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Assessment of isolates of entomogenous fungi collected from spruce bark beetle *Ips typographus* L. (Coleoptera, Scolytidae) in the Bohemian Forest

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Abstract
Entomogenous fungi *Beauveria bassiana*, *Paecilomyces farinosus*, *P. fumosoroseus*, and *Verticillium lecanii* were among the most frequently collected fungi from adults of *Ips typographus* in the Šumava National Park. New isolates were assessed and compared with comparative strains using standard *in vitro* and *in vivo* bioassays. Also, RAPD fingerprint was generated and analyzed within selected set of newly collected isolates. All isolates that have been assessed were completing developmental cycle on the solid media or in liquid artificial media and produced viable conidia that were virulent when tested on alternative insect host. RAPD analysis clustered all isolates of *B. bassiana* into one group that was distinctly separated from other fungi. Inside "*B. bassiana* group" separation of non-endemic strain BBA-M was clearly visible. RAPD analysis enables to distinguish *B. bassiana* isolates collected in the Bohemian Forest habitats from non-endemic isolates with values of similarity coefficients among isolates from Bohemian Forest and comparative non-endemic strain BBA-M ranging from 0.219–0.278.

Key words: Ips typographus, spruce bark beetle, entomogenous fungi, *Paecilomyces fumosoroseus*, *Paecilomyces farinosus*, *Beauveria bassiana*, *Verticillium lecanii*, bioassay, RAPD analysis

Introduction

Different species of the entomogenous fungi are known as fungal pathogens of the spruce bark beetle *Ips typographus* L. (Coleoptera, Scolytidae). Among many, entomogenous fungi *Beauveria bassiana* (Bals.) Vuill., *Paecilomyces farinosus* (Holm; Fr.) A.H.S. Brown et G. Smith, and *Paecilomyces fumosoroseus* (Wize) A.H.S. Brown et G. Smith (Deuteromycotina, Hyphomycetes) were recorded as primary pathogens that are naturally associated with the adults of *I. typographus*. (Vaupel & Zimmermann 1996). However, some other entomogenous fungi have been recorded as pathogenic to various species of scolytid beetles including species of the genera *Beauveria*, *Metarhizium*, *Paecilomyces*, *Tolyplocladium*, and *Verticillium* (Doberski 1981a, 1981b, Houle & al. 1987). Recently, nine species of entomogenous fungi were discovered as associated with spruce bark beetle at different sites in the Bohemian Forest (Landa & al. 2001). *Verticillium lecanii* (Zimm.) Viégas was the most frequent pathogen associated with the adults caught with pheromone traps, while *B. bassiana*, *P. farinosus*, and

P. fumosoroseus were among the most frequently collected fungi from spruce bark beetle adults in the bark. All above are polyphagous, geographically widespread fungi, which create large amount of various strains (GOETTEL & INGLIS 1997). Large differences in host range, pathogenicity, and characteristics such as germination, sporulation, growth rate and enzyme production have been demonstrated for most of entomogenous fungi (JENKINS & GRZYWACZ 2000). Strain selection, mutation, and genetic manipulation studies to combine and enhance characteristics important to epizootic potential are therefore fundamental to the future development of entomogenous fungi for insect control (DRUMMOND & HEALE 1988, ST LEGER & JOSHI 1999). Besides, genetic variability of various entomogenous fungi revealed by molecular markers is reported (e.g. TIGANO-MILANI & al. 1995, OBORNÍK & al. 1995, LEXOVÁ & al. 1998). Different methods are used to evaluate the individual isolates of entomogenous fungi. This study was aimed to assess various isolates of entomogenous fungi, which were collected from naturally infected adults of *I. typographus* and compare some characteristics of those isolates with comparative strains of the same species.

MATERIAL AND METHODS

Fungal Isolates

Beauveria bassiana isolate BBA-M (re-isolated from microbial insecticide MYCOTROL®, Mycotech Corporation, MT, USA); *Paecilomyces farinosus* isolate PFA-CCM (item 8023, Czech Collection of Microorganisms, Faculty of Science, Masaryk University Brno, CR); *Paecilomyces fumosoroseus* strain PFR 97 (ATTC 20874, stock culture from Mid-Florida Research & Educational Center, University of Florida, FLA, USA) and Batch-189 (re-isolate from microbial insecticide PFR 97™ 20%WDG, Thermo Trilogy Corp., MD, USA); and *Verticillium lecanii* strains VLE-M (re-isolate from microbial insecticide MYCOTAL®, Koppert B.V., the Netherlands) and Vle 112 (Collection of Entomogenous fungi, Faculty of Agriculture, University of South Bohemia, České Budějovice, CR) were used as the comparative strains. All other isolates (13) that were used in this study were collected from naturally infected adults of spruce bark beetle at various sites of the Šumava National Park during 1999 (LANDA & al. 2001) (Table 1).

Fungal Maintenance

Stock cultures were maintained as dry alginate pellets that were formulated by mixing biomass of submerged cultures of monospore isolates with sterile wheat bran and aqueous sodium alginate (EYAL & al. 1994). Dry pellets were stored in plastic vials (approx. 400–500 pellets per strain) in freezer (–23±1°C). For experiments, all strains were maintained using two-step procedure. First, alginate pellets were aseptically plated on a surface of 2% water agar in sterile Petri dishes and cultured at 25°C until adequate superficial growth and sporulation of fungi was observed (4–5 days). Activated pellets were soaked in sterile 0.05% Tween 80 solution to obtain basic conidial suspension which was successively used either as inoculum for submerged cultures or to inoculate surface of solid media to produce surface cultures.

In vitro bioassays

All fungi were maintained on potato dextrose agar (PDA, Difco). Cultures were prepared by depositing drop of conidial suspension with sterile inoculating loop at the center of PDA surface in a sterile petri dish (90×15 mm). When inoculated, cultures were placed into air-conditioned room and incubated at 25±1.0°C with photoperiod 12/12 for 14 days. Ten cultures have been prepared for each tested fungal isolate. Radial growth of each culture was measured

Table 1. – Specification, origin and source of fungal isolate

Species	Isolate	Origin, site	Source
Beauveria bassiana	Bba 6	BF1, Modrava	*I. typographus*, adult
B. bassiana	Bba 7	BF, Strážný	*I. typographus*, adult
B. bassiana	Bba 11	BF, Kvilda	*I. typographus*, adult
B. bassiana	Bba 12	BF, Kvilda	*I. typographus*, adult
B. bassiana	Bba 13	BF, Modrava	*I. typographus*, adult
B. bassiana	Bba 14	BF, Modrava	*I. typographus*, adult
B. bassiana	BBA-M 2	USA	Mycotrol®
Paecilomyces farinosus	Pfa 1	BF, Modrava	*I. typographus*, adult
P. farinosus	Pfa 5	BF, Modrava	*I. typographus*, adult
P. farinosus	Pfa 8	BF, Modrava	*I. typographus*, adult
P. farinosus	PFA-CCM 3	Czech Republic	CCM 8023
Paecilomyces fumosoroseus	Pfr 2	BF, Modrava	*I. typographus*, adult
P. fumosoroseus	Pfr 4	BF, Stožec	*I. typographus*, adult
P. fumosoroseus	Pfr 10	BF, Kvilda	*I. typographus*, adult
P. fumosoroseus	PFR 97 4	USA	stock culture
P. fumosoroseus	Batch 189 2	USA	PFR97™ 20% WDG
Verticillium lecanii	Vle 9	BF, Kvilda	*I. typographus*, adult
V. lecanii	VLE-M 2	the Netherlands	Mycotal®
V. lecanii	Vle-112[5]	Czech Republic	CEF – 120

[1] BF – Bohemian Forest, National Park, Czech Republic
[2] Re-isolated from sample of commercial biopreparation
[3] Czech Collection of Microorganisms (CCM), Faculty of Science, Masaryk University Brno, Czech Republic
[4] Thermo Trilogy Corporation, Columbia, Maryland, USA – stock culture for bio-insecticide PFR97™ 20% WDG
[5] Collection of Entomogenous Fungi, Department of Plant Production, Faculty of Agriculture, University of South Bohemia, České Budějovice, Czech Republic

(1.0-mm accuracy) and results were expressed as mean diameter of 14 days old cultures.

Spore viability was determined after a conidial suspension was obtained by rinsing the PDA plates with 0.05% Tween 80 and the suspension was poured into sterile glass tube and homogenized on a vortex mixer. All samples were properly adjusted to the final concentration of 5.0×10^6 conidia/ml after concentration was determined using a hemacytometer (Improved Neubauer). Spore viability was assayed in a standard germination test. For this purpose, ten drops of adjusted suspension were placed on surface of sterile microscope slide coated with a thin layer of 2.0% water agar and incubated for 16 (% germination) and 24 (mean GI) hours at 25°C. Percentage germination was determined by viewing a minimum of 200 conidia using compound microscope. The numbers of viable (germ tube present) and nonviable (no germ tube present) spores were counted after 16 hours of incubation. Additionally, status of each fungal isolate was assayed again after 24 hours. The second assessment involved evaluation of the status of development of each individual conidia using rating scale ranging from 0 (none activity) to 3.0 (full sporulation) with intermediate values – 0.5 (for details see Landa & al. 1994 and Fig. 1.).

In vivo bioassay

The standard laboratory bioassay (Landa & al. 1994) involved placing drops of adjusted

Table 2. – Main *in vitro* characteristics of comparative strains and isolates of entomogenous fungi collected from naturally infected adults of *Ips typographus*.

Species	Isolate	Radial growth (mm ± STDV)	Spores/culture	Germination (%)	Mean GI ± STDV
B. bassiana	Bba 6	32.3 ± 1.14	7.14 × 108	83.5	0.71 ± 0.456
B. bassiana	Bba 7	53.7 ± 3.10	2.98 × 108	63.8	0.46 ± 0.387
B. bassiana	Bba 11	41.8 ± 1.55	5.88 × 108	91.1	0.84 ± 0.531
B. bassiana	Bba 12	39.7 ± 2.02	8.63 × 108	100	2.00 ± 0.000
B. bassiana	Bba 13	35.8 ± 2.81	6.20 × 108	95.4	0.98 ± 0.509
B. bassiana	Bba 14	49.5 ± 2.46	5.49 × 108	84.1	0.48 ± 0.260
B. bassiana	BBA-M	42.2 ± 2.85	7.53 × 108	100	1.76 ± 0.112
P. farinosus	Pfa 1	51.6 ± 4.18	6.12 × 108	45.3	0.37 ± 0.459
P. farinosus	Pfa 5	49.1 ± 2.85	4.10 × 108	97.2	1.06 ± 0.347
P. farinosus	Pfa 8	42.3 ± 3.56	5.08 × 108	100	1.47 ± 0.422
P. farinosus	PFA-CCM	58.8 ± 2.16	3.88 × 108	92.7	0.88 ± 0.365
P. fumosoroseus	Pfr 2	55.6 × 2.08	7.88 × 108	100	2.00 ± 0.000
P. fumosoroseus	Pfr 4	49.5 × 1.80	6.54 × 108	89.3	0.89 ± 0.464
P. fumosoroseus	Pfr 10	50.3 × 2.45	7.70 × 108	84.1	0.71± 0.438
P. fumosoroseus	PFR 97	57.8 × 1.15	1.54 × 109	100	1.83 ± 0.133
V. lecanii	Vle 9	46.9 ± 0.65	1.20 × 109	100	2.13 ± 0.188
V. lecanii	VLE-M	43.8 ± 1.39	5.82 × 108	92.2	1.08 ± 0.464

Table 3. – Similarity matrix of analyzed isolates of entomogenous fungi – Nei & Li similarity coefficient (randomly selected primers 1, 2, 5, 8, 10–13 from kit OPF).

Bba 6	1							
Bba 7	0.840	1						
Bba 12	0.927	0.840	1					
Bba 13	0.918	0.833	0.965	1				
BBA-M	0.247	0.278	0.219	0.237	1			
PFR 97	0.154	0.156	0.185	0.176	0.107	1		
Vle 9	0.135	0.137	0.162	0.156	0.031	0.175	1	
Vle 112	0.056	0.057	0.056	0.054	0.065	0.111	0.476	1
	Bba 6	**Bba 7**	**Bba 12**	**Bba 13**	**BBA-M**	**PFR 97**	**Vle 9**	**Vle 112**

conidial suspension on a sterile microscope slide (30 drops per slide, 10 drops in each of three rows running the length of each slide) using a sterile inoculating loop. Early 4th instar nymphs of greenhouse whitefly *Trialeurodes vaporariorum* Westwood (Homoptera, Aleyrodidae) were placed in the middle of each conidial drop (1 nymph per drop) and a total of 25 nymphs were placed on one slide. Five drops were left without nymphs to serve as a control of contamination. Each single slide with nymphs was placed in a wet chamber (sterile plastic petri dish with wet filter paper on bottom). Wet chambers were placed into growth chamber and incubated for 7 days at 25°C under constant light. Each fungal isolate was assayed based on 4 wet chambers (total of 100-nymphs per tested isolate). Development of fungal isolates was recorded daily within a period of 4 days. The rating system was used to assess the de-

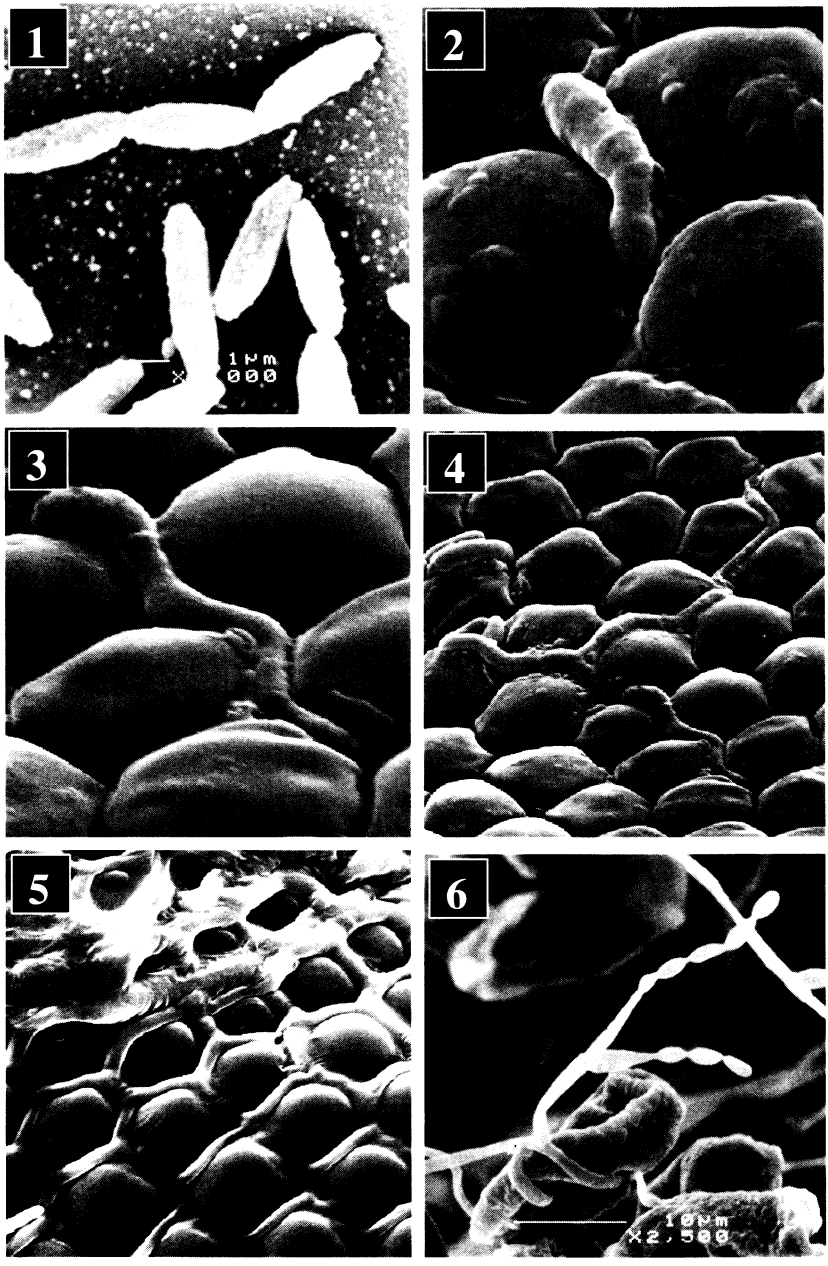

Fig 1. – Evaluation of development of entomogenous fungi: FDI 0 – dormant conidia (1), FDI 0.5 – germinating conidium (2), FDI 1 – primary germ tube (3), FDI 1.5 – presence of secondary branches forming mycelium (4), FDI 2 – superficial growth of mycelium (5), and FDI 3 – sporulation (demonstrated with entomogenous fungus *Paecilomyces fumosoroseus*, SEM microscopy, photo by Lance S. Osborne & Z. Landa).

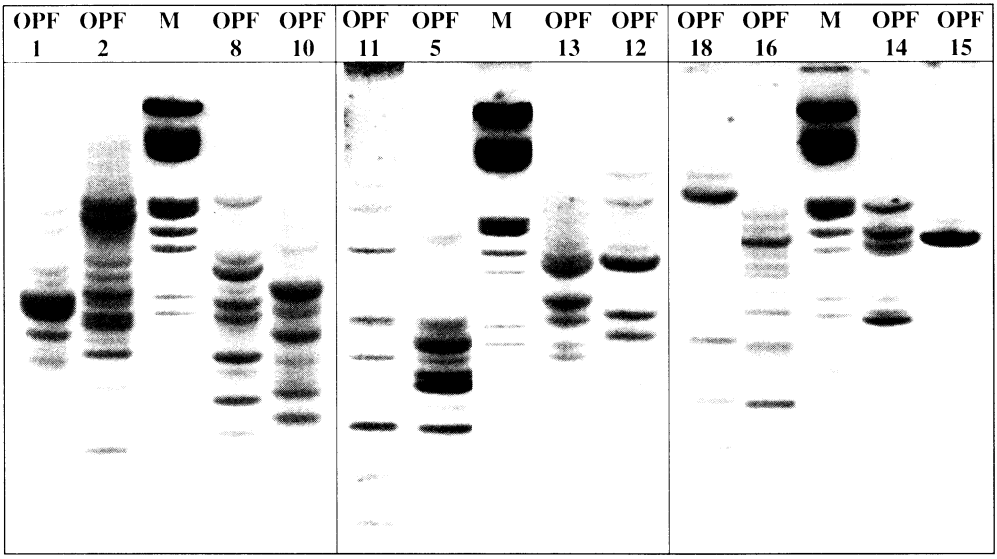

Fig. 2. – RAPD fingerprint of *Beauveria bassiana* strain Bba 6 collected from an adult of spruce bark beetle at Modrava site (M – marker) in the Šumava National Park .

gree of fungal development on the host. When assessed, each nymph was rated individually under compound microscope and the stage of fungus development on the nymph was recorded according to rating index ranging from 0 (none activity) to 3 (full sporulation of fungus on host surface) (LANDA & al. 1994). The results were expressed as a mean FDI value (± STDV) for all nymphs in treatment, separately for each day of bioassay.

Re-isolation of *P. fumosoroseus* from adult of spruce bark beetle *I. typographus*

Water suspension of microbial insecticide PFR 97™20%WDG – commercial batch PA 189 (2g/1000ml) was applied on surface of trap-tree (freshly chopped down spruce bark tree) at the Šumava National Park, site Modrava. Bark samples were cut off from treated trap-tree 3 weeks after application. Thereafter, the adults of spruce bark beetle *Ips typographus* with symptoms of mycosis were collected from bark, placed on surface of 2% water agar in Petri dish and maintained for 2–3 days until distinctive proliferation of fungus on host cuticle was evident. Then, *P. fumosoroseus* growing on infected adults was purified using one-two consequent isolations through PDA plates. (LANDA & al. 2001). Purified isolate was used to produce biomass in submerged culture and proceed for DNA extraction and RAPD analysis as described bellow.

DNA extraction and RAPD analysis

Submerged cultures were used to produce fungal biomass for DNA extraction. Sterile flasks (250 ml) filled with 100 ml of potato-dextrose-broth (PDB SIGMA) were inoculated with 10 ml of adjusted spore suspension and shaken continuously on laboratory rotary shaker (200 rpm, amplitude 5.25°C) for 5-days.

Modified method of DNA extraction (TIGANO-MILANI & al. 1995) was used in this study. Approximately 100 mg of biomass obtained from submerged culture was used for DNA extraction. Biomass was frozen at –20°C for 24 h and then ground in 1.5 ml microfuge tube

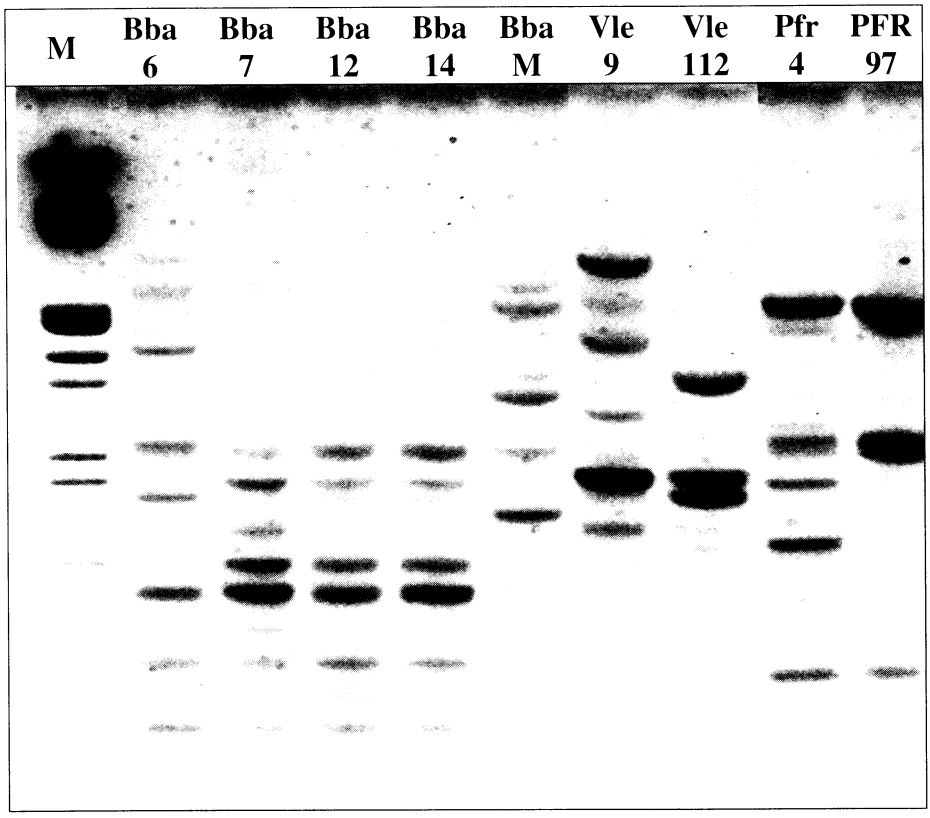

Fig. 3. – Comparison of RAPD patterns of various entomogenous fungi collected from *I. typographus* adults in the Šumava National Park (primer OPF 11, M – marker).

with sterile plastic stick. The homogenized mixture was suspended in 500 µl sterile lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 100 mM EDTA), then 50 µl 10% (w/v) SDS was added, and the tubes were shaken gently. After one hour at 37°C, 75 µl 5 M NaCl was added and mixed, 60-µl cetyltrimethylammonium bromide (CTAB) solution (10%, w/v, CTAB in 0.7 M 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 14000 rpm for 10 min were washed with 70% (v/v) sterile ethanol, dried and resuspended in 100 µl of sterile distilled water (Lexová & al. 1989). The extracted DNA was assessed on 1% TAE (Tris/Acetic Acid/EDTA) agarose gel stained with etidium bromide (Sambrook & al. 1989).

The RAPD reactions were performed in a 25 µl volumes of reaction mix: 0.2mM of each dNTPs (Promega), 0.5 µM primer (Operon Technologies), 1U Taq-polymerase (Finnzyme), 1 x polymerase recommended buffer (Finnzyme) and 1µl template DNA, using Termocycler PTC 1160 (MJ-Research). The temperature profile for all reactions was 92°C for 3 min in 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. Reaction products were checked by loading full reaction mixture onto 2% TAE agarose gel with ethidium bromide. Gels were photographed by Polar-

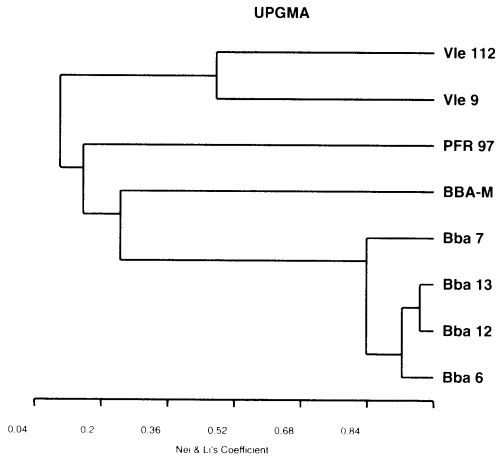

Fig 4. – Genetic distances of analysed strains determined from the RAPD analysis based on primers OPF 1, 2, 5, 8, 10, 11, 12 and 13.

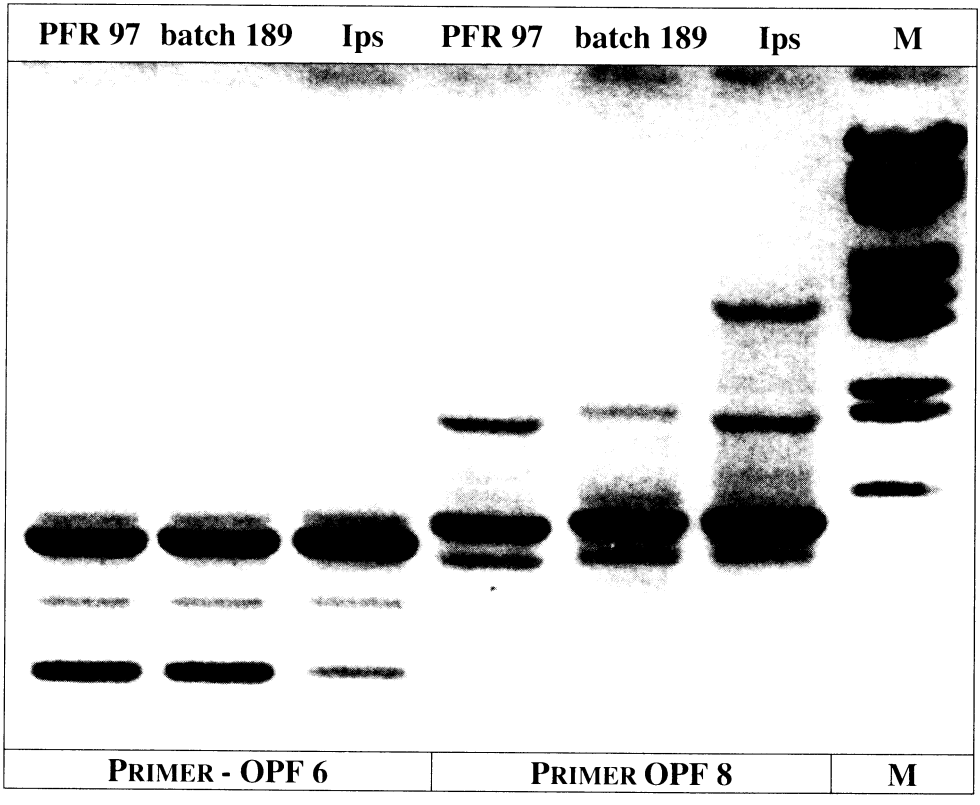

Fig. 5. – RAPD pattern of stock culture of *Paecilomyces fumosoroseus* strain *PFR 97* compared with patterns of isolates from bio-insecticide (batch 189) and from infected spruce bark beetle adult – *Ips typhographus* (*Ips*) (M – marker).

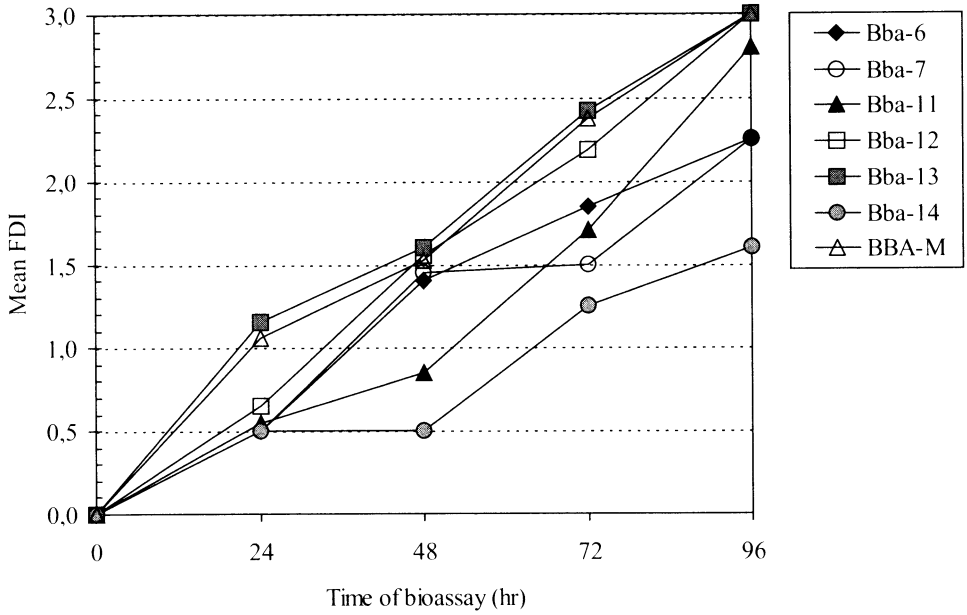

Fig. 6. – Development of comparative strain of *Beauveria bassiana* and strains collected from naturally infected adults of spruce bark beetle *Ips typographus*.

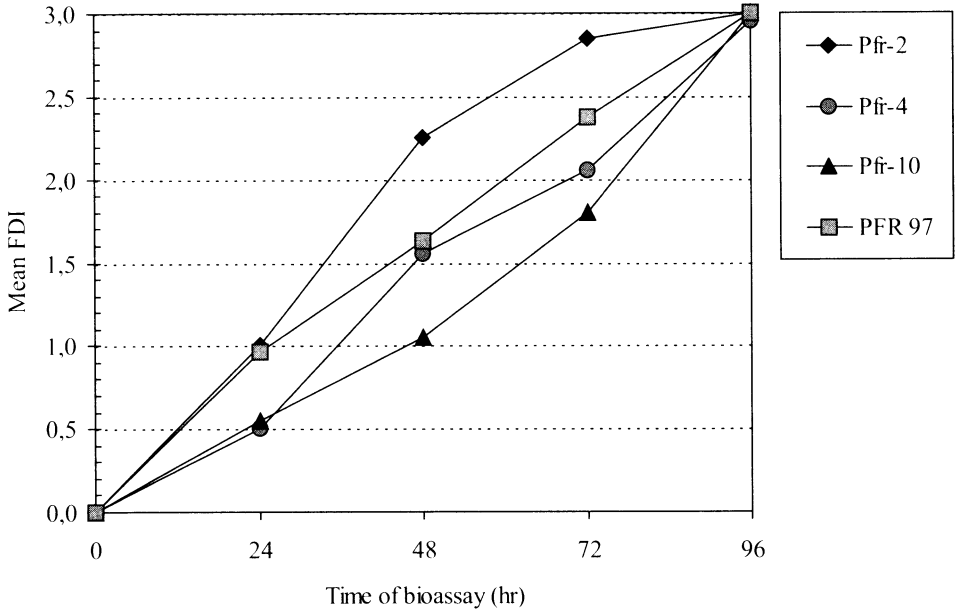

Fig. 7. – Development of comparative strain of *Paecilomyces fumosoroseus* and strains collected from naturally infected adults of spruce bark beetle *Ips typographus*.

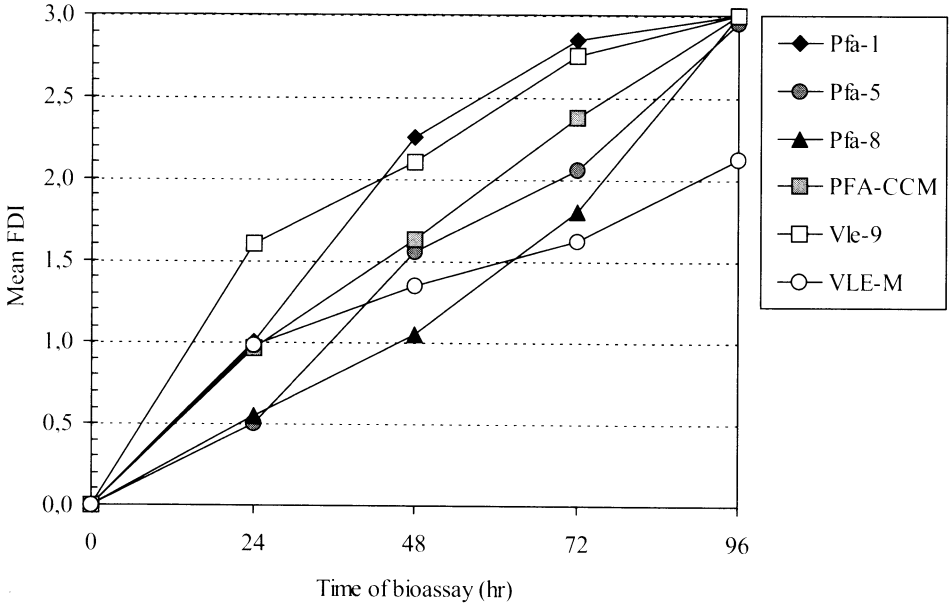

Fig. 8. – Development of comparative strains of *Paecilomyces farinosus* and *Verticillium lecanii* and strains collected from naturally infected adults of spruce bark beetle *Ips typographus*.

oid gel camera, the pictures were digitized (600 dpi, Adobe PhotoShop) and processed using Gel Manager for Windows (LEXOVÁ & al. 1998)

Preliminary genetic screening was done with set of twenty 10-mer primers (Operon Technologies, Kit OPF) that were used to characterize selected isolates (Bba 6, Bba 7, Bba 12, Bba 13 and Vle 9). The sizes of RAPD products were estimated using Lambda/EcoR I+Hind III marker (Promega). Genetic distances were generated based on binary characteristics that were revealed with eight randomly selected primers OPF 1, 2, 5, 8, and 11–13. Nei & Li similarity coefficients (ROSEWICH & al. 1999) were calculated from matrix of presence and absence of bands using statistical program MVSP 3.1. The matrix of similarity coefficients was then subjected to cluster analysis using the unweighted average linkage (UPGMA) algorithm.

RESULTS

All isolates of entomogenous fungi used in this study was easy to propagated as surface cultures on solid defined medium (PDA) and as submerged cultures in liquid medium (PDB). Evaluation of *in vitro* characteristic and results of *in vivo* laboratory bioassay indicate relevant differences among isolates in comparable group that has been restricted by fungal species. Among isolates of *B. bassiana* negative correlation between radial growth and sporulation was recorded. Most intensively growing isolates (Bba 7 and Bba 14) were also the less conidia producing one. Vice versa, small cultures creating isolates were the most conidia producing (Bba 6, Bba 12 and Bba 13). Comparative strain BBA-M showed moderate radial growth and high sporulation. Among all *B. bassiana*, two isolates – the comparative strain BBA-M (100 % germination, mean GI – 1.76) and newly collected isolate Bba 12 (100% germination, mean GI – 2.00) displayed very high viability. All other isolates performed signif-

icantly lower values of viability. Moreover, some newly collected isolates of *B. bassiana* (Bba 12 and Bba 13) performed also highly virulent when assessed based on FDI (Table 2, Fig. 6). Similarly, among *P. farinosus* isolates, two newly collected isolates (Pfa 5 and Pfa 8) performed as more and high viable conidia producing when compared with comparative strain PFA-CCM (Table 2, Fig. 8), while among isolates of *P. fumosoroseus* the comparative stock strain PFR 97 performed as best in all of assessed parameters. Nevertheless, new isolate Pfr 2 also displayed intensive radial growth and high viability of produced conidia (Table 2, Fig. 7). Finally, *V. lecanii* (isolate Vle 9), which was collected from naturally infected adult of *I. typographus* performed as more vigorous than comparative strain VLE-M. In particularly, production of conidia was almost doubled and development expressed as mean GI was also significantly faster (mean GI 2.13 compared with 1.08).

Among all species of entomogenous fungi that were collected from naturally infected adults of *I. typographus*, highly virulent isolates were discovered. Concretely, two isolates of *B. bassiana* (Bba 12 and Bb 13) and one isolate of *P. fumosoroseus* (Pfr 1) performed equally to commercial strains BBA-M and PFR 97. Besides, mean FDI values of *V. lecanii* isolate Vle 9 indicate even more virulent status when compared with the comparative strain VLE-M. Fig. 1.

RAPD fingerprint of *B. bassiana* isolate Bba 6 was generated with twelve primers from kit OPF. Selected primers produced 108 repeatable fragments for this isolate. The average number of fragments produced per primer was 13.5 (Fig. 2). Consequently, set of *B. bassiana* (Bba 6, Bba 7, Bba 12 and Bba 14) and *V. lecanii* (Vle 9) isolates that were collected from adults of *I. typographus* has been analyzed for DNA polymorphism. Primers OPF 1, 2, 5, 8, and 10–13, that generated the most informative RAPD patterns were selected for construction of specific fingerprints and evaluation of diversity of tested isolates. Isolate specificity of RAPD patterns was verified by comparing with RAPD spectra of comparative strains BBA-M, Vle 112 and PFR 97 (Fig. 3). RAPD analysis showed high correspondence among major binary fragments in spectrum of *B. basiana* isolates. RAPD markers revealed low diversity among *B. bassiana* isolates due to few different minor bands in RAPD patterns. Distances among these isolates according to values of Nei & Li similarity coefficients fluctuated from 3.5 to 16.7% (Table 3). The cluster analysis based on distance matrix was performed to generate dendrograms, enabling to assess predictable value of chosen set of primers for identification on the isolate level. All isolates of *B. bassiana* were clustered into one group that was distinctly separated from all other fungi. Inside "*B. bassiana* group" separation of non-endemic strain BBA-M is clearly visible (Fig. 4). This result indicates that RAPD analysis enables to distinguish *B. bassiana* isolates originated from the Bohemian Forest from non-endemic isolates with values of similarity coefficients among isolates from the Bohemian Forest and BBA-M ranging from 0.219–0.278 (Table 3). However, limited set of isolates only was used and analyzed in this study and more detailed analysis of polymorphism must be done on expanded spectrum of genotypes (isolates) to verify this preliminary results.

Isolate of *P. fumosoroseus* that was re-isolated after application of PFR 97 from newly infected adult of *Ips typographus* was compared with stock culture of PFR 97 and isolate which originate from commercial microbial insecticide Batch 189. There were any changes of RAPD spectra detected when two primers (OPF 6 and 8) were used for elementary RAPD analysis and all three isolates performed as the identical (Fig. 4).

Discussion

The incidence of entomogenous fungi directly associated with the adults of spruce bark beetle and soils closely related to sites with presence of *I. typographus* has been monitored within a

period 1998–2000. (LANDA & al. 2001). The results of monitoring demonstrate importance of search for new isolates of entomogenous fungi in their natural habitats. Nevertheless, throughout the literature, there are numerous references to attenuation or enhancement of the virulence of entomopathogenic fungi based on various laboratory techniques. Frequently, repeated subculturing in axenic culture or passings through particular insect host is recommended (e.g. HALL 1980, SAMŠIŇÁKOVÁ & KÁLALOVÁ 1983). It is generally believed, that the virulence of entomogenous fungi increases with successive passages through susceptible host and, conversely, decreases following successive subculturing on artificial medium (IGNOFFO & al. 1982). Nevertheless, the deuteromycetous entomogenous fungi, e.g. *Beauveria bassiana*, *Metarhizium anisopliae*, *Paecilomyces farinosus*, *P. fumosoroseus*, and *Verticillium lecanii* have worldwide distributions that predetermine immense potential of individual isolate (BIDOCHKA & al. 1998, ZIMMERMAN 1993). Therefore, search out for new fungal isolates at their natural habitats, followed by standard evaluation of newly collected isolates must be perceived as the acceptable alternative to laboratory manipulations with particular isolate.

It was found that among isolates of all entomogenous fungi that have been collected from infected adults of *I. typographus* are distinctive differences, which were detected in all *in vitro* and *in vivo* assays that have been used for evaluation. In spite of differences, all isolates that have been assessed were completing developmental cycle on solid media or in liquid artificial media and produced viable conidia that were virulent when tested on alternative insect host. It implies, that all newly collected isolates accomplished fundamental requirements that are defined before new strain is selected for further development as microbial agent (JENKINS & GRZYWACZ 2000). Besides, when compared with comparative strains (that are already used as the active ingredient of different commercial bio-insecticides), some of new collected isolates performed either equally or even better in some of assessed parameters. For example, among isolates of *B. bassiana*, new isolates Bba 12 and Bba 13 produced highly viable conidia and performed as very virulent. Similarly, new isolate of *V. lecanii* (Vle 9) performed significantly better in both assessed parameters when compared with strain VLE-M. This indicates, that among isolates that are collected at natural habitats may be discovered unique strains that represent specific quality and may be used for further development.

Construction of strain-specific and isolate-specific fingerprint allows characterising of extremely variable biological units on the molecular level. Polymorphism revealed by RAPD markers could be used as genetic characteristics for identification of the isolate or variety, to determinate genetic relationships or for population studies on different taxonomic groups of organisms (WILLIAMS & al. 1993, HARDYS & al. 1992, SAMEC 1993, TIGANO-MILANI & al. 1995, MCDONALD 1997). Cited problems with lower reproducibility of RAPD patterns or problematic interpretation of complex banding patterns reported by WILLIAMS & al. (1993), HARDYS & al. (1992), SAMEC (1993), BACKELJAU & al. (1995), TIGANO-MILANI & al. (1995) and MCDONALD (1997) may be overcome by optimization of reaction conditions and selection of appropriate 10-mer primers. Also, precise digital processing of electrophoresis gels is necessary to avoid problems with reproducibility of obtained data. RAPD data might be confirmed also by some other methods (e.g. RFLP, AFLP, DNA sequencing) focused on more specific DNA markers (BACKELJAU & al. 1995, MCDONALD 1997).

This study confirmed that various entomogenous fungi may be distinguished reliably even at the isolate level when assessed based on appropriately selected set of 10-mer primers. Concretely, isolates of *B. bassiana* (Bba 6, Bba 7, Bba 12 and 14) displayed RAPD spectrum which was identical in the major bands, but relevant differences were illustrated by dendrogram, that were caused by non-identical minor bands generated by primers OPF 1, 11, 13 and 16. According to preliminary data on RAPD polymorphism of *B. bassiana* might be declared, that isolates which were discovered and collected at the region of the Bohemian Forest is

possible differentiate based on RAPD pattern from non-indigenous isolates. RAPD method was already used for construction of specific fingerprint of different isolates of entomogenous fungi (OBORNÍK & al. 1997, LEXOVÁ & al. 1998). These results suggest that RAPD method could provide a valuable tool, which could be routinely used not only in microbiological laboratories, but also on level of producers of microbial pesticides. However, more detailed characterization of DNA polymorphism of this group would be necessary to identify individual isolates of *B. bassiana* reliably. Evidently, RAPD method offers an interesting tool that could be utilised in the characterisation and identification of fungal strains and/or isolates and their genetic diversity.

Additional applied utility of RAPD method was demonstrated when partial RAPD fingerprint was used to determine entomogenous fungus *P. fumosoroseus* after passed through adult of *Ips typographus*. This case study shows that RAPD technique could be used as a suitable tool for evaluation of genetic stability of strains re-isolated from ecosystem after introduced as commercial bio-preparation based on particular fungal strain as the active ingredient. This level of identification is particularly important as it provides a mechanism for tracking the progress and fate of the particular fungal strain in the environment, which is inevitable when fungal biopreparation have to be introduced or re-introduced into ecosystems at the protected areas (MENN & HALL 1999, JENKINS & GRZYWACZ 2000).

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