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Development of a PCR based marker system for easy identification and classification of aerobic endospore forming bacilli

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Abstract

Restriction fragment length analysis of 16S rRNA gene of 52 different aerobic endospore forming Bacilli (AEFB) strains with HaeIII enzyme has revealed the presence of a 460 bp long fragment in 50 AEFB strains. BLAST analysis revealed that the fragment was 463 bp long and it was located at 3' end of 16S rRNA gene. Further specificity of this fragment for AEFB strains was checked by PCR and *in silico* methods. In PCR based method a primer pair (463 F and 463R) specific to this fragment was designed and this primer pair has shown amplification of 463 bp fragment in AEFB strains only. In *in silico* methods homology of primer pair and presence of restriction enzyme site in 16S rRNA genes were checked in 268 species of AEFB. Almost all species of AEFB have shown positive results for both of the tests. Further multiple alignments of 463 bp sequences of different species of AEFB have shown that it is a good marker for identification and classification of AEFB.

Keywords: AEFB; 16S rRNA gene; HaeIII; Specificity of fragment; PCR; *In silico*

Introduction

Aerobic endospore-formers have long been considered to be important components of the soil bacterial community (Mandic-Mulec and Prosser 2011). There is a great diversity of physiology among the aerobic spore formers. Their collective features include degradation of all substrates derived from plant and animal sources including cellulose, starch, pectin, proteins, agar, hydrocarbons and others, antibiotic production, nitrification, denitrification, nitrogen fixation, facultative lithotrophy, autotrophy, acidophily, alkaliphily, psychrophily, thermophily and parasitism. Endospore formation, universally found in this group, is thought to be a strategy for survival even under adverse soil environment, where these bacteria predominate (Kumar et al. 2012). To get the beneficial effects of these AEFB it becomes very necessary to know how much diverse and abundant these microbes are in different soil ecosystems. Since 1990s various approaches based on phenotypic and genotypic characteristics have been applied to identify and classify the members of class Bacilli. Few decades before genus *Bacillus* was the only representative

of class Bacilli among aerobic spore formers. Development of cultivation independent approaches have attracted microbiologist towards the molecular approaches for examining the microbes in a better way. Among different molecular methods, 16S rRNA gene sequencing is the best one. Since 1991, several new genera of aerobic spore formers like *Amphibacillus* (Niimura et al. 1990), *Paenibacillus* (Ash et al. 1991, 1993), *Alicyclobacillus* (Wisotzkey et al. 1992), *Aneurinibacillus* (Shida et al. 1996), *Brevibacillus* (Shida et al. 1996), *Gracilibacillus* (Waino et al. 1999), *Salibacillus* (Waino et al. 1999), *Virgibacillus* (Heyndrickx et al. 1998), *Filobacillus* (Schlesner et al. 2001), *Geobacillus* (Nazina et al. 2001), *Jeotgalibacillus* and *Marinibacillus* (Yoon et al. 2001) and *Ureibacillus* (Fortina et al. 2001) have been created based on this method. For phylogenetic arrangement of these newly discovered taxa various markers based on 16S rDNA have been developed by different scientists (Priest et al. 1988; Ash et al. 1991; Gurtler and Stanisich 1996; Daffonchio et al. 1998a, b; Goto et al. 2000; Stackebrandt and Swiderski 2002; Xu and Cote 2003; De Clerck et al. 2004; Vardhan et al. 2011). Primer set developed by Garbeva et al. (2003) was found to be 100% specific for many of species of *Bacillus* and related genera. After a gap of years, Vardhan et al. (2011)

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developed a set of primers for identification of hyper variable region of 16S rDNA in different *Bacillus* species and partial sequencing of this hyper variable region behaves as an index for easy identification of species related to genera *Bacillus*.

With development of more advanced approaches to find cultivable and noncultivable diversity of microbes, lot of new species and genera, belonging to AEFB are discovering day by day. So, need of new marker systems is always there for proper identification and classification of these lineages. Hence the main objective of present study was to develop a simple and easy identification and classification tool for *Bacillus* and related genera which is an extension of research related to bacilli. The restriction digestion of amplified 16S rRNA gene by HaeIII enzyme has given a fragment of around 460 bp length in all species of *Bacillus* and related genera. Sequence information of this fragment (downloaded from NCBI) was used to find exact length of the fragment (463 bp) and to develop specific primers for amplification of this fragment in AEFB genera. Further sequence information and multiple alignment of 463 bp long sequences of different species of AEFB genera has revealed that this is an easy tool for identification and classification of the members of *Bacillus* and related genera. Another beneficial information provided by our study is that almost all species of *Bacillus* and related genera have restriction enzyme sites for Hae III enzyme which give a product of 460 bp. Restriction enzyme site for HaeIII are present at different positions in other bacterial lineages, therefore give product of different size after restriction digestion which clearly discriminate the *Bacillus* and related genera from others.

Material and methods

Bacterial strains

All of the bacterial strains used in the present study are Bacilli isolated from the rhizospheric soil of *Phyllanthus amarus* which were identified by 16S rRNA gene sequencing in our previous research work (Kadyan et al. 2013). Taxonomic information and accession numbers of isolates have been given in Table 1.

16S rRNA gene amplification and restriction digestion by HaeIII enzyme

Gene coding for 16S rRNA gene of all of the 52 AEFB strains along with 10 reference strains (*Shigella Flexneri* ATCC12022, *Proteus mirabilis* ATCC43071, *Staphylococcus aureus* ATCC259323, *E. Coli* ATCC25922, *Salmonella typhimurium* ATCC13311, *Klebsiella pneumonia* ATCC 700603, *Pseudomonas fluorescens* MTCC1749, *Serratia marcescens* MTCC4822, *Bacillus subtilis* MTCC7193, and *Staphylococcus aureus* MTCC7443) was amplified by using universal primers i.e. B27f (5'-AGAGTTTGATCCTGGCT

CAG-3') and U1492R (5'-GGTTACCTTGTTACGAC TT-3') in thermal cyclor (Biorad). Further reaction mixture for restriction digestion was prepared by mixing 8.5 µl of purified PCR products, 5 U of restriction endonuclease, HaeIII (Fermentas) and 1.0 µl of 10X recommendation buffer. Reaction mixture was incubated overnight in water bath at 37°C. Restriction digested DNA was analysed by horizontal electrophoresis in 2% agarose gels with 100 bp DNA marker. The gels were visualized on a gel documentation system (Alpha Innotech). Photograph of gel has been shown in Figure 1(a&b).

Restriction pattern analysis and designing of oligonucleotide primers

Restriction pattern analysis of HaeIII digested 16S rRNA gene has shown the presence of a fragment having length around 460 bp (Figure 1a) in all of the bacterial species belonging to *Bacillus* and related genera (except *Bacillus arsenicus*, *Paenibacillus taiwanensis* and 9 reference strains related to other bacterial lineages) (Figure 1b). On the basis of these observations it was assumed that this 460 bp fragment was specific for *Bacillus* and related genera. To find out the exact location and sequence information of this fragment, 16S rRNA gene sequence of all of the *Bacillus* isolates taken in our study was downloaded from NCBI gene bank database. All of the 16S rRNA gene sequences were checked for HaeIII enzyme cut sites (GG↓CC). Sequence between two cut sites having length of around 460 bp was found in all of the 16S rRNA gene sequences at same position. Further length of this region was found to be 461-463 bp. Primer pair specific to this region was designed by using software, Primer 3.0 and further synthesized from the facility available at Eurofins Genomics India Pvt. Ltd., Bangalore.

Sequence specificity of primer pair and occurrence of restriction enzyme site

The specificity of oligonucleotide primers was checked by PCR amplification of the 463 bp fragment in all of the 52 AEFB isolates along with 10 reference strains (*Shigella Flexneri* ATCC12022, *Proteus mirabilis* ATCC43071, *Staphylococcus aureus* ATCC259323, *E. Coli* ATCC25922, *Salmonella typhimurium* ATCC13311, *Klebsiella pneumonia* ATCC 700603, *Pseudomonas fluorescens* MTCC1749, *Serratia marcescens* MTCC4822, *Bacillus subtilis* MTCC7193, and *Staphylococcus aureus* MTCC7443). Reaction conditions for PCR were, initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 20 seconds, extension at 72°C for 30 seconds and at last final extension at 72°C for 7 minutes. Theoretically primer pair was checked for its specificity in 16S rRNA gene sequences (downloaded from NCBI) in different species of *Bacillus* and related genera i.e. 153 different species of *Bacillus*, 20 *Virgibacillus*, 15

Table 1 Strain names and NCBI accession numbers of 52 AEFB strains isolated from rhizospheric soil of *Phyllanthus amarus*

Strain code	Bacterial isolate	Accession number	Strain code	Bacterial isolate	Accession number
1.P3	<i>B. marisflavi</i> JP44SK40	JX129227	15.P2	<i>B. subtilis</i> subsp. <i>spizizenii</i> JP44SK24	JX144714
2.P1	<i>B. megaterium</i> JP44SK1	JX144691	16.P1	<i>B. simplex</i> JP44SK25	JX144715
2.P2	<i>B. megaterium</i> JP44SK2	JX144692	16.P2	<i>B. simplex</i> JP44SK26	JX144716
3.P1	<i>Lysinibacillus sphaericus</i> JP44SK3	JX144693	17.P3	<i>B. cereus</i> JP44SK27	JX144717
3.P2	<i>Lysinibacillus sphaericus</i> JP44SK4	JX144694	18.P3	<i>B. aquimaris</i> JP44SK28	JX144718
3.P3	<i>B. megaterium</i> JP44SK5	JX144695	19.P1	<i>B. simplex</i> JP44SK29	JX144719
4.P1	<i>B. licheniformis</i> JP44SK6	JX144696	19.P2	<i>B. simplex</i> JP44SK30	JX144720
5.P3	<i>Paenibacillus taiwanensis</i> JP44SK7	JX144697	20.P1	<i>B. simplex</i> JP44SK31	JX144721
6.P1	<i>B. mycoides</i> JP44SK8	JX144698	20.P2	<i>B. simplex</i> JP44SK32	JX144722
6.P3	<i>B. mycoides</i> JP44SK9	JX144699	23.P1	<i>B. cereus</i> JP44SK33	JX144723
7.P1	<i>B. aryabhatai</i> JP44SK11	JX144701	23.P2	<i>B. cereus</i> JP44SK34	JX144724
7.P2	<i>B. megaterium</i> JP44SK10	JX144700	23.P3	<i>B. megaterium</i> JP44SK35	JX144725
7.P3	<i>Lysinibacillus xylanilyticus</i> JP44SK52	JX155769	24.P1	<i>B. mycoides</i> JP44SK36	JX144726
8.P1	<i>B. simplex</i> JP44SK12	JX144702	24.P3	<i>B. cereus</i> JP44SK37	JX144727
8.P2	<i>B. simplex</i> JP44SK13	JX144703	25.P2	<i>B. aryabhatai</i> JP44SK38	JX144728
8.P3	<i>B. arsenicus</i> JP44SK14	JX144704	26.P3	<i>B. megaterium</i> JP44SK39	JX144729
9.P3	<i>B. marisflavi</i> JP44SK15	JX144705	27.P1	<i>Brevibacillus laterosporus</i> JP44SK41	JX155758
10.P3	<i>B. firmus</i> JP44SK16	JX144706	27.P3	<i>B. cereus</i> JP44SK42	JX155759
11.P1	<i>B. firmus</i> JP44SK17	JX144707	30.P1	<i>B. cereus</i> JP44SK43	JX155760
11.P3	<i>B. megaterium</i> JP44SK18	JX144708	31.P3	<i>Jeotgalibacillus</i> sp. JP44SK56	KC012993
12.P3	<i>B. flexus</i> JP44SK19	JX144709	36.P3	<i>B. cereus</i> JP44SK44	JX155761
13.P1	<i>B. megaterium</i> strain JP44SK21	JX144711	37.P3	<i>B. cereus</i> JP44SK45	JX155762
13.P3	<i>B. firmus</i> JP44SK20	JX144710	38.P3	<i>Terribacillus saccharophilus</i> JP44SK46	JX155763
14.P2	<i>Brevibacillus laterosporus</i> JP44SK51	JX155768	41.P3	<i>Terribacillus goriensis</i> JP44SK47	JX155764
14.P3	<i>B. cereus</i> JP44SK22	JX144712	43.P3	<i>B. cereus</i> JP44SK49	JX155766
15.P1	<i>B. subtilis</i> subsp. <i>spizizenii</i> JP44SK23	JX144713	44.P3	<i>B. mycoides</i> JP44SK50	JX155767

Geobacillus, 1 *Filobacillus*, 4 *Jeotgalibacillus*, 5 *Ureibacillus*, 21 *Alicyclobacillus*, 5 *Amphibacillus*, 5 *Aneurinibacillus*, 16 *Brevibacillus*, 9 *Gracilibacillus*, 5 *Paenibacillus*, 5 *Lysinibacillus* and 4 *Terribacillus*. A number of other bacterial lineages of Gram positive and negative bacteria were also checked for primer specificity which includes genera from phylum Firmicutes (other than Bacilli), Actinobacteria, Alpha Proteobacteria, Beta Proteobacteria and Gamma Proteobacteria.

Multiple alignment of 463 bp long partial 16S rDNA sequence of different species of Bacilli

To check the ability of marker for classification of Bacilli, we have done the multiple alignments of specific, 463 bp long sequences of 16S rRNA gene of 52 strains (taken in our study) with the reference sequences downloaded from NCBI. Multiple alignment of very closely related species of genus *Bacillus* (29 different species of *Bacillus*) lying in two nearby clusters in all species living tree by Yarza et al.

(2010) has also been done to check the differentiation ability of this sequence. Software Clustal X 2.0 (Larkin et al. 2007) was used for alignment of different sequences and further alignment file was used in molecular evolutionary genetic analysis software version 5.1 (MEGA 5.1) (Tamura et al. 2011) for construction of phylogenetic tree.

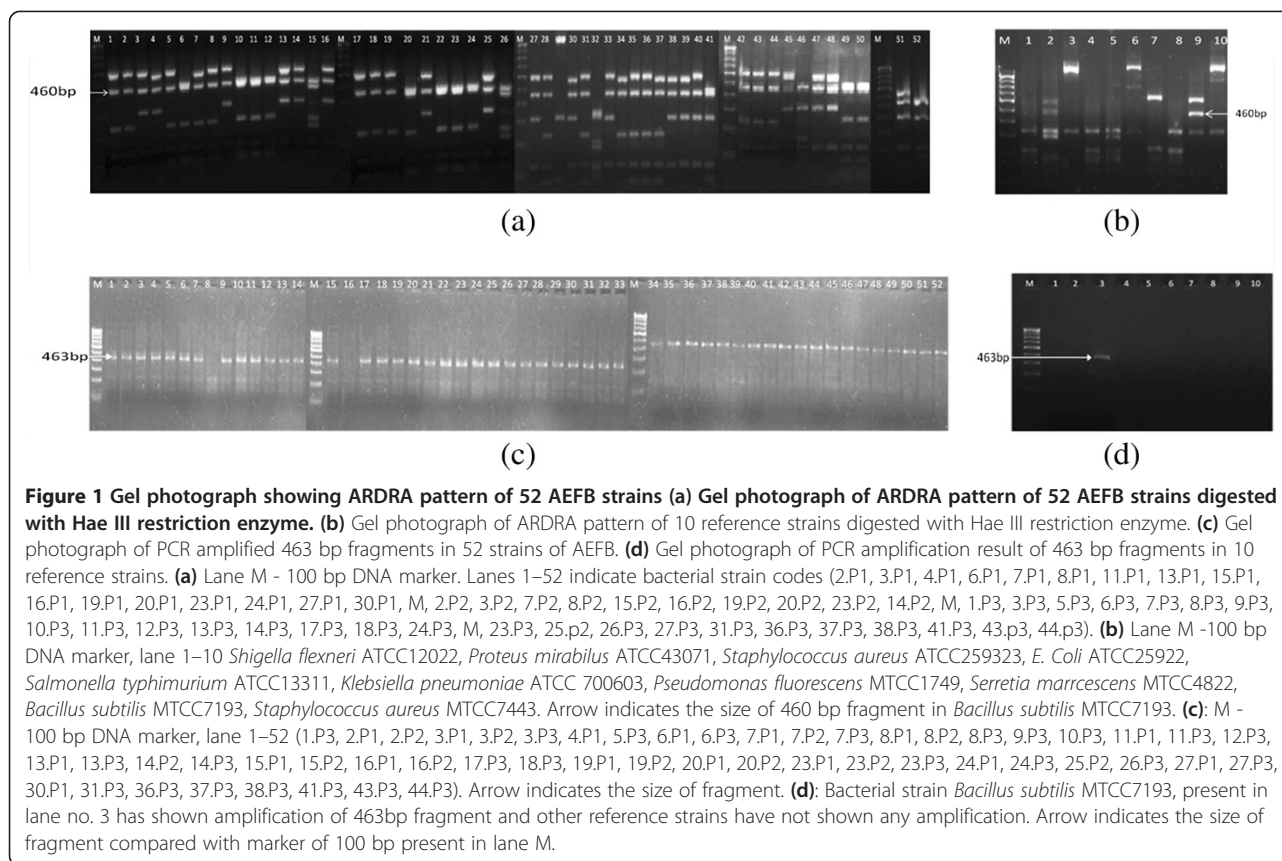
Results

Oligonucleotide primers

Bacillus and related genera specific primers designed in our study were named as 463 F (5'CTAAACTCAAAG GAATTGACG3') and 463R (5'AATACGTTCCCGG GCCTT3').

PCR amplification of 463 bp sequence

PCR amplification has confirmed the specificity of the primer pair in 52 AEFB strains and 10 reference strains. Out of total, 50 strains belonging to *Bacillus* and related genera have shown the amplification of the specific



region. However, the region was not amplified in *Bacillus arsenicus*, *Paenibacillus taiwanensis* and 9 reference strains (Figure 1c & d).

Sequence homology of primers in 16S rRNA gene sequences of *Bacillus* and related genera

Primer sequences were found to be 100% similar with the 16S rRNA gene sequences (downloaded from NCBI) of 120 species of genera *Bacillus*, 13 *Geobacillus*, 1 *Filobacillus*, 4 *Jeotgalibacillus*, 5 *Ureibacillus*, 7 *Alicyclobacillus*, 2 *Brevibacillus* and 5 *Lysinibacillus*. Number of other bacterial lineages of Gram positive and negative bacteria which includes genera from phyla Firmicutes (*Staphylococcus chromogenes* D83360, *Streptococcus pyogenes* AB002521, *Enterococcus faecalis* AB012212, *Clostridium populeti* X71853, *Listeria monocytogenes* X56153), Actinobacteria (*Corynebacterium diphtheria* X84248, *Mycobacterium tuberculosis* X58890, *Nocardia asteroides* AF430019, *Streptomyces lavendulae* subsp. *Lavendulae* D85116), Alpha proteobacteria (*Rhizobium leguminosarum* U29386, *Azospirillum lipoferum* Z29619, *Acetobacterium woodii* X96954), Beta proteobacteria (*Burkholderia cepacia* U96927, *Bordetella pertussis* U04950) and Gamma Proteobacteria (*Pseudomonas aeruginosa* X06684, *Escherichia coli* X80725, *Klebsiella pneumoniae* X87276, *Shigella*

dysenteriae X96966) have not shown any sequence homology (Table 2).

Multiple alignments of 463 bp sequences of different strains of *Bacilli*

Dendrogram prepared on the basis of alignment of 463 bp sequence has been given in Figure 2(a&b). Dendrogram prepared for 52 different strains of *Bacillus* and related genera (taken in our study) and some reference sequences downloaded from NCBI has been shown in Figure 2(a). Dendrogram has been divided in to 7 different groups (I-VII). Group I contains strains belonging to species *Bacillus aquimaris* and *marisflavi*. Strains belonging to genera *Lysinibacillus* (*sphaericus* and *xylanilyticus*) and *Jeotgalibacillus* are present in Group II. Group III contains strains belonging to Genera *Terribacillus* (*sacharophilus* and *goriensis*), *Bacillus subtilis* sub sp. *spizizinii* and *Bacillus licheniformis*. Group IV contains strains belonging to species, *Bacillus mycoides* and *Bacillus cereus*. Group V contains strains belonging to genera *Paenibacillus* and *Brevibacillus* and strains belonging to species *Bacillus simplex* and *Bacillus firmus* have shared the group VI. *Bacillus arsenicus* has not shown any grouping with any other species or genera and *Bacillus megaterium* and *Bacillus flexus* have shared a single group VII while some strains of *Bacillus megaterium*, *Bacillus flexus* and *Bacillus*

Table 2 % similarity of 463 bp sequence of 16S rRNA gene of type sp. (*Bacillus subtilis*) with 16S rRNA sequences of different AEFB strains (downloaded from NCBI), primer sequences in these AEFB strains, presence and absence of restriction enzyme site and position of specific fragment in AEFB strains

Sr. no.	Name of bacteria	NCBI accession no.	Sequence of primer pair in different AEFB strains	% similarity of 463 bp sequence and presence of restriction enzyme site	Position of 463 bp sequence in 16S rRNA gene
1	<i>Alicyclobacillus sacchari</i>	AB264020	AAT CCG TTC CCG GGCCTT CTGAAACTCAAAGGAATTGACG	88%+	913-1374
2	<i>Alicyclobacillus acidiphilus</i>	AB076660	AAT CCG TTC CCG GGCCTT CTGAAACTCAAAGGAATTGACG	88%-	913-1374
3	<i>Alicyclobacillus acidoterrestris</i>	AB042057	AAT CCG TTC CCG GGCCTT CTGAAACTCAAAGGAATTGACG	88%+	910-1371
4	<i>Alicyclobacillus hesperidum</i>	AJ133633	AAT CCG TTC CCG GGCCTT CTGAAACTCAAAGGAATTGACG	88%+	884-1345
5	<i>Alicyclobacillus fastidiosus</i>	AB264021	AAT CCG TTC CCG GGCCTT CTGAAACTCAAAGGAATTGACG	88%+	910-1371
6	<i>Alicyclobacillus vulcanalis</i>	AY425985	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	88%+	894-1355
7	<i>Alicyclobacillus sendaiensis</i>	AB084128	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	88%+	885-1346
8	<i>Alicyclobacillus contaminans</i>	AB264026	AAT CCG TTC CCG GGCCTT CTGAAACTCAAAGGAATTGACG	88%+	925-1386
9	<i>Alicyclobacillus acidocaldarius</i> subsp. <i>acidocaldarius</i> (Type sp)	AJ496806	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	87%+	902-1363
10	<i>Alicyclobacillus aeris</i>	FM179383	AAT CCG TTC CCG GGCCTT CTGAAACTCAAAGGAATTGACG	88%+	911-1372
11	<i>Alicyclobacillus pomorum</i>	AB089840	AAT CCG TTC CCG GGCCTT CTGAAACTCAAAGGAATTGACG	88%+	911-1372
12	<i>Alicyclobacillus disulfidooxidans</i>	AB089843	AAT CCG TTC CCG GGCCTT CTGAAACTCAAAGGAATTGACG	85%+	911-1372
13	<i>Alicyclobacillus tolerans</i>	Z21979	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	87%-	906-1365
14	<i>Alicyclobacillus ferrooxydans</i>	EU137838	AAT CCG TTC CCG GGCCTT CTGAAACTCAAAGGAATTGACG	88%+	913-1374
15	<i>Alicyclobacillus cycloheptanicus</i>	AB042059	AAT CCG TTC CCG GGCCTT CTGAAACTCAAAGGAATTGACG	88%+	911-1372
16	<i>Alicyclobacillus macrosporangiidus</i>	AB264025	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	87%-	927-1388
17	<i>Alicyclobacillus kakegawensis</i>	AB264022	AAT CCG TTC CCG GGCCTT CTGAAACTCAAAGGAATTGACG	86%+	924-1385
18	<i>Alicyclobacillus shizuokensis</i>	AB264024	AAT CCG TTC CCG GGCCTT CTGAAACTCAAAGGAATTGACG	87%+	924-1385
19	<i>Alicyclobacillus herbarius</i>	AB042055	AAT CCG TTC CCG GGCCTT CTGAAACTCAAAGGAATTGACG	87%+	924-1385
20	<i>Alicyclobacillus pohliae</i>	AJ564766	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	88%+	904-1363
21	<i>Alicyclobacillus tolerans</i>	Z21979	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	87%-	906-1365
22	<i>Amphibacillus sediminis</i>	AB243866	AATACGTTCCCGGG TC T CTGAAACTCAA AGA AATTGACG	96%-	928-1386
23	<i>Amphibacillus jilinensis</i> ,	FJ169626	AATACGTTCCCGGG TC T CTGAAACTCAA AGA AATTGACG	95%-	948-1406
24	<i>Amphibacillus tropicus</i>	AF418602	AATACGTTCCCGGG TC T CTGAAACTCAA AGA AATTGACG	95%-	905-1362
25	<i>Amphibacillus fermentum</i>	AF418603	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGG TC T	93%-	910-1368

Table 2 % similarity of 463 bp sequence of 16S rRNA gene of type sp. (*Bacillus subtilis*) with 16S rRNA sequences of different AEFB strains (downloaded from NCBI), primer sequences in these AEFB strains, presence and absence of restriction enzyme site and position of specific fragment in AEFB strains (Continued)

26	<i>Amphibacillus xylanus</i> , type sp.	D82065	AATACGTTCCCGGGTCTT CTGAAACTCAAAAAGAATTGACG	94%-	948-1406
27	<i>Aneurinibacillus aneurinilyticus</i> type sp.	X94194	AATACGTTCCCGGGTCTT CTGAAACTCAAAGGAATTGACG	91%-	903-1369
28	<i>Aneurinibacillus migulanus</i>	X94195	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGTCTT	90%-	903-1359
29	<i>Aneurinibacillus danicus</i>	AB112725	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGTCTT	91%-	903-1354
30	<i>Aneurinibacillus thermoaerophilus</i>	X94196	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGTCTT	92%-	904- 1361
31	<i>Aneurinibacillus terranovensis</i>	AJ715385	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGTCTT	91%-	897-1353
32	<i>Brevibacillus centrosporus</i>	D78458	GTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	91%+	917-1377
33	<i>Brevibacillus choshinensis</i>	AB112713	GTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	91%+	894-1354
34	<i>Brevibacillus reuszeri</i>	AB112715	GTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	91%+	894-1354
35	<i>Brevibacillus parabrevis</i>	AB112714	GTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	91%+	894-1354
36	<i>Brevibacillus brevis</i> type sp.	AB271756	GTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	91%+	896-1356
37	<i>Brevibacillus formosus</i>	AB112712	GTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	91%+	894-1354
38	<i>Brevibacillus agri</i>	AB112716	GTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	91%+	895-1355
39	<i>Brevibacillus limnophilus</i>	AB112717	GTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	91%+	909-1369
40	<i>Brevibacillus invocatus</i>	AF378232	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	91%+	896-1356
41	<i>Brevibacillus panacihumi</i>	EU383033	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	91%+	902-1362
42	<i>Brevibacillus borstelensis</i>	AB112721	GTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	92%+	894-1354
43	<i>Brevibacillus ginsengisoli</i>	AB245376	GTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	92%+	873-1333
44	<i>Brevibacillus laterosporus</i>	D16271	GTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	91%+	896-1356
45	<i>Brevibacillus fluminis</i>	EU375457	GTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	91%+	896-1356
46	<i>Brevibacillus levickii</i>	AJ715378	GTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	91%+	897-1357
47	<i>Brevibacillus thermoruber</i>	Z26921	GTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	92%+	915-1376
48	<i>Gracilibacillus lacisalsi</i>	DQ664540	CTGAAACTCAAAAAGAATTGACG AATACGTTCCCGGGCCTT	94%+	933-1393
49	<i>Gracilibacillus thailandensis</i>	FJ182214	CTGAAACTCAAAAAGAATTGACG AATACGTTCCCGGGCCTT	94%+	942-1402
50	<i>Gracilibacillus saliphilus</i>	EU784646	CTGAAACTCAAAAAGAATTGACG AATACGTTCCCGGGCCTT	94%+	917-1377
51	<i>Gracilibacillus orientalis</i>	AM040716	CTGAAACTCAAAAAGAATTGACG AATACGTTCCCGGGCCTT	93%+	931-1391

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52	<i>Gracilibacillus dipsosauri</i>	AB101591	CTGAAACTCAAA AGA ATTGACG AATACGTTCCCGGGCCTT	95%+	923-1383
53	<i>Gracilibacillus ureilyticus</i>	EU709020	CTGAAACTCAAA AGA ATTGACG AATACGTTCCCGGGCCTT	95%+	923-1383
54	<i>Gracilibacillus boracitolerans</i>	AB197126	CTGAAACTCAAA AGA ATTGACG AATACGTTCCCGGGCCTT	94%+	935-1395
55	<i>Gracilibacillus halotolerans</i> type sp.	AF036922	CTGAAACTCAAA AGA ATTGACG AATACGTTCCCGGGCCTT	94%+	934-1394
56	<i>Gracilibacillus halophilus</i>	EU135704	CTGAAACTCAAA AGA ATTGACG AATACGTTCCCGGGCCTT	94%+	924-1384
57	<i>Paenibacillus polymyxa</i> type sp.	D16276	AATACGTTCCCGGG T CCTT CTGAAACTCAAAGGAATTGACG	90%-	913-1375
58	<i>Paenibacillus antarcticus</i>	AJ605292	AATACGTTCCCGGG T CCTT CTGAAACTCAAAGGAATTGACG	90%-	915-1374
59	<i>Paenibacillus macquariensis</i> subsp. <i>macquariensis</i>	X60625	AATACGTTCCCGGG T CCTT CTGAAACTCAAAGGAATTGACG	90%-	935-1394
60	<i>Paenibacillus macquariensis</i> subsp. <i>defensor</i>	AB360546	AATACGTTCCCGGG T CCTT CTGAAACTCAAAGGAATTGACG	90%+	936-1395
61	<i>Paenibacillus glacialis</i>	EU815294	AATACGTTCCCGGG T CCTT CTGAAACTCAAAGGAATTGACG	91%-	934-1393
62	<i>Virgibacillus pantothenicus</i> type sp.	D16275	AATACGTTCCCGGG T CCTT CTGAAACTCAAAGGAATTGACG	95%-	919-1375
63	<i>Virgibacillus proomii</i>	AJ012667	CTGAAACT AAAAGA ATTGACG AATACGTTCCCGGG T CCTT	95%-	916-1372
64	<i>Virgibacillus salexigens</i>	Y11603	CTGAAACTCAAA AGA ATTNACG AATACGTTCCCGGGCCTT	95%+	921-1379
65	<i>Virgibacillus marismortui</i>	AJ009793	AATACGTTCCCGGGCCTT CTGAAACTCAAA AGA ATTGACG	95%+	947-1407
66	<i>Virgibacillus salarius</i>	AB197851	AATACGTTCCCGGGCCTT CTGAAACTCAAA AGA ATTGACG	95%+	949-1409
67	<i>Virgibacillus olivae</i>	DQ139839	AATACGTTCCCGGGCCTT CTGAAACTCAAA AGA ATTGACG	95%+	948-1409
68	<i>Virgibacillus halodenitrificans</i>	AY543169,	CTGAAACTCAAA AGA ATTGACG AATACGTTCCCGGGCCTT	95%+	926-1386
69	<i>Virgibacillus koreensis</i>	AY616012	CTGAAACTCAAA AGA ATTGACG AATACGTTCCCGGGCCTT	97%+	926-1386
70	<i>Virgibacillus halophilus</i>	AB243851	CTGAAACTCAAA AGA ATTGACG AATACGTTCCCGGGCCTT	94%+	926-1386
71	<i>Virgibacillus sediminis</i>	AY121430	CTGAAACTCAAA AGA ATTGACG AATACGTTCCCGGGCCTT	96%-	946-1406
72	<i>Virgibacillus xinjiangensis</i>	DQ664543	CTGAAACTCAAA AGA ATTGACG AATACGTTCCCGGGCCTT	96%-	894-1354
73	<i>Virgibacillus chiguensis</i>	EF101168	CTGAAACTCAAA AGA ATTGACG AATACGTTCCCGGGCCTT	96%-	919-1375
74	<i>Virgibacillus dokdonensis</i>	AY822043	CTGAAACTCAAA AGA ATTGACG AATACGTTCCCGGG T CCTT	96%-	927-1383
75	<i>Virgibacillus carmonensis</i>	AJ316302	CTGAAACTCAAA AGA ATTGACG AATACGTTCCCGGGCCTT	95%+	925-1385
76	<i>Virgibacillus necropolis</i>	AJ315056	CTGAAACTCAAA AGA ATTGACG AATACGTTCCCGGGCCTT	95%+	925-1385
77	<i>Virgibacillus arcticus</i>	EF675742	CTGAAACTCAAA AGA ATTGACG AATACGTTCCCGGGCCTT	95%+	809- 1269

Table 2 % similarity of 463 bp sequence of 16S rRNA gene of type sp. (*Bacillus subtilis*) with 16S rRNA sequences of different AEFB strains (downloaded from NCBI), primer sequences in these AEFB strains, presence and absence of restriction enzyme site and position of specific fragment in AEFB strains (Continued)

78	<i>Virgibacillus byunsanensis</i>	FJ357159	CTGAAACTCAAA AGA AATTGACG AATACGTTCCCGGGCCTT	95%+	923-1383
79	<i>Virgibacillus salinus</i>	FM205010	CTGAAACTCAAA AGA AATTGACG AATACGTTCCCGGGCCTT	95%+	932-1392
80	<i>Virgibacillus subterraneus</i>	FJ746573	AATACGTTCCCGGGCCTT CTGAAACTCAAA AGA AATTGACG	91%+	905-1362
81	<i>Virgibacillus kekensis</i>	AY121439	CTGAAACTCAAA AGA AATTGACG AATACGTTCCCGGGCCTT	95%+	945-1405
82	<i>Geobacillus stearothermophilus</i> type sp.	AB021196,	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	912-1376
83	<i>Geobacillus kaustophilus</i>	X60618	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	92%+	933-1390
84	<i>Geobacillus lituanicus</i>	AY044055	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	92%+	933-1397
85	<i>Geobacillus thermoleovorans</i>	Z26923	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	918-1382
86	<i>Geobacillus thermocatenulatus</i>	AY608935	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	940-1404
87	<i>Geobacillus jurassicus</i>	AY312404	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	915-1379
88	<i>Geobacillus uzenensis</i>	AF276304	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	92%+	907-1370
89	<i>Geobacillus subterraneus</i>	AF276306	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	931-1395
90	<i>Geobacillus thermodenitrificans</i>	AY608961	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	939-1409
91	<i>Geobacillus debilis</i>	AJ564616	AATACGTTCTCGGGCCTT CTGAAACTCAAAGGAATTGACG	91%-	936-1398
92	<i>Geobacillus toebii</i>	AF326278	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	910-1374
93	<i>Geobacillus thermoglucosidasius</i>	AY608981	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	939-1405
94	<i>Geobacillus caldxylosilyticus</i>	AF067651	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	94%+	925-1389
95	<i>Geobacillus tepidamans</i>	AY563003	G TGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	872-1334
96	<i>Geobacillus vulcani</i>	AJ293805	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	920-1384
97	<i>Filobacillus milosensis</i>	AJ238042,	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	94%+	915-1375
98	<i>Jeotgalibacillus alimentarius.</i>	AF281158	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	911-1373
99	<i>Jeotgalibacillus salarius</i>	EU874389	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	910-1372
100	<i>Jeotgalibacillus campisalis</i>	AY190535	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	908-1370
101	<i>Jeotgalibacillus marinus</i>	AJ237708	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	918-1380
102	<i>Ureibacillus thermosphaericus</i>	AB101594	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	92%+	931-1392
103	<i>Ureibacillus composti</i>	DQ348071	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	92%+	929-1390

Table 2 % similarity of 463 bp sequence of 16S rRNA gene of type sp. (*Bacillus subtilis*) with 16S rRNA sequences of different AEFB strains (downloaded from NCBI), primer sequences in these AEFB strains, presence and absence of restriction enzyme site and position of specific fragment in AEFB strains (Continued)

104	<i>Ureibacillus thermophilus</i>	DQ348072	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	91%+	931-1392
105	<i>Ureibacillus suwonensis</i>	AY850379	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	92%-	918-1379
106	<i>Ureibacillus terrenus</i>	AJ276403	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	92%-	900-1361
107	<i>Lysinibacillus boronitolerans</i>	AB199591	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	898-1360
108	<i>Lysinibacillus xylanilyticus</i>	FJ477040	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	826-1288
109	<i>Lysinibacillus fusiformis</i>	AJ310083	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	920-1382
110	<i>Lysinibacillus sphaericus</i>	AJ310084	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	920-1382
111	<i>Lysinibacillus parviboronicapiens</i>	AB300598	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	910-1372
112	<i>Terribacillus goriensis</i>	DQ519571	CTGAAACTCAAAA AGA AATTGACG AATACGTTCCCGGGCCTT	94%+	895-1355
113	<i>Terribacillus saccharophilus</i>	AB243845	CTGAAACTCAAAA AGA AATTGACG AATACGTTCCCGGGCCTT	94%+	922-1382
114	<i>Terribacillus halophilus</i>	AB243849	CTGAAACTCAAAA AGA AATTGACG AATACGTTCCCGGGCCTT	95%+	922-1382
115	<i>Terribacillus aidingensis</i>	FJ386524	CTGAAACTCAAAA AGA AATTGACG AATACGTTCCCGGGCCTT	95%+	922-1382
116	<i>Bacillus massiliensis</i>	AY677116	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	93%+	908-1370
117	<i>Bacillus cecembensis</i>	AM773821	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	93%+	931-1393
118	<i>Bacillus odysseyi</i>	AF526913	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	93%+	931-1393
119	<i>Bacillus decisifrondis</i>	DQ465405	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	85%+	843-1305
120	<i>Bacillus psychrodurans</i>	AJ277984	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	95%-	918-1380
121	<i>Bacillus psychrotolerans</i>	AJ277983	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	95%-	903-1365
122	<i>Bacillus insolitus</i>	AM980508	GAGGGTTC CCCGGGCCTT CTGAAACTCAAAGGAATTGACG	94%+	917-1378
123	<i>Bacillus beijingensis</i>	EF371374	AATACGTTCCCGGGTCTT CTGAAACTCAAAGGAATTGACG	96%-	929-1387
124	<i>Bacillus ginsengi</i>	EF371375	AATACGTTCCCGGGTCTT CTGAAACTCAAAGGAATTGACG	97%-	929-1387
125	<i>Bacillus aquimaris</i>	AF483625	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	99%+	910-1372
126	<i>Bacillus vietnamensis</i>	AB099708	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	98%+	903-1365
127	<i>Bacillus marisflavi</i>	AF483624	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	100%+	909-1371
128	<i>Bacillus seohaeanensis</i>	AY667495	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	98%+	872-1334
129	<i>Bacillus mycoides</i>	AB021192	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	97%+	907-1367

Table 2 % similarity of 463 bp sequence of 16S rRNA gene of type sp. (*Bacillus subtilis*) with 16S rRNA sequences of different AEFB strains (downloaded from NCBI), primer sequences in these AEFB strains, presence and absence of restriction enzyme site and position of specific fragment in AEFB strains (Continued)

130	<i>Bacillus weihenstephanensis</i>	AB021199	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	97%+	925-1385
131	<i>Bacillus thuringiensis</i>	D16281	AATACGTTCCCGGGCCTT CTGAAACTCAAAGGAATTGACG	97%+	911-1371
132	<i>Bacillus pseudomycooides</i>	AF013121	CTGAAACTCAAAGGATTTGACG AATACGTTCCCGGGCCTT	95%+	932-1392
133	<i>Bacillus funiculus</i>	AB049195	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	98%+	919-1379
134	<i>Bacillus panaciterrae</i>	AB245380	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	904-1364
135	<i>Bacillus flexus</i>	AB021185	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	98%+	923-1385
136	<i>Bacillus megaterium</i>	D16273	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	98%+	910-1372
137	<i>Bacillus koreensis</i>	AY667496	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	847-1309
138	<i>Bacillus aerius</i>	AJ831843	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	922-1382
139	<i>Bacillus aerophilus</i>	AJ831844	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	927-1387
140	<i>Bacillus stratosphericus</i>	AJ831841	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	927-1387
141	<i>Bacillus sonorensis</i>	AF302118	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	908-1368
142	<i>Bacillus amyloliquefaciens</i>	AB255669	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	909-1369
143	<i>Bacillus siamensis</i>	GQ281299	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%-	931-1352
144	<i>Bacillus methylotrophicus</i>	EU194897	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	898-1358
145	<i>Bacillus subtilis</i> subsp. <i>subtilis</i>	AJ276351	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	919-1379
146	<i>Bacillus subtilis</i> subsp. <i>spizizenii</i>	AF074970	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	907-1367
147	<i>Bacillus vallismortis</i>	AB021198	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	924-1384
148	<i>Bacillus mojavensis</i>	AB021191	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	920-1380
149	<i>Bacillus atrophaeus</i>	AB021181	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	909-1369
150	<i>Bacillus pumilus</i>	AY876289	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	879-1339
151	<i>Bacillus safensis</i>	AF234854	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	879-1339
152	<i>Bacillus altitudinis</i>	AJ831842	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	934-1394
153	<i>Bacillus ginsengihumi</i>	AB245378	TT GAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	98%-	914-1376
154	<i>Bacillus acidiproducens</i>	EF379274	TT GAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%-	879-1341
155	<i>Bacillus acidicola</i>	AF547209	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	99%+	934-1396

Table 2 % similarity of 463 bp sequence of 16S rRNA gene of type sp. (*Bacillus subtilis*) with 16S rRNA sequences of different AEFB strains (downloaded from NCBI), primer sequences in these AEFB strains, presence and absence of restriction enzyme site and position of specific fragment in AEFB strains (Continued)

156	<i>Bacillus oleronius</i>	AY988598	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	934-1396
157	<i>Bacillus sporothermodurans</i>	U49078	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	904-1366
158	<i>Bacillus carboniphilus</i>	AB021182	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	910-1372
159	<i>Bacillus chungangensis</i>	FJ514932	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	94%+	890-1352
160	<i>Bacillus endophyticus</i>	AF295302	AATACGTTCCCGGGTCTT CTGAAACTCAAAGGAATTGACG	96%-	906-1362
161	<i>Bacillus isabelliae</i>	AM503357	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	98%+	912-1372
162	<i>Bacillus shackletonii</i>	AJ250318	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	98%+	909-1371
163	<i>Bacillus circulans</i>	AY043084	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	902-1364
164	<i>Bacillus nealsonii</i>	EU656111	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	928-1390
165	<i>Bacillus korensis</i>	EU603328	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	98%+	889-1351
166	<i>Bacillus sibiralis</i>	AF071856	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	905-1367
167	<i>Bacillus benzoevorans</i>	X60611	AATACGTTCCCGGGTCTT CTGAAACTCAAAGGAATTGACG	95%-	931-1386
168	<i>Bacillus firmus</i>	D16268	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	907-1369
169	<i>Bacillus infantis</i>	AY904032	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	871-1333
170	<i>Bacillus oceanisediminis</i>	GQ292772	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	861-1323
171	<i>Bacillus kribbensis</i>	DQ280367	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%-	919-1381
172	<i>Bacillus horneckiae</i>	EU861362	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	98%+	796-1258
173	<i>Bacillus badius</i>	X77790	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%-	913-1370
174	<i>Bacillus smithii</i>	Z26935	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	924-1383
175	<i>Bacillus aeolius</i>	AJ504797	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	94%-	901-1361
176	<i>Bacillus coagulans</i>	AB271752	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%-	911-1373
177	<i>Bacillus alveayuensis</i>	AY605232	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	934-1396
178	<i>Bacillus thermoamylovorans</i>	L27478	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	92%+	930-1391
179	<i>Bacillus fordii</i>	AY443039	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	94%+	892-1354
180	<i>Bacillus fortis</i>	AY443038	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	94%+	927-1389
181	<i>Bacillus farraginis</i>	AY443036	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	94%+	838-1300

Table 2 % similarity of 463 bp sequence of 16S rRNA gene of type sp. (*Bacillus subtilis*) with 16S rRNA sequences of different AEFB strains (downloaded from NCBI), primer sequences in these AEFB strains, presence and absence of restriction enzyme site and position of specific fragment in AEFB strains (Continued)

182	<i>Bacillus galactosidilyticus</i>	AJ535638	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	904-1367
183	<i>Bacillus ruris</i>	AJ535639	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%-	901-1363
184	<i>Bacillus lentus</i>	AB021189	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	928-1390
185	<i>Bacillus novalis</i>	AJ542512	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	908-1370
186	<i>Bacillus vireti</i>	AJ542509	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	908-1370
187	<i>Bacillus bataviensis</i>	AJ542508	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	98%+	908-1370
188	<i>Bacillus drentensis</i>	AJ542506	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	98%+	844-1306
189	<i>Bacillus soli</i>	AJ542513	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	908-1370
190	<i>Bacillus fumarioli</i>	AJ250056	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	909-1371
191	<i>Bacillus niacini</i>	AB021194	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	98%+	921-1383
192	<i>Bacillus pocheonensis</i>	AB245377	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	98%+	910-1372
193	<i>Bacillus boroniphilus</i>	AB198719	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	930-1392
194	<i>Bacillus selenatarsenatis</i>	AB262082	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	870-1332
195	<i>Bacillus jeotgali</i>	AF221061	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	908-1370
196	<i>Bacillus thioparans</i>	DQ371431	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	908-1370
197	<i>Bacillus foraminis</i>	AJ717382	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	922-1384
198	<i>Bacillus canaveralius</i>	DQ870688	CTGAAACTCAAAGGAATTGACG SEQUENEWAS SHORT	97%	887-1323
199	<i>Bacillus infernus</i>	U20385	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	921-1383
200	<i>Bacillus methanolicus</i>	AB112727	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	909-1372
201	<i>Bacillus butanolivorans</i>	EF206294	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	914-1380
202	<i>Bacillus simplex</i>	AJ439078	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	98%+	920-1379
203	<i>Bacillus muralis</i>	AJ316309	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	909-1371
204	<i>Bacillus psychrosaccharolyticus</i>	AB021195	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	900-1362
205	<i>Bacillus asahii</i>	AB109209	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	909-1373
206	<i>Bacillus indicus</i>	AJ583158	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	918-1381
207	<i>Bacillus cibi</i>	AY550276	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	896-1358

Table 2 % similarity of 463 bp sequence of 16S rRNA gene of type sp. (*Bacillus subtilis*) with 16S rRNA sequences of different AEFB strains (downloaded from NCBI), primer sequences in these AEFB strains, presence and absence of restriction enzyme site and position of specific fragment in AEFB strains (Continued)

208	<i>Bacillus idriensis</i>	AY904033	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	889-1351
209	<i>Bacillus niabensis</i>	AY998119	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	904-1366
210	<i>Bacillus fastidiosus</i>	X60615	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	930-1386
211	<i>Bacillus litoralis</i>	AY608605	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	908-1370
212	<i>Bacillus herbersteinensis</i>	AJ781029	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	98%+	908-1370
213	<i>Bacillus galliensis</i>	FM162181	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	908-1370
214	<i>Bacillus alkalitelluris</i>	AY829448	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	94%+	911-1373
215	<i>Bacillus humi</i>	AJ627210	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%	910-1372
216	<i>Bacillus halmapalus</i>	X76447	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	98%+	908-1370
217	<i>Bacillus horikoshii</i>	AB043865	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	929-1391
218	<i>Bacillus cohnii</i>	X76437	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	910-1372
219	<i>Bacillus acidiceler</i>	DQ374637	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	916-1376
220	<i>Bacillus luciferensis</i>	AJ419629	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	909-1369
221	<i>Bacillus azotoformans</i>	AB363732	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	909-1370
222	<i>Bacillus taeanensis</i>	AY603978	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	917-1378
223	<i>Bacillus macauensis</i>	AY373018	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	88-1350
224	<i>Bacillus rigui</i>	EU939689	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%-	903-1365
225	<i>Bacillus solisalsi</i>	EU046268	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%-	887-1349
226	<i>Bacillus gelatini</i>	AJ551329	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	909-1371
227	<i>Bacillus arsenicus</i>	AJ606700	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	94%-	928-1390
228	<i>Bacillus barbaricus</i>	AJ422145	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%-	882-1342
229	<i>Bacillus algicola</i>	AY228462	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	931-1393
230	<i>Bacillus hwajinpoensis</i>	AF541966	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	909-1371
231	<i>Bacillus decolorationis</i>	AJ315075	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	94%+	909-1371
232	<i>Bacillus okuhidensis</i>	AB047684	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	874-1335
233	<i>Bacillus lehensis</i>	AY793550	AATACGTTCCCGGGTCTT CTGAAACTCAAAGGAATTGACG	95%-	939-1395

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234	<i>Bacillus oshimensis</i>	AB188090	AATACGTTCCCGGGTCTT CTGAAACTCAAAGGAATTGACG	95%-	937-1393
235	<i>Bacillus patagoniensis</i>	AY258614	AATACGTTCCCGGGTCTT TTGAAACTCAAAGGAATTGACG	95%-	913-1369
236	<i>Bacillus clausii</i>	X76440	AATACGTTCCCGGGTCTT CTGAAACTCAAAGGAATTGACG	95%-	913-1369
237	<i>Bacillus gibsonii</i>	X76446	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	912-1372
238	<i>Bacillus murimartini</i>	AJ316316	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	913-1373
239	<i>Bacillus plakortidis</i>	AJ880003	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	906-1366
240	<i>Bacillus pseudocaliphilus</i>	X76449	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	909-1371
241	<i>Bacillus trypoxylicola</i>	AB434284	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	911-1373
242	<i>Bacillus alcalophilus</i>	X76436	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	909-1371
243	<i>Bacillus bogoriensis</i>	AY376312	CTGAAACTCAAAGGAATTGAGC AATACGTTCCCGGGCCTT	97%+	911-1374
244	<i>Bacillus akibai</i>	AB043858	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	950-1411
245	<i>Bacillus krulwichiae</i>	AB086897	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	94%+	912-1374
246	<i>Bacillus okhensis</i>	DQ026060	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	916-1378
247	<i>Bacillus wakoensis</i>	AB043851	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	930-1392
248	<i>Bacillus hemicellulosilyticus</i>	AB043846	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	940-1402
249	<i>Bacillus macyae</i>	AY032601cpf	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	916-1378
250	<i>Bacillus alkalinitrilicus</i>	EF422411	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	919-1381
251	<i>Bacillus pseudofirmus</i>	X76439	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	97%+	910-1372
252	<i>Bacillus qingdaonensis</i>	DQ115802	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	913-1375
253	<i>Bacillus halochares</i>	AM982516	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%-	881-1343
254	<i>Bacillus aidingensis</i>	DQ504377	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%-	946-1407
255	<i>Bacillus salarius</i>	AY667494	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	858-1320
256	<i>Bacillus persepolensis</i>	FM244839	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	940-1402
257	<i>Bacillus agaradhaerens</i>	X76445	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGTCTT	96%+	925-1385
258	<i>Bacillus neizhouensis</i>	EU925618	TTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	96%+	905-1367
259	<i>Bacillus beveridgei</i>	FJ825145	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	94%+	944-1409

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260	<i>Bacillus chagannorensis</i>	AM492159	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	94%+	945-1407
261	<i>Bacillus saliphilus</i>	AJ493660	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	919-1381
262	<i>Bacillus aurantiacus</i>	AJ605773	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	929-1381
263	<i>Bacillus vedderi</i>	Z48306	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%-	905-1367
264	<i>Bacillus cellulosilyticus</i>	AB043852	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%-	924-1386
265	<i>Bacillus clarkii</i>	X76444	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	95%+	926-1328
266	<i>Bacillus polygoni</i>	AB292819	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	94%-	945-1408
267	<i>Bacillus horti</i>	D87035	CTGAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	93%+	923-1378
268	<i>Bacillus mannanilyticus</i>	AB043864	AATACGTTCCCGGG TC T CTGAAACTCAAAGGAATTGACG	96%-	955-1413
	Actinobacteria (High GC content gram positive bacteria)				
269	<i>Corynebacterium diphtheriae</i>	X84248	CT AAA ACTCAAAGGAATTGACG AATACGTNCCCGGGCCTT	83%-	880-1341
270	<i>Mycobacterium tuberculosis</i>	X58890	CTAAA AA CTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	85%-	1541-2002
271	<i>Nocardia asteroides</i>	AF430019	CT AAA ACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	84%-	875-1376
272	<i>Streptomyces lavendulae</i> subsp. <i>lavendulae</i>	D85116	CT AAA ACTCANAGGAATTGACG AATACGTTCCCGGGCCTT	81%-	893-1361
	Low GC content Firmicutes (gram +ve)				
273	<i>Staphylococcus chromogenes</i>	D83360	AATACGTTCCCGGG TC T CTGAAACTCAAAGGAATTGACG	92%+	913-1371
274	<i>Streptococcus pyogenes</i>	AB002521	TT GAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	89%+	890-1350
275	<i>Enterococcus faecalis</i>	AB012212	TT GAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	91%+	939-1395
276	<i>Clostridium populeti</i>	X71853	AT GAAACTCAAAGGAATTGACG AATACGTTCCCGGG TC T	86%-	903-1359
277	<i>Listeria monocytogenes</i>	X56153	AATACGTTCCCGGGCCT N TT GAAACTCAAAGGAATTGACG	94%+	936-1392
	Alpha proteobacteria				
278	<i>Rhizobium leguminosarum</i>	U29386	TTA AAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	86%-	913-1371
279	<i>Azospirillum lipoferum</i>	Z29619	TTA AAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	84%-	845-1305
280	<i>Acetobacterium woodii</i>	X96954	TT GAAACTCAAAGGAATTGACG AAT G CGTCCCGGGTCTT	90%-	840-1305
	Beta proteobacteria				
281	<i>Burkholderia cepacia</i>	U96927	AATACGTTCCCGGG TC T TTA AAACTCAAAGGAATTGACG	82%-	870-1322

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282	<i>Bordetella pertussis</i>	U04950	TTAAAAC TCAAGGAATTGACG AATACGTTCCCGGGTCTT	81%-	922-1375
	Gamma proteobacteria				
283	<i>Pseudomonas aeruginosa</i>	X06684	AATACG TCCCCGGCCTT TTAAAAC TCAATGAATTGACG	86%-	923-1384
284	<i>Escherichia coli</i>	X80725	TTAAAAC TCAATGAATTGACG AATACGTTCCCGGGTCTT	83%-	921-1379
285	<i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i>	X87276	TTAAAAC TCAATGAATTGACG AATACGTTCCCGGGTCTT	82%-	920-1381
286	<i>Shigella dysenteriae</i>	X96966	TTAAAAC TCAATGAATTGACG AATACGTTCCCGGGTCTT	83%-	908-1362

aryabhatai have not shown any grouping with any other strain. Second dendrogram (Figure 2b) containing 29 different closely related species has been divided in to two major clusters and only one species *Bacillus siamensis* GQ281299 has not shown any grouping with any other member. 7 bacterial species i.e. *Bacillus aquaemaris* AF483625, *Bacillus marisflavi* AF483624, *Bacillus seohaenensis* AY667495, *Bacillus vietnamensis* AB099708, *Bacillus flexus* AB021185, *Bacillus megaterium* D16273, *Bacillus koreensis* AY667496 lie in one cluster. Other, 21 bacterial strains have shared the other major cluster.

Discussion

When we use molecular approaches to study microbial communities then the use of universal primers is not fully successful in finding the clear picture of community. Various researchers have faced such type of problems as Van Elsas et al. (2002) faced the problem when they studied two 16S rDNA clone libraries (one from grassland and one from arable land) prepared with bacterial primers and most of the isolated strains were found to be belonging to phylum Proteobacteria and the number of clones related to Bacilli were very few. When the same microbial communities were studied by Garbeva et al. (2003), by using *Bacillus* specific primers then a lot of *Bacillus* clones were isolated. The problem faced by universal primers can be overcome by the use of group specific primers and various researchers have used group specific primers in their studies to overcome this problem. Heuer and Smalla (1997) used Actinomycete specific primers to monitor Actinomycete communities in the potato rhizosphere. Similarly Boon et al. (2001) used several group specific nested PCR systems to identify a lot of groups under same DGGE conditions. So the need of group specific primers is there to find out the diversity and identity of the members of a specific group.

In the present research we have focused on identification and classification of AEFB by using a specific fragment of 16S rRNA gene. So in the following session we

have discussed the research related to identification and classification of bacilli by using 16S rRNA gene. As Many researchers have developed a lot of different *Bacillus* specific primers i.e. Goto et al. (2000), synthesized a *Bacillus* specific prime pair which was used to amplify a 275 bp sequence near the 5' end of 16S rDNA gene and this sequence was very specific for identification and classification of *Bacillus* strains. Garbeva et al. (2003) developed a *Bacillus* specific primer pair (Bac F and Bac R). Specificity of both primers was checked independently and some species of *Bacillus* and other related genera have shown 100% similarity with primer Bac F and likewise the reverse primer has shown similarity with 31 different species of *Bacillus* and related genera. Vardhan et al. (2011) developed a primer pair specific for amplification of a hyper variable region in 16S rDNA gene of *Bacillus* and related genera.

In the present study we found that a restriction digestion product of 16S rRNA gene (460 bp) by HaeIII enzyme was specific for *Bacillus* and related genera. Position of this fragment was near the 3' end of 16S rDNA gene and primer pair specific to this 463 bp fragment has been designed. Primer pair when checked for specificity has shown amplification of a 463 bp long fragment in strains belonging to genera *Bacillus*, *Lysinibacillus*, *Terribacillus*, *Brevibacillus* and *Jeotgalibacillus*. No any amplification was seen in two AEFB strains i.e. *Bacillus arsenicus* and *Paenibacillus taiwanensis* and 9 different strains of bacterial lineages other than AEFB (Figure 1c&d). Reason for no amplification of this fragment in *Bacillus arsenicus* and *Paenibacillus taiwanensis* is may be due to the reason that during the course of evolution these have faced some variations because of which the restriction enzyme sites for Hae III enzymes were deleted at that position and primer pair designed in the present study includes the restriction site which causes the non specificity for primer.

Results of our study indicate that primer pair designed here is specific for *Bacillus* and related genera and not for other bacterial lineages. Primer pair when checked for

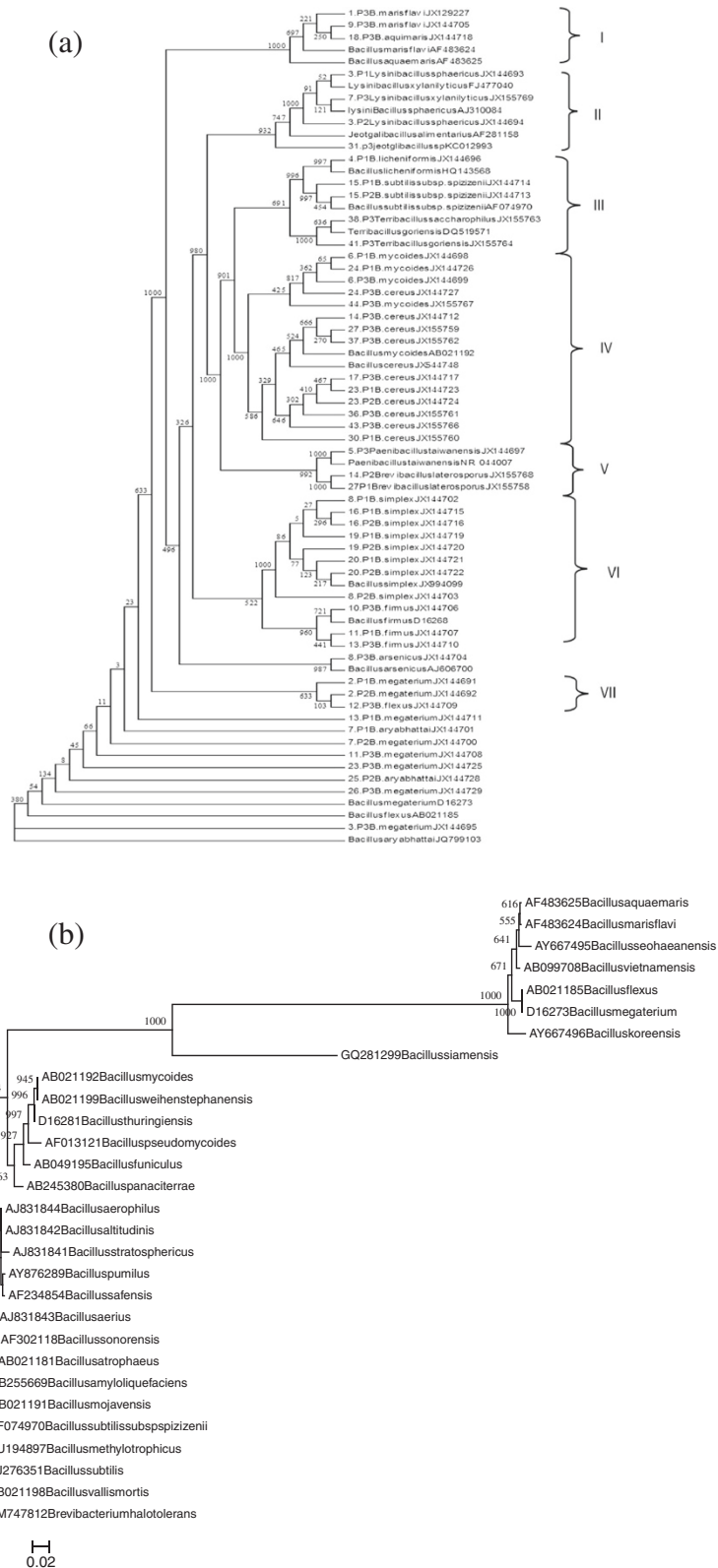


Figure 2 Dendrograms showing the phylogenetic relationship (a) 52 AFB1 strains with reference sequences (b) 29 closely related species of genera *Bacillus* based on 463 bp long 16S rRNA sequences.

homology (*in silico*) has shown 100% homology with 16S rDNA sequences of 120 species related to genera *Bacillus*. *Bacillus* species which do not have shown 100% similarity of these primers have acquired anomalous positions in the classification based on 16S rRNA gene (Yarza et al. 2010). While some species i.e. *B. pseudomycooides* AF013121, *B. ginsengihumi* AB245378, *B. acidiproducens* EF379274, *B. endophyticus*, AF295302, *B. benzoevorans*, X60611, *B. horneckiae* EU86136 have shown anomalous positions with other bacterial lineages according to classification systems based on 16S rRNA gene (Yarza et al. 2010) in spite of having homology with primer pair in our study. Bacilli strains other than the genus *Bacillus* have also shown the primer pair similarity and these genera are *Virgibacillus* (7), *Geobacillus* (5), *Filobacillus* (1), *Jeotgalibacillus* (4) and *Ureibacillus* (5). Almost all the species checked for primer pair homology has shown 100% similarity except *Virgibacillus pantothenticus* D16275, *Virgibacillus proomii* and AJ012667. All of these genera belong to the family Bacillaceae except *Jeotgalibacillus* which belong to the family Planococcaceae. *Bacillus* related genera which don't have shown primer pair similarity are *Alicyclobacillus* (7), *Amphibacillus* (5), *Aneurinibacillus* (5), *Brevibacillus* (16), *Gracilibacillus* (9) and *Paenibacillus* (5). Only a few members of these genera have shown homology with primer pair and these are *Alicyclobacillus acidocaldarius* AJ496806, *Alicyclobacillus tolerans* Z21979, *Brevibacillus invocatus* AF378232, *Brevibacillus panacihum*. Genera which do not have shown primer specificity belong to different species other than Bacillaceae except *Amphibacillus*, *Gracilibacillus* and *Terribacillus*. In our study genera belonging to family Bacillaceae have shown primer specificity and genera belonging to family other than Bacillaceae have not shown primer specificity except some genera which have shown primer specificity in reverse order.

Phylogenetic relationship based on 463 bp sequence of 52 bacilli strains (taken in our study) along with reference sequences (downloaded from NCBI) (Figure 2a) has shown that different bacterial strains belonging to same species and genera have shared a single group except some strains belonging to *Bacillus megaterium*, *B. aryabhatai* and *B. flexus*. As strains belonging to species *Bacillus megaterium* have not grouped in one cluster. Out of total 8 strains of *B. megaterium*, only two strains belonging to species *B. megaterium* have made grouping with *B. flexus*. Another 6 strains of *B. megaterium* and two strains of *B. aryabhatai* have not shown any grouping with any other strain, however all these eight strains lie below *B. megaterium* and *B. flexus* group. This shows that different strains of *B. megaterium* and *B. aryabhatai* (close relative of *B. megaterium*) have remarkable strain to strain genetic variations. Grouping of strains belonging to *Bacillus* related genera in between the

strains related to *Bacillus* indicates that during the course of evolution these genera have been evolved from the older one genera i.e. *Bacillus* which is similar to the classifications according to others (Xu and Cote 2003; Yarza et al. 2010; Vardhan et al. 2011). Further the phylogenetic relationship of some closely related strains of genera *Bacillus*, sharing a single cluster in the all species living tree (Yarza et al. 2010) have shown the same phylogenetic relationship in our study (Figure 2b). The only exception is *Bacillus siamensis* GQ281299 which has not shown any grouping with any other *Bacillus* species. However, in all species living tree this strain has shown relationship with other *Bacillus* species which lie in the lower cluster in our study (Figure 2b).

From the present study we can conclude that the restriction digestion of 16S rRNA gene by HaeIII enzyme and amplification of 463 bp fragment with specific primers designed in our study are easy methods for identification of *Bacillus* and related genera. Further the sequence information and multiple alignment of 463 bp fragment of *Bacillus* and related genera have been proved to be a good identification and classification tool for *Bacillus* and related genera.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

SK carried out experimental research work. MP has contributed in interpretation of data and preparation of manuscript. KS has participated in sequence alignment. JPY has supervised the research work, prepared and edited the manuscript. All authors read and approved the final manuscript.

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