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MOLECULAR IDENTIFICATION AND SCAR DETECTION OF ENTOMOPATHOGENIC FUNGI OF THE GENUS *BEAUVERIA*

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Abstract

In recent years, there has been increasing interest in identifying and studying beneficial microorganisms that can be used in organic farming as fertilizers and plant protection agents. Entomopathogenic fungi with an endophytic nature of the genus *Beauveria* are quite promising and their identification and detection in the plant and soil samples are of great importance. Molecular identification of fungi of the genus *Beauveria* was conducted by sequencing the internal transcribed spacer 1 and 5.8S ribosomal RNA gene (ITS) regions with universal primers. As a result of the sequencing, it was found that the analysed strains belong to four genera of *Beauveria* with the following percentage distribution: *B. bassiana* (68.18%), *B. pseudobassiana* (13.64%), *B. varroae* (9.09%) and *B. tenella* (9.09%). The largest percentages of the strains were identified as *B. bassiana*. Sequence-characterized amplified regions (SCAR) have been applied for the detection of *Beauveria* strains in the field samples. The efficiency and ability of three pairs of SCAR markers to differentiate the studied strains were tested. The applied SCAR markers generated fragments with expected length in more than 90% of the investigated strains and could be used as markers for the detection of the fungi from the genus *Beauveria* in field samples. In the selected experimental conditions only *B. varroe* 501 and *B. tenella* 593 lack fragments with used SCAR markers.

Key words: *Beauveria*, identification, SCAR markers

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Introduction. The species of the genus *Beauveria* are entomopathogenic fungi with an endophytic nature and occupy a key place in modern plant protection and organic farming. In addition to proven insecticidal activity, they also exhibit antifungal properties. In recent years, part of the research has been aimed at evaluating the potential of entomopathogenic fungi for a double effect, as biocontrol agents against phytopathogenic fungi, as well as bioinsecticide [1].

Insecticidal fungi of the genus *Beauveria* are applied and successfully colonize the tissues of several plants without affecting their physiological development [2–4]. Even endophytic strains have a growth-promoting effect on colonized plants and they outperformed non-inoculated controls [5].

To exploit these beneficial properties in agriculture, *Beauveria* species must be identified and studied. Classical microbiology methods such as plating into the selective culture media and subsequent microscopy are time-consuming and not as sensitive as modern molecular techniques [6]. Sequencing determines the precise order of nucleotides in DNA and is one of the most reliable means of identifying the different microorganism isolates. 18S rDNA sequences (18S rDNA) have been used for phylogenetic analyses and determining relationships between distantly and closely related taxa. ITS regions are also used for the identification of microorganisms. As opposed to coding regions of rDNA, ITS regions vary greatly due to mutations (deletions, insertions and point mutations) [7].

In the last two decades, different molecular marker techniques have been widely used to study genetic diversity and a more complete understanding of the genetic profile and differentiation of the isolates [6, 8].

One of the advantages of the molecular markers is that the polymorphic fragments could be easily converted into SCAR markers. SCAR markers were developed for the molecular detection of *Beauveria* fungi; these markers were chosen from polymorphic patterns obtained with 3 primers via RAPD-PCR [9]. SCAR markers capable of distinguishing insect pathogenic fungi from field samples have been developed for *Beauveria* species [9, 10]. This makes SCAR markers a valuable tool from a practical point of view for rapid identification after the treatment of *Beauveria* isolate in field samples.

The aim of the work is the molecular identification of fungal isolates as well as testing the efficiency and ability of three pairs of SCAR markers to differentiate the studied strains.

Materials and methods. The study was conducted at the Agricultural University of Plovdiv in 2021 and 2022.

Fungal isolates. The fungal isolates were provided by Prof. Slavimira Draganova from the Agricultural Academy – Bulgaria, Institute of Soil Science, Agrotechnologies and Plant Protection (ISSAPP). The study included 23 isolates (214, 262, 270, 287, 336, 340, 426-8, 487, 495, 501, 561-re, 592, 593, 623, 626, 629, 640, 644, 648, 717, 733, 743, 759). The mycelial growth of the entomopathogenic fungi was enhanced by temperature 27 °C and yeast extract agar

(YEA) (Oxoid, UK) as was reported by James et al. [11]. Fungal suspensions starting from dry conidia were grown on Sabouraud dextrose agar (Oxoid, UK) and YEA medium (Oxoid, UK) in the dark at 27 °C. The hosts of the analyzed isolates have been published in previous studies [12, 13], isolates: 262, 270, 287, 495, 640 – host *Zabrus tenebrioides*, isolates 336, 340, 487 – hosts *Lema melanopus* L. and *Lema lichenis* Voet., isolate: 426-8 – host *Acrida cinerea*, isolates: 501, 592, 593, 626, 648 – hosts *Cydia pomonella* and *Cydia funebrana*, isolates: 717, 743, 759, 561-re – hosts *Leptinotarsa decemlineata* (larva, adult), isolates 623, 629 – host *Trialeurodes vaporariorum* [12]. The strain 214 was isolated from *Agrilus mokrzeckii* (Coleoptera, Buprestidae, *Agrilus*) on red oil roses (*Rosa damascena* Mill.), strain 644 was isolated from *Tanymecus dilaticollis* Gyll. (Coleoptera, Curculionidae, *Tanymecus*), and strain 733 was isolated from *Leptinotarsa decemlineata* (Coleoptera, Chrysomelidae, *Leptinotarsa*) [13].

Molecular methods*: *DNA isolation. DNA isolation was performed using the HiPurA™ Fungal Genomic DNA Purification Kit (Canvax, Spain) following the manufacturer's protocol. Concentrations of isolated DNA samples were determined on an agarose gel by comparison with standard concentrations of lambda DNA (Thermo Fisher Scientific Inc., USA) and by Qubit Fluorometric quatification (Qubit, Thermo Fisher Scientific Inc., USA).

SCAR marker analyses. PCR reactions were conducted with three primer pairs of SCAR markers developed by Castrillo et al. [9] for *Beauveria bassiana* detection and testing their applicability to the studied isolates. Amplification reactions were performed in a total volume of 10 µL. Ready PCR mixes RedTaq DNA polymerase (Canvax, Spain) were used to conduct the PCR reactions. A DNA template of 30–50 ng was used in the PCR reactions with three pairs of primers SCA14 445, SCA15 441 and SCB9 677 were performed (Table 1).

Table 1

Sequences of primers used in this study

SCAR primers	Primer sequence (5′-3′)	Annealing temperature	Authors
OPA14 F_{445}	TCT GTG CTG GCC CTT ATC G	63 °C	[9]
OPA14 R_{445}	TCT GTG CTG GGT ACT GAC GTG		
OPA15 F_{441}	TTC CGA ACC CGG TTA AGA GAC		
OPA15 R_{441}	TTC CGA ACC CAT CAT CCT GC		
OPB9 F_{677}	TGG GGG ACT CGC AAA CAG		
OPB9 R_{677}	TGG GGG ACT CAC TCC ACG		
ITS and NL primers	**Primers sequencing**	**Annealing temperature**	**Authors**
ITS5	GGAAGTAAAAGTCGTAACAAGG	55 °C	[14]
ITS4	TCCTCCGCTTATTGATATGC		
NL 4	GGTCCGTGTTTCAAGACGG		[15]

Conditions of PCR reactions: 2 min initial denaturation at 94 °C; 10 cycles of 94 °C for 15 s, 63 °C for 30 s and 72 °C for 45 s; followed by 15 cycles of 94 °C for 15 s, 63 °C for 30 s, and 72 °C for 45 s, and a final extension at 72 °C for 7 min [9].

Sequencing analyses. PCR analyses were performed on the 18S and 5.8S region of ribosomal RNA gene and of each strain with primers ITS 5 [14], ITS 4 [14] and NL 4 [15]. PCR analyses were performed in a final volume of 20 µL containing 1 µL (30–50 ng) of DNA and 8 µL of Red-Taq DNA MIX (Canvax, Spain), 1 µL each of 10 µM of both primers. The PCR reaction included: 2 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 55 °C, and 2 min at 72 °C with a final extension of 10 min at 72 °C. Amplified fragments were excised from the gel and purified with a gel isolation kit. The PCR products of internal transcribed spacer (ITS) region of each strain were sent for sequencing in Microsynth Seqlab GmbH, Germany.

Electrophoresis conditions. The results of the PCR reactions were visualized by electrophoresis in a 1% agarose gel (1X TAE) stained with SafeView (NBS Biologicals, UK) at 70 V. A GeneRuler 1 kb DNA Ladder was used for fragment length comparison.

Bioinformatics programs and databases. For data analysis the databases of the National Center for Biotechnology Information (NCBI) were used. The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between protein or nucleotide sequences. The program was used to compare nucleotide sequences to sequences in a database and calculate the statistical significance of the matches. The results are deposited in the NCBI database under submission SUB10981546 (OM366244 – OM366265, `https://www.ncbi.nlm.nih.gov/nuccore/?term=OM366244:OM366265`) from 24.01.2022.

Results and discussion. Morphological characterization of some cases is not enough to correctly identify strains at the species level [16]. The sequencing method can be used for more accurate identification of *Beauveria* sp. [7].

ITS sequence data sets were constructed for 22 strains of *Beauveria* spp. Fifteen strains were identified as *Beauveria bassiana* (214, 262, 270, 340, 561-re, 592, 623, 626-2, 629, 644, 648, 717, 733, 743, and 759), three strains as *Beauveria pseudobassiana* (287, 487, and 495), two strains as *Beauveria varroae* (426-8 and 501) and two strains as *Beauveria tenella* (336 and 593). The ITS sequence data and the accession in the GeneBank are shown in Table 2.

To be identified in their application in agriculture and organic farming, in addition to sequencing, it is necessary to look for other fast molecular methods for their recognition and their differentiation from the rest of the natural microflora.

Castrillo et al. [9] applied RAPD markers to detect and estimate the density of the applied commercial strain of *B. bassiana* (strain GHA) in field samples. Based on the most effective RAPD markers, three pairs of SCAR primers have been developed. The established primer pairs are highly sensitive and capable of detecting *B. bassiana* GHA genomic DNA in concentration of 100 pg in the soil [9].

Table 2

Molecular identification of the strains

Molecular identification of *Beauveria* No.	ID	Accession number of the nucleotide sequences
214	*Beauveria bassiana*	OM366244 [13]
262	*Beauveria bassiana*	OM366245
270	*Beauveria bassiana*	OM366246
287	*Beauveria pseudobassiana*	OM366264
340	*Beauveria bassiana*	OM366255
426-8	*Beauveria varroae*	OM366254
487	*Beauveria pseudobassiana*	OM366256
495	*Beauveria pseudobassiana*	OM366247
501	*Beauveria varroae*	OM366248
561-re	*Beauveria bassiana*	OM366249
592	*Beauveria bassiana*	OM366250
593	*Beauveria tenella*	OM366251
623	*Beauveria bassiana*	OM366252
626-2	*Beauveria bassiana*	OM366253
629	*Beauveria bassiana*	OM366257
336	*Beauveria tenella*	OM366265
644	*Beauveria bassiana*	OM366261 [13]
648	*Beauveria bassiana*	OM366258
717	*Beauveria bassiana*	OM366262
733	*Beauveria bassiana*	OM366259 [13]
743	*Beauveria bassiana*	OM366260
759	*Beauveria bassiana*	OM366263

In the present study, PCR reactions were performed with the three SCAR primer pairs to test their applicability to the isolated strains.

In the research by Castrillo et al. [9] marker $SCA14_{445}$ showed the greatest specificity and generated fragments only in GHA strain, in ARSEF 6444 and strains collected from field samples from GHA-treated areas. While SCAR markers $SCA15_{441}$ and $SCB9_{677}$ are polymorphic and generate fragments of similar length as strain GHA was amplified also in other *B. bassiana* strains even after increasing specificity [9].

In the present study, the $SCA14_{445}$ marker resulted in the amplification of fragments with expected length only in nine of the isolated strains. Our results were in accordance with Castrillo et al. [9] and $SCA15_{441}$ marker showed the lowest specificity and a fragment of expected length was detected in 17 of the tested strains. With $SCB9_{677}$ marker, a fragment of expected length was generated in 14 of the strains (Fig. 1).

Electrophoresis results			
Isolates	SCAR 445 bp	SCAR 441 bp	SCAR 677 bp
214	-	+	+
262	-	-	+
270	-	-	+
287	-	+	-
336	-	+	-
340	-	+	-
426-8	-	+	-
487	-	+	-
495	-	+	-
501	-	-	-
561-re	-	-	+
592	-	+	-
593	-	-	-
623	+	+	+
626	+	+	+
629	+	+	+
640	+	+	+
644	+	-	+
648	+	+	+
717	+	+	+
733	+	+	+
743	-	+	+
759	+	+	+

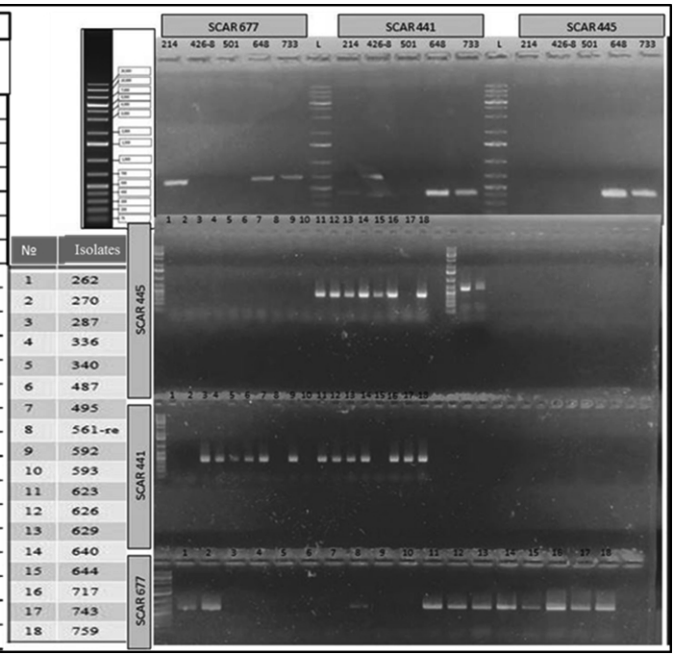

Fig. 1. Results of electrophoresis of 23 *Beauveria* strains with three pairs of SCAR markers ($SCA14_{445}$, $SCA15_{441}$, and $SCB9_{677}$ [9])

With the applied PCR conditions only in eight of the analyzed strains (623, 626, 629, 640, 648, 717, 733, and 759) amplified specific fragments with all three pairs of SCAR markers tested. *B. varroe* 501 and *B. tenella* 593 lack amplification of fragments with selected three pairs of primers (Fig. 1). The applied SCAR markers generated fragments of the expected length in more than 90% of the tested strains and could be used as markers in the application of the representatives of the genus *Beauveria* to plants.

Conclusions. Based on a comparative analysis of the obtained sequences, the highest percentage of the studied strains were identified as *B. bassiana*. The SCAR markers were effective in most tested *Beauveria* species and could be used as markers in the application of the studied *Beauveria* species to plants.

REFERENCES

[1] Yun H., D. Kim, W. Gwak et al. (2017) Entomopathogenic fungi as dual control agents against both the pest *Myzus persicae* and phytopathogen *Botrytis cinerea*, Mycobiol., **45**(3), 192–198.

[2] Petkova M., V. Spasova-Apostolova, V. Masheva, N. Tahsin (2020) Comparison of endophytic colonization of Bulgarian variety of tobacco by entho-

mopathogenic fungi – *Beauveria bassiana* and *Beauveria brongniartii*, Ecologia Balkanica, **12**(1), 31–40.
[3] Petkova M., V. Spasova-Apostolova, V. Masheva et al. (2021) Endophytic colonization of Solanaceae family plants by fungal entomopathogen *Beauveria bassiana* strain 339 to control Colorado potato beetle (*Leptinotarsa decemlineata* Say), Bulg. J. Agric. Sci., **27**(Suppl. 1), 143–149.
[4] Spasova-Apostolova V. V., V. B. Masheva, M. K. Petkova, N. T. Tahsin (2021) Endophytic colonization of tobacco plants (*N. tabacum*, L., ssp. *Orientalis*) by the strain 538 of entomopathogenic fungus *Beauveria bassiana*, C. R. Acad. Bulg. Sci., **74**(6), 928–936.
[5] Barra-Bucarei L., M. G. González, A. F. Iglesias et al. (2020) *Beauveria bassiana* multifunction as an endophyte: Growth promotion and biologic control of *Trialeurodes vaporariorum*, (Westwood) (Hemiptera: Aleyrodidae) in tomato, Insects **11**(591), https://doi.org/10.3390/insects11090591.
[6] Biswas Ch., P. Dey, B. S. Gotyal, S. Satpathy (2015) A method of multiplex PCR for detection of field released *Beauveria bassiana*, a fungal entomopathogen applied for pest management in jute (*Corchorus olitorius*), World J. Microbiol. Biotechnol., **31**, 675–679.
[7] Mondal S., S. Baksi (2018) Strain-specific identification of *Beauveria bassiana* isolated from a novel habitat, using rDNA-based sequence analogy, Egypt. J. Biol. Pest Control, **28**, https://doi.org/10.1186/s41938-018-0033-4.
[8] Prabhukarthikeyan S. R., U. Keerthana, S. Archana, T. Raguchander (2017) Analysis of genetic diversity among different strains of *Beauveria bassiana* by RAPD-PCR, J. Biol. Control, **31**(1), 18–24.
[9] Castrillo L. A., J. D. Vandenberg, S. P. Wraight (2003) Strain-specific detection of introduced *Beauveria bassiana* in agricultural fields by use of sequence-characterized amplified region markers, J. Invertebr. Pathol., **82**(2), 75–83.
[10] Hegedus D. D., G. G. Khachatourians (1996) Identification and differentiation of the entomopathogenic fungus *Beauveria bassiana* using polymerase chain reaction and single-strand conformation polymorphism analysis, J. Invertebr. Pathol., **67**(3), 289–299.
[11] James R. R., B. A. Croft., B. T. Shaffer, B. Lighthart (1998) Impact of temperature and humidity on host-pathogen interactions between *Beauveria bassiana* and a Coccinellid, Environ. Entomol., **27**(6), 1506–1513.
[12] Petkova M., V. V. Spasova-Apostolova (2024) Biological activities of 20 strains of entomopathogenic fungi of the genus *Beauveria*, Bulgarian J. Soil Sci. Agrochem. Ecol., **58**(1), 27–38, (in Bulgarian).
[13] Radev Z., V. Spasova-Apostolova, M. Petkova (2024) Bio-insecticidal efficacy of *Beauveria bassiana* Vuill. strains against *Tettigonia viridissima* L. in tobacco, C. R. Acad. Bulg. Sci., **77**(9), 1417–1422.
[14] White T. J., T. Bruns, S. Lee, J. Taylor (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Protocols and Applications – A Laboratory Manual, San Diego, Academic Press, 315–322.
[15] Kurtzman C. P., C. J. Robnett (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences, Antonie van Leeuwenhoek, **73**(4), 331–371.
[16] Poeaim S., S. Hangtrakul, K. Soytong (2014) Genetic diversity of *Beauveria* sp. isolated in Thailand, Int. J. Agric. Technol., **10**(3), 607–616.

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