Characterization and Identification of Entomopathogenic and Mycoparasitic Fungi using RAPD-PCR Technique

LENKA BIELIKOVÁ[1], ZDENĚK LANDA[2], LANCE S. OSBORNE[3] and VLADISLAV ČURN[4]

University of South Bohemia, [1]Faculty of Agriculture – Department of Genetics and [2]Faculty of Biology – Department of Plant Production, České Budějovice, Czech Republic; [3]Mid-Florida Research & Educational Center, IFAS, University of Florida, Apopka, Florida, USA; [4]University of South Bohemia, Faculty of Agriculture – Department of Fodder Plants, České Budějovice, Czech Republic

Abstract

BIELIKOVÁ L., LANDA Z., OSBORNE L.S., ČURN V. (2002): **Characterization and identification of entomopathogenic and mycoparasitic fungi using RAPD-PCR technique**. Plant Protect. Sci., **38**: 1–12.

Entomopathogenic and mycoparasitic fungi were characterised by RAPD technique, with special attention to evaluate the genetic stability of strains that are used as active ingredients in commercial biopesticides. Strain-specific fingerprints were constructed for *Paecilomyces fumosoroseus* – strain PFR 97 Apopka, *Gliocladium virens* – strain GL 21 and *Verticillium lecanii* – strain MYCOTAL. Genetic stability and homogeneity was confirmed among re-isolates that were obtained from commercial batches of bio-insecticide PFR 97™ 20%WDG and bio-fungicide SoilGard™12G that had been produced in 1995–1999. RAPD analysis indicated the genetic identity of *V. lecanii* strains re-isolated from the two different bio-insecticides MYCOTAL® and VERTALEC®. The usefulness of RAPD technique was demonstrated when *P. fumosoroseus* strain PFR 97 Apopka was reliably identified after having passed through adults of the spruce bark beetle *Ips typographus*, and by analysis of the relationship between fungi of the genus *Gliocladium*.

Keywords: *Gliocladium* spp.; *Gliocladium virens*; *Paecilomyces fumosoroseus*; *Verticillium lecanii*; entomopathogenic fungi; mycoparasitic fungi; identification using RAPD-PCR

Fungal biopesticides used in biological control already cover a wide range of fungal genera and applications, including use as antagonists to fungi and agents to control insect pests. Once selected for development as microbial control agents, fungal isolates must be formally identified on the subspecies level (JENKINS & GRZYWACZ 2000). This will place the fungal isolate within a taxonomic rank, initially at the species level, but many fungal species are widely distributed in the environment worldwide and thus have an enormous number of particular strains. For instance, more than 700 species of fungi from about 90 genera are known as insect pathogens, with many subspecies, pathotypes, strains and isolates within some of these (CHARNLEY 1989). Individual isolates of a particular entomopathogenic and mycoparasitic fungus may display considerable specialisation in host range, and it is becoming increasingly apparent that identification at the species level is no longer adequate (HUMBER 1997).

Several methods have been used to describe the variation within a species of entomopathogenic and mycoparasitic fungi. These include morphological characteristics of spores and colonies, extracellular protein profiles, pathogenicity and growth or nutrient requirements (SAMSON 1981). Furthermore, immunotaxonomic and chemotaxonomic methods have been used, though only with limited success (BIDOCHKA *et al*. 1994). Obviously, tax-

Supported by the Ministry of Education, Youth and Sports of the Czech Republic, Grand No. CEZ: J06/98:122200002/2 and Project 0856.

onomic procedures are becoming more and more complex and it is generally accepted that some forms of molecular identification techniques are needed in addition to the traditional morphological characteristics formally used to classify fungal species (BRIDGE & ARORA 1998). Different molecular techniques were used for various applications and on different entomopathogenic and mycoparasitic fungi (HEGEDUS & KHACHATOURIANS 1996; MOR *et al*. 1996; FUKATSU *et al*. 1997; MCDONALD 1997; CASTLE *et al*. 1998), including identification of fungal isolates based on polymorphism of DNA using RAPD-PCR technique (CAETANO-ANNOLES *et al*. 1991; EDEL 1998; ST LEGER & JOSHI 1999).

The RAPD (random amplified polymorphic DNA) technique was described in 1990 (WILLIAMS et al. 1990). It is a modification of PCR (polymerase chain reaction) and allows to reveal polymorphism within completely unknown samples without the need of probe hybridisation or DNA sequencing. Only one short oligonucleotide primer (6–12 bases) is used for the reaction, and the sequence of primers is fully arbitrary. The product of a reaction is a spectrum of DNA fragments differing from each other in length and nucleotide sequence. The total number of products and the length of each depend on the template DNA and primer used and is specific for a particular combination. The application of RAPD markers is similar to those of other methods based on detection of DNA polymorphism. It can be used also for characterisation of a fungal isolate by constructing a specific fingerprint or for genetic stability testing of an individual isolate. RAPD has already been used to estimate the diversity of a population, for genotype characterisation or constructing the molecular phylogeny of closely related taxons (WILLIAMS *et al*. 1990; HARDYS *et al*. 1992; SAMEC 1993; TIGANO-MILANI *et al*. 1995b; OBORNÍK *et al*. 1997; VAKALOUNAKIS & FRAGKIADAKIS 1999).

The filamentous fungi analyzed in this study belong to the *Deuteromycetes*. These conidial fungi produce their asexual spores on exposed hyphae rather than in a closed fruiting structure. Exceptionally, conidial fungi (anamorphs) occur together with their sexual state (teleomorphs) and both morphs have different scientific names (HUMBER 1997). Hyphomycetes are distinguished by the morphology of their conidia and conidiogenous cells and by the identity of their hosts. However, it is almost impossible to distinguish individual isolates using only morphological characters because of limited distinctive characteristics (SAMSON 1974; SAMŠIŇÁKOVÁ *et al*. 1983). Moreover, neither standard laboratory bioassays nor interactions with their natural hosts offer sufficient information to identify fungi on the subspecies level (OSBORNE & LANDA 1992).

The aim of this study was to characterise the entomopathogenic fungi *Paecilomyces fumosoroseus* (Wize) Brown et Smith and *Verticillium lecanii* (Zimm.) Viégas, and mycoparasitic fungi of the genus *Gliocladium* by RAPD technique, with special attention to evaluate the genetic stability of strains that are used as active ingredients in commercial bio-insecticides and bio-fungicides.

MATERIALS AND METHODS

Fungal isolates

Paecilomyces fumosoroseus strain PFR 97 Apopka (ATTC 20874, stock culture from the collection of Mid-Florida Research & Educational Centre, Apopka, IFAS University of FL, USA); *Gliocladium virens* strain GL 21 (stock culture, Thermo Trilogy Corporation, Columbia, MD, USA), and a strain of *Verticillium lecanii* re-isolated from microbial insecticide MYCOTAL® (Koppert B.V., The Netherlands) were used as comparative strains. Eleven isolates of *P. fumosoroseus* were obtained after re-isolation from various batches of microbial insecticide PFR 97™20%WDG that had been produced in the period 1995–1999. Similarly, two isolates of *G. virens* were re-isolated from microbial fungicide SoilGard™12G (commercial batches produced in 1996 and 1998), and one isolate of *V. lecanii* originated from a commercial batch of the microbial insecticide VERTALEC® (Koppert B.V., The Netherlands). All other isolates used in this study were acquired from various Czech Collections of Microorganisms (for details see Table 1).

Maintenance of fungi

For long periods all isolates were stored as dry alginate pellets, which had been formulated by mixing the centrifuged submerged biomass of monospore isolates of each isolate with sterile wheat bran and aqueous sodium alginate (EYAL *et al*. 1994). Dry pellets were stored in separate plastic vials (approx. 400–500 pellets per isolate) at –23 ± 1.0°C. For the experiments, all isolates were maintained using a two-step procedure. First, dry alginate pellets were plated aseptically on 2% water agar in sterile Petri dishes and cultured at 25°C for 4–5 d to induce growth of the fungi on the surface of the pellets. Once adequate growth and sporulation of the fungi was visible, the activated pellets were rinsed in sterile 0.05% Tween 80 solution to obtain a spore suspension that was then adjusted to a standard concentration (1.0×10^7 spores per 1 ml) and used as the inoculum for submerged cultures. The latter were used to produce fungal biomass for DNA extraction. Sterile flasks (250 ml) filled with 100 ml of PDB (potato-dextrose-broth, SIGMA) were inoculated with 10 ml of adjusted spore suspension and shaken continuously on a rotary shaker (200 RPM, amplitude 5) at 25°C for 3 d (*Gliocladium* spp.) or 4 d (*P. fumosoroseus* and *V. lecanii*).

Table 1. Description of fungal isolates used

Species	Isolate	Source – Origin
Paecilomyces fumosoroseus	PFR 97 Apopka	ATTC 20874, stock culture [A]
	A33	re-isolate from PFR 97™20%WDG (1995) [B]
	119	re-isolate from PFR 9797™20%WDG (1996) [B]
	178	re-isolate from PFR 9797™20%WDG (1997) [B]
	189–196	re-isolates from PFR 9797™20%WDG (1998) [B]
Gliocladium virens	GL 21	stock culture [B]
	SG1	re-isolate from SoilGard™12 G (1996) [B]
	SG2	re-isolate from SoilGard™12 G (1998) [B]
	CCM 8042 [C]	collection item
Gliocladium roseum	CCM 8070	collection item
Gliocladium sp.	CCF 783 [D]	collection item
Verticillium lecanii	MYCOTAL	re-isolate from bioinsecticide MYCOTAL [E]
	VERTALEC	re-isolate from bioinsecticide VERTALEC [E]
	CCEF 111 [F]	collection item
	CCEF 113	collection item
	CCEF 116	collection item

[A] IFAS Univ.ersity of Florida, Mid-Florida Research & Educational Centre, Apopka, Florida, USA
[B] Thermo Trilogy Corporation, Columbia, Maryland, USA
[C] CCM – Czech Collection of Microorganisms, Faculty of Science, Masaryk University Brno, CR
[D] CCF – Culture Collection of Fungi, Department of Botany, Fac. Natural Sciences, Charles University Prague, CR
[E] Koppert B.V, The Netherlands
[F] CCEF – Culture Collection of Entomogenous Fungi, Department of Plant Production, University of South Bohemia, České Budějovice, CR

Sub-culturing of *P. fumosoroseus* through spruce bark beetle *Ips typographus*

The bio-insecticide PFR 97™20%WDG (batch No. 189) was applied as a water suspension (2 g/1000 ml) on the surface of a trap-tree (a freshly felled spruce) at Modrava in the National Park of the Bohemian Forest. After 3 weeks, bark samples were cut from the treated trap-tree and placed into moist chambers (sterile plastic boxes with moistened filter paper on the bottom), and these were kept in growth cabinets (25°C, photoperiod 12/12 hrs) for the next 5 d. Thereafter, the adults of the spruce bark beetle *Ips typographus* L. (Coleoptera, Scolytidae) that were infected with *P. fumosoroseus* were collected from the bark, placed on the surface of 2% water agar in a Petri dish and kept for 2–3 days until distinctive proliferation of the fungus on the surface of the host cuticle. The fungus was then isolated and purified after transfer and sub-culture on PDA plates; biomass for DNA extraction was produced in submerged culture (PDB) as described above.

DNA extraction (modified method described by TIGANO-MILANI *et al.* [1995a])

Approximately 100 mg of biomass obtained from submerged culture was used for DNA extraction. The biomass was frozen at –20°C for 24 h and then ground in a 1.5 ml microfuge tube with a sterile plastic stick. The homogenized mixture was suspended in 500 µl sterile lysis buffer (50mM Tris/HCl, 150mM NaCl, 100mM EDTA), then 50 µl 10% (w/v) SDS was added, and the tubes were shaken gently. After 1 h at 37°C, 75 µl 5M NaCl was added and mixed, 60-µl cetyltrimethylammonium bromide (CTAB) solution (10%, w/v, CTAB in 0.7M NaCl) was added and mixed again, the suspension was incubated at 65°C for 20 min and centrifuged for 2 min at 8000 RPM. The supernatant was extracted with an equal volume of chloroform/isoamylalcohol (24:1 v/v), and 0.6 volume of 2-propanol was added to precipitate the nucleic acids. The pellets obtained by centrifugation at 14 000 RPM for 10 min were washed with 70% (v/v) sterile ethanol, dried and resuspended in 100 µl of sterile distilled water. The extracted DNA was assessed on 1% TAE (Tris/Acetic Acid/EDTA) agarose gel stained with ethidium bromide (SAMBROOK *et al.* 1989).

RAPD analysis

The RAPD reactions were performed in 25 µl volumes of the reaction mix: 0.2mM of each dNTPs (Promega), 0.5 µM primer (Operon Technologies), 1U Taq-polymerase (Finnzyme), 1× polymerase recommended buff-

er (Finnzyme) and 1 µl template DNA, using Termocycler PTC 1160 (MJ-Research). The temperature profile for all reactions was 92°C for 3 min at the initial step followed by 45 cycles of 92°C for 1 min, 35°C for 2 min, 72°C for 3 min, with a final extension at 72°C for 10 min. The reaction products were checked by loading all of the reaction mixture onto 2% TAE agarose gel with ethidium bromide. The gels were photographed by Polaroid gel camera and pictures were digitised (600 dpi, Adobe Photoshop) and processed using Gel Manager.

Table 2. Amount of RAPD-PCR amplified fragments per primer for comparative strains of *Paecilomyces fumosoroseus*, *Gliocladium virens* and *Verticillium lecanii*

Primer	Sequence 5´to 3´	Total amount of RAPD amplified fragments		
		P. fumosoroseus PFR 97 Apopka	*G. virens* GL 21	*V. lecanii* MYCOTAL
OPF-02	GAGGATCCCT	12	–	–
OPF-05	CCGAATTCCC	15	–	–
OPF-06	GGGAATTCGG	–	10	–
OPF-07	CCGATATCCC	7	8	–
OPF-08	GGGATATCGG	11	13	–
OPF-09	CCAAGCTTCC	–	10	11
OPF-10	GGAAGCTTGG	–	8	–
OPF-11	TTGGTACCCC	–	11	–
OPF-12	ACGGTACCAG	12	–	–
OPF-13	GGCTGCAGAA	14	7	–
OPF-14	TGCTGCAGGT	–	8	–
OPF-15	CCAGTACTCC	–	7	–
OPF-16	GGAGTACTGG	14	13	8
OPF-20	GGTCTAGAGG	10	–	–
OPB-01	GTTTCGCTCC	12	–	–
OPB-02	TGATCCCTGG	13	–	–
OPB-03	CATCCCCCTG	11	–	–
OPB-04	GGACTGGAGT	12	–	–
OPB-10	CTGCTGGGAC	15	–	–
OPB-12	CCTTGACGCA	–	–	12
OPB-14	TCCGCTCTGG	–	–	12
OPB-16	TTTGCCCGGA	9	–	9
OPB-18	CCACAGCAGT	–	–	9
OPA-06	GGTCCCTGAC	–	–	15
OPA-08	GTGACGTAGG	–	–	13
OPA-15	TTCCGAACCC	–	–	16
OPA-17	GACCGCTTGT	–	–	12
OPA-19	CAAACGTCGG	–	–	10
Total amount of fragments		167	95	127

Three primer sets, each with twenty 10-mer primers (Operon Technologies, kits OPA, OPB and OPF) were used to create specific fingerprints of the comparative strains (*P. fumosoroseus* PFR 97 Apopka, *G. virens* GL21 and *V. lecanii* MYCOTAL®) (Table 2). The set of primers was selected for each comparative strain to generate specific patterns of bands and construct a strain-specific fingerprint. The sizes of RAPD products were estimated using Lambda/EcoR I+Hind III marker (Promega) and quantified after processing with Gel Manager.

A set of primers was selected and used to compare and assess the genetic stability of *P. fumosoroseus* isolates obtained from various commercial batches of bio-insecticide PFR 97™20%WDG. Eight primers from kit OPF (3–6, and 9–12) were used to compare re-isolates from different batches of this commercial bio-pesticide that had been produced in the period 1995–1997 (batches No. A33, PA 119, and PA 178), and primers OPF 1, 6–9 and 11 were used to evaluate the genetic stability of *P. fumosoroseus* PFR 97 after re-isolation from commercial batches PA 189–196 that had been produced in 1998. For both, the comparative strain PFR 97 Apopka was used as the positive control. Also, the original stock culture (PFR 97 Apopka) and the re-isolate from the commercial batch that was applied to induce infection of spruce bark beetle (PFR 97™20%WDG – batch No. 189) were used as positive controls of the isolate that was purified after passage through adults of *I. typographus*. Primers OPF 6, 8, 12 and 15 were used to compare these three isolates of *P. fumosoroseus*.

Five primers from kit OPF (primers 6–10) were used to analyse two isolates of *G. virens* that originated from different commercial batches of the bio-fungicide SOILGARD™12G (produced in 1996 and 1998), and seven primers from the same kit (OPF 6–8, 11, 13, 15 and 16) were used to demonstrate variations among fungi of the genus *Gliocladium*.

Finally, two commercial strains of *V. lecanii* (re-isolated from bio-insecticides MYCOTAL® and VERTALEC®) were analyzed based on RAPD patterns generated with a set of seven primers from kit OPA (OPA 14–20), and single primer OPF 11 was used to demonstrate variability of partial RAPD patterns among different *V. lecanii* isolates.

RESULTS

The comparative strains were characterised by using RAPD markers, and strain-specific fingerprints were constructed based on binary characters revealed by three kits of twenty 10-mer primers of arbitrary sequences (Table 2). The entomopathogenic fungus *P. fumosoroseus* strain PFR 97 Apopka was characterized with eight 10-mer primers from kit OPF and six 10-mer primers from kit OPB. The primers revealed a total of 167 repeatable bands that were

used to construct strain-specific RAPD patterns (Table 3). Similarly, 10 primers from kit OPF were selected to construct a specific RAPD fingerprint of the mycoparasitic fungus *G. virens* strain GL 21 using 95 distinct repeatable bands that were revealed (Table 4). Finally, the entomopathogenic fungus *V. lecanii* strain MYCOTAL was characterised by 127 repeatable binary characters that were revealed with five primers from kit OPA, four primers from kit OPB and two OPF primers (Table 5).

The entomopathogenic fungus *P. fumosoroseus* was used in three different studies. First, the long-term genetic stability of the stock strain was assessed using three isolates that were re-isolated from different commercial batches of bio-insecticide PFR 97™ 20%WDG produced during 1995–1997. The RAPD analysis based on binary characters revealed by the selected eight OPF primers demonstrated that stock strain PFR 97 Apopka and all re-isolates were identical (Fig. 1). Likewise, genetic stability and homogeneity was verified among eight isolates of *P. fumosoroseus* that were re-isolated from different batches of PFR 97™20%WDG that had been produced and distributed during 1998; there were no differences between the RAPD patterns of these isolates which thus were considered as genetically identical (Fig. 2). Finally, *P. fumosoroseus* re-isolated from infected adults of *I. typographus* was compared with the original stock strain PFR 97 Apopka and with an isolate from the commercial batch of bio-insecticide PFR 97™20%WDG that was used to pass the pathogen through its natural insect host. In this study, four primers from kit OPF were used to generate the RAPD patterns. Repeatedly, no differences were detected when RAPD spectra of these three isolates were analysed (Fig. 3).

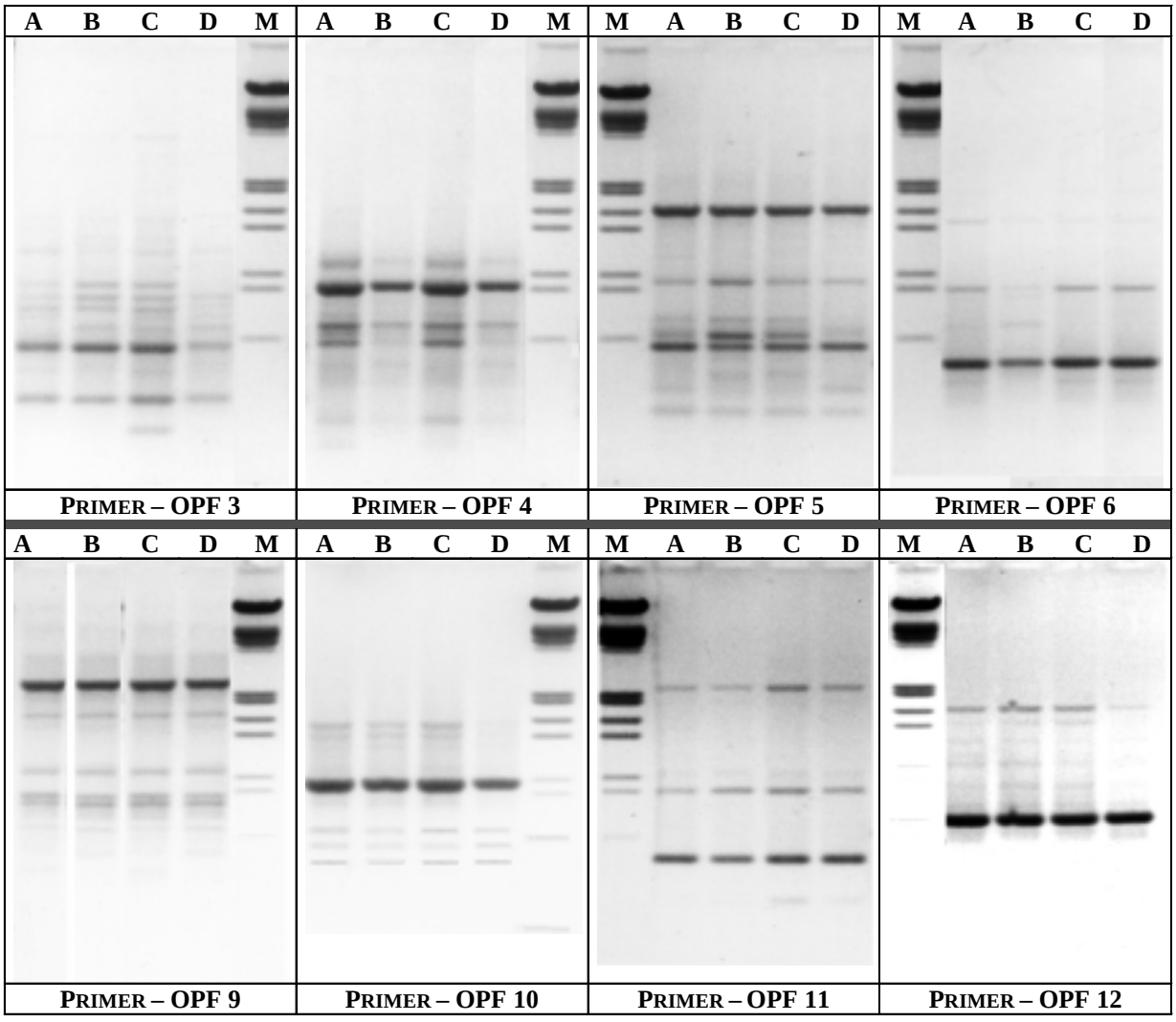


Fig. 1. Comparison of the RAPD patterns of *P. fumosoroseus* strains from various batches of commercial biopreparation PFR 97™20%WDG (A = comparative stock strain PFR 97, B = batch A33, C = batch 119, D = batch 178 and M = marker)

Table 3. Specific fingerprint of *Paecilomyces fumosoroseus*, strain PFR 97 Apopka, generated by a selected set of primers

A. Kit OPB

Primer	OPB 1	OPB 2	OPB 3	OPB 4	OPB 10	OPB 16
Size of amplified fragments (bp)	2055	2621	2886	2479	174	52698
	1589	2284	2065	2186	156	32428
	1315	1658	1175	1920	126	72010
	1195	1373	813	1455	121	21635
	1066	1014	642	1310	109	81214
	962	889	542	1180	937	1044
	846	728	494	927	829	772
	761	630	388	842	763	639
	649	572	348	791	681	549
	590	537	243	718	621	
	548	454	190	640	567	
	489	416		470	507	
	287				474	
					378	
					327	

B. Kit OPF

PRIMER	OPF 2	OPF 5	OPF 7	OPF 8	OPF 12	OPF 13	OPF 16	OPF 20
Size of amplified fragments (bp)	3544	2147	1998	2477	2777	2244	2237	2460
	3073	1550	1877	2207	2637	2216	1863	2003
	2574	1256	1290	2010	1732	1624	1751	1413
	1993	1050	868	1446	1410	1390	1368	1147
	1806	921	743	1275	1218	1175	1266	767
	1132	801	599	1116	1067	968	1084	573
	859	731	475	964	959	748	1024	416
	812	702		841	870	658	929	333
	718	550		758	765	580	845	258
	550	497		806	545	504	732	213
	516	446		494	487	431	637	
	437	408			225	364	560	
		368				305	424	
		311				146	333	
		263						

Long-term genetic stability and homogeneity of *G. virens* strain GL 21 was demonstrated when two isolates from commercial batches that were produced and distributed in 1996 and 1998 were analysed. The RAPD patterns created with five primers from kit OPF proved the identity of both isolates (Fig. 4). By contrast, significant differences in RAPD patterns were detected between various isolates of mycoparasitic fungi of the genus *Gliocladium* (e.g. *G. virens, G. roseum* and *Gliocladium* spp.) (Fig. 5). The dendrogram constructed from RAPD characteristics and generated from the similarity matrix indicates identity of *G. virens* GL 21 isolates and a close relationship of these with another strain of *G. virens* (CCM 8042). Simultaneously, all other isolates were considered as outliners and *P. fumosoroseus* was clearly distinguished and separated as an outgroup (Fig. 6).

A comparison of RAPD patterns generated with seven primers from kit OPA of re-isolates from two different bio-insecticides (Mycotal® and Vertalec®) based on the entomopathogenic fungus *V. lecanii* indicated genetic

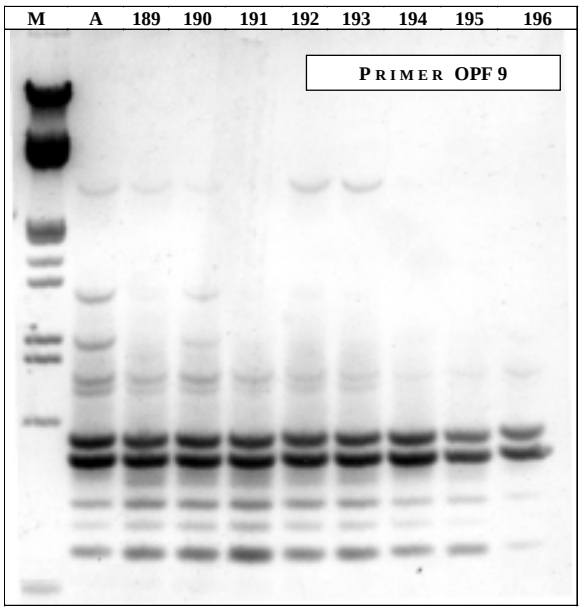


Fig. 2. Stability of the strain specific RAPD pattern demonstrated by several strains of *P. fumosoroseus* (M = marker, A = comparative stock strain, 189–196 all re-isolates from commercial batches of PFR 97™20%WDG (see Table 1)

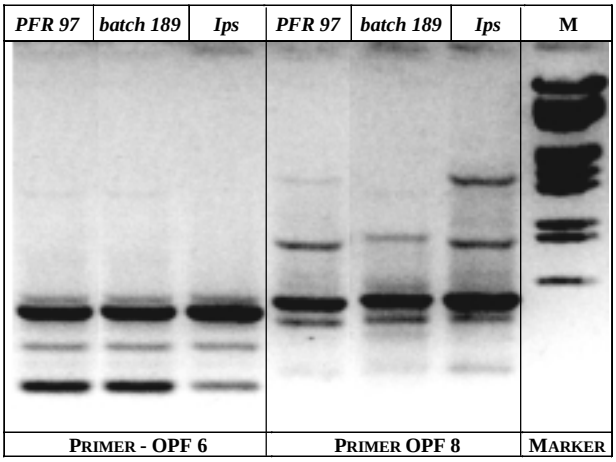


Fig. 3. RAPD pattern of *P. fumosoroseus* strain PFR 97 Apopka compared with patterns of isolates from standard biopreparation (batch 189) and from infected adult of spruce bark beetle *Ips typhographus* (Ips)

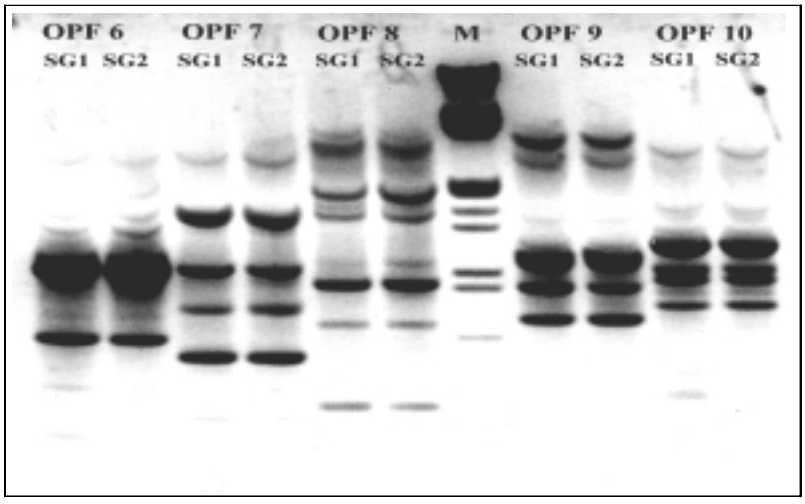


Fig. 4. Stability of the strain-specific RAPD pattern demonstrated by two re-isolates of *G. virens* from different batches of Soil-Gard™12G (SG1 = isolated in 1996, SG2 = isolated in 1998, M = marker)

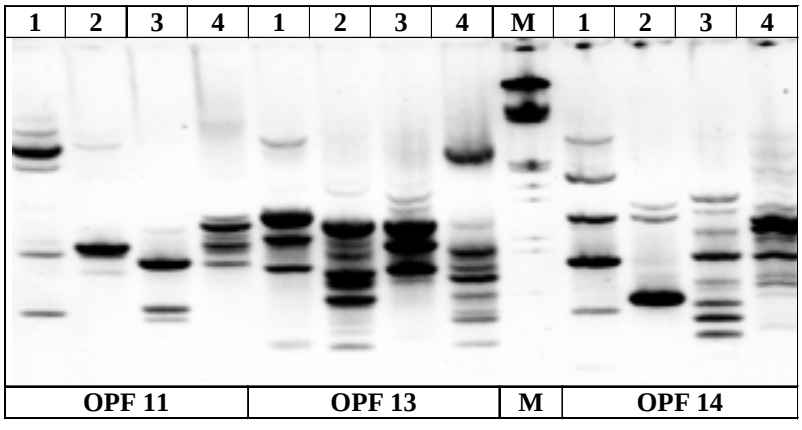


Fig. 5. Comparison of RAPD patters generated by mycoparasitic fungi of the genus *Gliocladium* (1 = *G. virens* GL 21, 2 = *Gliocladium* sp. CCF 783, 3 = *G. virens* CCM 8042, 4 = *G. roseum* CCM 8070)

Table 4. Specific fingerprint of *Gliocladium virens*, strain GL 21, generated by a selected set of primers, kit OPF

Primer	OPF6	OPF7	OPF8	OPF9	OPF10	OPF11	OPF13	OPF14	OPF15	OPF16
Size of amplified fragments (bp)	2524	3152	3246	2831	2317	2977	2793	2601	1650	3116
	1903	2480	2787	2297	2005	2406	1168	1663	1030	2249
	1565	1387	2592	1381	1573	2036	1114	1116	746	1434
	1358	941	1789	1073	1293	1612	943	1044	700	1355
	1018	670	1488	951	1039	1260	872	746	580	984
	929	436	1032	915	927	866	705	595	494	898
	859	402	870	784	734	771	335	472	345	810
	553	226	798	691	619	696		270		728
	305		621	354		641				670
	175		379	200		488				617
			314	275		442				554
			181			472				229

identity of both isolates (Fig. 7). On the other hand, obvious differences between various isolates of *V. lecanii* were discovered when the RAPD pattern was completed with only a single 10-mer primer OPF 11 (Fig. 8).

DISCUSSION

A range of biopesticides based on filamentous fungi is commercially available (MENN & HALL 1999). The regulatory environment has been generally favorable to fungal-based biopesticides, but recently new data requirements for bio-pesticide products were established, with special emphasis on the identification and detailed description of the fungal strain as an active ingredient of a particular biopesticide (NEALE & NEWTON 1999). Current legislation requires precise environmental and epizootiologial information even before none-native fungi are released, including when used for research purposes only (JEN-

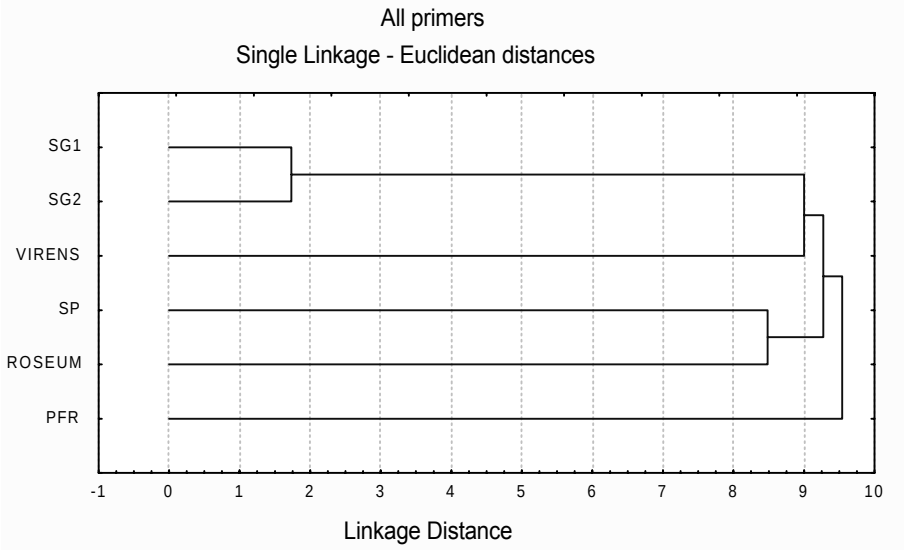
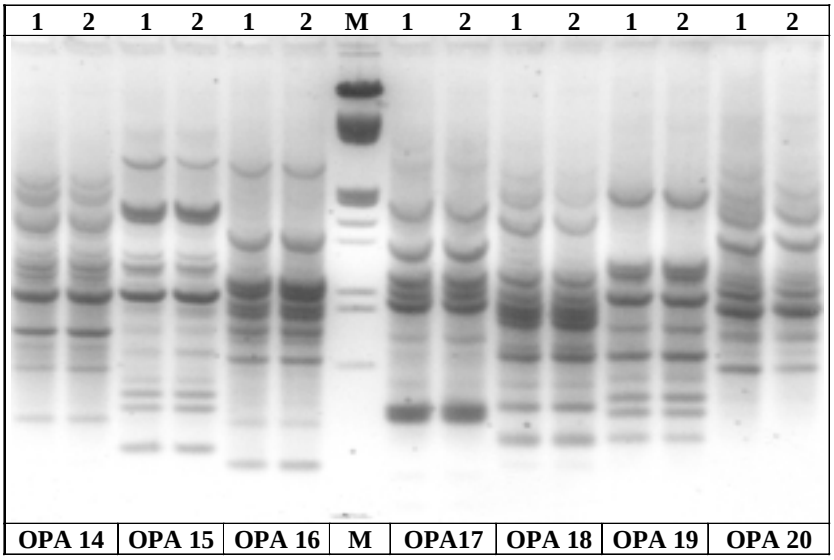


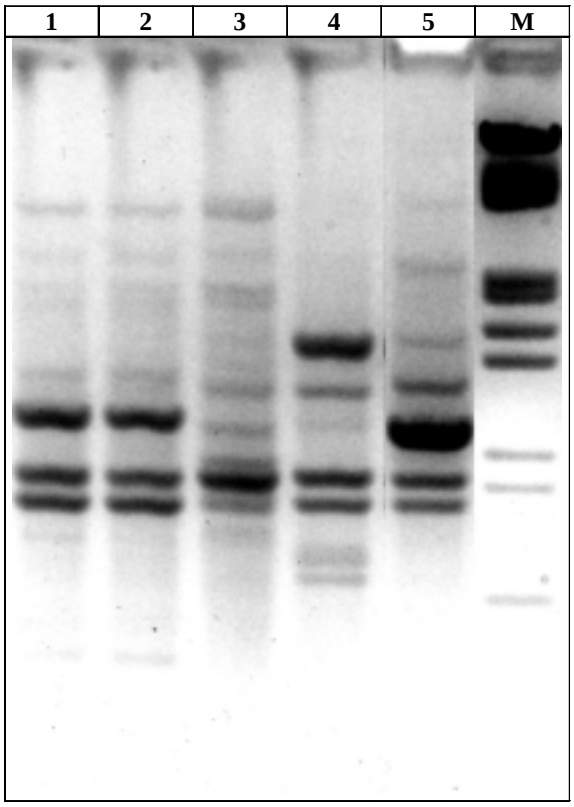
Fig. 6. Distribution of strains of mycoparasitic fungi of the genus *Gliocladium* within a dendrogram based on RAPD patterns[1]

[1]Patterns generated by primers from kit OPF-6, 7, 8, 11, 13, 15 & 16 (SG1 = *G. virens* GL21, SG2 = *G. virens* GL21, VIRENS = *G. virens* CCM 8042, SP = *Gliocladium* sp. CCF 783, ROSEUM = *G. roseum* strain CCM 8070 and PFR = *P. fumosoroseus* PFR 97 Apopka; for details see Table 1)



1 = strain isolated from microbial insecticide Mycotal, 2 = strain isolated from microbial insecticide Vertalec; M = marker

Fig. 7. Comparison of RAPD patterns for two strains of *Verticillium lecanii*



1 = Mycotal, 2 = Vertalec, 3 = CCEF 111, 4 = CCEF102, 5 = CCEF113, M = marker, see also Table 1

Fig. 8. Comparison of RAPD patterns of several isolates of *Verticillium lecanii* generated by primer OPF11

KINS & GRZYWACZ 2000). Besides, once selected for development and utilisation as a microbial control agent, the fungal isolate must remain stable and be clearly identifiable at the subspecies level. This level of identification is particularly important as it provides a mechanism for tracking the progress and fate of the agent in the environment, and to validate the purity and identity of the registered formulations and a standard reference that may be used to register or protect an individual isolate (JENKINS & GRZYWACZ 2000).

The standard RAPD method for evaluation of the fungi *Paecilomyces fumosoroseus* (TIGANO-MILANI *et al*. 1995a; OBORNÍK *et al*. 1997) and *Gliocladium virens* (LEXOVÁ *et al*. 1998) was modified with special attention to its routine and easy use. This procedure enables precise characterisation and identification of individual strains and eliminates problems with lower reproducibility of RAPD patterns or problematic interpretation of complex banding patterns as reported by WILLIAMS *et al*. (1993), HARDYS *et al*. (1992), SAMEC (1993), BACKELJAU *et al*. (1995), TIGANO-MILANI *et al*. (1995b) and MCDONALD (1997). Optimiaation of reaction conditions and selection of suitable primers overcame those disadvantages. RAPD products generated by each particular primer were highly reproducible and no variation was found in reactions from different DNA extractions. Also, precise digital processing of electrophoresis gels is necessary to avoid problems with reproducibility of the obtained data.

RAPD patterns of *P. fumosoroseus* isolates obtained from commercial batches A33, 119 and 178 showed no

Table 5. Specific fingerprint of *Verticillium lecanii*, strain re-isolated from MYCOTAL®, generated by selected sets of primers

Primer	OPA 6	OPA 8	OPA 15	OPA 17	OPA 19	OPB 12	OPB 14	OPB 16	OPB 18	OPF 9	OPF 16
Size of amplified fragments (bp)	2326	1510	2650	1673	1885	2855	1844	1762	2033	2456	2981
	1591	1300	1717	1224	1115	1904	1670	1369	1341	1609	2011
	1374	1117	1635	1002	1057	1296	1490	1296	1233	1343	1488
	1277	1046	1219	922	885	1041	1410	1233	1016	1137	1205
	1147	907	1115	842	733	903	1150	1173	899	1052	1128
	1041	721	1077	679	597	801	1131	944	788	747	927
	888	619	976	601	506	617	948	675	401	676	819
	851	573	905	482	430	560	907	593	342	581	783
	706	466	836	383	380	440	778		226	458	
	617	438	724	363	298	333	692			396	
	537	388	631	199	1885	322	466			301	
	415	355	538	138		148	423				
	320	271	495								
	237		441								
	144		392								
			268								

differences; all were identical with the pattern of the original stock strain PFR 97 Apopka. There were also no differences between the RAPD pattern of PFR 97 Apopka and those of isolates obtained from eight commercial batches (coded 189–196). Likewise, RAPD analysis did not reveal any polymorphism among isolates of *P. fumosoroseus* that had been used as active ingredients of commercial batches of PFR™20%WDG produced and distributed from 1995 to1998. These results confirmed the genetic stability of strain PFR 97 Apopka, because no significant changes in genome structure appeared during its long-term processing and use. Nevertheless, RAPD analysis enabled to detect and differentiate even negligible changes in the genome of PFR 97 Apopka. Distinct changes in RAPD patterns had been induced by 10 consecutive passes through a natural host, the two spotted spider mite *Tetranychus urticae* (Acarina, Tetranychidae) (unpublished data). It demonstrates that RAPD analysis is sufficiently sensitive to distinguish changes in genome structure caused by simple manipulation with the strain. On the other hand, no changes in RAPD fingerprint were detected after passage of PFR 97 Apopka through adults of spruce bark beetle *I. typographus*, re-isolated and purified through sub-culturing on PDA and compared with the original stock strain. This demonstrates the possibility to take advantage of the RAPD technique as a suitable tool to evaluate the genetic stability of strains after having been applied in and re-isolated from the ecosystem (JENKINS & GRZYWACZ 2000).

RAPD analysis of various isolates of GL 21 (*G. virens*) proved the genetic stability of this strain and found no changes in genome structure that might be related with long-term maintenance. Similar to other fungal bio-control agents (AVIS *et al*. 2001), RAPD markers could be recommended to monitor the genetic and environmental fate of GL 21 as the active ingredient of commercial biofungicides. In addition, the genetic assessment of fungi of the genus *Gliocladium* confirmed the possibility to use the RAPD technique to differentiate and identify these fungi even to the level of individual isolates, which is a precondition for both detecting the origin of a particular isolate and monitoring its fate in the environment.

Finally, the study aimed to compare two strains of *V. lecanii* confirmed data that had already been published earlier (MOR *et al*. 1996) about the genetic identity of *V. lecanii* strains which are used as active ingredients in two different bio-insecticides. RAPD analysis proved a very close relationship between re-isolates from the bio-insecticides MYCOTAL® and VERTALEC®, and clearly visible and detectable differences between these isolates and other strains of *V. lecanii*. Again, this level of identification seems particularly important as it provides a mechanism to validate the purity of a fungal strain in a formulation and that can be used as standard reference to register or protect an individual fungal isolate (JENKINS & GRZYWACZ 2000).

DNA polymorphism can be revealed by various molecular techniques, with some of them being even more sensitive (e.g. AFLP, RFLP, sequencing). Nevertheless, differentiation of fungal strains and species by RAPD certainly is an easy tool to detect polymorphism in a large number of samples at relatively low cost, and may be

routinely used as an obligate parameter for registration, quality control and to study the fate of a particular agent in the environment.

References

AVIS T.J., HAMELIN R.C., BELANGER R.R. (2001): Approaches to molecular characterization of fungal biocontrol agents: some case studies. Can. J. Plant Pathol., **23**: 8–12.

BACKELJAU T., DE BRUIN L., DE WOLF H., JORDAENS S.K., VAN DONGEN S., VERHAGEN R., WINNEPENNINIK X.B. (1995): Random amplified polymorphic DNA (RAPD) and parsimony methods. Cladistic, **11**: 119–130.

BIDOCHKA M.J., ST. LEGER R.J., ROBERTS D. (1994): Differentiation of species and strains of entomopathogenic fungi by random amplification of polymorphic DNA (RAPD). Curr. Genet., **25**: 107–113.

BRIDGE P.D., ARORA D.K. (1998): PCR for species definition. In: BRIDGE P.D., ARORA D.K., REDDY C.A., ELANDER R.P. (eds.): Application of PCR in Mycology. CAB Int., Wallingford: 63–84.

CAETANO-ANNOLES G., BASSAM B.J., GRESSUOFF P.M. (1991): DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. Biotechnology, **9**: 553–557.

CASTLE A., SPERANZINI D., RGHEI N., ALM G., RINKER D., BISSETT J. (1998): Morphological and molecular identification of *Trichoderma* isolates on North American mushroom farms. Appl. Envir. Microbiol., **64**: 133–137.

CHARNLEY A.K. (1989): Mycoinsecticides – present use and future prospects. In: Progress and Prospects in Insect Control. BCPC Monograph No. 43: 165–181.

EDEL V. (1998): PCR in mycology – an overview. In: BRIDGE P.D., ARORA D.K., REDDY C.A., ELANDER R.P. (eds.): Application of PCR in Mycology. CAB Int., Wallingford: 1–20.

EYAL J., MABUD A., FISCHBEIN K.L., WALTER J.F., OSBORNE L.S., LANDA Z. (1994): Assessment of *Beauveria bassiana* Nov. EO-1 Strain, which produces a red pigment for microbial control. Appl. Biochem. Biotechnol., **44**: 65–80.

FUKATSU T., SATO H., KURIYAMA H. (1997): Isolation, inoculation to insect host, and molecular phylogeny of an entomogenous fungus, *Paecilomyces tenuipes*. J. Invertebr. Pathol., **70**: 203–208.

HARDYS H., BALICK M., SCHIERWATER M. (1992): Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Molec. Ecol., **1**: 55–63.

HEGEDUS D.D., KHACHATOURIANS G.G. (1996): Identification and differentiation of the entomopathogenic fungus *Beauveria bassiana* using Polymerase Chain Reaction and Single-Strand Conformation Polymorphism Analysis. J. Invertebr. Pathol., **67**: 289–299.

HUMBER R.A. (1997): Fungi – Identification. In: LACEY L.A. (ed.): Manuals of Techniques in Insect Pathology. Acad. Press. Chapter V-1: 153–185.

JENKINS N.E., GRZYWACZ D. (2000): Quality control of fungal and viral biocontrol agents – assurence of product perfomance. Biocontrol Sci. Technol., **10**: 753–777.

LEXOVÁ L., DĚDIČOVÁ L., LANDA Z., ČURN, V. (1998): Evaluation of RAPD technique for identification and characterization of *Gliocladium virens* isolates. In: Sbor. ZF JU – fytot., 15, 2: 25–39.

MCDONALD B.A. (1997): The population-genetics of fungi – tools and techniques. Phytopathology, **87**: 448–453.

MENN J.J., HALL F.R. (1999): Biopesticides: Present Status and Future Prospects. In: HALL F.R. & MENN J.J. (eds.): Methods in Biotechnology. Vol. 5: Biopesticides – Use and delivery. Humana Press Inc., Totowa, NJ. Chapter 1: 1–10.

MOR H., GINDIN G., BEN-ZE'EV I.S., RACCAH B., GESCHTOVT N.U., AJTKHOZHINA N., BARASH I. (1996): Diversity among isolates of *Verticillium lecanii* as expressed by DNA polymorphism and virulence towards *Bemisia tabaci*. Phytoparasitica, **24**: 111–118.

NEALE M., NEWTON P. (1999): Registration – Regulatory Requirements in Europe. In: HALL F.R. & MENN J.J. (eds.): Methods in Biotechnology. Vol. 5: Biopesticides – Use and Delivery. Humana Press Inc., Totowa, NJ. Chapter 24: 453–471.

OBORNÍK M., LANDA Z., ČURN V. (1997): Molecular characterization of *Paecilomyces fumosoroseus* strain 97 by use of RAPD markers. In: Sbor. JCU ZF, XIV, 2: 49–55.

OSBORNE L.S., LANDA Z. (1992): Biological control of whiteflies with entomopathogenic fungi. Florida Entomol., **75**: 456–471.

SAMBROOK J., FRITSCH E.F., MANIATIS T. (1989): Molecular Cloning: A Laboratory Manual. 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

SAMEC P. (1993): DNA polymorphism and RAPD technology. Genet. a Šlecht., **4**: 291–320.

SAMSON R.A. (1974): *Paecilomyces* and some allied *Hyphomycetes*. Stud. Mycology, **6**: 1–119.

SAMSON R.A. (1981): Identification – Entomopathogenic Deuteromycetes. In: BURGES H.D. (ed.): Microbial Control of Pests and Plants Diseases 1970–1980. Acad. Press, London: 93–106.

SAMŠIŇÁKOVÁ A., KÁLALOVÁ S., FASSATIOVÁ O. (1983): Morfologické srovnání některých entomofágních hub rodů *Beauveria*, *Paecilomyces*, *Tolypocladium* a *Culicinomyces*. Ochr. Rostl., **19**: 195–204.

ST LEGER R.J., JOSHI L. (1999): The application of molecular techniques to insect pathology with emphasis on entomopathogenic fungi. In: LACEY L.A. (ed.): Manuals of Techniques in Insect Pathology. Academic Press. Chapter VIII-3: 365–394.

TIGANO-MILANI M.S., HONEYCUTT R.J., LACEY L.A., ASSIS R., MCCLELLAND M., SOBRAL B.W.S. (1995a): Genetic variability of *Paecilomyces fumosorose*us isolates revealed by molecular markers. J. Invertebr. Pathol., **65**: 274–282.

TIGANO-MILANI M.S., SAMSON R.A., MARTINS I., SOBRAL B.W.S. (1995b): DNA markers for differentiating isolates of *Paecilomyces lilacinus*. Microbiology, **141**: 239–245.

VAKALOUNAKIS D.J., FRAGKIADAKIS G.A. (1999): Genetic diversity of *Fusarium oxysporum* isolates from cucumber: differentiation by pathogenicity, vegetative compatibility, and RAPD fingerprinting. Phytopathology, **89**: 161–168.

WILLIAMS J.G.K., HANAFEY M.K., RAFALSKI J.A., TINGEY S.V. (1993): Genetic analysis using random amplified polymorphic DNA markers. Methods Enzymol., **218**: 704–741.

WILLIAMS J.G.K., KUBELIK A.R., LIVAK K.J., RAFALSKI J.A., TINGEY S.V. (1990): DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., **18**: 6531–6535.

Recieved for publication August 1, 2001
Accepted for publication December 10, 2001

Souhrn

BIELIKOVÁ L., LANDA Z., OSBORNE L.S., ČURN V. (2002): **Characteristika a identifikace entomopatogenních a mykoparazitických hub RAPD-PCR technikou**. Plant Protect. Sci., **38**: 1–12.

Pomocí techniky RAPD byla hodnocena genetická stabilita vybraných kmenů entomopatogenních a mykoparazitických hub. Specifický RAPD fingerpint byl zkonstruován pro *Paecilomyces fumosoroseus* kmen PFR 97 Apopka, *Gliocladium virens* kmen GL 21 a *Verticillium lecanii* kmen Mycotal. Genetická stabilita a homogenita kmenů byla demonstrována pomocí analýzy reizolátů získaných z komerčních šarží biopreparátů PFR 97™20%G a SoilGard™12G, které byly vyprodukovány v rozmezí let 1995–1999. RAPD analýza prokázala genetickou identitu kmenů *V. lecanii* reizolovaných ze dvou různých bioinsekticidů – Mycotal® a VERTALEC®. Praktické využití techniky RAPD bylo demonstrováno pomocí aplikovaných studií, ve kterých byl kmen *Paecilomyces fumosoroseus* PFR 97 Apopka spolehlivě identifikován i po pasáži přes přirozeného hostitele (dospělci lýkožrouta smrkového *Ips typographus*) a při identifikaci kmene GL 21 (*G. virens*) v sadě kmenů hub rodu *Gliocladium*.

Klíčová slova: *Gliocladium* spp.; *Gliocladium virens*; *Paecilomyces fumosoroseus*; *Verticillium lecanii*; entomopatogenní houby, mykoparazitické houby; RAPD-PCR

Corresponding author:

Prof. Ing. ZDENĚK LANDA, CSc., Jihočeská univerzita v Českých Budějovicích, Zemědělská fakulta, Katedra rostlinné výroby – oddělení ochrany rostlin, Studentská 13, 370 05 České Budějovice, Česká republika
tel. & fax: + 420 38 777 24 45, e-mail: zlanda@zf.jcu.cz