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Use of MALDI-TOF mass spectrometry in rapid identification of *Beauveria bassiana* and *Beauveria pseudobassiana*

Využitie MALDI-TOF hmotnostnej spektrometrie na rýchlu identifikáciu druhov *Beauveria bassiana* a *Beauveria pseudobassiana*

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Abstract

Species of the genus *Beauveria* are entomopathogenic fungi used in biological control to protect against insect pests. Identification of species is based on observation of morphological, phylogenetic and genetic differences. Currently, MALDI-TOF (Matrix Assisted Laser Desorption/Ionization- Time of Flight) mass spectrometry is often used in microbial diagnostic but identification of *Beauveria* species has not been tested before. The aim of this work was the possibilities of this method and to compare it with genetic analysis. Twenty strains of *B. bassiana* and *B. pseudobassiana* isolated from insect cadavers, mycoinsecticides and soil samples were used. Sequences of ITS (Internal Transcribed Spacer) region were used for genetic identification of strains. Sequences and results from protein analysis of isolates were compared with the reference strains of *B. bassiana* and *B. pseudobassiana*. Results of MALDI Biotyper softvare identification were correct for all strains. Identification was reliable with high log score (>2.1) for most of strains. Score value less than 1 was observed only in a single case - strain GHA isolated from mycoinsecticide Botanigard.The result of both analyses was also dendrogram (phylogenetic tree) which showed high degree of result consistence. MALDI-TOF identification of fungi from *Beauveria* genus is usable for fast and reliable identification of entomopathogenic fungi isolates acquired from environment and results can be utilized in conservation biocontrol strategies in agriculture and forest ecosystems.

Keywords: *Beauveria*, genetic analysis, spectrum analysis of proteins

Abstrakt

Rod *Beauveria* patrí medzi entomopatogénne huby využívané v biologickej regulácii hmyzích škodcov v poľnohospodárstve. Identifikácia druhov je založená na pozorovaní morfologických, fylogenetických a genetických rozdielov. V súčasnosti sa do popredia dostáva aj MALDI-TOF (Matrix Assisted Laser Desorption/Ionization-Time of Flight) hmotnostná spektrometria, ktorá v prípade rodu *Beauveria* nebola doposiaľ testovaná. Cieľom našej práce bolo zhodnotiť možnosť využívania tejto metódy na základe porovnania s genetickou analýzou. Použitých bolo 20 kmeňov *B. bassiana* a *B. pseudobassiana*, získaných izoláciou z kadáverov hmyzu, komerčne dostupných prípravkov a pôdnych vzoriek. Na genetickú identifikáciu kmeňov bol použitý región ITS (Internal Transcribed Spacer). Výsledné sekvencie a analýzy proteínových spektier vzoriek boli porovnávané s referenčnými kmeňmi *B. bassiana* a *B.pseudobassiana*. Výsledky identifikácie s využitím softvéru MALDI Biotyper boli správne pre všetky analyzované kmene. Identifikácia bola spoľahlivá s vysokým log skóre (>2.1) pre väčšinu kmeňov. Len v jedinom prípade – kmeň GHA, izolovaný z mykoinsekticídu Botanigard, bolo skóre nižšie ako 1. Výsledkom oboch analýz bol dendrogram (fylogenetický strom), ktorý poukázal na zhodnú klasifikáciu s referenčnými kmeňmi. MALDI-TOF identifikáciu húb z rodu *Beauveria* je možné využiť na rýchlu a spoľahlivú diagnostiku izolátov entomopatogénnych húb získaných z prostredia a výsledky využiť v systémoch biologickej ochrany lesných a poľnohospodárskych ekosystémov.

Kľúčové slová: analýza proteínových spektier, *Beauveria*, genetická analýza

Introduction

Entomopathogenic fungi are microorganisms which are commonly used in biological control of plant pests (Faria and Wraight, 2007). They colonize wide range of insect hosts because of their ability to penetrate the host cuticule and cause infectious diseases. The most important genera are *Beauveria, Metarhizium*, *Isaria* and *Lecanicillium* (Landa, 2002). Due to their target effects on various species of arthropods these fungal genera are used for the biological regulation of insects and other invertebrates such as mosquitoes, pest of agricultural crop including butterflies, wireworms or whiteflies as well as invasive species which cause ecological problems such as termites. In agricultural industry they are also used biopesticides such as BotaniGard, Naturalis, Boverol and Mycotrol (Kirkland et al., 2004). *Beauveria* (Bals.) Vuilll. (Ascomycota: Hypocreales) is a cosmopolitan anamorphic genus of soil borne facultative necrotrophic arthropod pathogens with high degree of pathogenicity (Eyeal et al., 1994). It includes the agronomically important species like *B. bassiana* and *B. pseudobassiana* (Rehner et al., 2011). Members of this family are found in different ecological habitats and include plant pathogens, entomopathogens and endophytes (White et al., 2003). Based on their ability to adapt to different environmental conditions like pH, temperature and soil types they can be also used in agriculture for protection against plant pathogens (Rehner et al., 2011). In addition *Beauveria* produce high amounts of biologically active secondary metabolites that

include non-ribosomal synthesized peptide antibiotics, non-peptide pigments and polyketides (Vey et al., 2001).

B. bassiana and *B. pseudobassiana* are cryptic species. Their differentiation is not based on their morphological characteristics. Only genetic analysis is reliable for recognition of *Beauveria* species. A multilocus phylogeny of *Beauveria* species based on partial sequences of five genes (ITS, BLOC, RPB1, RPB2, TEF) led to taxonomic revision of this genus and currently 17 species are described (Rehner et al., 2011).

MALDI-TOF MS is Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry. It is useful for a wide range of microorganisms (Guo et al., 2014). MALDI-TOF MS is applied in analysis of proteins (bacteria) and glycoproteins (fungi), lipids and nucleic acids. In microbiology it is used for the identification of Gram-positive and Gram-negative bacteria, bacterial spores, yeasts and fungi (Lacroix et al., 2014). Identification is based on comparison of obtained spectrum with reference strain from database (Trevino et al., 2012). The principle of analysis is to apply a suspension of spores or intact cells mixed with the matrix solution on the plate. During the drying process matrix crystals are joined to the sample. Next step is ionization of co-crystalized sample material by short laser pulses. The ions are accelerated and their time of flight is measured in a vacuum flight tube (Marvin et al., 2003).

The possibility of automation and fast molecular determination has made MALDI-TOF mass spectrometry a real alternative to microbiological and molecular methods. However, identification and classification of microorganisms using MALDI-TOF MS has been performed by many researchers (studies of Murray, 2010) while a limited number of studies have focused of fungal cells: Valentine et al. (2002), Hettic et al. (2008), Marinach-Patrice et al. (2009), Santos et al. (2010), or fungal spores: Li et al. (2000) – *Aspergillus*, Chen et al. (2005) – *Penicillium*, Kemptner et al. (2009) – *Fusarium,* Respins et al., (2010) – *Trichoderma*, Chowdappa et al. (2013) – *Alternaria* and Lopes et al. (2014) - *Metarhizium*. In case of *Beauveria* species MALDI-TOF mass spectrometry has not been tested before.

The aim of this work was to evaluate the use of spectrum analysis of proteins by MALDI-TOF mass spectrometry in identification of *Beauveria* cryptic species *B. bassiana* and *B. pseudobasiana* and to compare it to the traditionally used genetic identification.

Materials and methods

All together 22 strains of *Beauveria* were used in analysis of proteins and the genetic analysis. Sixteen strains were acquired from insect cadavers and plants, 4 strains from commercial mycoinsecticides, and 2 ex-type cultures were provided from the ARSEF fungal database (ARS Collection of Entomopathogenic Fungal Cultures, U.S. Department of Agriculture, ARS Plant Protection Research Inic, Ithaca, NY, USA).

In the first group (Table 1) were endophytic isolates of plants tissues (sign EF) and insect isolates (sign MK, EP, IK) from Institute of Forest Ecology and from forest biotope near the village Skýcov (SKJM) and Párovské Háje (SKMT).

Table 1. Basic characteristics of isolates from plants tissues and insects
Tabuľka 1. Základná charakteristika izolátov z rastlinných pletív a hmyzu

Strain	Species	Host	Origin
EF99	*B. bassiana*	*Viburnum utile*	Arborétum SAV
EF101	*B. bassiana*	*Rhodonendron decorum*	Arborétum SAV
EP72	*B. bassiana*	*Hylobius abietis*	Banská Štiavnica
IK10	*B. bassiana*	*Ips typographus*	Michalovo
MK34	*B. bassiana*	*Ips typographus*	Stará Lesná
MK225	*B. bassiana*	*Ips typographus*	Polcmanská Maša
SKJM004	*B. bassiana*	*Formica sp.*	Skýcov
SKJM007	*B. bassiana*	*Forficula auricularia*	Skýcov
SKJM011	*B. bassiana*	*Forficula auricularia*	Skýcov
SKJM013	*B. pseudobassiana*	*Lepidoptera sp.*	Skýcov
SKJM015d	*B. pseudobassiana*	*Coleoptera sp.*	Skýcov
SKJM017c	*B. pseudobassiana*	*Curculionidae sp.*	Skýcov
SKJM018	*B.pseudobassiana*	*Curculionidae sp.*	Skýcov
SKMT001	*B.pseudobassiana*	*Coleoptera sp.*	Párovské Háje
SKMT003	*B.pseudobassiana*	*Coleoptera sp.*	Párovské Háje
SKMT004	*B.pseudobassiana*	*Coleoptera sp.*	Párovské Háje

In the second group, isolates were obtained from free-range anti-pests. Information about mycoinsecticides e.g. name of product, corporation, sample name and species are shown in Table 2.

Table 2. Basic characteristic of isolates from commercial mycoinsecticides
Tabuľka 2. Základná charakteristika izolátov z voľnopredajných mykoinsekticídov

Strain	Species	Product	Corporation
GHA	*B. bassiana*	Botanigard	BioWorks
ESALQ PL63	*B. bassiana*	BoVeril	Koppert
Boverol	*B. bassiana*	Boverol	Fytovita
ATCC 74040	*B. bassiana*	Naturalis	Koppert

In the third group, reference strains of *B. bassiana* and *B. pseudobassiana* were included. Genotypes were selected from the ARSEF microscopic fungi database. Samples were delivered lyophilized and each isolate was stored at -20 °C. Names and number of isolates are shown in Table 3.

Table 3. Basic characteristic of reference isolates
Tabuľka 3. Základná charakteristika referenčných kmeňov

Sample	Species	Host	Origin
ARSEF 1564	*B. bassiana*	*Hyphantria cunea*	Italy
ARSEF 3405	*B. pseudobassiana*	*Lymantria dispar*	USA

Genetic analysis

Strains for genetic analysis were inoculated on Saboraud´s dextrose agar at 25 °C in darkness. DNA was extracted from 7 days old fungal culture. Approximately 50 mg of wet mycelium was scraped, placed in 200 µl of PrepMan solution (Life technologies) and homogenized with glass beads on BeadBug homogenizer (Benchmark scientific). One µl of supernatant was used as in PCR (Polymerase Chain Reaction) reaction. Nuclear gene region ITS was amplified and sequenced with primers ITS 5 (5´GGAAGTAAAAGTCGTAA GAAGG-3´) and ITS 4 (5´TCCTCCGCTTATTGATA TGC-3´) (White et al., 1990). Thirty µl of PCR mixture contained 3 µl 10X Dream Taq DNA buffer, 3 µl of 2 mM dNTP mix, 1 µl of $MgCl_2$, 1.2 µl each of the opposing amplification primers (10 mM), 0.1 µl Dream Taq DNA polymerase and 1 µl of genomic DNA. PCR amplification was performed in thermocycler MJ Mini (Biorad, USA). Cycling conditions were as follows: 95 °C for 60 s followed by 45 cycles of

95 °C for 30 s, annealing at temperature 56 °C for 30 s, 72 °C for 30 s and a final elongation at 72 °C for 5 min. Amplification products were sequenced with primer ITS 5. Sequencing was performed by Macrogen (South Korea). Acquired sequences were assembled and processed using the MEGA 7 (Molecular Evolutionary Genetics Analysis) software (Kumar et al., 2016). Alignment was made using MUSCLE (MUltiple Sequence Comparison by Log-Expectation) (Edgar, 2004). The acquired sequences as well as the reference sequences of all currently available Beauveria ex-type strains from Genbank database were used for phylogenetic analysis. A phylogenetic tree was constructed using method of maximum likelihood with substitute model Tamura-Nei (1993).

Spectrum analysis of proteins by MALDI-TOF mass spectrometry

In MALDI-TOF mass spectrometry strains which were cultivated in darkness at 25 °C for 4 days were used. Approximately 50 mg of wet mycelium was mixed with 300 µl of water and homogenized in homogenizer Genie 2 (Mobio Laboratories, USA). After that, 900 µl of ethanol were added and centrifuged for 2 minutes at maximum centrifugal force (24,400 ×g) in a Rotina 380R centrifuge (Hettich Zentrifugen, Germany). Next 30 µl of formic acid were added to pellet and vortexed for 1 minute. The last step was to add 30 µl of acetonitrile and to centrifuge solution for 2 minutes. One µl of mixed solution was pipetted onto a stainless steel MALDI target plate and left to dry. After drying matrix solution was added. Samples were measured by mass spectrometer MALDI-TOF MS Microflex LT (Bruker Daltonics, Czech Republic). After insertion of the MALDI plate, the spectrometer is controlled by the FlexControl software. The range of measured proteins was 1,960 – 20,137 Da. The spectrum was obtained from 500 shoots applied in plate. At least 8 spectra for each sample were obtained. Observed spectra were processed by FlexAnalysis 3.4 which verified the purity of peaks. Software MALDI Biotyper 3.1 for unification of the measured spectra was used. Sequence making (maximum 100 peaks) was performed by Biotyper pre-processing standard method with 25 Da algorithm (Savitzky, Golay, 1964) Accumulation of spectra was made using Mass Spectrum Projection (MSP). The similarity tree was constructed by Biotyper MSP Dendrogram Creation Standard Method.

Results and discussion

Beauveria species are microorganisms with high frequency of occurrence, as confirmed by the results of studies about their adaptability to different climatic conditions. Bride et al. (2005) confirmed their presence in Antarctica. A large occurrence was observed in Mexico (Pérez-Gonzáles, 2014).

In strains obtained from insect cadavers and forest biotope from Slovakia high representation of *Beauveria* species, mostly *B. bassiana* and *B. pseudobassiana* was obtained.

Genetic analysis

Genetic identification of strains and comparison to ex- types is shown in Figure 1. Using genetic analysis of ITS region off all 22 strains, 7 haplotypes were identified. Three haplotypes were found in *B. pseudobassiana* clade while 4 haplotypes were found within *B. bassiana* clade. Slovakian strains showed high degree of similarity and they were grouped in only 4 haplotypes. This can be linked to spatial distribution of sampling sites.

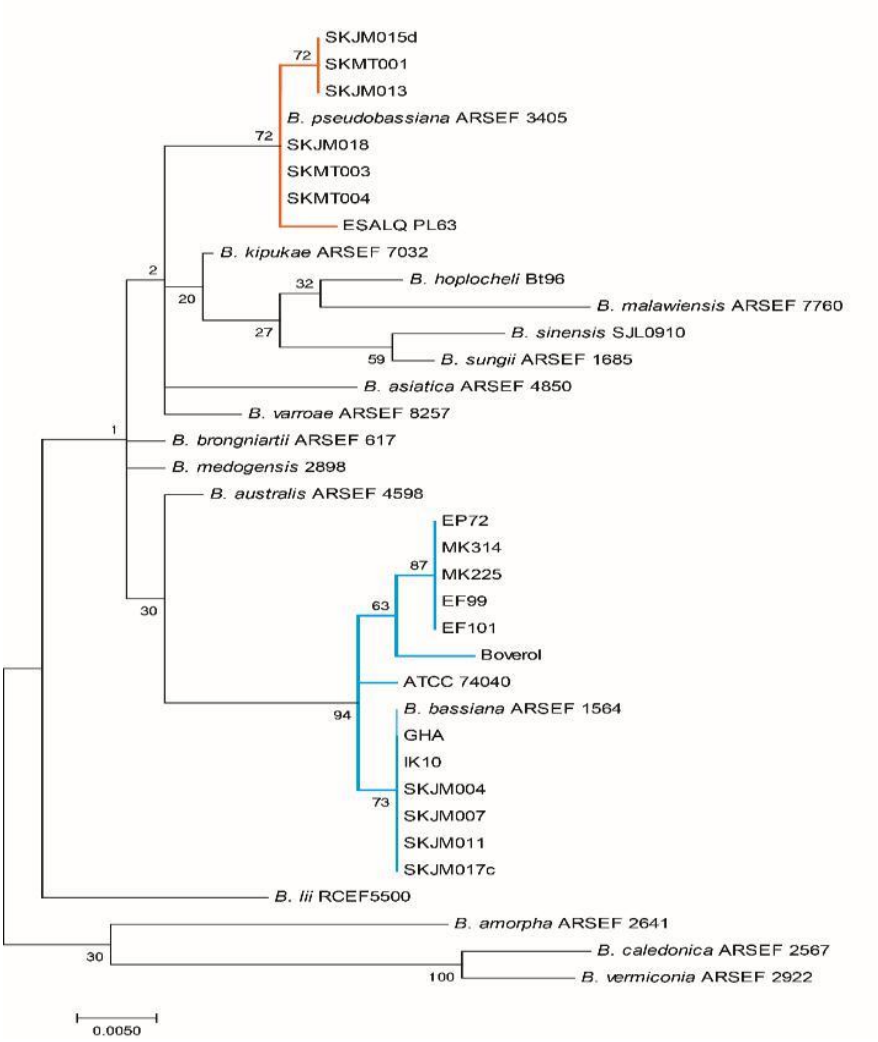

Figure 1. Maximum likehood dendrogram of ITS sequences showed similarity of *Beauveria* isolates

Obrázok 1. Maximum likehood dendrogram sekvencií ITS regiónu vyjadrujúci podobnosť izolátov *Beauveria*

Spectrum analysis of proteins by MALDI-TOF mass spectrometry

There are qualitative and quantitative differences between composition of fungal cell walls and spores. According to Kemptner et al. (2009) there are differences not only within different fungal species, but also between different strains of the same fungal species.

The success of species matching is dependent on the number of strains in the database. The results obtained in this study and in the study of Carolis et al. (2011) show that identification was possible for isolates with fewer reference spectra in the database.

Using MALDI-TOF MS Biotyper method, a clear difference between *B. bassiana* and *B. pseudobassiana* was identified. Each group formed well defined clade in MSP dendrogram. Only single strain GHA was not associated to reference ex types. However, when its spectrum was compared to ex types (3405 or 1564) identification by Biotyper software showed medium reliable identification to 1564. Results are presented in Figure 2.

Identification of fungal isolates was consistent with genetic analysis. Only in a single case identification was non-reliable. It was strain GHA isolated from mycoinsecticide Botanigard. In mycoinsecticides fungal strains are usually very strongly selected to gain best pathogenity together with high resistance to UV, temperature and other conditions. Despite this fact it is assumed that this strain is also highly protein-specific. Based on analysis of the protein spectrum, this strain cannot be classified. Based on studies of other species it can be assumed that the non-reliable identification of strains probably depends on length of cultivation or production of secondary metabolites.

To avoid production of secondary metabolites the analysis should be carried out immediately after cultures attain the specific parameters. Secondary metabolites usually do not interfere with sequential analysis of nucleic acids with exception of problems with extraction of pure DNA (Paterson et al., 2008; Hawksworth, 2009). However, metabolites may affect phenotypes and subsequently MALDI profiles (Santos et al., 2010). Metabolites production depends on inoculation and incubation. *Metarhizium* samples were inoculated on PDA and SDA medium (Lopes et al., 2014). De Respins et al. (2010) used 2 days old *Trichoderma* samples and Chowdappa et al. (2013) incubated *Alternaria* for 5 days. The optimal technique for *Beauveria* species has not been tested before. The strains were inoculated on SDA medium and incubated for 4 days. Mycelium was used instead of a liquid culture. Even the use of liquid culture did not improve identification of GHA strain.

Most strains of insect cadavers from village Skýcov and Párovské Háje (sign SKMT and SKJM) and strain ESALQ PL63 from mycoinsecticide BoVeril were assigned to the reference strain ARSEF 3405 (*B. pseudobassiana*). Strains from endophytic isolates from plant tissues (sign EF), insect isolates from Institute of Forest Ecology (sign MK, EP, IK) and two strains from mycoinsecticides Boverol (sign Boverol) and Naturalis (sign ATCC 74040) were assigned to the reference strain ARSEF 1564 *(B. bassiana).*

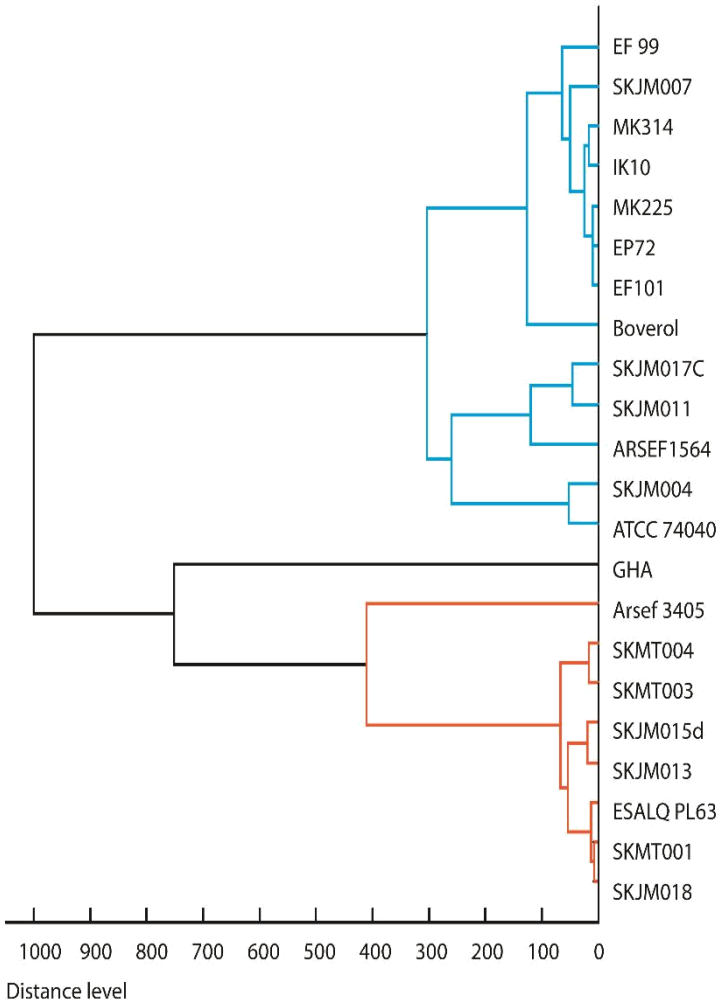

Figure 2. Mass Spectrum Projection dendrogram showed similarity of *Beauveria* isolates

Obrázok 2. Mass Spectrum Projection dendrogram vyjadrujúci podobnosť izolátov *Beauveria*

MALDI Biotyper software compares the spectra by generating a numerical value (score value) based on the similarities between the observed and stored data sets. This score value provides information about the validity of the identification. A score value above 2 is generally considered to be a valid species level identification. Values between 2 and 1.7 represented reliable genus level of identification. As it is shown in Table 4, mycelial sample of GHA strain (mycoinsecticide Botanigard) produced low quality spectrum with score value 0.787 (value that was considerably below the cut-off limit of 1.7). Fifteen strains produced high quality spectrum (value that was over 2) with those scores: ESALQ PL63 (2.132), Boverol (2.378), ATCC 74040 (2.339), EP72 (2.223), MK225 (2.082), MK314 (2.339), SKJM004 (2.035), SKJM011 (2.157), SKJM013 (2.238), SKJM015d (2.296), SKJM017c (2.173), SKJM018 (2.266), SKMT001 (2.414), SKMT003, (2.354), SKMT004 (2.396). Seven strains were classified as *Beauveria pseudobassiana* and 8 strains like *Beauveria*

bassiana. Last 4 strains produced quality spectrum with value between 1.9-2: EF99 (1.931), EF101 (1.941), IK10 (1.973), SKJM007 (1.983).

Table 4. Identification of *Beauveria* spp. strains using MALDI Biotyper software
Tabuľka 4. Identifikácia *Beauveria* spp. Kmeňov spoužitím Maldi Biotyper softvéru

Strain	Ex-type	Log score	Reliability
GHA	ARSEF 1564	0.787	-
ESALQ PL63	ARSEF 3405	2.132	+++
Boverol	ARSEF 1564	2.378	+++
ATCC 74040	ARSEF 1564	2.339	+++
EF 99	ARSEF 1564	1.931	++
EF 101	ARSEF 1564	1.941	++
EP 72	ARSEF 1564	2.223	+++
IK 10	ARSEF 1564	1.973	++
MK 225	ARSEF 1564	2.082	+++
MK 314	ARSEF 1564	2.339	+++
SKJM 004	ARSEF 1564	2.035	+++
SKJM 007	ARSEF 1564	1.983	++
SKJM 011	ARSEF 1564	2.157	+++
SKJM 013	ARSEF 3405	2.238	+++
SKJM 015d	ARSEF 3405	2.296	+++
SKJM 017c	ARSEF 1564	2.173	+++
SKJM 018	ARSEF 3405	2.266	+++
SKMT 001	ARSEF 3405	2.414	+++
SKMT 003	ARSEF 3405	2.354	+++
SKMT 004	ARSEF 3405	2.396	+++

The aim of this work was to test analysis of proteins by MALDI-TOFF mass spectrometry and compare it with genetic analysis. Pietro et al. (2013) compared MALDI-TOF MS with sequencing analysis of ITS region for identification of dermatophytes. Their results showed almost 99% match. In the case of Sendida et al. (2013) only 2.3% of yeast isolates could be analyzed using MALDI-TOF MS. In

study of Shin et al. (2015) were compared outcomes of 16S rDNA sequencing with analysis of proteins spectra in *Aeromonas* genus. They identified 92% of isolates on species level and 6% of isolates on genus level by MALDI-TOF mass spectrometry. Results of this experiment predict this method as an effective way of identifying cryptic species of *Beauveria* genus with 95% of reliable identification.

Conclusion

It can be evaluated that the analysis of protein spectra using MALDI-TOF mass spectrometry is highly effective. Similarity with genetic analysis using ITS region was observed for 20 isolates. Compared to genetic analysis it is financially significantly less demanding and similarly effective. After integration into the MALDI software this database can be widely used and shared on other sites. Expansion of the MALDI-TOF mass spectrometry database with *Beauveria* and other fungal species with agronomical or clinical importance will help to enhance the utility of this analysis for the identification of unknown fungal pathogens.

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