ISSN 0026-2617, Microbiology, 2015, Vol. 84, No. 2, pp. 236–246. © Pleiades Publishing, Ltd., 2015.

EXPERIMENTAL ARTICLES

Comparison of Molecular and Phenetic Typing Methods to Assess Diversity of Selected Members of the Genus *Bacillus*[1]

P. Rai, A. Sharma, P. Saxena, A. P. Soni, H. Chakdar[2], P. L. Kashyap, A. K. Srivastava, and A. K. Sharma

ICAR-National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau, Uttar Pradesh-275101, India

Received September 14, 2014

Abstract—A comparison of different molecular typing methods viz. ERIC-PCR, BOX-PCR and ARDRA along with carbohydrate utilization pattern was carried out to analyze their discriminatory power and suitability for assessing diversity of selected *Bacillus* isolates. ERIC-PCR generated 61 bands ranging from 0.56 to 3.9 kb while BOX-PCR resulted in 127 bands ranging from 0.16 to 3.9 kb. Restriction analysis of 16S rDNA with *Alu*I and *Hae*III produced 56 and 67 bands ranging from 0.14 to 0.54 and 0.12 to 0.96, respectively. Clustering of isolates based on the ERIC, BOX and ARDRA pattern clearly showed the superiority of the former two methods to reveal the intrageneric and intraspecific diversity. Carbohydrate utilization pattern showed that most preferred sugar was Fructose while Xylose, Rhamnose and D-Arabinose were least preferred by the isolates used for the study. Clustering based on carbohydrate utilization was also able to differentiate among the isolates which showed 100% similarity based on ARDRA profiles. This study clearly shows that typing methods exploiting the repetitive elements distributed over the genome are more useful for assessing genetic diversity. Moreover, metabolic diversity of the bacterial groups may also be useful instead of using single locus specific marker systems for revealing the diversity.

Keywords: *Bacillus*, Repetitive elements, 16S rDNA, genetic diversity, phenetic diversity

DOI: 10.1134/S0026261715020113

The genus Bacillus constitutes of phenotypically heterogeneous, physiologically, metabolically and phylogenetically diverse species [1, 2]. Numerous attempts have been made to identify and classify the members of this gram positive, spore forming bacteria based on their morphometric characters, sporulation, utilization and degradation of various compounds, rDNA homology, DNA base composition etc. [2–4 (3–5)]. With the development of advanced molecular tools, further phenotypic and genetic characterization of the members of the *Bacillus* have led to the creation of new genera like *Amphibacillus, Alicyclobacillus, Paenibacillus, Aneurinibacillus, Brevibacillus, Virgibacillus, Gracilibacillus, Salibacillus, Filobacillus, Geobacillus, Ureibacillus, Jeotgalibacillus* and *Marinibacillus* [6–12].

16S rDNA sequencing technology with the availability of huge number of reference sequences in public domain databases (like NCBI, DDBJ, EMBL, RDP etc.) have provided an excellent opportunity for identification and characterization of microorganisms at species and subspecies levels [13]. Restriction patterns of 16S ribosomal DNA have also been exploited as a rapid identification tool for different bacteria [14–18]. PCR based techniques using repetitive elements like BOX, REP, $(GTG)_5$ and ERIC (Enterobacterial Repetitive Intergenic Consensus) have recently been used extensively for genetic characterization of both gram-positive and gram-negative bacteria and population genetic studies. The repetitive extragenic palindromic (REP) elements are palindromic units containing a variable loop in stem-loop structure [19]. ERIC sequences are characterized by central, conserved palindromic structures [20] while BOX elements consist of differentially conserved subunits, namely boxA, boxB, and boxC [21] of which only the boxA-like subunit sequences are highly conserved among diverse bacteria [22]. Methods based on such repetitive elements have also been used for studying the diversity in the ecosystem, presenting the phylogenetic relationship between strains and discriminating between microorganisms those are genetically close to each other [23].

In this study, we compared three different typing methods based on ERIC, BOX element, restriction profiling of 16S rDNA along with carbohydrate utilization to assess the genetic as well phenetic diversity of twenty strains of different *Bacillus* species and to evaluate their discriminatory power for the analysis of diversity.

[1] The article is published in the original.

[2] Corresponding author; e-mail: hillol.chakdar@gmail.com

Table 1. List of *Bacillus* isolates used for the study

SI. No.	NAIMCC Accession no.	Name	Isolate history
1	B-00072	*Bacillus amyloliquefaciens*	Isolated from Kasargod, Kerala
2	B-00076	*B. brevis*	Isolated from Assam
3	B-00080	*B. circulans*	Isolated from Uttar Pradesh
4	B-00082	*B. circulans*	NCIM isolate no. 5057
5	B-00083	*B. coagulans*	NCIM isolate no. 2030
6	B-00089	*B. laterosporus*	ATCC isolate; ATCC-64; Reclassified as *Brevibacillus laterosporus* (type strain)
7	B-00091	*B. licheniformis*	NCIM isolate no. 2042
8	B-00093	*B. licheniformis*	Isolated from *Cyamopsis* rhizopshere from Rajasthan
9	B-00096	*B. marcescens*	NCIM isolate no. 5037
10	B-00111	*B. sphaericus*	ATCC4525; Reclassified as *Lysinibacillus sphaericus*
11	B-00119	*B. subtilis*	NCIM isolate no. 2192
12	B-00120	*B. subtilis*	NCIM isolate no. 2236
13	B-00132	*B. thuringiensis*	Isolated from Andhra Pradesh
14	B-00133	*B. thuringiensis*	Isolated from Andhra Pradesh
15	B-00744	*B. cereus*	Isolated from Maharashtra
16	B-00771	*B. pumilus*	Isolated from lake water of Leh, Jammu & Kashmir; Psychrotolerant
17	B-00776	*B. pumilus*	Isolated from lake water of Leh, Jammu & Kashmir; Psychrotolerant
18	B-00840	*B. subtilis*	Isolated from Vermicomposts in Maharashtra
19	B-00846	*B. subtilis*	Isolated from Vermicomposts in Maharashtra
20	B-00870	*B. subtilis*	Isolated from rhizospheric soils of eastern Uttar Pradesh

#—NCIM: National Collection of Industrial Microorganisms, India; ATCC: American Type Culture Collection, USA.

MATERIALS AND METHODS

Cultures. Glycerol stocks of twenty cultures of *Bacillus* listed in Table 1 were obtained from National Agriculturally Important Microbial Culture Collection (NAIMCC), NBAIM, U.P., India. Bacterial cultures from different geographical origin and ecological habitats were chosen including multiple strains of bacterial species.

Growth and maintenance. Cultures were revived by streaking on Nutrient Agar (HiMedia, India) plates and incubating at 35 ± 2°C temperature for 24–48 hours. Bacterial cultures were subcultured on Nutrient Agar slants for further studies. For DNA extraction and carbohydrate utilization study, inoculum was prepared by growing the cultures in 5 mL Nutrient Broth (HiMedia, India) in screw capped tubes and incubating overnight at 35 ± 2°C.

Carbohydrate utilization. Utilization of sixteen sugars and nine sugar alcohols (Table 2) and consequent acid production was studied using HiCarbo kit (HiMedia, India). This study was carried out following the manufacturer's protocol.

DNA extraction and quantification. For DNA extraction, cultures were grown in Nutrient broth under the conditions described in Growth and Maintenance section. 5 mL culture suspension was centrifuged to obtain the pellets and DNA was extracted using the protocol described by Ausubel et al. [24]. Quantification and purity check of DNA samples were done spectrophotometrically.

BOX PCR. BOX A1R primer (5'-CTACGGCAAGGCGACGCCTGACG-3') was used for this purpose [22]. Amplification reactions were performed in a reaction volume of 25.0 μL containing 2.5 μL 10× PCR buffer with $MgCl_2$, 1.5 μL 25 mM dNTP mixture, 1.2 μL of BOX A1R primer (10 pM), 0.5 units *Taq* DNA polymerase, 2 μL template DNA and 17.65 μL sterile distilled water. Thermal cycling was achieved in G-STORM thermal cycler with the conditions described by Rademaker and De Bruijn [23]. 10 μL of amplified products along with molecular weight marker (Step Up 1 kb ladder, Bangalore Genei, India) were electropohresed on 1.2% agarose gel (Sigma) at 75 volts for 1.5 hours. Then gel was stained with ethidium bromide (0.5 μg/mL) solution for 1 min and de-stained in water for 30 min. Amplified products were visualized under UV light and gel photograph was documented using gel documentation unit (BioRad, USA).

ERIC-PCR. ERIC-PCR was performed using specific ERIC primers viz. ERIC 1R (5'-ATG-

Table 2. Utilization of different sugars and sugar alcohols by the *Bacillus* strains studied

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
B-00120	–	–	+	+	+	–	+	+	–	+	–	+	–	+	–	–	–	–	+	+	–	–	–	–	+
B-00080	–	–	–	+	+	–	–	–	–	+	+	+	–	+	–	–	–	–	–	+	–	–	–	–	–
B-00096	–	–	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B-00846	–	–	+	+	+	–	–	+	–	+	+	+	–	+	–	–	–	+	–	+	–	–	–	–	+
B-00093	–	–	+	+	–	–	–	+	–	+	+	–	–	+	–	–	–	+	+	+	–	–	–	–	+
B-00091	–	–	–	+	+	–	–	–	–	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–
B-00083	–	–	–	+	+	–	–	–	–	+	+	+	–	–	–	–	+	–	–	–	–	–	–	–	–
B-00119	–	–	+	+	+	–	–	+	–	+	+	+	–	+	–	–	–	+	+	+	–	–	–	–	+
B-00771	+	–	+	+	+	–	–	+	–	+	+	+	+	+	–	+	+	+	+	+	–	–	–	–	+
B-00776	–	–	+	+	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
B-00072	+	–	+	+	+	–	+	+	+	+	+	+	–	+	–	–	–	–	+	+	–	–	–	–	+
B-00082	–	–	–	+	+	–	–	+	–	+	+	+	–	+	–	–	–	–	–	+	–	–	–	+	–
B-00076	–	–	+	+	+	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
B-00089	+	+	+	+	+	–	–	+	–	–	+	+	–	–	–	–	–	–	+	+	–	–	–	–	+
B-00132	–	–	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B-00133	–	–	+	+	+	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
B-00111	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
B-00744	–	–	–	+	–	–	–	–	–	+	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–
B-00870	–	–	–	+	+	–	+	+	–	+	+	+	–	+	–	–	–	+	+	+	–	–	–	–	+
B-00840	–	–	+	+	+	–	–	+	–	+	+	+	–	+	–	–	–	+	+	+	–	–	–	–	+

1. Lactose; 2. Xylose; 3. Maltose; 4. Fructose; 5. Dextrose; 6. Galactose; 7. Raffinose; 8. Trehalose; 9. Melibiose; 10. Sucrose; 11. L-Arabinose; 12. Mannose; 13. Rhamnose; 14. Cellobiose; 15. Melezitose; 16. D-Arabinose; 17. Dulcitol; 18. Inositol; 19. Sorbitol, 20. Mannitol; 21. Adonitol; 22. Arabitol; 23. Erythritol; 24. Xylitol; 25. Inulin; +—indicates positive results for carbohydrate utilization while, – indicates negative results.

TAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') [25]. Amplification was performed in a reaction volume of 25 μL containing 2.5 μL 10× PCR buffer with $MgCl_2$, 1.5 μL 25 mM dNTP mixture, 0.6 μL of each primer (10 pM), 0.5 units *Taq* DNA polymerase, 2 μL template DNA and 19.3 μL sterile distilled water. Thermal cycling was achieved in G-STORM thermal cycler under the conditions described by De Bruijn (1992) after minor modifications [26]. Electrophoresis, gel staining with ethidium bromide, visualization of bands and documentation were carried out as described in the previous section.

Amplified ribosomal DNA restriction analysis. (a) Amplification of 16S rRNA gene: universal primer pairs viz. pA (5'-AGTTTGATCCTGGCTAG-3') and pH (5'-AGGAGGTGATCCAGCCGCA-3') reported by Edwards et al. (1989) [27] were used to amplify the 16S rRNA gene. PCR amplifications were performed as described by Vardhan et al. [28]. Electrophoresis, gel staining with ethidium bromide, visualization of bands and documentation were carried out as described in the previous sections.

(b) Restriction digestion of amplified 16S rRNA gene products: two restriction enzymes viz. *Alu*I and *Hae*III were procured from Promega (USA). Restriction digestions were performed in 25 μL reactions following the manufacturer's instructions. 10 μL of digested products along with molecular weight marker (Step Up 100 bp ladder, Bangalore Genei, India) were run on 2.5% agarose gel (Sigma). Gel staining with ethidium bromide, visualization of bands and documentation were carried out as described in the previous sections.

Statistical analysis. Pair wise genetic similarities among the isolates under study were determined using Jaccard's coefficient [29]. The size and number of bands were determined using FluorChem5500 (Alpha InnoTech, USA) for this purpose. Cluster analyses were carried out on similarity estimates using the unweighted pair-group method with arithmetic average (UPGMA) using NTSYS_pc, version 2.02 [30]. To test the goodness of fit of clustering, cophenetic

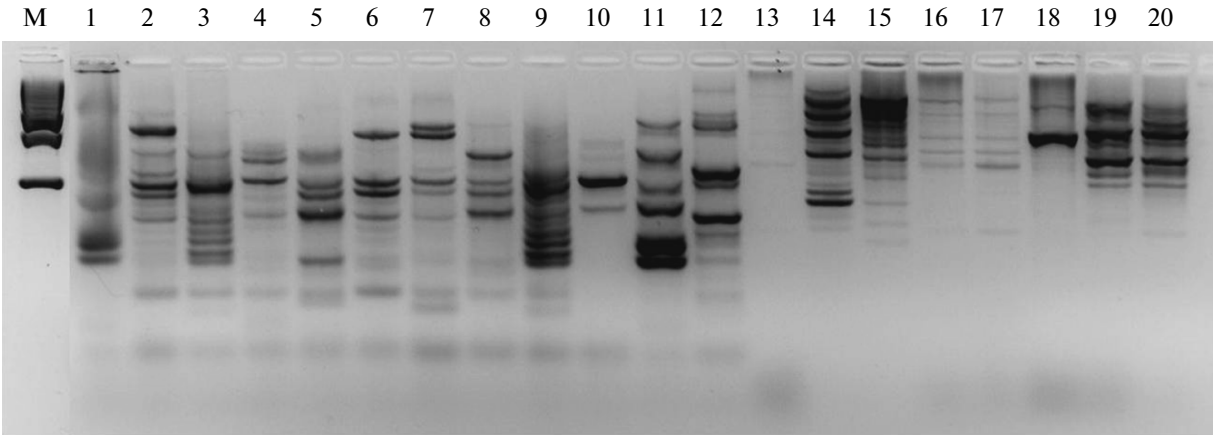

Fig. 1. BOX-PCR profiles of the twenty *Bacillus* strains studied.

Lane 1 = 1 kb ladder	Lane 2 = B-00120	Lane 3 = B-00080	Lane 4 = B-00096	Lane 5 = B-00846	Lane 6 = B-00093	Lane 7 = B-00091
Lane 8 = B-00083	Lane 9 = B-00119	Lane 10 = B-00771	Lane 11 = B-00776	Lane 12 = B-00072	Lane 13 = B-00082	Lane 14 = B-00076
Lane 15 = B-00089	Lane 16 = B-00132	Lane 17 = B-00133	Lane 18 = B-00111	Lane 19 = B-00744	Lane 20 = B-00870	Lane 21= B-00840

correlation coefficient or cophenetic value was estimated as described by Chakdar and Pabbi [31] using NTSYS_pc program. The degree of fit was interpreted subjectively—$0.9 \le r$ is very good fit, $0.8 \le r \le 0.9$ is good fit, $0.7 \le r \le 0.8$ is poor fit and $r < 0.7$ is very poor fit.

RESULTS AND DISCUSSION

BOX PCR generated a total 127 bands ranging from 160 bp to 3.9 kb for all the twenty Bacilli studied (Fig. 1). Maximum numbers of bands (11) were observed for *B. pumilus* B-00771 while lowest was (02) in case of *B. brevis* B-00076 (Table 3). Dendrogram generated through BOX PCR fingerprints revealed two major clusters, one containing nineteen while the other contained only one strain viz. *B. cereus* B-00744 (Fig. 2). The former cluster had two sub-clusters, one containing eighteen strains and the other contained one i.e. *B. brevis* B-00076. Similarity coefficient among the strains ranged from 0.025 to 0.72. Although multiple isolates from different *Bacillus* species like *subtilis, circulans, thuringiensis* were used but interestingly only *B. subtilis* B-00840 and *B. subtilis* B-00870 were clustered together with a similarity of 67.5%. The clustering based on BOX PCR fingerprints was found to be good fit ($r = 0.82$).

61 bands were generated through ERIC-PCR of all the *Bacillus* strains and band sizes ranged from 240 bp to 3.9 kb (Fig. 3). *B. laterosporus* B-00089 did not show any amplification during ERIC-PCR and among rest of the nineteen strains, *B. subtilis* B-00870 showed maximum number (09) of bands while *B. subtilis* B-00846 showed lowest number (01) of bands (Table 3). Like BOX-PCR, the dendrogram generated based on the ERIC-PCR fingerprints also showed two major clusters containing eighteen and two strains respectively (Fig. 4). The former major cluster contained two sub-clusters containing eleven and seven strains respectively. The Jaccard's similarity coefficient ranged from 0.025 to 0.50. Interestingly, like BOX-PCR, *B. sphaericus* B-00111 and *B. thuringiensis* B-00133 clustered together with a similarity of 32.5% in case of ERIC-PCR also. The cophenetic correlation coefficient for clustering based on ERIC-PCR was 0.77 indicating poor fit of the clustering.

Restriction digestion of 16S rDNA with *Alu*I produced 56 bands (140 to 540 bp) (Fig. 5, Table 3). Most of the strains showed 3 bands upon digestion but *B. pumilus* B-00771 and *B. laterosporus* B-00089 showed two bands while *B. subtilis* B-00870 showed a single band of 200 bp. When the 16S rDNA was digested with *Hae*III, 67 bands ranging from 120 to 960 bp were generated. *B. subtilis* B-0080 produced only one band of 500 bp, *B. brevis* B-00076 produced two bands while other strains produced 3–4 bands each. Based on restriction pattern 16S rDNA (digested with both *Alu*I and *Hae*III) bacterial isolates were clustered in two major clades, one with nineteen strains and the other with only one strain i.e. *B. subtilis* B-00870 (Fig. 6). In the former major cluster, two sub-clusters were found, one with eighteen strains and the

Table 3. Amplicons produced by the studied bacilli during ARDRA, ERIC- and BOX-PCR

SI. No.	Bacteria	No. of Bands				Size range of bands, kb			
		BOX-PCR	ERIC-PCR	ARDRA		BOX-PCR	ERIC-PCR	ARDRA	
				*Alu*I	*Hae*III			*Alu*I	*Hae*II
1	B-00120	05	07	03	04	0.29–3.2	0.68–3.8	0.19–0.50	0.12–0.67
2	B-00080	08	03	03	04	0.18–3.0	0.57–1.4	0.18–0.47	0.12–0.62
3	B-00096	09	02	03	04	0.18–1.6	0.56–0.92	0.19–0.43	0.13–0.96
4	B-00846	05	01	03	03	0.16–1.9	3.1	0.20–0.50	0.25–0.64
5	B-00093	06	02	03	03	0.16–1.6	1.0–1.5	0.20–0.50	0.24–0.62
6	B-00091	06	02	03	04	0.18–2.2	0.62–1.6	0.21–0.50	0.12–0.62
7	B-00083	08	02	03	04	0.14–2.4	0.63–1.6	0.20–0.52	0.12–0.60
8	B-00119	06	01	03	04	0.18–2.5	1.0	0.20–0.50	0.12–0.58
9	B-00771	11	03	02	03	0.17–1.98	0.75–2.6	0.14–0.20	0.23–0.60
10	B-00776	04	01	03	04	0.65–2.0	3.2	0.20–0.54	0.21–0.92
11	B-00072	06	03	03	04	0.28–2.5	0.88–3.9	0.20–0.50	0.21–0.89
12	B-00082	07	04	03	03	0.29–3.0	0.81–2.4	0.19–0.48	0.21–0.56
13	B-00076	02	04	03	02	1.4–2.5	1.1–3.4	0.19–0.48	0.21–0.86
14	B-00089	09	No amplification	02	04	0.48–3.6	No amplification	0.32–0.35	0.19–0.62
15	B-00132	08	05	03	03	0.5–3.9	0.42–2.6	0.20–0.46	0.19–0.86
16	B-00133	05	02	03	03	1.7–3.9	0.58–1.2	0.20–0.46	0.19–0.86
17	B-00111	06	01	03	03	0.49–3.9	1.4	0.14–0.50	0.21–0.86
18	B-00744	02	04	03	04	2.2–3.6	0.24–1.2	0.19–0.50	0.19–0.89
19	B-00870	05	09	01	01	0.94–3.3	0.55–3.7	0.20	0.50
20	B-00840	08	05	03	03	0.44–3.4	0.41–2.4	0.18–0.50	0.18–0.48
	Total no. of bands	127	61	56	67				

other with only *B. pumilus* B-00771. The similarity coefficient ranged from 0.125 to 1.00. In congruence with ERIC and BOX PCR, the restriction profiling of ribosomal DNA also clustered *B. sphaericus* B-00111 and *B. thuringiensis* B-00133 together with a similarity of 62.5%. In contrast to the BOX- and ERIC-PCR, the dendrogram generated based on the ARDRA profile showed clustering of *B. subtilis* B-00120, *B. subtilis* B-00119, *B. circulans* B-00080 and *B. coagulans* B-00083 together and clustering of *B. subtilis* B-00846, *B. licheniformis* B-00093 and *B. licheniformis* B-00091 together with 100% similarity. ARDRA based clustering was found to be very good fit ($r = 0.92$).

Out of twenty five sugar and sugar alcohols used, acid production could be observed from all the sugars but Melezitose and Galactose while sugar alcohols like Adonitol, Arabitol and Erythritol were not used by any of the strains (Table 2). Most preferred sugar was Fructose while Xylose, Rhamnose and D-Arabinose were least preferred among the sugars used (Table 2). Dendrogram based on the patterns of sugar and sugar alcohol utilization showed two clusters—one with nineteen strains and another with only one isolate viz. *B. sphaericus* B-00111 (Fig. 7). The former cluster in turn showed two sub-clusters—one with fourteen strains and the other with five strains. In the former sub-cluster the strains viz. *B. subtilis* B-00119 and *B. subtilis* B-00840 showed 100% similarity while in the later sub-cluster *B. brevis* B-00076 and *B. thuringiensis* B-00133 showed 100% similarity. The similarity among all the strains ranged from ~15 to 100%. The degree of the goodness of fit of the clustering based on carbohydrate utilization pattern was found to be very good fit ($r = 0.92$).

In a classical study, Priest et al. used numerical classification based on a series of phenetic characters for classifying 368 *Bacillus* strains [3]. But phenotypic characters are always influenced by environmental conditions which limits their use for discriminatory purposes. Apart from the phenetic diversity exhibited by the members of the genus *Bacillus*, they are genetically diverse also which have been reported in a number of studies [32–34]. Molecular approaches are increasingly being used for rapid species identification

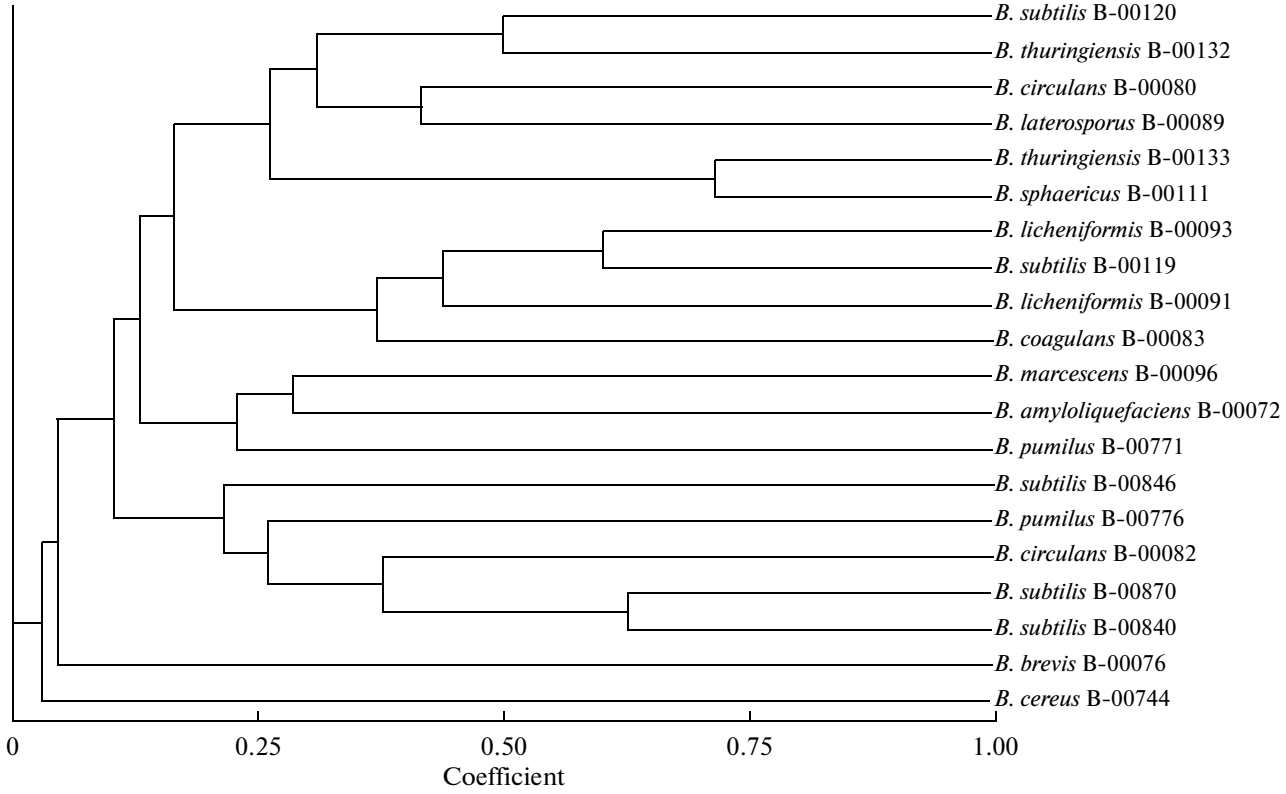

Fig. 2. Clustering of the studied bacilli based on BOX-PCR profiles.

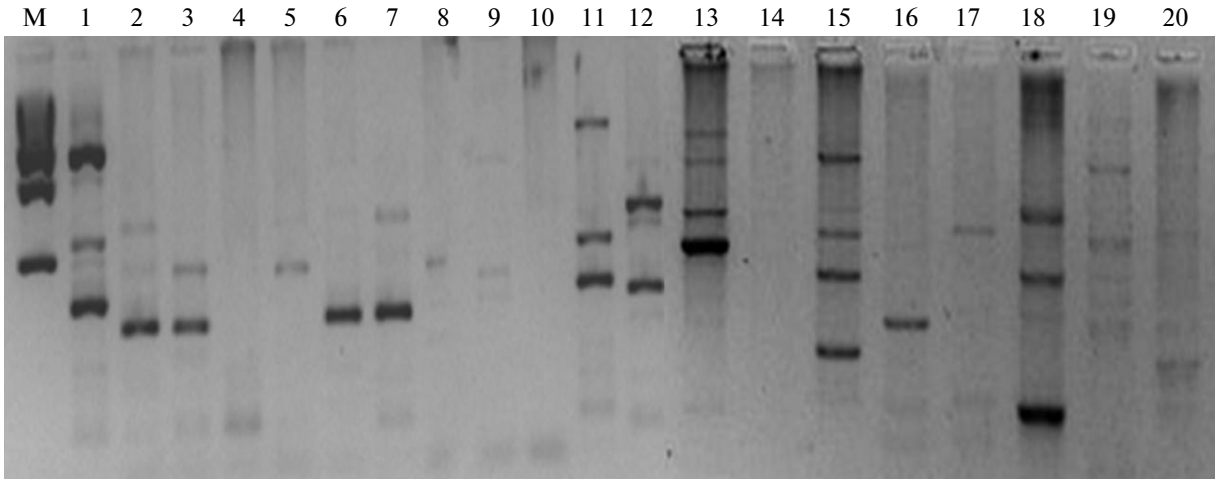

Fig. 3. ERIC-PCR profiles of the twenty *Bacillus* strains studied.

Lane 1 = 1 kb ladder	Lane 2 = B-00120	Lane 3 = B-00080	Lane 4 = B-00096	Lane 5 = B-00846	Lane 6 = B-00093	Lane 7 = B-00091
Lane 8 = B-00083	Lane 9 = B-00119	Lane 10 = B-00771	Lane 11 = B-00776	Lane 12 = B-00072	Lane 13 = B-00082	Lane 14 = B-00076
Lane 15 = B-00089	Lane 16 = B-00132	Lane 17 = B-00133	Lane 18 = B-00111	Lane 19 = B-00744	Lane 20 = B-00870	Lane 21 = B-00840

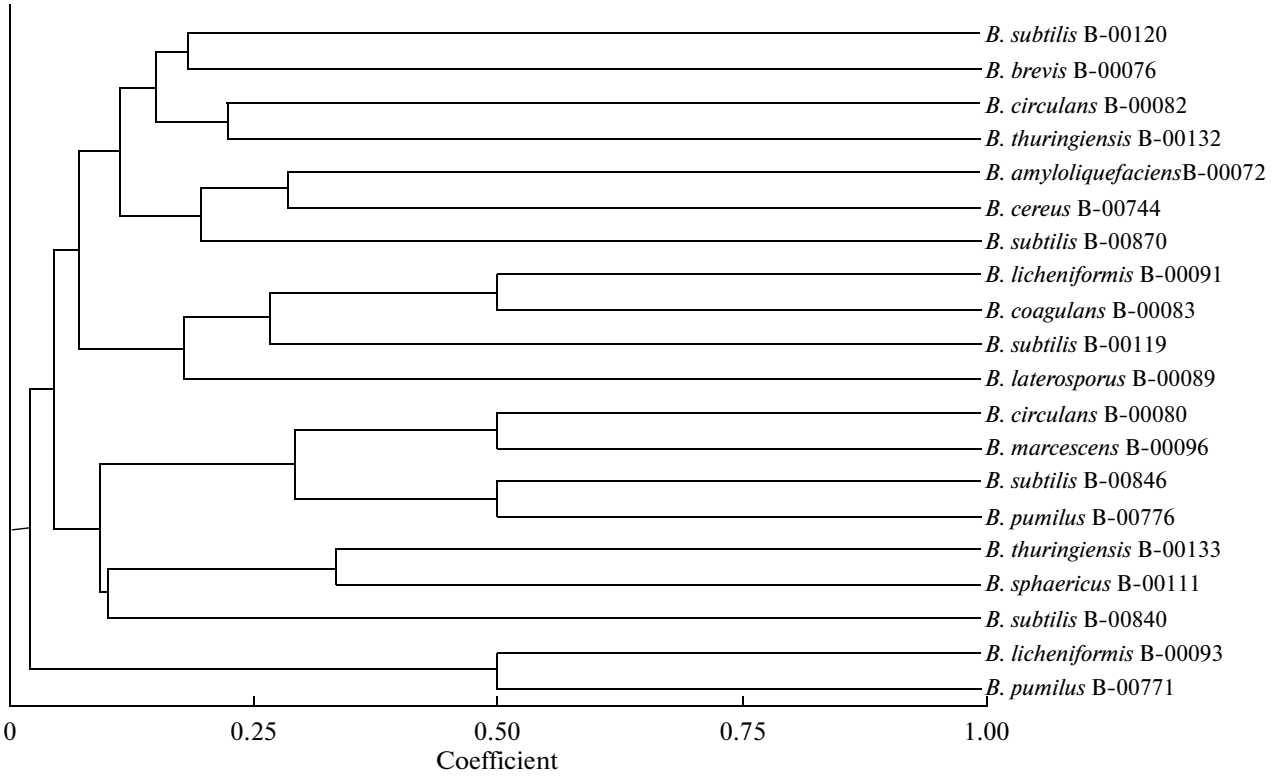

Fig. 4. Clustering of the studied bacilli based on ERIC-PCR profiles.

exploiting this enormous intra- and inter-specific diversity. Although, 16S rDNA sequencing has emerged as the ultimate identification tool, tools like multi-locus sequence typing have now become more handy and trendy tools for assessing the phylogenetic relationships in a better way. Exploiting repetitive genetic elements which are wide spread over the bacterial genomes, can also be a good and powerful recourse to identify and discriminate different bacterial species. This method can be used to generate more accurate information because it is capable of screening several parts of the bacterial genome [22, 25]. Amplified ribosomal DNA restriction analysis is another tool which can be helpful for differentiating among the bacterial species. But definitely it does not have the advantage of considering genome wide diversity and selection of ideal restriction enzyme for digestion becomes a tricky thing.

In the present study the comparison among the different molecular typing methods viz. ARDRA, BOX-PCR and ERIC-PCR reveals that the discriminatory power of the BOX and ERIC-PCR is comparatively higher than the ARDRA which is evident from the number bands generated and the range of similarity coefficient obtained after cluster analysis The results of this study also revealed that the numbers of BOX elements (127 bands in 20 strains) are more than double of the ERIC elements (61 bands in 20 strains) which was expected BOX elements are more likely in gram positive bacteria. Again the genome wide coverage of repetitive elements makes ERIC- and BOX-PCR better tool than ARDRA for bacterial diversity analysis which is well supported by the results obtained in the study. The range of similarity coefficient in cluster analysis based on BOX-PCR (0.25 to 0.72) and ERIC-PCR (0.025 to 0.50) was comparatively lower reflecting more diversity as compared to ARDRA (0.125 to 1.00. Frietas et al. (2008) reported that among different repetitive element based PCR systems viz. $(GTG)_5$, BOX- and ERIC-, the later two were more efficient than the former one and particularly ERIC-PCR resulted in similar clustering of *Bacillus* isolates as obtained with 16S rDNA sequence based phylogenetic analysis [35]. They also reported ERIC-PCR as a powerful tool for examining genetic relationship among the unknown *Bacillus* isolates. In congruence with results reported by Freitas et al. (2008), our results also showed that PCR based on repetitive elements have better discriminatory power for intra-generic as well as intra-specific diversity of *Bacillus*. However, the poor fitness of the clustering for ERIC-PCR and lack of amplification in one strain (*B. laterosporus* B-00089) in the present study indicates that PCR based on ERIC elements may not be always very trustworthy for discriminating *Bacillus* members. Clustering analysis based on ARDRA showed 4 strains (*B. subtilis* B-00120, *B. subtilis* B-00119, *B. circulans* B-00080, *B. coagulans* B-00083 together) in one cluster and another 3 strains (*B. subtilis* B-00846, *B. licheniformis* B-00093 and *B. licheniformis* B-

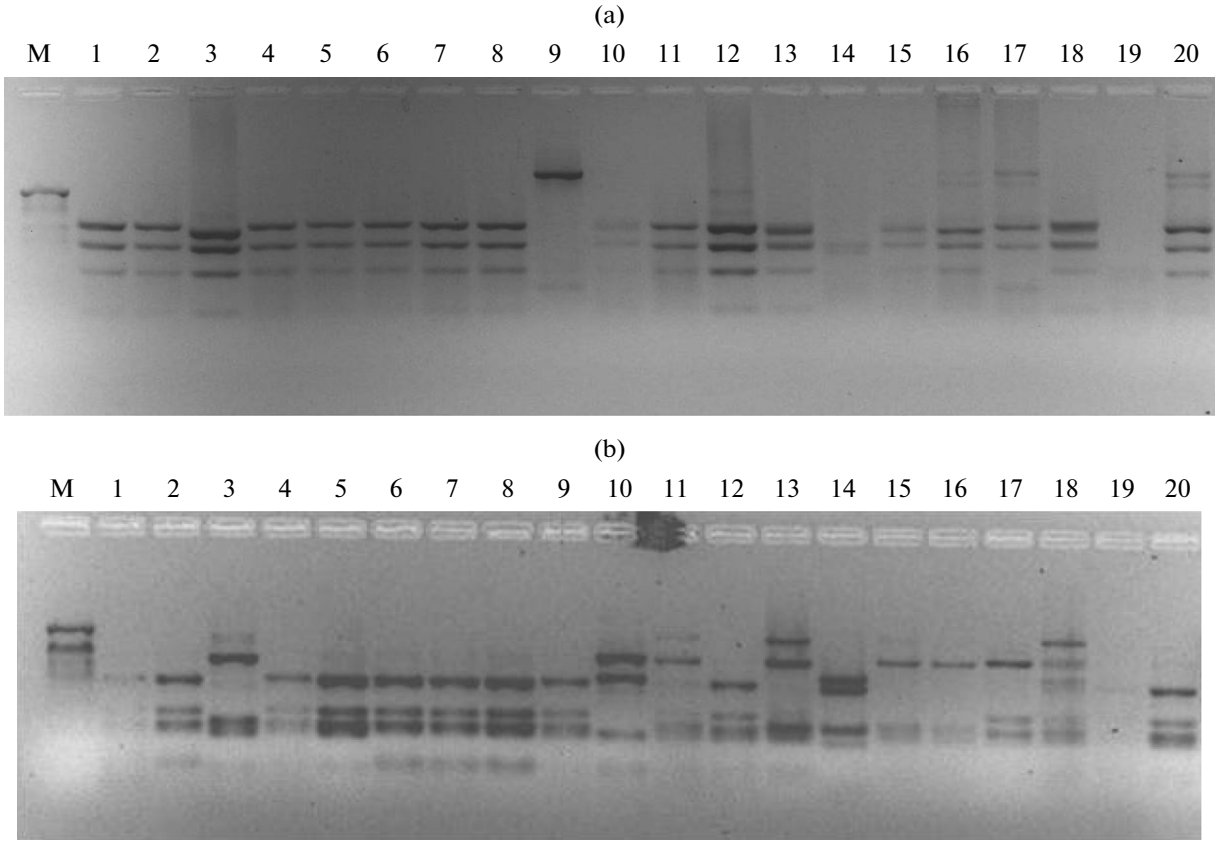

Fig. 5. ARDRA profiles of the twenty *Bacillus* strains after (a) *Alu*I digestion and (b) *Hae*III digestion.

Lane 1 = 100 bp ladder	Lane 2 = B-00120	Lane 3 = B-00080	Lane 4 = B-00096	Lane 5 = B-00846	Lane 6 = B-00093	Lane 7 = B-00091
Lane 8 = B-00083	Lane 9 = B-00119	Lane 10 = B-00771	Lane 11 = B-00776	Lane 12 = B-00072	Lane 13 = B-00082	Lane 14 = B-00076
Lane 15 = B-00089	Lane 16 = B-00132	Lane 17 = B-00133	Lane 18 = B-00111	Lane 19 = B-00744	Lane 20 = B-00870	Lane 21 = B-00840

00091) in another cluster with 100% similarity while the BOX and ERIC-PCR both were able to differentiate among them. It will be very pertinent to mention here that, in an earlier work by Vardhan et al. (2010) using few of the present *Bacillus* isolates for rapid identification, it was reported that isolates of *B. coagulans* (B-00083), *B. circulans* (B-00080), *B. subtilis* (B-00119) and *B. licheniformis* (B-00091) were clustered together based on the *Alu*I restriction pattern of 16S rDNA [28]. Even different strains of one species could be differentiated based on the ERIC and BOX PCR which was not possible with ARDRA. However, none of the clustering methods show any grouping based on geographical origin or documented functional attributes. Results obtained in the present study reveals that the genus *Bacillus* is genetically highly diverse and moreover, the strains of same species possess considerable genetic diversity among themselves. The clustering based on carbohydrate utilization pattern also revealed that the genus is metabolically highly diverse which can be effectively used for discriminating among the members of the genus. It will be worthy to mention here that the strains clustered together with 100% similarity in case of ARDRA, could also be differentiated based on carbohydrate utilization pattern. Although, the environmental influences on the metabolic activities can't be ruled out, under standard conditions carbohydrate utilization pattern can be easily used for rapid discrimination.

High genetic and metabolic diversity of the genus *Bacillus* points towards the possible use of both the features for intra-generic and intra-specific differentiation. However, the careful selection of the appropriate approach is key to the accurate discrimination. The results of the present study clearly indicates that BOX-PCR is a better tool than ERIC-PCR or

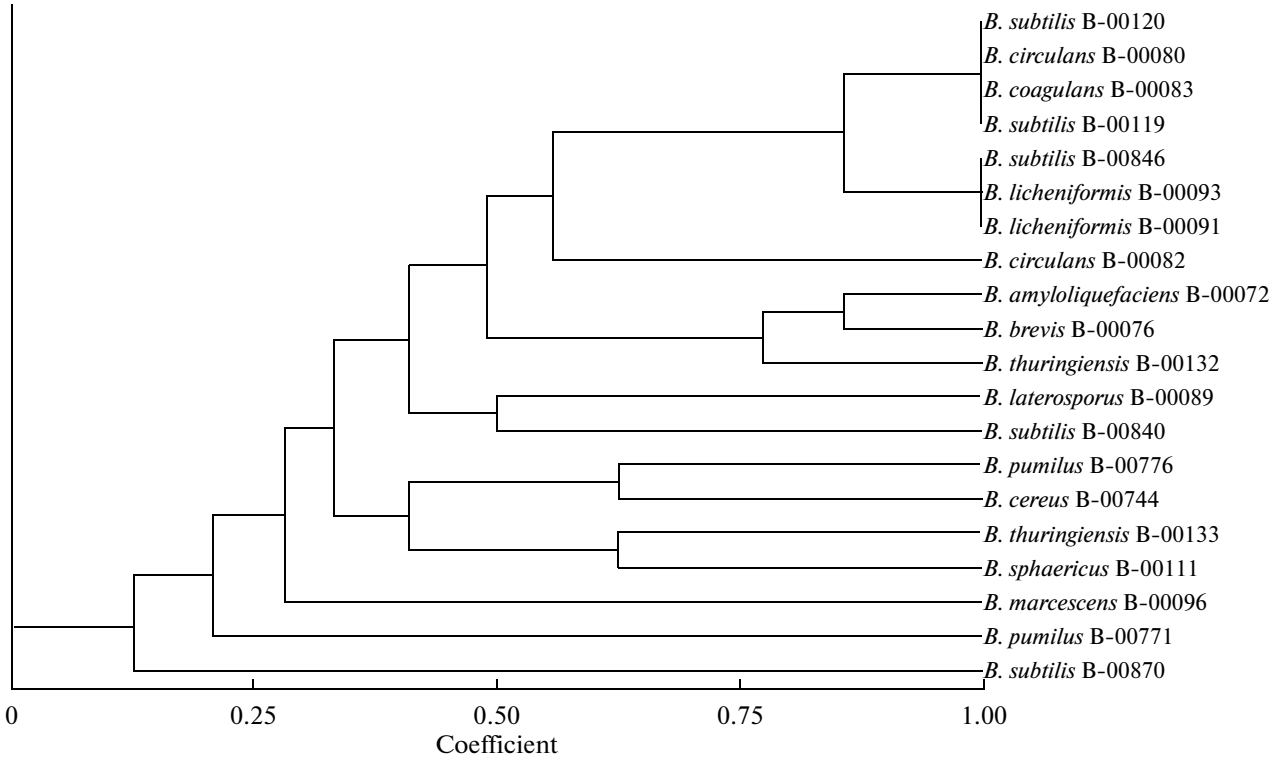

Fig. 6. Clustering of the studied bacilli based on restriction patterns of amplified 16S rDNA.

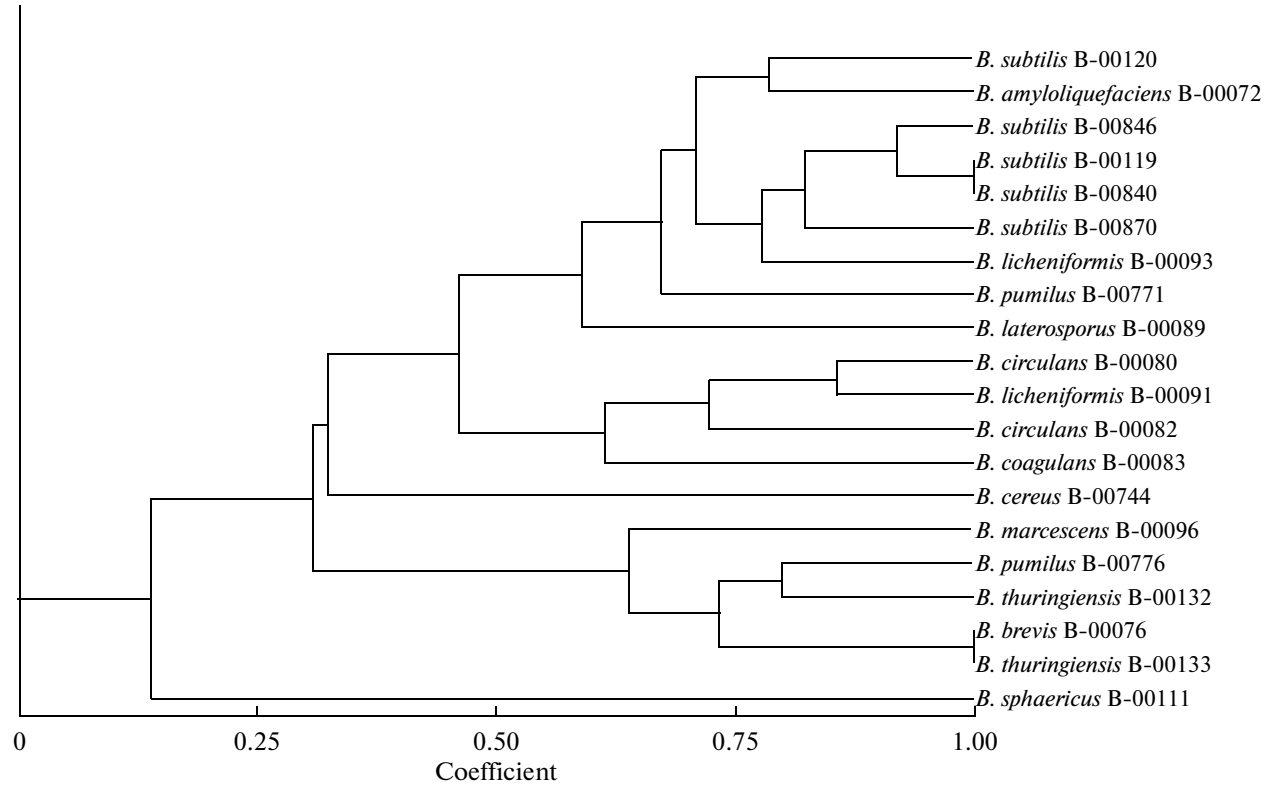

Fig. 7. Clustering of the studied bacilli based on carbohydrate utilization patterns.

ARDRA or metabolic profiling in terms of intra-generic and intra-specific discrimination, however, metabolic profiling like utilization pattern of carbohydrates may also be useful for discriminating closely related bacterial strains. In view of the increasing amendments in the classification of the *Bacillus* and reports of new species, it is further required to study more number of strains as well as compare other methods like MLST, ribotyping, whole cell protein profiling with the conventional ones to identify a single powerful tool to reveal the real diversity of the genetically heterogeneous genus like *Bacillus*.

ACKNOWLEDGMENTS

Authors gratefully acknowledge former In-Charge, NAIMCC, Dr. Sudheer Kumar for providing the bacterial cultures. Authors duly acknowledge Director, NBAIM for providing the resources and facilities for conducting the study.

REFERENCES

1. Claus, D. and Berkeley, R.C.W., Genus *Bacillus* Cohn 1872, 174AL, in *Bergey's Manual of Systematic Bacteriology,* Sneath, P.H.A., Mair, N.S., Sharpe, M.E., and Holt, J.G., Eds., Baltimore: Williams & Wilkins, 1986, vol. 2, pp. 1105–1139.
2. Zeigler, D.R. and Perkins, J.B., The genus *Bacillus*, in *Practical Handbook of Microbiology*, Goldman, E. and Green, L.H., Eds., Boca Raton: CRC, 2009.
3. Priest, F.G., Goodfellow, M., and Todd, C., A numerical classification of the genus *Bacillus, J. Gen. Microbiol.*, 1988, vol. 134, pp. 1847–1882.
4. Rossler, D., Ludwig, W., Schleifer, K.H., Lin, C., Mcgill, T.J., Wisotzkey, J.D., Jurtshuk, P., Jr., and Fox, G.E., Phylogenetic diversity in the genus *Bacillus* as seen by 16S rRNA sequencing studies, *Syst. Appl. Microbiol.*, 1991, vol. 14, pp. 266–269.
5. Ash, C., Farrow, J.A.E., Wallbanks, S., and Collins, M.D., Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences, *Lett. Appl. Microbiol.*, 1991, vol. 13, pp. 202–206.
6. Niimura, Y., Koh, E., Yanagida, F., Suzuki, K.I., Komagata, K. and Kozaki, M., *Amphibacillus xylanus* gen. nov., sp. nov., a facultatively anaerobic sporeforming xylan-digesting bacterium which lacks cytochrome, quinone, and catalase, *Int. J. Syst. Bacteriol.*, 1990, vol. 40, pp. 297–301.
7. Wisotzkey, J.D., Jurtshuk, P., Jr., Fox, G.E., Deinhard, G., and Poralla, K., Comparative sequence analyses on the 16S rRNA (rDNA) of *Bacillus acidocaldarius, Bacillus acidoterrestris*, and *Bacillus cycloheptanicus* and proposal for creation of a new genus, *Alicyclobacillus* gen. nov., *Int. J. Syst. Bacteriol.*, 1992, vol. 42, pp. 263–269.
8. Ash, C., Priest, F.G., and Collins, D., Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus *Paenibacillus, A. van. Leeuwenhoek*, 1993, vol. 64, pp. 253–260.
9. Shida, O., Takagi, H., Kadowaki, K., and Komagata, K., Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov., *Int. J. Syst. Bacteriol.*, 1996, vol. 46, pp. 939–946.
10. Heyndrickx, M., Lebbe, L., Kersters, K., de Vos, P., Forsyth, G., and Logan, N.A., *Virgibacillus*: a new genus to accommodate *Bacillus pantothenticus* (Proom and Knight 1950). Emended description of *Virgibacillus pantothenticus, Int. J. Syst. Bacteriol.*, 1998, vol. 48, pp. 99–106.
11. Wainø, M., Tindall, B.J., Schumann, P., and Ingvorsen, K., *Gracilibacillus* gen. nov., with description of *Gracilibacillus halotolerans* gen. nov., sp. nov.; transfer of *Bacillus dipsosauri* to *Gracilibacillus dipsosauri* comb. nov., and *Bacillus salexigens* to the genus *Salibacillus* gen. nov., as *Salibacillus salexigens* comb. nov., *Int. J. Syst. Bacteriol.*, 1999, vol. 49, pp. 821–831.
12. Schlesner, H., Lawson, P.A., Collins, M.D., Weiss, N., Wehmeyer, U., Volker, H., and Thomm, M., *Filobacillus milensis* gen. nov., sp. nov., a new halophilic spore-forming bacterium with Orn-D-Glu type peptidoglycan, *Int. J. Syst. Evol. Microbiol.*, 2001, vol. 51, pp. 425–431.
13. Adiguzel, A., Ozkan, H., Baris, O., Inan, K., Gulluce, M,. and Sahin, F., Identification and characterization of thermophilic bacteria isolated from hot springs in Turkey, *J. Microbiol. Methods*, 2009, vol. 79, pp. 321–328.
14. Laguerre, G., Allard, M.R., Revoy, F., and Amarger, N., Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes, *Appl. Environ. Microbiol.*, 1994, vol. 60, pp. 56–63.
15. Taghavi, M., Hayward, C., Sly, L.I., and Fegan, M., Analysis of the phylogenetic relationships of strains of *Burkholderia solanacearum, Pseudomonas syzygii*, and the blood disease bacterium of banana based on 16S rRNA gene sequences, *Int. J. Syst. Evol. Microbiol.,* 1996, vol. 46, pp. 10–15.
16. Lyra, C., Hantula, J., Vainio, E., Rapala, J., Rouhiainen, L., and Sivonen, K., Characterization of cyanobacteria by SDS-PAGE of whole-cell proteins and PCR-RFLP of the 16S rRNA gene, *Arch. Microbiol.*, 1997, vol. 168, pp. 176–184.
17. Lu, J.J., Perng, C.L., Lee, S.Y., and Wan, C.C., Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid, *J. Clin. Microbiol.*, 2000, vol. 38, pp. 2076–2080.
18. Joung, K.B. and Cote, J.C., Evaluation of ribosomal RNA gene restriction patterns for the classification of *Bacillus* species and related genera, *J. Appl. Microbiol.*, 2002, vol. 92, pp. 97–108.
19. Stern, M.J., Ames, G.F.L., Smith, N.H., Robinson, E.C., and Higgins, C.F., Repetitive extragenic palindromic sequences: a major component of the bacterial genome, *Cell*, 1984, vol. 37, pp. 1015–1026.
20. Hulton, C.S.J., Higgins, C.F., and Sharp, P.M., ERIC sequences: a novel family of repetitive elements in the

genomes of *Escherichia coli, Salmonella typhimurium* and other enterobacteria, *Mol. Microbiol.*, 1991, vol. 5, pp. 825–762.

21. Martin, B., Humbert, O., Camara, M., Guenzi, E., Walker, J., Mitchell, T., Andrew, P., Prudhomme, M., Alloing, G., Hakenbeck, R., Morrison, D.A., Boulnois, G.J., and Claverys, J.P., A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae, Nucl. Acids Res.*, 1992, vol. 20, pp. 3479–3483.
22. Versalovic, J., Schneider, M., de Bruijn, F.J., and Lupski, J.R., Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction, *Methods. Mol. Cell. Biol.*, 1994, vol. 5, pp. 25–40.
23. Rademaker, J.L.W. and de Bruijn, F.J., Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer assisted pattern analysis, in *DNA Markers: Protocols, Applications and Overviews*, Caetano-Anolles, G. and Gresshoff, P.M., Eds., New York: Wiley, 1997, pp. 1–26.
24. Ausubel, F.M., Brent, R., Moore, R.E., Seidman, J.G., and Smith, J.A., *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*, New York: Wiley, 2003.
25. Versalovic, J., Koeuth, T., and Lupski, J.R., Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes, *Nucl. Acids. Res.*, 1991, vol. 9, pp. 6823–6831.
26. De Bruijn, F., Use of repetitive (Repetitive Extragenic Palindromic and Enterobacterial Repetitive Intergeneric Consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria, *Appl. Environ. Microbiol.,* 1992, vol. 58, pp. 2180–2187.
27. Edwards, U., Rogall, T.H., Blocker, H., Emde, M., and Bottger, E.C., Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA, *Nucl. Acids. Res.*, 1989, vol. 17, pp. 7843–7853.
28. Vardhan, S., Kaushik, R., Saxena, A.K., and Arora, D.K., Restriction analysis and partial sequencing of the 16S rRNA gene as index for rapid identification of Bacillus species, *A. van Leuwenhoek*, 2010, vol. 99, pp. 283–296.
29. Jaccard, P., Nouvelles recherché sur la distribution florale, *Bulletin de la Society Vaudoise des Sciences Naturalles*, 1908, vol. 44, pp. 223–270.
30. Rohlf, F.J., *NTSYS pc Numerical Taxonomy and Multivariate Analysis System. Version 1.80*, Setauket: Exeter Software, 1995.
31. Chakdar, H. and Pabbi, S., Morphological characterization and molecular fingerprinting of *Nostoc* strains by multiplex RAPD, *Microbiology* (Moscow), 2012, vol. 81, pp. 768–778.
32. Carlson, C.R., Caugant, D.A., and Kolsto, A., Genetic diversity among *Bacillus cereus* and *Bacillus thuringiensis* strains, *Appl. Environ. Microbiol.*, 1997, vol. 60, pp. 1719–1725.
33. Sorokulova, I.B., Reva, O.N., Smirnov, V.V., Pinchuk, I.V., Lapa, S.V., and Urdaci, M.C., Genetic diversity and involvement in bread spoilage of *Bacillus* strains isolated from flour and ropy bread, *Lett. Appl. Microbiol.*, 2003, vol. 37, pp. 169–173.
34. Stratilo, C.W. and Bader, D.E., Genetic diversity among *Bacillus anthracis* soil isolates at fine geographic scales, *Appl. Environ. Microbiol.*, 2012, vol. 78, pp. 6433–6437.
35. Freitas, D.B., Reis, M.P., Lima-Bittencourt, C., Costa, P.S., Assis, P.S., Chartone-Souza, E., and Nascimento, A.M.A., Genotypic and phenotypic diversity of *Bacillus* spp. isolated from steel plant waste, *BMC Res. Notes*, 2008, vol. 1, p. 92.