, Rhodes & Larkin 1999).

In quality control, several techniques are commonly used to test for the presence of contaminants. For example, plating samples of inoculum on selective or non-selective growth media and observing the macroscopic appearance of contaminants is a widely used technique (Hantula, Dusabenyagasani & Hamelin 1996, Bouix & Leveau 1999). However, phenotypic characteristics such as morphology are often insufficient in discriminating between the desired biocontrol agent and an unwanted contaminant (Markovic & Markovic 1998). Furthermore, a quality control system demands even more precise tools when testing for genetic stability. In the 1980s, cytogenetic and isozyme analyses were widely accepted as reliable tools for this purpose

* Corresponding author.

Table 1. Fungal isolates and species used in this study.

Species	Designation	Isolates[a]	Substrate of origin	Geographic origin
Pseudozyma flocculosa	PF-A22 UL	CBS 167.88	*Trifolium pratense*	Ontario, Canada
P. flocculosa	PF-A22 UL (1)	Avis *et al.* (2001a)	Rose	Québec, Canada
P. flocculosa	PF-A22 UL (RM)	ATCC 74320	Not applicable	Québec, Canada
P. flocculosa	PF-A22 UL (CS)	Avis *et al.* (2001a)	Not applicable	Québec, Canada
P. flocculosa	PF-H	Avis *et al.* (2001a)	Unknown	Netherlands
P. flocculosa	PF-BC	Avis *et al.* (2001a)	*Cucumis sativus*	British Columbia, Canada
P. rugulosa	PR	CBS 170.88	*Zea mays*	Ontario, Canada
P. antartica	PAN	CBS 516.83	Sediment	Antarctica
P. aphidis	PAP	CBS 517.83	Aphid secretion	Germany
P. prolifica	PP	CBS 319.87	*Scirpus microcarpus*	Canada
Sporothrix inflata	SI	ATCC 24422	Soil	Germany
S. nivea	SN	ATCC 76232	Waste water	Saskatchewan, Canada
Tilletiopsis minor	TM	CBS 264.82	Erysiphaceae	Germany
T. washingtonensis	TW	ATCC 96156	*Cucumis sativus*	British Columbia, Canada

[a] CBS, Centraalbureau voor Schimmelcultures; and ATTC, American Type Culture Collection.

(Stacey *et al.* 1991). When DNA fingerprinting with Jeffreys' probe (Jeffreys, Wilson & Thein 1985) was introduced, it offered improved sensitivity in testing genetic stability in several organisms. In recent years, new and improved DNA fingerprinting techniques seem to offer an even greater sensitivity and precision than the previously employed techniques (Weising *et al.* 1995). It therefore seems that DNA fingerprinting would be the tool of choice for quality assessment and monitoring of biocontrol agents throughout their developmental process.

The yeast-like fungus *Pseudozyma flocculosa* (Boekhout, Fell & O'Donnell 1995) is a biocontrol agent that has completed the majority of steps towards registration as the biofungicide Sporodex for the control of powdery mildews (Bélanger & Labbé 2002). Recently, Avis *et al.* (2001a) established the genetic identity and diversity of *P. flocculosa* isolates and other closely related fungi. Many DNA fragments were characterized from this work and distinctive ribosomal DNA sequences, hypervariable microsatellite loci, and a partially sequenced species-specific intron were identified. These DNA sequences have the potential to be used in a quality control setting.

At present, it is of utmost importance to develop specific and reliable quality control tools for the continuing development of *P. flocculosa* as a biopesticide. Our objectives were therefore to construct molecular quality control tools to: (1) evaluate the innocuousness of the biopesticide; (2) monitor genetic stability that could have an effect on the integrity and, potentially, the biocontrol property of the antagonist; and (3) rapidly assess the identity of the biocontrol agent.

MATERIALS AND METHODS

Biological materials

The fungal isolates used in this study are listed in Table 1. All were derived from single-spore cultures and were maintained on slants of potato dextrose agar at 4 °C. Additional fungal material from 11 sequential mass productions of *Pseudozyma flocculosa* isolate PF-A22 UL (CS) was included. In addition, this same isolate was subjected to repeated 3 d subculturing over 6 months. Cells were cultured in potato dextrose broth on a rotary shaker (150 rpm) at 25 °. The resulting fungal biomass was centrifuged at 10 000 rpm for 10 min and the culture medium was discarded. Fungal cells were washed with sterile distilled water and centrifuged for an additional 10 min. The water was discarded and the fungal biomass was transferred to sterile 1.5 ml microtubes. The cells were lyophilized and stored at −20 ° until use.

DNA extraction

Genomic DNA was extracted from 40 mg of lyophilized fungal cells with an extraction kit (Fast DNA kit; Q-BIOgene, Carlsbad, CA). Briefly, the lyophilized samples were transferred to 2.0 ml tubes containing the provided 0.5 cm ceramic sphere and garnet matrix. Next, 100 µl of deionized sterile water was added to the tubes and the samples were processed for five repetitions of 10 s at speed setting 4 with the FastPrep instrument (Q-BIOgene). The extraction procedure was then performed according to the manufacturer's instructions. Finally, all DNA was diluted 1:100 in sterile water, a dilution determined to yield the most reproducible PCR amplification.

Multiplex PCR

Multiple sequence amplification in a single PCR reaction was performed using three previously described rDNA regions (Avis *et al.* 2001a): the region containing both ITS 1 and 2, partial sequences of the mtLSU, and partial sequences of the nSSU. The multiplex mixture contained 0.2 µM of ITS1f (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes & Bruns 1993)

and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers (White *et al.* 1990), 0.2 μM of NS51 (5′-GGGGAGTATGGTCGCAAGGC-3′) and NS61 (5′-TCAGTGTAGCGCGCGTGCGGC-3′) primers (Avis *et al.* 2001a), 1.2 μM of ML5 (5′-CTCGGCAAATTATCCTCATAAG-3′) and ML6 (5′-CAGTAGAAGCTGCATAGGGTC-3′) primers (White *et al.* 1990), 100 μM of each deoxynucleoside triphosphate and 2× of reaction buffer including 1.5 mM $MgCl_2$ (Boehringer Mannheim Biochemica, Mannheim). Reactions were performed using 2 units of Taq DNA polymerase (Boehringer Mannheim Biochemica) and 0.5 μl of template DNA in a 12.5 μl reaction volume. The microtubes were placed in the thermal cycler only once it had reached its initial denaturing temperature. The thermal cycler (MJ-Research, Watertown, MA) was programmed for an initial denaturation step of 95 ° for 3 min, 35 cycles of 92 ° for 30 s, 55 ° for 30 s, and 72 ° for 1 min. The amplifications were completed by a 10 min final extension at 72 °. Amplicons were visualized on 1.5% agarose gels in Tris-acetate-EDTA buffer at 3 V cm^{-1} for 1.5 h. Gels were stained with ethidium bromide and photographed under UV-light.

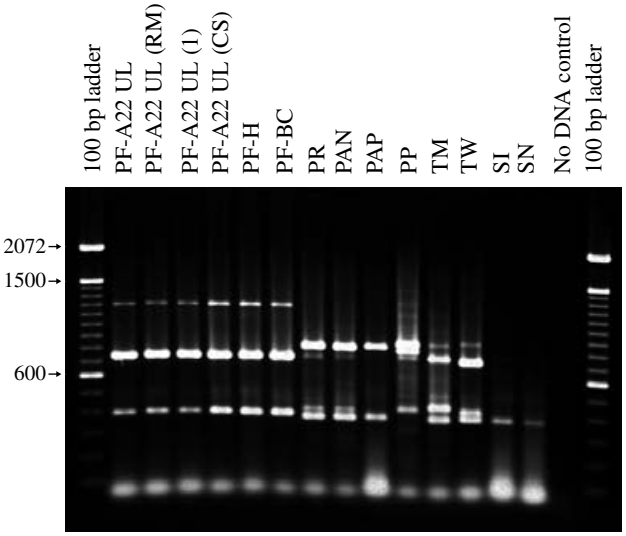

Fig. 1. Multiplex PCR fingerprints generated by three sets of primers (NS, ITS and ML). Lanes 1 and 17, 100 bp ladder (Invitrogen Canada, Burlington, ON); lane 2, *Pseudozyma flocculosa* A22 UL; lane 3, *P. flocculosa* A22 UL (RM); lane 4, *P. flocculosa* A22 UL (1); lane 5, *P. flocculosa* A22 UL (CS); lane 6, *P. flocculosa* strain H; lane 7, *P. flocculosa* strain BC; lane 8, *P. rugulosa*; lane 9, *P. antarctica*; lane 10, *P. aphidis*; lane 11, *P. prolifica*; lane 12, *Tilletiopsis minor*; lane 13, *T. washingtonensis*; lane 14, *Sporothrix inflata*; lane 15, *S. nivea*; lane 16, no DNA control.

Randomly amplified microsatellites (*RAMS*)

RAMS fingerprints of repeated subcultures of isolate PF-A22 UL (CS) were obtained using single primer amplification with GT (5′-VHVGTGTGTGTGTGT-GTG-3′) and CCA (5′-DDBCCACCACCACCACCA-3′) primers (Hantula *et al.* 1996), where V = A, C, or G; H = A, C, or T; D = A, G, or T; B = C, G, or T. PCR was performed using the Expand Long Template PCR System (Boehringer Mannheim Biochemica) in 25 μl volume containing 1× supplied buffer (including 1.5 mM $MgCl_2$), 100 μM of each deoxynucleoside triphosphate, 0.4 μM of primer, 2% DMSO, 1 unit of Taq Expand DNA polymerase and 1 μl of the template DNA. The thermal cycler was programmed for an initial denaturation step of 95 ° for 10 min, 34 cycles of 94 ° for 30 s, 58 ° for 1 min, and 68 ° for 2 min. The amplifications were completed by an 8 min final extension at 68 °, and visualized as before.

Reverse intron PCR (*RIP*)

A region of rDNA containing a partial sequence of the large mitochondrial subunit (mtLSU) was amplified. PCR was performed using the Taq DNA polymerase system (Boehringer Mannheim Biochemica) in volumes of 25 μl containing 1× supplied reaction buffer (including 1.5 mM $MgCl_2$), 100 μM of each deoxynucleoside triphosphate, 1 μM of ML7 (GACCCTATGCAGCTTCTACTG) and ML8 (TTATCCCTAGCGTAACTTTTATC) primers (White *et al.* 1990), 1 unit of Taq DNA polymerase and 1 μl of the template DNA. The thermal cycler was programmed for an initial denaturation step of 95 ° for 3 min, 35 cycles of 92 ° for 30 s, 58 ° for 30 s, and 72 ° for 1 min. The amplifications were completed by a 10 min final extension at 72 °, and visualized as before.

PCR products (partial mtLSU) were purified on QIAquick minispin columns (Qiagen, Hilden) and directly sequenced using an ABI 373A Stretch automated sequencer (Applied Biosystems, Mississauga, ON). Sequences were aligned with ClustalW, available on-line from the Baylor College of Medicine (http://searchlauncher.bcm.tmc.edu/multi-align/).

The reverse primer MLINTA (5′-GATATATGCCCGGGAAAG-3′) and the forward primer MLINTB (5′-GATTATCTGGAGGGTTTTCA-3′) were designed by aligning sequences of an intron included in the mtLSU of *Psendozyma flocculosa* based on sequences from ML5-ML6 (Avis *et al.* 2001a) and ML7-ML8 (this study) (Fig. 1). Primers were designed using Primer3, available on-line at the Whitehead Institute for Biomedical Research (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

PCR was carried out using the Expand Long Template PCR System (Boehringer Mannheim Biochemica) in volumes of 12.5 μl containing 1× supplied reaction buffer (including 1.5 mM $MgCl_2$), 100 μM of each deoxynucleoside triphosphate, 1 μM of each primer, 1 unit of Taq Expand DNA polymerase and 0.5 μl of template DNA. The PCR microtubes were placed in the thermal cycler once it had reached its denaturating temperature. The cycling parameters were

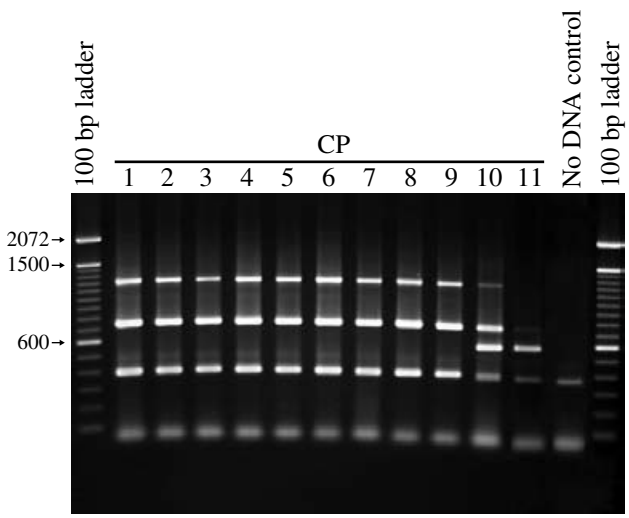

Fig. 2. Multiplex PCR fingerprints generated by three sets of primers on 11 sequential commercial scale productions (CP) of *Pseudozyma flocculosa* PF-A22 UL (CS). 100 bp ladder as in Fig. 1.

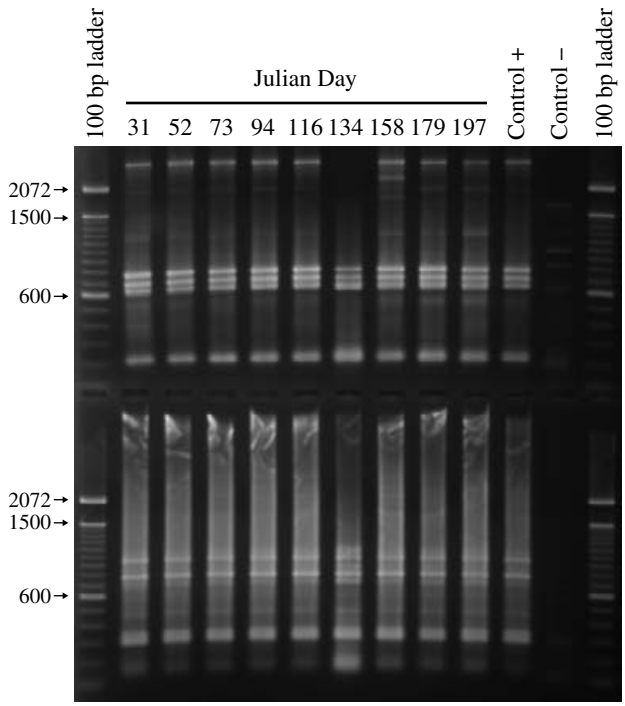

Fig. 3. Random amplified microsatellite generated by GT (top) and CCA (bottom) primers on *P. flocculosa* isolates subcultured over a 6 month period (lanes 2–10). Lanes 1 and 13, 100 bp ladder (as in Fig. 1.); lanes 2–10, sequential mass production of PF-A22 UL (CS) in Julian days; lane 11, standard DNA from PF-A22 UL (CS); lane 12, no DNA control.

the following: an initial denaturation step at 95 ° for 3 min, 35 cycles consisting of 30 s at 92 °, 30 s at 45 °, and 1 min at 72 °, and a final extension step of 10 min at 72 °, and visualized as before.

RESULTS

Multiplex PCR fingerprints were able to distinguish *Pseudozyma flocculosa* from other *Pseudozyma* spp., *Tilletiopsis* spp., and *Sporothrix* spp. (Fig. 1). In *P. flocculosa*, all isolates produced the three expected amplicons: the 390 bp nSSU, 730 bp ITS, and 1200 bp mtLSU fragments. The 1200 bp fragment was absent from other *Pseudozyma* spp., *Tilletiopsis* spp., and *Sporothrix* spp., revealing its uniqueness to the *P. flocculosa* isolates. *Sporothrix* spp., gave a pale amplicon of roughly 370 bp.

When multiplex PCR was performed on the eleven commercial scale productions of PF-A22 UL (CS), nine samples (CP1-CP9; Fig. 2) yielded the expected *P. flocculosa*-specific PCR products. However, an additional fragment of approximately 590 bp appeared in CP 10. This band reveals the presence of a contaminant in the biomass product. Subsequently, the next PF-A22 UL (CS) production (CP 11) revealed the absence of all three *P. flocculosa*-specific fragments and the appearance of a 590 bp PCR product belonging to another fungus contaminating the biomass.

RAMS of the subcultured PF-A22 UL (CS) isolate revealed the same fingerprint patterns previously obtained with *P. flocculosa* isolates (Avis *et al.* 2001a) and no differences in major amplicons were revealed, with the exception of the loss of the fragment over 2072 bp with amplification with GT primer after 134 d subculturing (Fig. 3). However, 24 d later the band was recovered. The last subcultured PF-A22 UL (CS) isolate (197 d) compared to the positive control is identical for both GT and CCA patterns.

The nucleotide sequence obtained from the PCR products generated by ML7 and ML8 primers allowed the complete sequence of the intron beginning in the previous region of the mitochondrial large rDNA to be obtained and enabled the construction of a RIP system (Fig. 4). RIP revealed differences in banding patterns among *Pseudozyma* spp., *Tilletiopsis* spp., and *Sporothrix* spp. There were no PCR products amplified by this set of primers in *T. washingtonensis*. Also, RIP demonstrated a difference within the *P. flocculosa* species (Fig. 5). Interestingly, within *P. flocculosa* isolates, the first four isolates, being derived PF-A22 UL, were distinguished by two major fragments of approximately 710 bp and 1700 bp and one minor fragment at 600 bp. However in the case of PF-H and PF-BC, the isolates from different geographic origins, the presence of additional minor bands (650 and 550 bp) and the disappearance of one major band, respectively (1700 bp; Fig. 5) indicated that RIP was more discriminating than the multiplex PCR (Fig. 1).

DISCUSSION

The biocontrol properties of *Pseudozyma flocculosa* against powdery mildews have brought this yeast-like

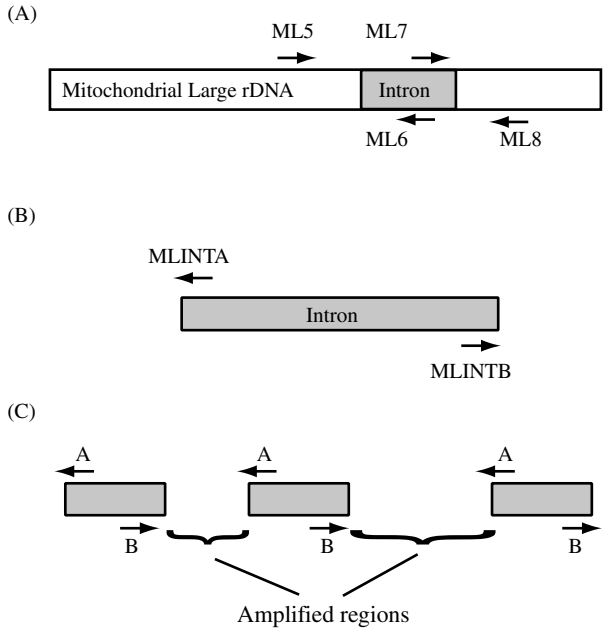

Fig. 4. Development of reverse intron PCR (RIP). (A) Position of an intron in the mitochondrial large rDNA region; (B) relative position of the MLINT primers; and (C) regions amplified by these primers.

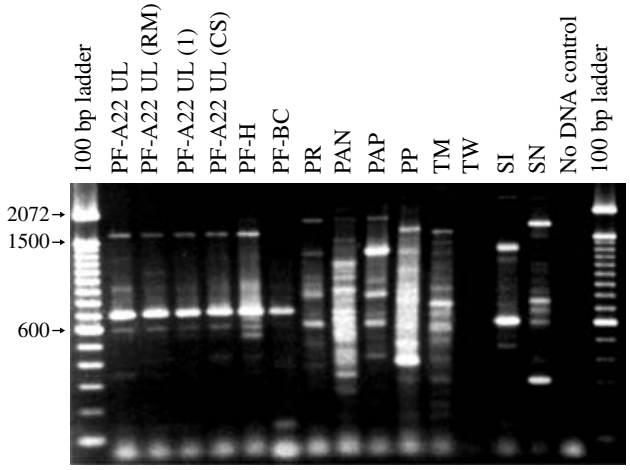

Fig. 5. Reverse intron PCR (RIP) fingerprints generated by MLINT primers (100 bp ladder and isolates as in Fig. 1).

fungus through a series of steps towards commercialization as a biofungicide. Mass production of a biocontrol agent such as *P. flocculosa* requires an effective quality control system ensuring the safety, viability and effectiveness of the biofungicide. This reliability is of major importance for consistency in commercial production as well as for registration purposes. In this study, new sensitive approaches to detect contaminants, monitor genetic stability, and rapidly assess identity of *P. flocculosa* were developed as part of a global quality control strategy that will assist with the development of *P. flocculosa* as a biofungicide.

In a previous study, the identity and classification of most known *Pseudozyma* species were determined (Avis *et al.* 2001a). In this work, nucleotide sequences of three previously characterized rDNA regions were used together in a multiplex PCR reaction in an effort to rapidly assess the identity and innocuousness of *P. flocculosa* isolates. When comparing *Pseudozyma* spp. with the multiplex PCR, *P. flocculosa* isolates were identifiable from other species. In particular, a 1200 bp fragment was absent from other *Pseudozyma*, *Tilletiopsis*, and *Sporothrix* species. Although a faint 1240 bp fragment appeared in *P. prolifica*, this is due to unspecific priming and the fragment does not represent the mtLSU as in *P. flocculosa*. These results demonstrate the reliability of the fingerprints developed and the sensitivity of multiplex PCR in distinguishing *P. flocculosa* from other species that could contaminate a mass produced product.

For instance, this same multiplex PCR reaction allowed the detection of a fungal contaminant that appeared in two samples of biomass product. Although a faint fragment of 380 bp appeared in CP11 and the control, this was determined to originate from contaminated water during PCR and should be disregarded. The two contaminated samples in this study were easily detected as an unexpected amplicon of 590 bp appearing in addition to (CP 10) or in place of (CP 11) the three expected bands (Fig. 3). These samples were taken from biomass that had already been through some levels of quality control, such as microscopy and plating, but did not show direct evidence of contamination. Therefore, the contaminants could only be detected when tested by the multiplex PCR reaction, demonstrating its importance as part of the quality control procedure for *P. flocculosa* isolate PF-A22 UL (CS).

Microsatellite fingerprinting of *P. flocculosa* (Avis *et al.* 2001a) and other fungi (McKay *et al.* 1999) previously have shown differences among isolates of various geographic origins. Considering the sensitivity of these hypervariable fingerprints and the larger portion of the genome that can be looked into when compared to rDNA, a 6 month subcultured isolate of the commercial strain of *P. flocculosa* (PF-A22 UL (CS)) was tested with these molecular tools. The two RAMS fingerprints used in this study suggest that, over a 6 month period or after 65 repeated mass subcultures, no mutations were detected in these multiple regions, which would indicate a high level of genetic stability of *P. flocculosa*. Although a fragment of over 2000 bp had disappeared in the GT-primed production after 134 d, the fragment reappeared in the following subculture indicating a problem with the PCR amplification rather than the loss of genetic integrity. In addition, the CCA pattern is consistent within the 197 d of subculture including the 134 d old subculture and is identical to the positive control. Nonetheless, the primers used only screen a part of the fungal genome and do not ensure that there are no mutations throughout the entire genome. Once again, these fingerprints complement a

variety of tests already in place as part of the quality control strategy of the biofungicide.

The presence of an intron revealed by partial sequencing of the mtLSU of *P. flocculosa* has allowed the development of a novel technique, named here as reverse intron PCR (RIP), to fingerprint closely related species. It has been demonstrated that introns act as mobile genetic elements that can be found throughout the genome (Lambowitz & Belfort 1993) and such mobile genetic elements could, therefore, allow for the generation of variable fingerprints (Ness *et al*. 1993). Other techniques such as enterobacterial repetitive intergenic consensus PCR and repetitive extragenic palindromic PCR proved to be efficient in generating fingerprints where primers built from consensus sequences of mobile elements were used to amplify regions confined between each consensus sequence (Louws *et al*. 1994). Along these lines, RIP uses primers to amplify between specific introns in the genome (Fig. 4C). Fingerprints generated by the set of primers with *P. flocculosa* are indeed different from the fingerprints of other *Pseudozyma* species. In addition, the fingerprints derived from *P. flocculosa* PF-A22 UL isolates proved to be identical, while those of isolates PF-H and PF-BC were different. This suggests that these two isolates are different from PF-A22 UL with respect to the distribution and/or number of this intron in their genome. Considering that PF-A22 UL, PF-H and PF-BC were isolated in different parts of the world, this confirms the discriminating power of RIP. In the case of *T. washingtonensis*, no amplicons were obtained by RIP indicating that this fungus would not contain the specific intron found in *P. flocculosa*. This new technique can find practical applications to differentiate closely related species and isolates of *Pseudozyma*, as well as of other genera. RIP does give specific fingerprints for fungi containing this intron and provides an additional, rapid means for the identification of *P. flocculosa*.

This study has demonstrated that the development of diverse molecular tools (fingerprinting techniques) will help monitor *P. flocculosa* in its commercially produced form, to test for genetic stability as well as contamination throughout the biofungicide production process. Future work with molecular markers will continue in order to sample a higher percentage of the *P. flocculosa* genome for greater precision in genetic stability testing. Also, further experimentation will attempt to include other primers as part of the multiplex PCR system, such as primers specific to the commercial isolate of *P. flocculosa*, as well as oligonucleotides priming biologically relevant DNA stretches, such as genes implicated in biocontrol. These tests will contribute towards a global quality control strategy where the biofungicide will be assessed rigorously to ensure its reliability, safety and efficacy. The tests will also prove invaluable in the registration process of *P. flocculosa* as a biocontrol agent of powdery mildew fungi.

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