Molecular and Physiological Analysis of the Powdery Mildew Antagonist *Pseudozyma flocculosa* and Related Fungi

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ABSTRACT

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A number of phenotypic and genotypic characteristics were used to ascertain the identity and diversity of *Pseudozyma flocculosa*, a natural antagonist of powdery mildews that has received little attention in terms of taxonomy. To this end, several putative isolates of *P. flocculosa* as well as several closely related species were analyzed. Ribosomal DNA sequences distinguished *P. flocculosa* from other *Pseudozyma* spp. and identified two previously unknown *Pseudozyma* isolates as *P. flocculosa*. Random amplified microsatellites revealed three distinct *P. flocculosa* strains among the tested isolates. Biocontrol properties and antifungal metabolite production were limited to the *P. flocculosa* spp. Results produced useful molecular markers to (i) distinguish *P. flocculosa* from other related fungi, (ii) identify different strains within this species, and (iii) aid in the construction of isolate-specific molecular tools that will assist in research and development of *P. flocculosa* as a biocontrol agent of powdery mildew fungi.

Additional keywords: antifungal fatty acids.

Pseudozyma flocculosa (Traquair, L. A. Shaw & Jarvis) Boekhout & Traquair (syn.: *Sporothrix flocculosa* Traquair, L. A. Shaw & Jarvis) (6) is a yeastlike fungus with strong antagonistic activity against powdery mildew fungi (3,11,12,16). Cytochemical observations revealed that the antagonist induces rapid collapse of powdery mildew conidial chains and cytoplasmic disintegration of fungal cells (13). The biocontrol agent produces extracellular fatty acids with antifungal properties (4,7). Bioassay of these fatty acids confirmed that they induce the same toxic effect in fungal cells as *P. flocculosa* itself (1,14), confirming their importance in the biocontrol potential of the antagonist.

Current development of this fungus as a biocontrol agent highlights the importance of assessing the identity and diversity of *P. flocculosa* isolates. First and foremost, taxonomic confusions hamper basic research and development of the fungal antagonist as a biocontrol agent. The fungus was initially classified in the genus *Sporothrix* (20), though recent studies have suggested that it is more closely related to the genus *Pseudozyma* (2,5). Assessing identity and diversity is also of great importance in the selection of suitable biocontrol candidates as well as in authentication of *P. flocculosa* isolates for industrial procedures and registration purposes (9). Indeed, the efficacy of different isolates may vary with changes in crops and geographic regions of intended use. This may require the re-evaluation of the most effective isolates of the fungus as these conditions evolve in agronomic practices (17). Furthermore, authentication of fungal isolates is critical for consistent results in efficacy in research and commercial trials as well as in the process of mass production in an industrial setting (19,23). In all of these processes, it is essential to have means for the discrimination of *P. flocculosa* from other closely related organisms and to assess variability within the *P. flocculosa* spp. In this context, DNA fingerprinting offers a sensitive and reliable approach to genetically characterize the biocontrol agent.

Our objectives were, therefore, (i) to assess ribosomal DNA (rDNA) sequences and random amplified microsatellite (RAMS) fingerprints as tools in identity/diversity studies of *P. flocculosa* and related fungi, and (ii) to correlate this information with biological activity in an attempt to provide a basis of selection of proper candidates for biocontrol programs.

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MATERIALS AND METHODS

Biological materials. Fungal isolates listed in Table 1 were derived from single-spore cultures and were maintained on slants of potato dextrose agar (PDA) at 4°C. Cells were cultured in potato dextrose broth (PDB) on a rotary shaker (150 rpm) at 25°C. The fungal biomass was centrifuged at 10,000 rpm for 20 min, and the culture medium was discarded. Fungal cells were washed with sterile distilled water and centrifuged for an additional 20 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°C until use.

DNA extraction. Genomic DNA was prepared as follows. Lyophilized fungi (≈10 mg) were mixed with an equal amount of diatomaceous earth (Sigma Chemical Co., St. Louis) and ground with a pestle. Six hundred microliters of extraction buffer (100 mM Tris-HCl at pH 9.5, 2% cetyltrimethylammonium bromide, 1.4 M NaCl, 1% polyethylene glycol 8000, 20 mM EDTA, and 1% β-mercaptoethanol) was added to the macerated cells and incubated at 65°C for 1 h. The mixture was extracted with 600 µl of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged at 10,000 × *g* for 5 min. The supernatant was transferred to a new microtube and re-extracted with 400 µl of phenol/chloroform/isoamyl alcohol. The supernatant was precipitated with 1 vol of cold isopropanol and centrifuged at 10,000 × *g* for 5 min. The precipitate was washed with 70% ethanol, vacuum-dried, and resuspended in 50 µl of Tris-EDTA buffer (10 mM Tris-

HCl and 1 mM EDTA, pH 8). All DNA was diluted (1:100) to a dilution determined to yield the most reproducible polymerase chain reaction (PCR) amplification.

PCR amplification conditions. Three regions of rDNA were amplified (Table 2): the entire region containing both internal transcribed spacers (ITS), partial sequences of the large mitochondrial subunit (mtLSU), and partial sequences of the small nuclear subunit (nSSU). PCR reactions were carried out by the *Taq* DNA polymerase system (Boehringer Mannheim Biochemica, Mannheim, Germany) in volumes of 25 µl containing 1× the supplied reaction buffer (including 1.5 mM $MgCl_2$), 100 µM each deoxynucleoside triphosphate, 1 µM each primer, 1 unit of *Taq* DNA polymerase, and 1 µl of the template DNA. Amplifications were performed in a thermal cycler (MJ Research Inc., Watertown, MA) programmed for an initial denaturation step at 95°C for 3 min, 35 cycles at 92°C for 30 s, 58 (ITS and mtLSU) or 52°C (nSSU) for 30 s, and 72°C for 1 min. The amplifications were completed with a 10-min final extension at 72°C.

RAMS fingerprints were produced by single-primer amplification with GT and CCA primers (Table 2). PCR was carried out (Expand Long Template PCR; Boehringer Mannheim Biochemica) in 25-µl volumes containing 1× the supplied buffer (including 1.5 mM $MgCl_2$), 100 µM each deoxynucleoside triphosphate, 0.4 µM primer, 2% dimethyl sulfoxide, 1 unit of *Taq* Expand DNA polymerase, and 1 µl of the template DNA. The thermal cycler was programmed for an initial denaturation step at 95°C for 10 min, 34 cycles at 94°C for 30 s, 58°C for 1 min, and 68°C for 2 min. The amplifications were completed with an 8-min final extension at 68°C. Amplicons were visualized on 1.5% agarose gels in Tris-acetate-EDTA buffer at 3 V/cm for 1.5 h. Gels were stained with ethidium bromide and photographed under UV light.

Nucleotide sequencing determination. rDNA PCR products (ITS, partial mtLSU, and partial nSSU) were purified on minispin columns (QIAquick; Qiagen, Hilden, Germany) and directly sequenced using an aflatoxin biosynthesis inhibitor automated sequencer (373A Strech; Applied Biosystems, Mississauga, Ontario, Canada). Multiple sequence alignment was performed with Clustal W, available on-line from the Baylor College of Medicine. Following visual inspection, final alignment of the sequences was performed by hand. Nucleotide sequences are available in the GenBank database as Accession Nos. AF294690 to AF294724.

Phylogenetic analysis. Phylogenetic analyses were performed by phylogenetic analysis using parsimony (PAUP 4.0b1; Sinauer Associates, Sunderland, MA) on rDNA sequences of the entire ITS region, partial mtLSU, and partial nSSU, both as individual and combined data sets. Indels were coded as single events. Unweighted parsimony analyses were performed on the individual data sets, excluding uninformative characters, using the heuristic search option with 1,000 random addition sequences with MULTREES on and tree bisection-reconnection branch swapping. Maximum parsimony analysis of the combined data set was by the branch-and-bound option in PAUP for exact solutions. The nSSU data set was comprised of a 14-taxon matrix, including sequences from *Sporothrix schenckii* (GenBank Accession No. M85053). The combined rDNA data set was comprised of an 11-taxon matrix, from which the three *Sporothrix* spp. were excluded. *Saccharomyces* sp. (GenBank Accession No. AB040998) and *Tilletiopsis washingtonensis* were selected as outgroups for rooting the nSSU and combined rDNA trees, respectively. Clade stability was assessed by 1,000 parsimony bootstrap replications. Neighbor-joining trees were also inferred with uncorrected "P" and maximum-likelihood distance methods. Concordance of the three rDNA data sets was evaluated with the partition-homogeneity test implemented with PAUP, using 1,000 random repartitions. The Kishino-Hasegawa likelihood test implemented in PAUP was used to compare various constrained and unconstrained topologies (Table 3).

Production of antifungal fatty acids. Isolates were tested for the production of 9-heptadecenoic and 6-methyl-9-heptadecenoic acids, two antifungal fatty acids produced by *P. flocculosa* (1). Fungi were cultured in PDB for 3 days on a rotary shaker (150 rpm) at 25°C, followed by a 28-day still culture, protected from light. Culture media was separated from fungal biomass by centrifugation at 10,000 rpm for 20 min. Culture media was extracted (3 × 50 ml) with chloroform (4). The combined organic phases were roto-evaporated and taken to dryness with a stream of nitrogen. The oily residue, containing both antifungal fatty acids, was derived with Phenacyl-8 (Pierce Chemical Company, Rockford, IL) (8). Phenacyl ester fatty acids were analyzed by reverse-phase high pressure liquid chromatography (Nova-Pak C-18 column, 60 Å, 4 µm, 3.9 × 300 mm) coupled to a photodiode array detector (Waters Limited, Mississauga, Ontario, Canada). The eluent was a gradient of 80 to 100% acetonitile, acidified with 0.1% H_3PO_4, at a 1.0 ml/min flow rate, as follows: 80 to 100% over 15 min, 100%

TABLE 2. Polymerase chain reaction-amplified regions and primers

Genomic region[a]	Primer	Sequence[b]	Ref.[c]
rDNA			
Entire ITS	ITS1f	5′-CTTGGTCATTTAGAGGAAGTAA-3′	(10)
	ITS4	5′-TCCTCCGCTTATTGATATGC-3′	(22)
Partial mtLSU	ML5	5′-CTCGGCAAATTATCCTCATAAG-3′	(22)
	ML6	5′-CAGTAGAAGCTGCATAGGGTC-3′	(22)
Partial nSSU	NS51	5′-GGGGGAGTATGGTCGCAAGGC-3′	TS
	NS61	5′-TCAGTGTAGCGCGCGTGCGGC-3′	TS
RAMS			
GT repeat	GT	5′-VHVGTGTGTGTGTGTGTG-3′	(15)
CCA repeat	CCA	5′-DDBCCACCACCACCACCA-3′	(15)

[a] ITS = internal transcribed spacer, mtLSU = mitochondrial large subunit, nSSU = small nuclear subunit, and RAMS = random amplified microsatellites.
[b] The following designations are used for degenerate sites: V (G, A, or C), H (A, T, or C), D (G, A, or T), and B (G, T, or C).
[c] TS = this study.

TABLE 1. Fungal isolates and species used in this study

Species	Designation	Isolate[a]	Host of origin	Geographic origin
Pseudozyma flocculosa	PF-A22	CBS 167.88	*Trifolium pratense*	Ontario, Canada
Pseudozyma flocculosa	PF-1	This study	Rose	Québec, Canada
Pseudozyma flocculosa	PF-RM	ATCC 74320	Not applicable	Québec, Canada
Pseudozyma flocculosa	PF-IS	This study	Not applicable	Québec, Canada
Pseudozyma sp.	PH	This study	Unknown	Netherlands
Pseudozyma sp.	PBC	This study	*Cucumis sativus*	British Columbia, Canada
Pseudozyma rugulosa	PR	CBS 170.88	*Zea mays*	Ontario, Canada
Pseudozyma antartica	PAN	CBS 516.83	Sediment	Antarctica
Pseudozyma aphidis	PAP	CBS 517.83	Aphid secretion	Germany
Pseudozyma prolifica	PP	CBS 319.87	*Scirpus microcarpus*	Canada
Sporothrix inflata	SI	ATCC 24422	Soil	Germany
Sporothrix nivea	SN	ATCC 76232	Wastewater	Saskatchewan, Canada
Tilletiopsis washingtonensis	TW	ATCC 96156	*Cucumis sativus*	British Columbia, Canada

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

held for 5 min, and 100 to 80% over 2 min. 9-Heptadecenoic and 6-methyl-9-heptadecenoic acids were identified in fungal culture medium extracts by comparing retention times and UV spectra with authentic standards (1). The experiment was repeated three times for each fungus.

Biocontrol activity of fungal isolates. Fungal isolates were assayed for their ability to antagonize *Sphaerotheca fuliginea* (Schlechtend.:Fr.) Pollacci, causal agent of cucumber powdery mildew. Foliar disks (5 cm) were cut from *Sphaerotheca fuliginea*-infected cucumber leaves and placed on 20-20-20 agar containing 2 g/liter of 20-20-20 'all purpose' soluble fertilizer and 8 g/liter of bacto-agar. Disks were cut from the leaf portions covered by at least 95% with colonies of *Sphaerotheca fuliginea*. Spores of tested isolates (1×10^6 spores per ml) were placed in water with 0.02% Aqua-Aid (Ken Crowe Inc., Montréal, Québec, Canada). Spores were sprayed directly on the infected cucumber disks and monitored daily over a 7-day period for antifungal activity evaluated as collapse of *Sphaerotheca fuliginea* conidial chains. Aqua-Aid alone (0.02%) served as a control. The experiment was repeated three times for each fungus.

RESULTS

The partial nSSU of rDNA data set consisted of 812 nucleotide characters, 65 of which were parsimony informative. These data yielded a single most parsimonious tree (MPT) (Fig. 1). Neighbor-joining trees were found using the uncorrected P and maximum-likelihood distance options and were topologically concordant with the MPT. Clade stability, as assessed by 1,000 bootstrap replications, identified strong support of two independent groups: the *Pseudozyma* clade that included the four *P. flocculosa* isolates, PBC, and PH, and the *Sporothrix* clade.

When the *Sporothrix* spp. were excluded, there was extensive variability within the three rDNA sequences in all tested *Pseudozyma* spp. The ITS rDNA data set revealed that PF-A22, PF-1, PF-RM, PF-IS, PBC, and PH had identical sequences, with the exception of a 2-bp insertion at positions 130 (T) and 131 (T) for PH. Sequences comprising the 1,183-bp partial mtLSU of rDNA data set contained a large intron of 733 bp for the four *P. flocculosa* isolates, PBC, and PH at the 3′ end of these sequences. Partial sequences of the nSSU of rDNA revealed that *P. prolifica* contained a large intron between position 64 and 480. Results of the partition-homogeneity test ($P = 0.072$) indicated that the three rDNA trees reflected the same underlying phylogeny. The three data sets were thus combined and analyzed by several tree building programs.

Maximum parsimony yielded five equally parsimonious trees for the combined data set (Fig. 2). Neighbor-joining trees were topologically concordant with the MPTs. Clade stability, as assessed by 1,000 bootstrap replications, identified strong support of multiple independent grouping of all *Pseudozyma* spp. as well as grouping the two unidentified *Pseudozyma* spp. (PBC and PH) in the *P. flocculosa* clade in a polytomy set of relationships. Topological constraints forcing the monophyly of different *P. flocculosa* groups were not significantly different than the unconstrained MPT when subjected to the Kishino-Hasegawa likelihood test ($P = 0.05$) (Table 3).

RAMS analysis revealed extensive differences in microsatellite fingerprints between a group consisting of the four *P. flocculosa* isolates, PBC, and PH and a second group comprised of the other *Pseudozyma* spp. GT primer yielded differences in banding patterns between the four *P. flocculosa* isolates from Ontario and Québec (PF-A22, PF-1, PF-RM, and PF-IS) and the isolates from British Columbia (PBC) and the Netherlands (PH) (Fig. 3). With all other major fragments the same, a PCR product of approximately 620 bp was present in the four isolates from Ontario and Québec, whereas in PH and PBC, the "equivalent" fragment was of approximately 600 bp. Moreover, CCA primer provided an amplicon positioned at 1,500 bp in PH and PBC that was absent in the four *P. flocculosa* isolates (Fig. 4). With CCA primer it was also possible to differentiate the PH and PBC isolates from each other. Indeed, the PBC isolate yielded a fragment of approximately 600 bp that was absent in the PH isolate. Neither RAMS primer distinguished differences in banding patterns between the four *P. flocculosa* isolates from Ontario and Québec.

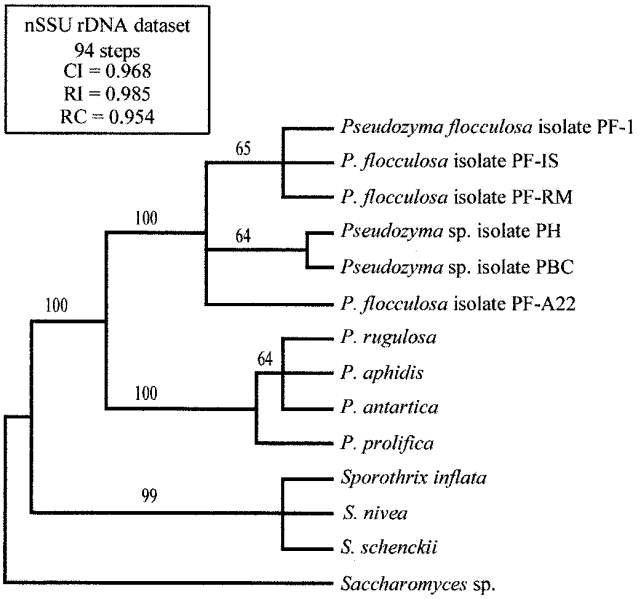


Fig. 1. Single most parsimonious tree inferred from partial sequences of the small nuclear subunit (nSSU) of rDNA. Bootstrap replication frequencies >50% are indicated above nodes.

TABLE 3. Likelihood analysis of constrained and unconstrained trees from the combined rDNA data set

Tree[a]	Tree length (steps)[b]	No. of trees[c]	Log likelihood (L)[d]			
			ln L	Difference[e]	SD	P[f]
Unconstrained	654	5	–6814.55668	(Best)	…	…
PF-A22, PF-1, PF-RM, and PF-IS monophyletic	654	2	–6814.55668	0.00000	0.00000	1.0000
PF-A22, PF-1, PF-RM, PF-IS, and PBC monophyletic	654	2	–6818.28770	–3.73102	3.72747	0.3169
PF-A22, PF-1, PF-RM, PF-IS, and PH monophyletic	655	9	–6821.11201	–6.55533	6.53826	0.3161
PF-A22, PF-1, PF-RM, PF-IS, PBC, and PH monophyletic	654	5	–6818.28770	–3.73102	3.72747	0.3169

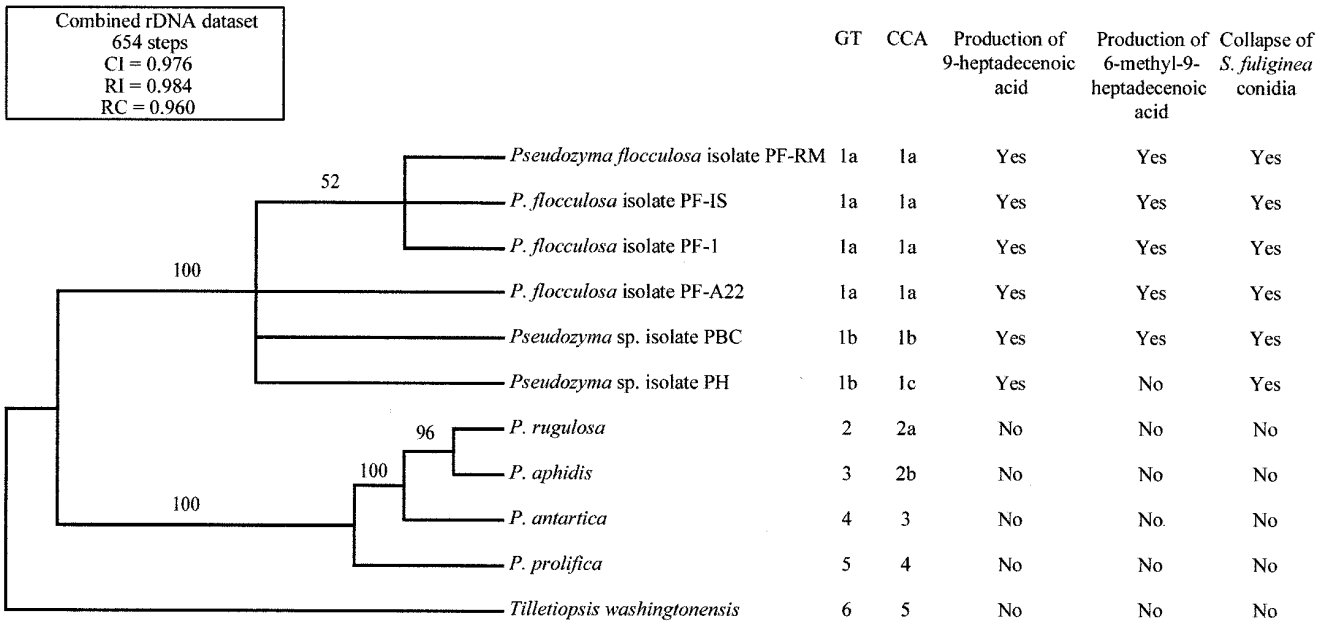
[a] Monophyly constraints enforced with phylogenetic analysis using parsimony (PAUP).
[b] Under indel coding excluding uninformative characters.
[c] Only the best tree from each constraint was included in the test.
[d] Transition/transversion ratio estimated via maximum likelihood for the Kishino-Hasegawa test.
[e] Difference in ln likelihood (L) between the best tree and constrained trees.
[f] Probability of obtaining more extreme T value, using the two-tailed test, with the null hypothesis that there is no difference between the two trees. Values are significant at $P < 0.05$.

Analysis of both antifungal fatty acids revealed that PF-A22, PF-1, PF-RM, PF-IS, PBC, and PH produced 9-heptadecenoic acid (Fig. 2). 6-Methyl-9-heptadecenoic acid was produced by PF-A22, PF-1, PF-RM, PF-IS, and PBC. PH did not produce 6-methyl-9-heptadecenoic acid. None of the other tested fungi produced either of the antifungal fatty acids.

When selected fungi were bioassayed against *Sphaerotheca fuliginea*, only the four *P. flocculosa* isolates, PBC, and PH induced collapse of *Sphaerotheca fuliginea* conidial chains (Fig. 2). The remaining fungi showed no evidence of biocontrol activity against *Sphaerotheca fuliginea* under our experimental conditions.

DISCUSSION

P. flocculosa is a recently characterized yeastlike fungus with the potential for biological control of powdery mildew disease.



	GT	CCA	Production of 9-heptadecenoic acid	Production of 6-methyl-9-heptadecenoic acid	Collapse of *S. fuliginea* conidia
Pseudozyma flocculosa isolate PF-RM	1a	1a	Yes	Yes	Yes
P. flocculosa isolate PF-IS	1a	1a	Yes	Yes	Yes
P. flocculosa isolate PF-1	1a	1a	Yes	Yes	Yes
P. flocculosa isolate PF-A22	1a	1a	Yes	Yes	Yes
Pseudozyma sp. isolate PBC	1b	1b	Yes	Yes	Yes
Pseudozyma sp. isolate PH	1b	1c	Yes	No	Yes
P. rugulosa	2	2a	No	No	No
P. aphidis	3	2b	No	No	No
P. antartica	4	3	No	No	No
P. prolifica	5	4	No	No	No
Tilletiopsis washingtonensis	6	5	No	No	No

Fig. 2. Strict consensus of the five most parsimonious trees based on the combined data set of three rDNA sequences. Bootstrap replication frequencies >50% are indicated above nodes. GT = GT-primed microsatellite fingerprint; and CCA = CCA-primed microsatellite fingerprint.

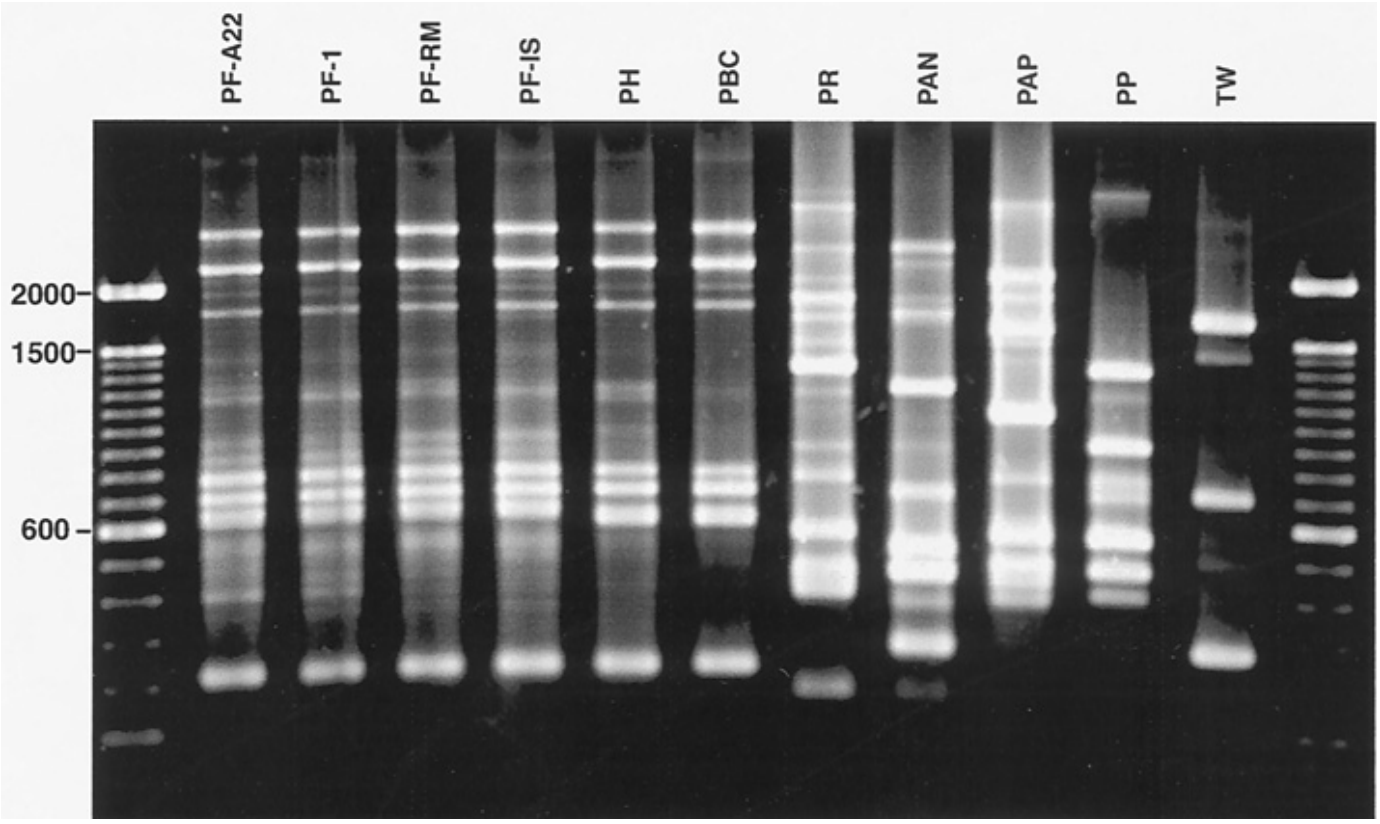


Fig. 3. Random amplified microsatellite fingerprints generated by GT primer. Designation of isolates is given in Table 1. Lanes 1 and 13 are molecular size markers (100-bp ladder).

When selecting fungi for a specific purpose or function it is essential to correctly classify and identify isolates for the selection of appropriate candidates in given situations. This precise identification is also necessary to ensure consistency in experimental, industrial, and commercial processes. In this study, a mixture of phenotypic and genotypic characteristics were studied to ascertain the identity and diversity of the known *P. flocculosa* isolates in order to gain insight into the strains that are of interest in biocontrol programs.

P. flocculosa was initially classified in the genus *Sporothrix* based on classical mycology techniques (20). Recent studies have suggested that the fungus is in fact from the genus *Pseudozyma*, based, in part, on sequences of the large nuclear subunit (nLSU) of rDNA (2,5). In this work, the nSSU of rDNA was used to directly compare *Pseudozyma* isolates, including *P. flocculosa*, with three *Sporothrix* spp. These results conclusively demonstrate that *P. flocculosa* is indeed distant from *Sporothrix* spp. and is more closely related to the genus *Pseudozyma*.

Cladistic analysis of the combined data set of three rDNA sequences clearly demonstrated that the tested *Pseudozyma* fungi are distributed into five distinct species based on their high bootstrap values (95% or greater). This is consistent with previous reports that have used the nLSU of rDNA (2,5). Moreover, the Kishino-Hasegawa likelihood test forcing the monophyly of the unknown *Pseudozyma* isolates from British Columbia (PBC) and the Netherlands (PH) with the four *P. flocculosa* isolates indicated that both PBC and PH should be considered as part of the *P. flocculosa* species.

Microsatellite fingerprinting of *P. flocculosa* isolates suggested that the four isolates from Ontario and Québec are very closely related. These results were expected because these four isolates were initially derived from a single strain. Also, microsatellite fingerprints of PBC and PH indicated that they are both distinct strains of *P. flocculosa*. Previous results with random amplified polymorphic DNA analysis also revealed that PH was distinct from PBC and PF-A22, although this analysis could not distinguish the latter two isolates between each other (21).

Under the test conditions used in this study, biocontrol activity against *Sphaerotheca fuliginea*, causal agent of cucumber powdery mildew, was limited to the six *P. flocculosa* isolates, including PH and PBC, indicating that biocontrol properties are not a general characteristic of *Pseudozyma* spp. but are specific to certain species. Although *P. rugulosa* has been reported to antagonize *Sphaerotheca fuliginea* (11), this was not demonstrated in our experiments.

Two of the antifungal fatty acids that mediate the biocontrol properties of *P. flocculosa* were produced in all *P. flocculosa* isolates, with the exception of PH, which only produces 9-heptadecenoic acid. This may play an important role in the selection of the most prolific *P. flocculosa* isolate in biocontrol programs. *P. rugulosa* produces a related antifungal fatty acid, 4-methyl-7,11-heptadecadienoic acid, but did not produce either of the two antifungal fatty acids tested in this study. This may well affect the general biocontrol property of *P. rugulosa*, which is less effective than *P. flocculosa* in controlling powdery mildew (11).

Overall, this study indicates that *P. flocculosa* can be genetically and biochemically discriminated from other related fungi. Current results demonstrated that there are three distinguishable *P. flocculosa* strains capable of biocontrol activity against *Sphaerotheca fuliginea*. Although this biocontrol fungus has a very limited genetic base, i.e., there are few known isolates, these findings provide a basis to identify, authenticate, and monitor these isolates, and possibly others yet to be discovered. This is of paramount importance in the production and release of *P. flocculosa* in a biocontrol program. For example, as experimental and commercial-scale testing of these isolates against other powdery mildew fungi as well as in different cultural and geographical conditions continues, these molecular markers will be useful in the selection of the most effective isolates in a given situation. Moreover, should more than one isolate of *P. flocculosa* be mass-produced in an industrial setting, these molecular markers would be instrumental in the detection of cross contamination between isolates in a quality control test (18). These findings could permit the construction of molecular tools based on isolate-specific sequences. This may allow the possible detection of mutations that could, if followed by selection or genetic drift, alter the genetic integrity and, potentially, the biocontrol property of the fungus. The discovery of specific and useful genetic markers in this study will, thus, be instrumental in gaining insight into the reliable use of a *P. flocculosa*-based biological fungicide against powdery mildew diseases.

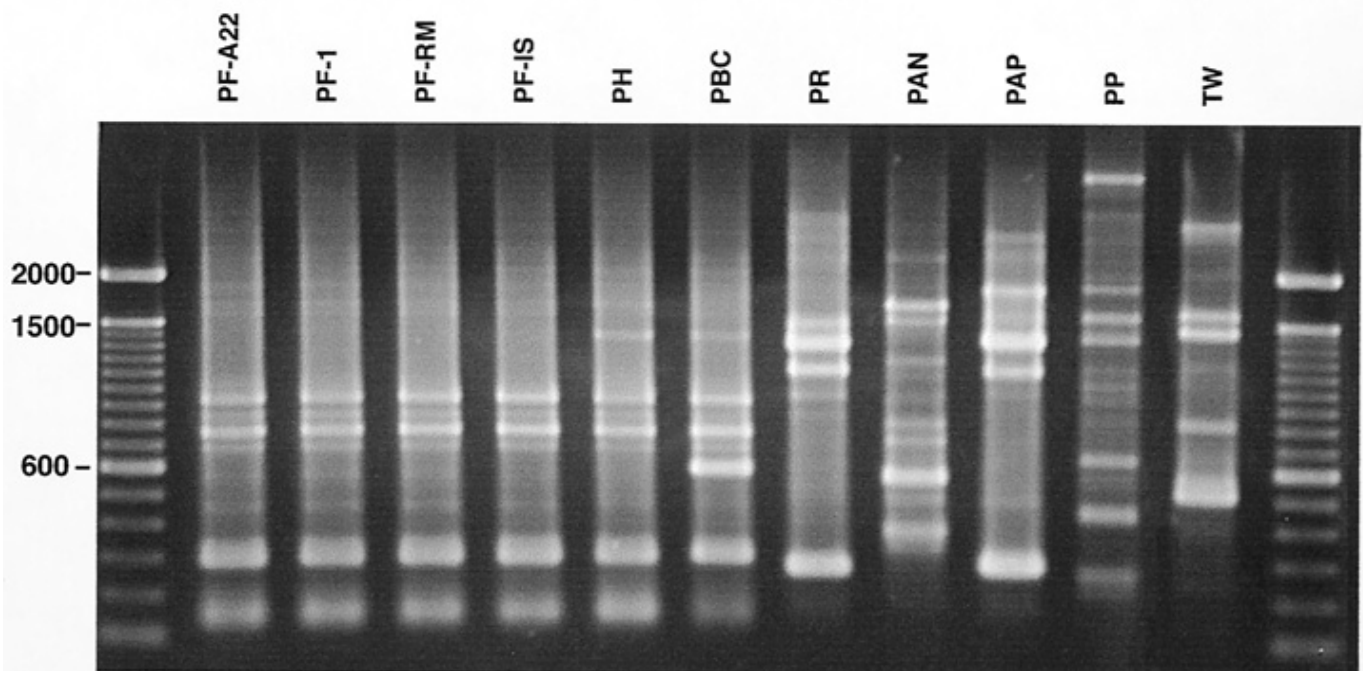


Fig. 4. Random amplified microsatellite fingerprints generated by CCA primer. Designation of isolates is given in Table 1. Lanes 1 and 13 are molecular size markers (100-bp ladder).

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