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Original scientific paper

GENOTYPING OF *BACILLUS* SPP. ISOLATE COLLECTION FROM NATURAL SAMPLES

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The aim of this study was genotyping and identification of collection of 164 *Bacillus* spp. isolates, from samples of soil, manure, and straw gathered from across Serbia, using Pulse field gel electrophoresis (PFGE) combined with sequencing of *tuf* gene, one of the housekeeping genes. The PFGE analysis with *Not*I enzyme was used to determine phylogenetic relationships of isolates and referent strains. Four large groups of *Bacillus* spp. were distinguishable: cereus, subtilis, pumilus and megaterium and within enormous genetic diversity. *Bacillus subtilis* Marburg referent strain did not group with rest of the strains from the subtilis group (*Bacillus subtilis* ATCC6633 and *Bacillus atrophaeus* ATCC9372). Strains from the cereus group were distinguished and closely grouped together. One representative isolate from each of 21 distinct PFGE groups was identified by sequencing of *tuf* gene. Eight different species were identified among chosen isolates: *B. amyloliquefaciens, B. subtilis, B. pumilus, B. safensis, B. megaterium, B. cereus, B. anthracis* and *B. thuringiensis*. Our results showed that PFGE analysis combined with sequencing of one of the housekeeping genes could be used for characterization of large collections of *Bacillus* isolates. The determination of *tuf* gene recommended itself to be an adequate and sufficient analysis for obtaining very clear and unambiguous results, with high resolution of separation of *Bacillus* species.

Keywords: Bacillus identification, PFGE, *tuf* gene

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INTRODUCTION

Bacillus spp. are rod-shaped, endospore forming, predominantly Gram-positive, aerobes or facultative aerobes. Mainly are isolated from soil or from environments that are associated, directly or indirectly with soil, but also found in water, food and clinical specimens. In the latest edition of Bergey's Manual of Systematic Bacteriology 142 species were included in genus *Bacillus* (LOGAN and DE VOS, 2009). Albeit the fact that *Bacillus cereus* and *Bacillus thuringiensis* are closely related but also completely different from *Bacillus subtilis* (PRIEST, 1993), determination of *Bacillus* spp. isolates at species level encounters with number of issues. *Bacillus amyloliquefaciens* is closely related to *B. subtilis* and the other two species, which compose the *B. subtilis* group, *Bacillus licheniformis* and *Bacillus pumilus.* These strains share many common properties and only few characteristics have been found by which they can be discriminated (LOGAN and DE VOS, 2009). Likewise, *B. cereus, B. thuringiensis* and *B. anthracis* are genetically also very close, as shown by gene sequence analysis (HELGASON *et al.*, 2000). Furthermore, within species, genetic diversity is still very high. Currently, 16S rDNA is basic standard for taxonomy of the bacteria. However, resolution of the different 16S rDNA groups within the genus *Bacillus sensu lato* is steel far from clear. Many species fall into several distinct groups such as the "*subtilis* group", "*cereus* group" and "*sphaericus* group" that can be even phenotypically distinguishable, but issue of identification for many strains remains (LOGAN and DE VOS, 2009). Several approaches towards resolution of molecular phylogeny of *Bacillus* were proposed over the years. Besides sequencing of complete 16S rDNA gene and 5' hypervariable region of 16S rDNA (GOTO *et al.*, 2000) some fingerprinting methods including RAPD (PINCHUK *et al.*, 2002; BERIĆ *et al.*, 2009), Rep-PCR (DA SILVA and VALICENTE, 2013) and PFGE (RIVERA and PRIEST, 2003; DREAN and FOX, 2015) were proposed and tested. Multilocus sequence analysis of the core genes (MAIDEN, 2006) even though very informative for many species, *Bacillus* spp. including, although predominantly within *B. cereus* group (HELGASON *et al*., 2004) is expensive and not very practical for large collections of strains.

The PFGE in *Bacillus* study is used for analyzing if pathogenic species are clonal, which is accustomed application of this method (BARTOSZEWICZ *et* al., 2008; BRANQUINHO *et al*., 2014). In our work we employ this technique, combining it with the sequencing of one of the core genes (gene for elongation factor Tu) to genetically distinguish collection of 164 strains of *Bacillus* spp. isolated from natural samples of soil, straw and manure from across Serbia.

MATERIALS AND METHODS

Bacterial strains and Bacillus spp. isolates

The *Bacillus* spp. used in this work are part of the larger collection belonging to the Laboratory of Microbiology, Faculty of Biology, isolated from different localities in Serbia. The sources of isolates used in this study were soil, hay and manure. Soil samples were collected from public areas, open fields, parks and private areas. The method used for isolating *Bacillus* spp. was as described in Berić et al. (2009). Shortly, after thermal inactivation of vegetative cells (80°C for 10 min), samples were incubated at 30°C for 48 h on Luria–Bertani (LB) agar plates until pure cultures are obtained (distinct single colonies) for each isolate. The preliminarily characterization, at the genus level, was conducted by microscopic appearance, Gram staining and catalase testing. Bacterial isolates and strains used in this work are shown in Table 1.

Table 1. The list of strains used in this work

Strain	Source of reference
164 isolates - *Bacillus* sp.	Stanković 2003. and Berić 2010. – Chair of microbiology, Faculty of Biology
***B. subtilis* Marburg**	Chair of microbiology, Faculty of Biology
***B. atropheus* ATCC 9372**	Chair of microbiology, Faculty of Biology
***B. subtilis* ATCC 6633**	Chair of microbiology, Faculty of Biology
***B. cereus* ATCC 10876**	Chair of microbiology, Faculty of Biology
***B. thuringiensis* HD1 ΔCryB**	Chair of microbiology, Faculty of Biology
***B. megaterium* OP3-4S**	Laboratory for molecular microbiology, IMGGI

Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis was performed with 164 *Bacillus* isolates and 6 reference strains adapted from KOJIC *et al.* (2006). Cells were grown in LB broth at 37°C to early logarithmic phase and centrifuged at 13000 rpm for 2 min, then washed with EET buffer (100 mM EDTA (SERVA), 10 mM EGTA (SERVA), 10 mM Tris–HCl, pH 8.0) and re-suspended in the same buffer to obtain 1×10^9 CFU/mL determined spectrophotometrically (OD at 610 nm). The cell suspension was warmed up to 42°C, where after it was mixed with an equal volume of pre-heated solution of 2% m/v InCert™ agarose in EET buffer (Lonza, Rockland, MA, USA), poured in 100 μL block modules, and allowed to solidify at 4°C and after that incubated for 24h at 37°C. Incubation with shaking of agarose blocks with incorporated cells was performed for 30 min at 50°C in 10 volumes of EET buffer containing SDS (0.5% m/v) and proteinase K (0.5 mg/mL). Subsequently agarose blocks were washed two times with 100 volumes of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and two times with sterile distilled water (SDW) containing 0.1 mM PMSF (phenylmethylsulfonyl fluoride), each time for 30 min, at room temperature. Prepared agarose blocks were sliced, and each slice was incubated for 30 min in 300μL of the 1×buffer O (UAB Fermentas, Thermo Scientific) at room temperature. After that, the buffer was removed and replaced with fresh O buffer (100 μL) containing 20 units of the *Not*I restriction enzyme (UAB Fermentas, Thermo Scientific). DNA digestion was carried out for 3 h at 37°C. Stop solution (40 % m/v sucrose, 10 mM EDTA, 0.01% (m/v) bromophenol blue, pH 8.0) was used for arresting the reaction and samples were kept at 4°C, until analysis. PFGE was performed with a 2015 Pulsafor unit (LKB Instruments, Broma, Sweden) equipped with a hexagonal electrode array. Agarose gels (1.2% m/v) were run in 0.5×TBE running buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) for 18 h at 280 V at 9°C. Pulse times were increased by step from 8s first 8 h to 19s additional 10 h during electrophoresis. The gels were stained with ethidium bromide (10 mM) and photographed under UV illumination (λ=260nm). Lambda phage concatemers were used as molecular size markers and prepared according to the protocol of WATERBURY and LANE (1987).

Analysis of PFGE profiles

The pictures of developed gels were marked with aid of Phoretix 1D software (TotalLab, free trial license). Database with 164 *Bacillus* spp. isolates and 6 *Bacillus* spp. referent strains was

created with Phoretix 1D Pro software program (TotalLab, free trial license). The database of profiles was used for creation of dendrogram using Band difference and Complete Linkage algorithm (DEFAYS, 1977).

DNA manipulation

Genomic DNA from the *Bacillus* spp. strains was isolated as described earlier (DIMKIĆ *et al.*, 2013). For detection and amplification of *tuf* gene previously described method was used (CAAMANO-ANTELO *et al.*, 2015). PCR reaction mixture was prepared in the following manner: 1 µL of total DNA; 0.1 µL of 5 U/µL KAPA Taq DNA polymerase (KAPA Biosystems, Boston, USA); 2.5 µL of 10 × KAPA Taq buffer; 2.0 µL of $MgCl_2$ (25 mM); 0.5 µL of dNTP mix suspension (10 mM of each); and 2.5 µL of each primers tufGPF (5′-ACGTTGACTGCCCAGGACAC-3′) and tufGPR (5′-ATACCAGTTACGTCAGTTGTACGGA-3′). The PCR conditions were as follows: initial denaturation step at 95°C for 8 min, followed with 35 cycles, each with 30 s of denaturation at 95°C, annealing at 55°C for 1 min and 30 s of extension at 72°C; and final extension step at 72°C for 10 min. After checking on agarose gel and prior to sequencing, PCR products were purified with the QIAquick PCR Purification Kit/250 (QIAGEN GmbH, Hilden, Germany) and sent to commercial service for sequencing in Macrogen sequencing service (Amsterdam, Netherlands).

Phylogenetic analysis

The obtained sequences were searched for homology with gene sequences deposited in the GenBank database through the National Center for Biotechnology Information's Blast search program 2.5.0 for nucleotides (http://www.ncbi.nlm.nih.gov/). All sequences were aligned using the CLUSTAL W multiple alignments (THOMPSON *et al.*, 1994) implemented in BioEdit 7.1.3.0 free software (HALL, 1999). The phylogenetic tree was constructed in MEGA 6.0 software (KUMAR *et al.*, 2008) using the Neighbor-joining method based on a pair-wise distance matrix with the Kimura two-parameter nucleotide substitution model. The topology of the trees was evaluated by the bootstrap resampling method with 1000 replicates and *Clostridium botulinum* NR_036786 was included as an outgroup.

RESULTS AND DISCUSSION

In this study, genetic diversity of large collection of *Bacillus* spp. was analyzed using fingerprinting method of PFGE analysis combined with sequencing of *tuf* gene for chosen isolates. The collection of over two hundred *Bacillus* spp. isolates from different location and habitats in Serbia was accumulated over the years (STANKOVIĆ, 2003; BERIĆ *et al*., 2009; BERIĆ, 2010; STANKOVIĆ *et al*., 2012). The preliminary identification of *Bacillus* isolates is simply and quick. It is sufficing to perform Gram and Schaeffer Fulton staining and catalase test after enrichment procedure (incubation at 80°C for 10 min). Further identification, at species level, could be performed using several strategies. For PFGE analysis of all *Bacillus* strains, isolates from environment as well as 6 referent strains, *Not*I restriction enzyme was used. This enzyme recognizes the sequence GCGGCCGC and breaks chromosomal DNA of *Bacillus* spp. isolates from our collection into 6-24 discriminated fragments. Bands in the size range from 48.5 kb to 1000 kb were produced, with the majority of the bands between 48 and 727 kb. Immense diversity among PFGE profiles was observed and 21 different pulsotype was observed (Fig 1).

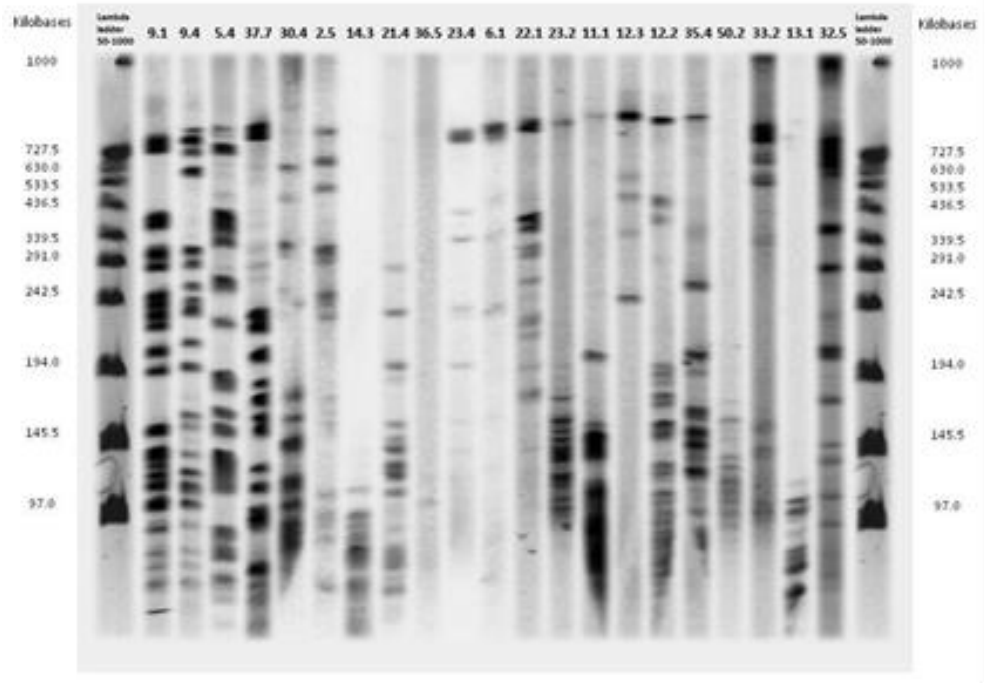

Fig. 1 Composite picture of PFGE profiles obtained from 21 *Bacillus* spp. isolates after analysis and arrangement of 170 profiles into groups. The profiles of the representative isolates and Lambda phage concatemers as molecular size markers were shown.

Based on the diversity of genetic profiles obtained, dendrogram that reconstruct phylogenetic relationships within collection and with the referent strains was constructed using Complete linkage and is shown in Fig. 2. It can be noticed that *B. subtilis* Marburg referent strain didn't group with rest of the strains from the „subtilis group" (*B.subtilis* ATCC6633 and *B. atrophaeus* ATCC9372). Strains from the „cereus group" distinguished itself and closely grouped together.

After assigning cutoff distance to 0.18, twenty-one group of band profiles distinct itself on the phylogenetic tree (Fig. 2). From each PFGE group one representative isolate was chosen for further identification by sequencing of gene for elongation factor (Tab. 2). All isolates gave one distinct DNA band of appropriate length of about 790 bp (data not shown). Purified amplified DNA was sequenced and after BLAST analysis, we obtained phylogenetic tree shown in Fig. 3.

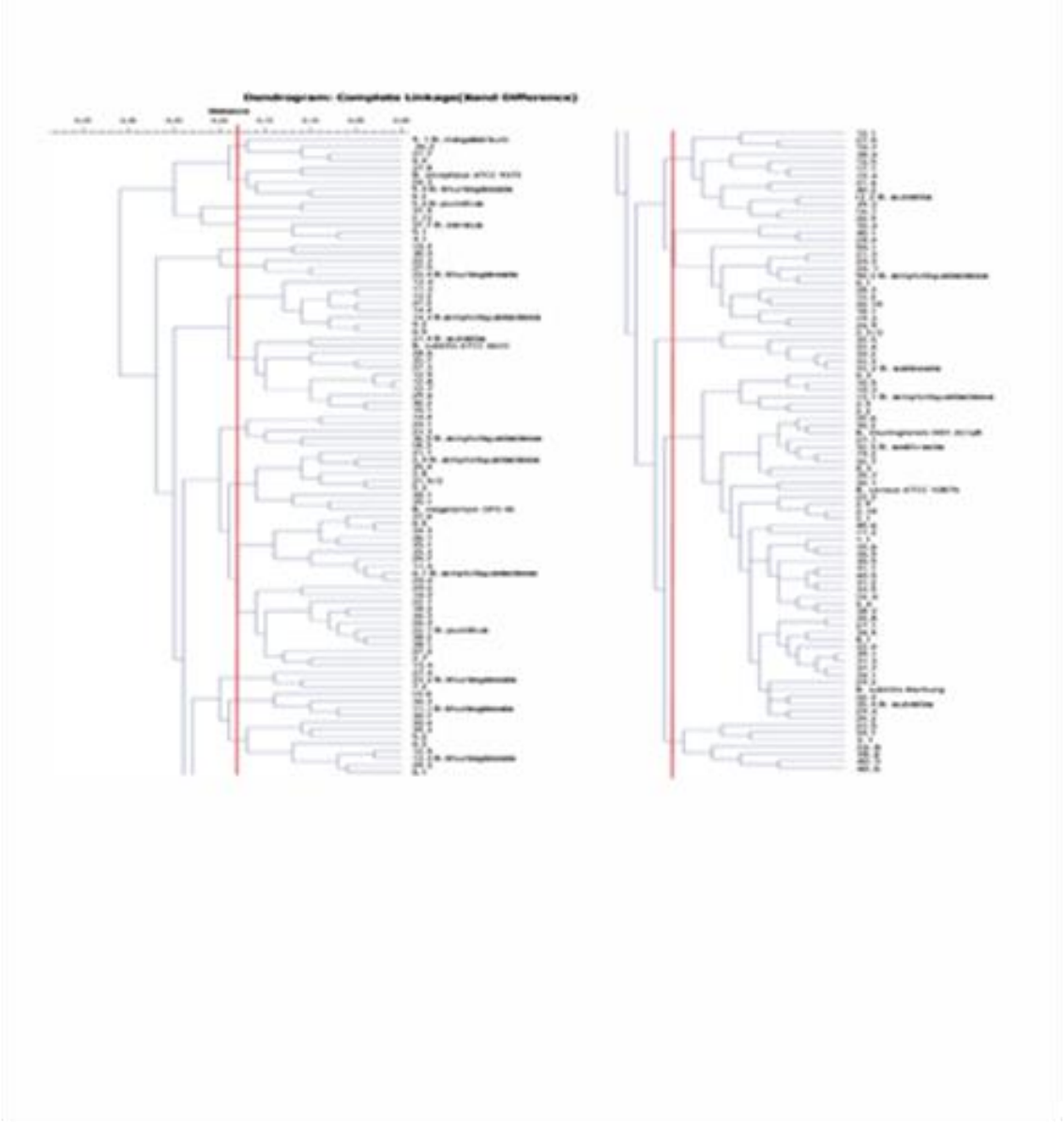

Fig. 2 The dendrogram that reconstructs phylogenetic relationships within collection of *Bacillus* spp. isolates. Complete Linkage method based on genomic profiles obtained after *Not*I digestion and PFGE separation was used. The referent strains are given in full species name. Red vertical line shows cutoff distance of 0.18 that was used for division of profiles into PFGE groups. Next to the denomination of representative isolates from each group, species name (as identified according to sequence of *tuf* gene) was shown.

Very clear and unambiguous grouping of isolates with related strains was achieved. Especially satisfactory results were obtained in dividing *B. subtilis* and *B. amyloliquefaciens* isolates as well as *B. thuringiensis* and *B. cereus* into separate clades on the phylogenetic tree. Isolates: SS-6.1, SS-13.1, SS-14.3, SS-36.5, VPS-50.2 and SS-2.5 showed closest phylogenetic relationship with *B. amyloliquefaciens* while SS-12.2, SS-21.4 and SS-35.4 were most similar with *B. subtilis*. Isolates denoted as *B. cereus*, *B. thuringiensis* and *B. anthracis* grouped close together, but actually, cereus and thuringiensis definitely were separated by the sequence of *tuf* gene, that is hard to achieve using sequence of 16S rRNA gene or even hypervariable regions of 16S rRNA gene. However, isolates SS-30.4, SS-37.7 and SS-32.5 could be assigned as both, *B. anthracis* and *B. cereus* e.g. clear distinction could not be made. Only one isolate (four in total, given the results of PFGE grouping) showed high degree of similarity with *B. megaterium* and clearly was distinct from other isolates. Isolate SS-5.4, SS-22.1 and SS-33.2 exhibited similarity with two species that are usually classified within subtilis group, *B. pumilus* and *B. safensis*, but apparently separated from subtilis clade.

Table 2a. Summary information about representative Bacillus spp. isolates

Isolate	Location	Source	PFGE group	*tuf* identification
SS-9.1	Paraćin courtyard	Soil	I	*B. megaterium* NBRC 15308 ATCC 14581
SS-9.4	Paraćin courtyard	Soil	II	*B. thuringiensis* MYBT18246
SS-5.4	Točane	Straw	III	*B. pumilus* ATCC 14884
SS-37.7	Ovča	Soil	IV	*B. cereus* ATCC 9634
SS-30.4	Location 30	Manure	V	*B. cereus* ATCC 9634
SS-23.4	Valjevo	Soil	VI	*B. thuringiensis* MYBT18246
SS-14.3	Paraćin dock	Soil	VII	*B. amyloliquefaciens* subsp. *plantarum* UCMB5113
SS-21.4	Mala Plana creek	Soil	VIII	*B. subtilis* subsp. *subtilis* NCIB 3610 ATCC 6051
SS-36.5	Erdevik	Soil	IX	*B. amyloliquefaciens* subsp. *plantarum* UCMB5113
SS-2.5	Bresnica	Straw	X	*B. amyloliquefaciens* ATCC 23842
SS-6.1	Paraćin	Soil	XI	*B. amyloliquefaciens* subsp. *plantarum* UCMB5113
SS-22.1	Valjevo	Soil	XII	*B. pumilus* ATCC 14884
SS-23.2	Valjevo - Petnica	Soil	XIII	*B. thuringiensis* MYBT18246
SS-11.1	Mala Plana arable land	Soil	XIV	*B. thuringiensis* MYBT18246
SS-12.3	Paraćin park	Soil	XV	*B. thuringiensis* MYBT18246

Table 2b. Summary information about representative Bacillus spp. isolates

Isolate	Location	Source	PFGE group	*tuf* identification
SS-12.2	Paraćin park	Soil	XVI	*B. subtilis* subsp. *subtilis* NCIB 3610 ATCC 6051
VPS.50.2	Beograd - Viša pedagoška	Soil	XVII	*B. amyloliquefaciens* subsp. *plantarum* UCMB5113
SS-33.2	Location 33	Manure	XVIII	*B. safensis* KCTC 12796BP
SS-13.1	Donje Vidovo	Soil	XIX	*B. amyloliquefaciens* subsp. *plantarum* UCMB5113
SS-32.5	Location 32	Manure	XX	*B. anthracis* SPV842_15
SS-35.4	Kaluđerica	Soil	XXI	*B. subtilis* subsp. *subtilis* NCIB 3610 ATCC 6051

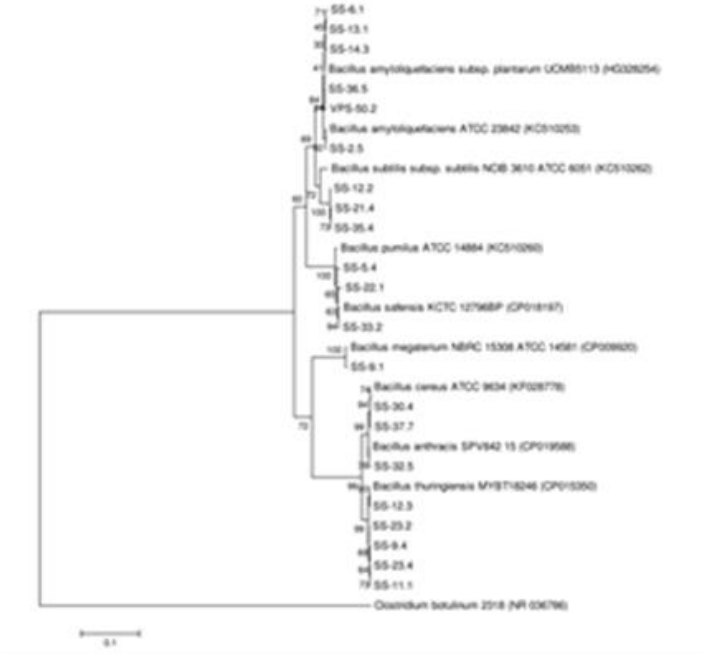

Fig. 3 Neighbor-joining phylogenetic tree based on *tuf* gene sequences (790 bp) showing the relationship of the tested isolates and related reference strains of the genus *Bacillus*. The topology of the trees was evaluated by the bootstrap resampling method with 1000 replicates and *Clostridium botulinum* NR_036786 was included as an outgroup.

When comparing results of sequence analysis with the ones obtained using profiling method of PFGE (Tab. 2 and Fig. 3), following could be observed. Although there was no such precision with identification and separation within *Bacillus* groups, groups of profiles were

sufficiently accurate to support general division into subtilis group (there was no apparent separation between subtilis and amyloliquefaciens isolates) and to a certain extent into cereus group. As already concluded OTLEWSKA *et al.* (2013), PFGE analysis could be used for diversification of strains but it is not suitable for taxonomic grouping of closely related strains of *B. cereus* and *B. thuringiensis*. In our study, actually, although *B. thuringiensis* profiles of isolates SS-9.4 and SS-23.4 group together with *B. cereus*, three groups of isolates (XII, XIV and XV) identified as *B. thuringiensis* formed separate clade branching far from former mentioned. The PFGE profiles of group I of isolates, represented with SS-9.1 (*B. megaterium*) were separated and closely branching with group II, with the representative isolate identified as *B. thuringiensis*. Similar can be observed, for isolates in question, on the phylogenetic tree in Fig. 3, where *B. megaterium* branches closely with cereus/thuringiensis/anthracis isolates. Only for PFGE groups III and XIII, with isolates identified as *B. pumilus* (SS-5.4 and SS-22.1) there was no expected grouping with subtilis group(s). In addition, PFGE group XXI, with seven isolates represented with SS-32.5, identified as belonging to *B. anthracis* showed no conjunction to other PFGE groups denominated as *B. cereus* and *B. thuringiensis*. Although methods, PFGE and sequencing of *tuf* gene are widely used separately, this kind of combined approach for identification purposes of bacilli was not used previously. Looking at isolates PFGE profiles and source and location of isolates sampling, any correlation between them was not found. This indicates enormous genetic diversity of *Bacillus* isolates, in any given environment.

CONCLUSION

In this work, we showed that PFGE analysis combined with sequencing of one of the housekeeping genes, gene for elongation factor Tu, could be used for characterization of large collections of *Bacillus* isolates. In addition, when choosing sequencing only for *Bacillus* spp. identification purposes, determination of *tuf* gene using very specific primers designed by CAAMANO-ANTELO *et al.* (2015) recommend itself to be an adequate and sufficient analysis for obtaining very clear and unambiguous results, with high resolution of separation of species.

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GENOTIPIZACIJA KOLEKCIJE *BACILLUS* SPP. IZOLOVANIH IZ UZORAKA IZ PRIRODE

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Izvod

Cilj ove studije je bio genotipizacija i identifikacija kolekcije od 164 izolata *Bacillus* spp. izolovanih iz zemljišta, stajskog đubriva i slame širom Srbije, korišćenjem elektroforeze u pulsirajućem polju (PFGE), kombinovane sa sekvenciranjem jednog od "housekeeping" gena, *tuf* gena. PFGE analiza sa *Not*I enzimom je korišćena da se odrede filogenetski odnosi izolata i referentnih sojeva. Razdvojile su se četiri velike grupe roda *Bacillus* spp: cereus, subtilis, pumilus i megaterium, sa velikim genetičkim diverzitetom i u samim grupama. *Bacillus subtilis* Marburg referentni soj nije se grupisao sa ostatkom sojeva iz subtilis grupe (*Bacillus subtilis* ATCC6633 i *Bacillus atrophaeus* ATCC9372). Sojevi iz cereus grupe su se izdvojili i tesno su se grupisali zajedno. Po jedan reprezentativni izolat svake od 21 različite PFGE grupe je identifikovan sekvenciranjem *tuf* gena. Ukupno osam različitih vrsta je identifikovano među izabranim izolatima: *B. amyloliquefaciens, B. subtilis, B. pumilus, B. safensis, B. megaterium, B. cereus, B. anthracis* i *B. thuringiensis*. Naši rezultati su pokazali da PFGE analiza kombinovana sa sekvenciranjem jednog od "housekeeping" gena može biti korišćena za karakterizaciju velikih kolekcija izolata roda *Bacillus*. Pristup identifikaciji u vidu umnožavanje i sekvenciranje *tuf* gena pokazao se kao adekvatna i potpuna analiza za dobijanje vrlo jasnih i nedvosmislenih rezultata, sa velikom rezolucijom u razdvajanju *Bacillus* vrsta.

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