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ORIGINAL PAPER

Phylogenetic diversity of isolates belonging to genera *Geobacillus* and *Aeribacillus* isolated from different geothermal regions of Turkey

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Abstract The phylogenetic diversity of 31 thermophilic bacilli belonging to genera *Geobacillus* and *Aeribacillus* were investigated which were isolated from various geothermal sites of Turkey. Twenty-seven of these isolates were found to be belonged within the genus *Geobacillus*, whereas 4 of them were identified as *Aeribacillus pallidus*. The comparative 16S rRNA gene sequence analyses revealed that the *A. pallidus* isolates displayed sequence similarity values from 98.0 to 99.6% to their closest relative. Furthermore, *Geobacillus* isolates showed sequence similarity values from 88.9 to 99.8% with the reference type strains. According to the phylogenetic analysis, isolates belonging to genus *Geobacillus* were diverged into nine clusters and among these isolates, 19 of them were identified as strains related to *G. caldoproteolyticus*, *G. thermodenitrificans*, *G. stearothermophilus*, *G. thermoglucosidasius* and *G. toebii* with the most abundant 13 isolates from *G. caldoproteolyticus*. Four of the *Geobacillus* isolates were named as unidentified mix group, as they found to be genetically very homogenous like their closely related type species: *G. thermoleovorans*, *G. vulcani*, *G. lituanicus*, *G. kaustophilus*, *G. caldovelox*, *G. caldotenax*, and *G. uralicus*. Moreover, the sequence comparisons of E173a, E265, C161ab and A142 isolates demonstrated that they represented novel species among genus *Geobacillus* as they shared lower than 96.7% sequence similarity to all the described type species. The *Alu*I-, *Hae*III- and *Taq*I-ARDRA results were in congruence with the 16S rRNA gene sequence analyses. By ARDRA results, the isolates were able to be differentiated and clustered, the discriminative restriction fragments of these isolates and type species were determined and the novelty of E173, E265, C161ab and A142 isolates could be displayed. Some differentiating phenotypic characters and the ability of amylase, glucosidase and protease production of these bacilli were also studied and biotechnologically valuable thermostable enzyme producing isolates were introduced in order to use in further studies.

Keywords Isolation · Thermophilic · *Geobacillus* · *Aeribacillus* · 16S rRNA gene · ARDRA

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Introduction

Since Cohn established the genus *Bacillus* in 1872, it has undergone to major taxonomic rearrangements, and many novel phylogenetic groups have been defined or reclassified as the new genera which were related to this group of bacteria (Stackebrandt et al. 1987; Logan et al. 2009). The investigations on phylogenetic divergence of the genus *Bacillus* and especially its thermophilic members indicated the need for further and extensive studies to place some of these bacilli in appropriate taxonomic levels (Ash et al. 1991; Rainey et al. 1994; Nazina et al. 2001). With the accumulation of further 16S rRNA gene sequence data, *Bacillus* has been divided into more manageable and better-defined groups (Logan et al. 2009). Molecular analysis

showed that the majority of such thermophilic bacteria described in the literature belonged to the genus *Bacillus* genetic group 5 (Ash et al. 1991; Nazina et al. 2001). As a consequence in 2001, the thermophilic, endospore-forming, aerobic bacteria belonging to *Bacillus* genetic group 5 were reclassified as being members of the genus *Geobacillus* (Nazina et al. 2001). The members of the genus *Geobacillus* can exhibit growth at temperatures ranging from 35 to 78°C, and are widespread in various geographical locations. They have been defined as a phylogenetically coherent group of thermophilic bacilli displaying very high intragenic similarity (96.5–99.2%) among their 16S rRNA gene sequences (Nazina et al. 2001; Nazina et al. 2004). Since then, new representatives of the genus *Geobacillus* have been described with the type species of *Geobacillus stearothermophilus* DSM 22^T and the genus comprises seventeen validly published species now. Finally, in 2010 Miñana-Galbis et al. reclassified *Geobacillus pallidus* (Scholz et al. 1988) Banat et al. 2004 as *Aeribacillus pallidus*, and a new genus: *Aeribacillus* was introduced and separated from genus *Geobacillus*.

Isolation of novel thermophilic bacilli has received considerable attention and also the interest in thermozymes is of importance for industrial applications (Zeikus et al. 1998). Thermophilic bacilli are the natural sources of many thermostable enzymes, such as amylases, glucosidases (Cihan et al. 2009), proteases (Chen et al. 2004), lipases (Lee et al. 1999), chitinases (Sandalli et al. 2008), xylanases (Touzel et al. 2000), restriction endonucleases (Pugatsch and Weber 1979) and DNA polymerases (Stenesh and Roe 1972). These thermozymes have been used in a number of industrial applications as they possess thermal stability to harsh industrial processes at high temperatures (Demirjian et al. 2001). During a previous research, we isolated more than five hundred bacilli from different geothermal regions of Turkey (Coleri et al. 2009). Of those from previous isolates, 115 bacilli were randomly selected and their 16S rRNA gene sequence analyses were carried out in order to study by a polyphasic approach. Among 115 endospore-forming bacilli, data including the 31 isolates belonging to genera *Geobacillus* and *Aeribacillus* are presented in this paper. The *Geobacillus* and *Aeribacillus* isolates were screened for their amylolytic, glucosidic and proteolytic activities which might have biotechnological potential. The taxonomic data of these thermophilic isolates presented in this research were derived from the phenotypic characteristics, 16S rRNA gene sequences and *Alu*I-, *Hae*III- and *Taq*I-Amplified Ribosomal DNA Restriction (ARDRA) patterns. This study contains comprehensive phenotypic and genotypic data derived from a large number of isolates belonging to two thermophilic and endospore-forming genera all of which were isolated from wide geothermal regions of Turkey.

Materials and methods

Sampling, isolation and growth conditions

A total of 28 water (2), sediment (8), soil (17) and tree branch (1) samples were collected aseptically from 9 hot springs and 7 high-temperature well pipelines located in two geographically separated areas in Turkey: Aegean Region and Middle Anatolian Region. Of those geothermal regions, Aydin (Region A; 27° 51′ E, 37° 51′ N), Manisa (Region B; 27° 26′ E, 38° 36′ N), Denizli (Region C; 29° 06′ E, 37° 46′ N) and Izmir (Region D; 27° 09′ E, 38° 25′ N) provinces are in the Aegean Region, whereas Nevsehir (Region E; 34° 43′ E, 38° 38′ N) and Ankara (Region F; 32° 52′ E, 39° 56′ N) province is located in the Middle Anatolian Region of Turkey. The water temperature and pH of these geothermal areas were measured between 50–100°C and 6.0–9.0, respectively.

One ml water and sediment sample or approximately 1 g soil and tree branch sample from each place was incubated in 5 ml of the MI medium containing 1% soluble starch (pH 7.0) at 60°C with 250 rpm shaking for 24 h to obtain the enrichment culture, after each sample was heat-treated at 80°C for 10 min to kill vegetative cells (Coleri et al. 2009). The turbid enrichments were streaked on plates of MI medium containing 3% agar and incubated aerobically at 60°C for 24–48 h. The single colonies showing different colony morphology were then picked and subcultured at least three times until a pure culture was obtained. The purity of the isolates was also confirmed by microscopic observation. All of the isolates were routinely maintained at 4°C on MI agar slants and stored at −80°C in MI broth cultures supplemented with 20% glycerol.

Isolates were designated according to their geothermal origin, the sample number taken from that origin and the number of the isolates obtained in that sample. The designation of the 31 isolates, their geothermal sampling locations and the reference strains used in this study are presented in Table 1.

Morphologic and physiologic characterization

The temperature range was determined by incubating the strains in MI medium at temperatures from 20 to 80°C for 24–72 h. After the temperature range was determined, all the incubation conditions were adjusted to the temperature requirements of the bacteria, from 50 to 65°C. The cell morphology, motility and spore formation were observed with freshly prepared wet mounts using phase-contrast microscopy. The active cultures grown in MI broth under shaking for 18–24 h were used when describing the cell morphology and motility. The formation of endospores was

Table 1 Diversity and origin of the thermophilic bacilli isolated from various geothermal regions of Turkey and the reference strains used in this study

Bacterial isolates	Origin	Number of the isolates and strains
A113[a], A142[b], A146[b], A333[a], A335[a]	Omerbeyli, Germencik, Aydin, Turkey	5
A353[b], A364[a], A392b[a], A394[a], A403[a], A404[a], A412b[a], A413[a]	Yavuzkoy, Salavatli, Aydin, Turkey	8
B84a[c]	Urganlı, Turgutlu, Manisa, Turkey	1
C161ab[a], C196[a], C226[a], C304[b]	Buharkent, Tekkehamam/Tekkekoy, Denizli, Turkey	4
D195[b], D494[b], D504[b], D621[a], D623[a], D642[a]	Dikili, Kaynarca, Kocaoba, İzmir, Turkey	6
D413[a]	Dikili, Camur Hot Spring, Izmir, Turkey	1
E134[d], E265[b], E334[d]	Altinsu, Kozakli, Nevsehir, Turkey	3
E173a[a], E173b[a]	Baglica Kozakli, Nevsehir, Turkey	2
F84a[b]	Kizilcahamam, Ankara, Turkey	1
	Total number of the bacterial isolates	31
Reference strains		
Geobacillus stearothermophilus DSM 22^T	Kindly provided by Prof. A. O. Belduz (Karadeniz Technical University, Turkey)	
Geobacillus stearothermophilus ATCC 43223	Kindly provided by Prof. A. O. Belduz	
Geobacillus stearothermophilus DSM 5934	DSMZ	
Geobacillus thermoglucosidasius DSM 2542^T	DSMZ	
Geobacillus thermodenitrificans DSM 465^T	DSMZ	
Geobacillus thermodenitrificans subsp. *calidus* DSM 22629^T	From our collection	
Bacillus licheniformis DSM 13^T	DSMZ	
Bacillus amyloliquefaciens DSM7^T	DSMZ	

[a] Soil sample, [b] sediment sample, [c] water sample, [d] branch of a tree in the hot spring

also tested by using MI broth cultures of 18–48 h supplemented with 5 mg/l $MnSO_4.4H_2O$.

The colony morphologies were determined using cultures grown aerobically on MI plates (supplemented with 3% agar) for 18–24 h. Gram staining and catalase activity were carried out by the methods of Claus and Berkeley as described (Claus and Berkeley 1986). The reference strains were used as control groups in all the phenotypic characterization tests, and the phenotypic analyses were carried out in triplicates.

Enzyme assays

All of the isolates were screened for their amylase, α-glucosidase and protease activities qualitatively on agar plates. Amylolytic activity was tested on MI agar plates after incubation for 48 h. Then the plates were treated with 0.2% I_2 in 2% KI, and isolates having starch digestion zones around their colonies were determined as amylolytic (Coleri et al. 2009). When determining α-glucosidase activity, a screening was carried out on MI plates by searching *para*-nitrophenol α-D-glucopyranoside (*p*NPG) activity on blotting filter paper as described previously (Cihan et al. 2010). The paper disk was incubated at 60°C and the yellow color formation, the color of which was caused by the reaction of α-glucosidase on the substrate, was observed and selected for the positive α-glucosidase reaction. In the screening of protease activity, isolates were growth on Skim Milk Agar (pH 7.0) plates (Denizci et al. 2004) for 72 h. Protease producing isolates which gave a clear zone around their colonies due to the hydrolysis of skim milk were selected. The diameters of halo zones and the amount of yellow color formation were also confirmed and compared with the reference strains which were able to produce the mentioned enzymes.

16S rRNA gene amplification and sequencing analyses

Genomic DNA was extracted from the cultures growing on MI plates for 18 h at 60°C by using genomic DNA purification kit (Fermentas). The gene encoding 16S rRNA was amplified by PCR with the 16S bacteria specific 27F forward and the 1492R reverse primer as described previously (Kuisiene et al. 2002). The amplification products were purified from agarose gel using Gel Extraction Kit (Omega Ezna). The sequences of the PCR-amplified 16S rRNA gene were determined by using ABI 3100 gene sequencer with Bigdye cycle sequencing kit. In the phylogenetic

analysis, homology search was carried out using the basic BLASTN search program at the NCBI Web-site. Phylogenetic analysis were performed using the maximum-likelihood and neighbor-joining methods with bootstrap values based on 1,000 replications and the phylogenetic tree (Saitou and Nei 1987) was constructed with the MEGA package version 4 (Tamura et al. 2007) according to Jukes-Cantor method (Jukes and Cantor 1969). For most of the isolates, their almost complete 16S rRNA genes were sequenced (1,383–1,407 bp) and the GenBank accesion numbers are given below.

The GenBank accession numbers: The 16S rRNA gene sequences presented in this study were obtained from the following isolates: A113 (FJ429596), A142 (FJ429990), A146 (FJ429568)^{p}, A333 (EU326497)^{p}, A335 (FJ429994), A353 (FJ429997), A364 (FJ429998), A392b (FJ430002), A394 (FJ430003), A403 (FJ429570)^{p}, A404 (FJ430005), A412b (FJ429571)^{p}, A413 (FJ430006), B84a (FJ430009), C161ab (FJ430012), C196 (FJ430014), C226 (FJ430015), C304 (FJ429574)^{p}, D195 (FJ430023), D413 (FJ430040), D494 (FJ430045), D504 (FJ430047), D621 (FJ430050), D623 (FJ430051), D642 (FJ430052), E134 (EU477771)^{p}, E173a (FJ430056), E173b (FJ430057), E265 (FJ429590), E334 (FJ429594)^{p}, F84a (EU477772)^{p}, (^{p}: the partial 16S rRNA gene sequences).

Amplified ribosomal DNA restriction analysis

ARDRA analysis of the 16S rRNA gene primed by 27F/1492R was carried out on the amplified PCR products by single enzyme digestion, according to the manufacturer's instructions, with Fast digest *Alu*I, *Hae*III and *Taq*I restriction enzymes (MBI Fermentas). The ARDRA profiles of the digested DNA were analyzed by electrophoresis through 2% (w/v) agarose gel using 1× TBE buffer at 120 V for 1.5 h (Caccamo et al. 2001). The ARDRA patterns were analyzed by the Bionumerics version 6.1 software packages (Applied Maths, Belgium). The experimental restriction fragments higher than 75 bp were included in the statistical analysis, in order to avoid confusion with primer dimmer bands. Similarities of the digitized profiles were calculated using Dice correlation and an average linkage (UPGMA) dendrogram was obtained. All the restriction analyses and their agarose gel electrophoresis were carried out in triplicates. In addition to experimental restriction analyses, the theoretical restriction mapping of the analyzed 16S rRNA gene sequences was also carried out by using an online restriction mapping service (http://restrictionmapper.org/). The developed theoretical-ARDRA groups were abbreviated as G-A-#; (*Geobacillus-Alu*I-group number), G-H-#; (*Geobacillus-Hae*III-group number) and G-T-#; (*Geobacillus-Taq*I-group number).

Results

Selection of *Geobacillus* and *Aeribacillus* isolates according to the 16S rRNA gene sequence analyses

The phylogenetic diversity of one hundred and fifteen aerobic and endospore forming isolates were analyzed using 16S rRNA gene nucleotide sequences in order to avoid the repeated examination in the same bacterial taxon. All the isolates were phylogenetically clustered on the basis of their individual 16S rRNA gene sequence homologies to their closest relatives. According to the phylogenetic analysis of these sequences, most of the identified isolates from geothermal regions of Turkey fell into *Bacillus* genetic group 5 along with thermophilic species. The other isolates clustered in *Bacillus* genetic group 1 and 3 with their mesophilic and facultative thermophilic counterparts. Comparison of the generated sequences with those in the GenBank database indicated that all were clustered among the 7 genera: *Anoxybacillus*, *Bacillus*, *Brevibacillus*, *Geobacillus*, *Aeribacillus*, *Paenibacillus*, and *Thermoactinomycetes* with 52, 16, 13, 27, 4, 1 and 2 isolates.

Among these isolates, thirty-one aerobic, thermophilic, endospore-forming isolates from genera *Geobacillus* and *Aeribacillus* were selected and taken into further researches in this study. As *G. pallidus* (Scholz et al. 1988) Banat et al. 2004 was reclassified as *A. pallidus* by Miñana-Galbis et al. in 2010, the ARDRA results of this reclassified group were taken into consideration together with the other isolates within the genus *Geobacillus*. These selected isolates were totally obtained from 16 geothermal sampling stations located in the Aegean Region and Middle Anatolian Region in Turkey. The 16S rRNA gene sequence data of 31 isolates have been deposited in the GenBank databases and their accession numbers in relation to the isolates were given in the material and methods section and in the phylogenetic tree which was obtained using the neighbor-joining method (Fig. 1).

The phylogenetic diversity of the isolates and type strains belonging to genera *Geobacillus* and *Aeribacillus*

Nearly all the validly published 20 type species from genus *Geobacillus* and one type species from genus *Aeribacillus* were included in comparative sequence analysis among with our isolates as presented in Fig. 1. The phylogenetic analyses revealed that the type species from genus *Geobacillus* showed 16S rRNA gene sequence similarities from 88.9 to 99.8%. Furthermore, some of the type species of genus *Geobacillus* were determined to share high 16S rRNA gene sequence similarities as they could not be

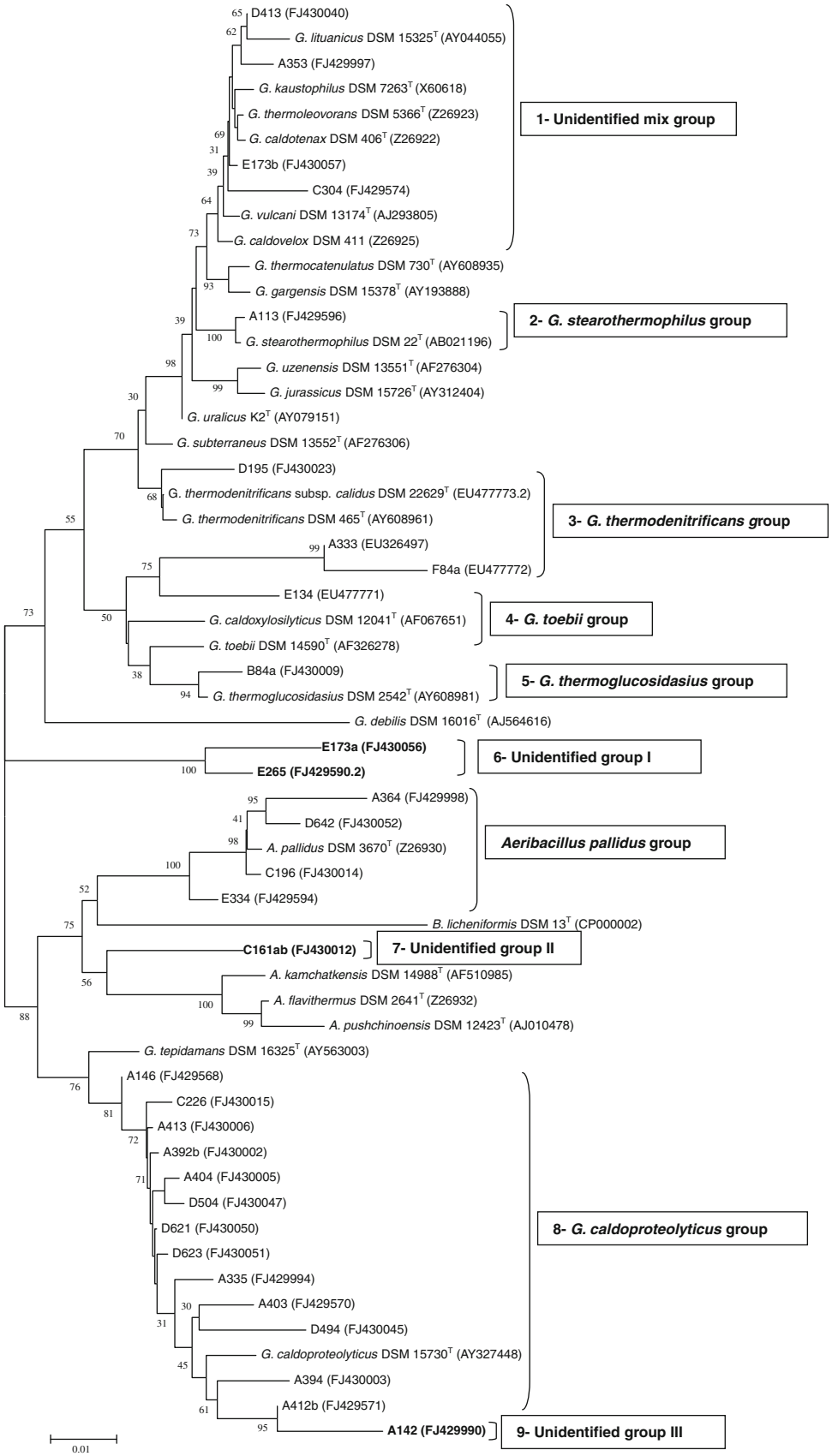

Fig. 1 A phylogenetic tree based on the 16S rRNA gene sequences between isolates belonging to genera *Geobacillus*, *Aeribacillus* and all the representative members from these genera. The tree was generated by neighbour-joining method. Boostrap values (%) are based on 1,000 replicates and shown for branches with more than 30% bootstrap support. *Bar* indicates 0.01 substitutions per 100 nucleotide positions

Table 2 The species groups of the *Geobacillus* and *Aeribacillus* isolates, the intragenic sequence similarity values and the number of the bacteria belonging to these groups derived from 16S rRNA gene nucleotide sequences

Genus	16S rRNA gene grouping	16S rRNA gene sequence similarities of the isolates to the closest relative (%)	Number of the isolates
Geobacillus	1-Unidentified mix group	>99.0 (similarity to more than 3 type strains)	4
	2-*G. stearothermophilus* group	99.3	1
	3-*G. thermodenitrificans* group	97.6–98.7	3
	4-*G. toebii* group	98.5	1
	5-*G. thermoglucosidasius* group	99.2	1
	6-Unidentified group I (E173a and E265)	94.3–95.6 (similarity to *G. toebii*)	2
	7-Unidentified group II (C161ab)	<96.3 (similarity to *G. caldoproteolyticus*)	1
	8-*G. caldoproteolyticus* group	97.7–99.9	13
	9-Unidentified group III (A142)	<96.7 (similarity to *G. caldoproteolyticus*)	1
		Isolates belonging to genus *Geobacillus*	27
Aeribacillus	1-*A. pallidus* group	98.0–99.6	4
		Isolates belonging to genus *Aeribacillus*	4
		Total	31

differentiated without DND-DNA hybridization analyses. According to these results: a 98.1–99.9% sequence similarity between type strains of *Geobacillus thermoleovorans* DSM 5366^T, *Geobacillus vulcani* DSM 13174^T, *Geobacillus lituanicus* DSM 15325^T, *Geobacillus kaustophilus* DSM 7263^T, *Geobacillus caldovelox* DSM411^T, *Geobacillus caldotenax* DSM 406^T and *Geobacillus uralicus* K2^T; a 99.3% similarity value between *Geobacillus thermodenitrificans* DSM 465^T and *Geobacillus subterraneus* DSM 13552^T; a 98.5% similarity between *Geobacillus toebii* DSM 14590^T and *Geobacillus thermoglucosidasius* DSM 2542^T; and a 98.2% sequence similarity value between *Geobacillus tepidamans* DSM 16325^T and *Geobacillus caldoproteolyticus* DSM 15730^T were observed.

The 16S rRNA gene sequence analysis revealed that 27 of the isolates were belonged to genus *Geobacillus* and 4 to genus *Aeribacillus* as showed in phylogenetic tree in Fig. 1. The phylogenetic analyses derived from the neighbor-joining method were essentially consistent with those obtained using the maximum-likelihood algorithm, thus only the phylogenetic tree revealed by the neighbor-joining algorithm is presented in this study. The divergence of the species in these two genera, their 16S rRNA gene sequence similarity values to their closest relatives and the number of the isolates belonging to the species groups are also given in Table 2. Among the isolates, four of the isolates were found to be belonged to *A. pallidus* type species with sequence similarity values from 98.0 to 99.6% to their closest relative. These isolates also showed sequence similarity values between 97.1 and 98.9% to each other.

The comparative sequence analyses also revealed that the sequence similarity values between isolates from genus *Geobacillus* and the reference type strains were determined as 88.6–99.3%. In addition, *Geobacillus* isolates demonstrated 16S rRNA gene sequence similarities from 89.0 to 99.8% to each other. Based on this phylogenetic analysis, isolates from genus *Geobacillus* clustered into 9 distinct lineages according to the similarity values to their closely related type species (Fig. 1). On the basis of the 16S rRNA gene sequence similarity values, 4 isolates demonstrated from 98.6 to 99.7% sequence similarity to *G. thermoleovorans*, *G. vulcani*, *G. lituanicus*, *G. kaustophilus*, *G. caldovelox*, *G. caldotenax* and *G. uralicus*. These isolates showed more than 99.0% sequence similarity to at least three of these mentioned closest relatives and could not be included to any of these species, unless DNA hybridization analyses were performed. Thus, these 4 isolates from Group 1 were named as unidentified mix group. Although *G. stearothermophilus* is commonly accepted as the most abundant thermophilic strain in the literature, there was only one isolate which was found to be belonging to this species (Group 2: *G. stearothermophilus* group). Group 3, 4 and 5 were consisted of species from *G. thermodentirificans* (3 isolates), *G. toebii* (1 isolate) and *G. thermoglucosidasius* (1 isolate). Group 6 was named as unidentified group I and contained 2 isolates: E173a and E265 which showed very low sequence similarity to only *G. toebii* type strain with similarity values of 94.3 and 95.6%, respectively. These isolates were thought to be novel species belonging to genus *Geobacillus* as they showed very low sequence similarities to their closest relative, as they displayed a 97.5% sequence similarity to each other and as they branched together and formed a distinct cluster from the other isolates and thermophilic bacilli type strains in the phylogenetic tree. Moreover, strain C161ab represented a novel species;

forming a distinct cluster among other isolates and type strains, and also sharing less than 96.3% full sequence similarity to only the *G. caldoproteolyticus* type species. Thus, isolate C161ab was clustered as Group 7; with a new group name of unidentified group II. Majority of our isolates (13) belonging to this genus were found to be from species *G. caldoproteolyticus* with sequence similarity values from 97.7 to 99.9% to this closest relative (Group 8: *G. caldoproteolyticus* group). And finally, a ninth group named as unidentified group III was consisted of isolate A142, which comprised the fourth novel species among the isolates from genus *Geobacillus*. This novel isolate solely showed a 96.6% sequence similarity to *G. caldoproteolyticus* and branched out from this type species in the phylogenetic tree.

Phenotypic characteristics of the *Geobacillus* and *Aeribacillus* isolates

All of the isolates and reference strains from genus *Geobacillus* were found to be Gram-positive or variable staining, endospore-forming and motile bacilli. They were also positive in catalase activity. In addition, all of the isolates were determined to be thermophilic with temperature ranges for growth from 30 to 70°C (T_{opt} = 50–70°C). Colony morphologies and spore formation differed depending on the species.

Some differentiating phenotypic characters of these isolates were investigated and presented in Table 3. Species of *A. pallidus* had circular and cream to pale yellow colored colonies. They had central to subterminally located ellipsoidal endospores in swollen or non swollen sporangia. Starch was not hydrolyzed. Isolates from *Geobacillus* unidentified mix group displayed round and cream to yellow colored colony morphology. Terminally located ellipsoidal endospores were observed in swollen or non swollen sporangia. They could hydrolyze starch as a dominant character. Furthermore, they had both amylolytic and glucosidic activity. Among the unidentified mix group isolates, α-glucosidase production of E173b, amylase production of D413 and both protease and amylase production of C304 were all important characters of these isolates. All the isolates from *G. caldoproteolyticus* had round and cream colored colonies on MI plates. They produced terminally located ellipsoidal or oval spores in swollen sporangia. All the *G. caldoproteolyticus* isolates could able to hydrolyzed starch. The isolates belonging to this species might have a biotechnological potential due to their amylolytic and proteolytic activities. D504, D621, D623 isolates were able to produce amylase. Furthermore, all the isolates produce extracellular proteases except A335 isolate. Of those from protease producers: D403, A412b, A392b, A394, A404 and A413 displayed a significant level of protease zones when compared with reference strains.

Table 3 Differentiating phenotypic characteristics of the isolates and type strains from the genera *Geobacillus* and *Aeribacillus*

Differentiating character	16S rRNA gene groups of the isolates belonging to genus *Geobacillus*									*Aeribacillus*
	1	2	3	4	5	6	7	8	9	10
Colony morphology	Circular, cream to yellow colored	Round cream colored	Cream colored, circular, flat with lobate edges and dry-powdery surface	Circular and yellowish	Circular and yellowish	Round cream colored	Cream colored	Round and cream	Cream colored	Circular, cream to pale yellow colored
Spore position	T	T	T	T	T	T	St	T	T	St, C
Spore shape	E	E	E	E	E	E	E	E, O	E	E
Temperature requirement (°C)	35–70	37–65	50–65	45–70	37–70	40–70	37–65	35–65	37–65	30–70
Amylase activity	+	+	+	+	+	–	+	+	+	–
Glucosidase activity	+	V	+	+	+	–	+	V	+	–
Protease activity	V	–	–	–	–	–	–	+	–	–

1 Unidentified mix group, *2 G. stearothermophilus* group, *3 G. thermodenitrificans* group, *4 G. toebii* group *5 G. thermoglucosidasius* group, *6* Unidentified group I (E173a and E265) *7* Unidentified group II (C161ab) *8 G. caldoproteolyticus* group *9* Unidentified group III (A142), 10 *A. pallidus* group)

E134 from *G. toebii* and B54a from *G. thermoglucosidasius* had circular colonies with yellowish pigmentation. Their spores were ellipsoidal, located terminally in swollen sporangia. They could hydrolyze starch. In addition, both of these isolates could produce significant amount of α-glucosidase. *G. thermodenitrificans* species had very typical colony morphology such as; cream colored, circular, flat with lobate edges and with a dry-powdery surface. They could produce terminally located ellipsoidal endospores in non swollen sporangia. Starch hydrolysis was a dominant character and all of the *G. thermodenitrificans* isolates and type strain were able to produce high levels of yellow color in α-glucosidase assay. A113 isolate and reference strains from *G. stearothermophilus* had round colonies in cream color, produced terminally located ellipsoidal endospores in non swollen sporangia, and could able to hydrolyzed starch. Although, both A113 and DSM 22^T could produce high amount of amylolytic zones, only DSM 22^T could able to form yellowish color in α-glucosidase reaction. E173a and E265 isolates from unidentified group I had round and cream colored colonies, produced terminally located ellipsoidal endospores in swollen sporangia. Starch hydrolysis was found to be positive on both of the isolates, but they were negative for amylolytic and glycosidic activity. C161ab isolate from unidentified group II and A142 isolate from unidentified group III possessed cream colored colonies, gave positive reaction in starch degradation, produced ellipsoidal endospores in swollen sporangia, but they differed from each other in their spore locations. While endospores of A142 located terminally, subterminal endospore location was observed in C161ab isolate.

*Alu*I-ARDRA groups of the isolates from genera *Geobacillus* and *Aeribacillus*

The amplified 16S rRNA genes of the isolates and reference strains from genus *Geobacillus* and *Aeribacillus* were digested with *Alu*I, *Hae*III and *Taq*I restriction enzymes. In Fig. 2, *Alu*I-, *Hae*III- and *Taq*I-ARDRA cluster analyses of some digitized banding patterns derived from representative *Geobacillus* and *Aeribacillus* isolates and reference strains were presented. The 16S rRNA gene groups, experimental and theoretical ARDRA groups of these representative strains were also indicated beside Fig. 2.

According to the cluster analysis of the 27 *Geobacillus*, 4 *Aeribacillus* isolates and reference strains based on the *Alu*I ARDRA profiles of the amplified 16S rRNA gene, totally 7 experimental and 10 theoretical patterns were observed according to these restriction fragments and some of these representatives were presented in Table 4. Although there were 10 theoretical groups, G-A-3 of D195, G-A-6 of A364 and G-A-10 of A142 could not be differentiated by the experimental analyses. As presented on a similarity dendrogram in Fig. 2, the experimental digitized banding patterns from *Geobacillus* and *Aeribacillus* isolates and type strains were diverged into 7 clusters. Cluster 1 diverged into two groups which contained strains belonging to *G. thermodenitrificans* and unidentified mix group. These two 16S rRNA gene groups could not be able to differentiate not only by experimental, but also by the theoretical-ARDRA analyses. The theoretical restriction group of cluster 1 was mostly compost of G-A-1. This group of isolates could be differentiated by the presence of a 75 bp (6th) restriction fragment. Furthermore, the absence of a 165 bp restriction fragment was a distinctive character of Cluster 1, which was determined in the isolates belonging to Cluster 2 and 7. The second cluster contained only isolates and strains from *G. stearothermophilus* which formed the G-A-2 theoretical group. In the restriction profiles, only isolates from cluster 2 and 7 shared the restriction fragment of 165 bp, but members of cluster 2 differed in cluster 7 by the presence of a 346 bp fragment. The third cluster was branched into two groups belonging to isolates from *G. thermoglucosidasius* and *G. toebii*. These isolates displayed a G-A-4 theoretical-ARDRA group and experimentally differed from the other clusters by the presence of a 175 bp (5th) restriction fragment. Unidentified group I members of E173a and E265 formed a forth cluster which contained a distinctive 150 bp (5th) restriction fragment and displayed a unique G-A-5 theoretical-ARDRA profile. The isolates from genus *Aeribacillus* branched out into a new cluster apart from the members of the genus *Geobacillus*: cluster 5. The presence of the first restriction fragment (648 bp) was the most distinctive character for the isolates from cluster 5, and they generally shared the G-A-7 theoretical-ARDRA profile. Cluster 6 comprised solely the C161ab isolate from unidentified group II. This group was unique by means of its second restriction fragment (239 bp) and by means of its G-A-8 theoretical-ARDRA profile. Finally, cluster 7 was composed of strains from *G. caldoproteolyticus* and the isolate A142 from unidentified group III. Although they showed different theoretical-ARDRA profiles such as G-A-9 and G-A-10, they displayed similar restriction patterns in experimental analysis. These isolates contained a 4th restriction fragment (165 bp) as in the case of cluster 2, but differed in cluster 2 by the absence of a 346 bp fragment.

*Hae*III-ARDRA groups of the isolates from genera *Geobacillus* and *Aeribacillus*

The *Hae*III-ARDRA analyses of the isolates and reference strains belonging to genus *Geobacillus* and *Aeribacillus* resulted in 6 experimental and 12 theoretical groups (Table 5), but these additional theoretical groups could not

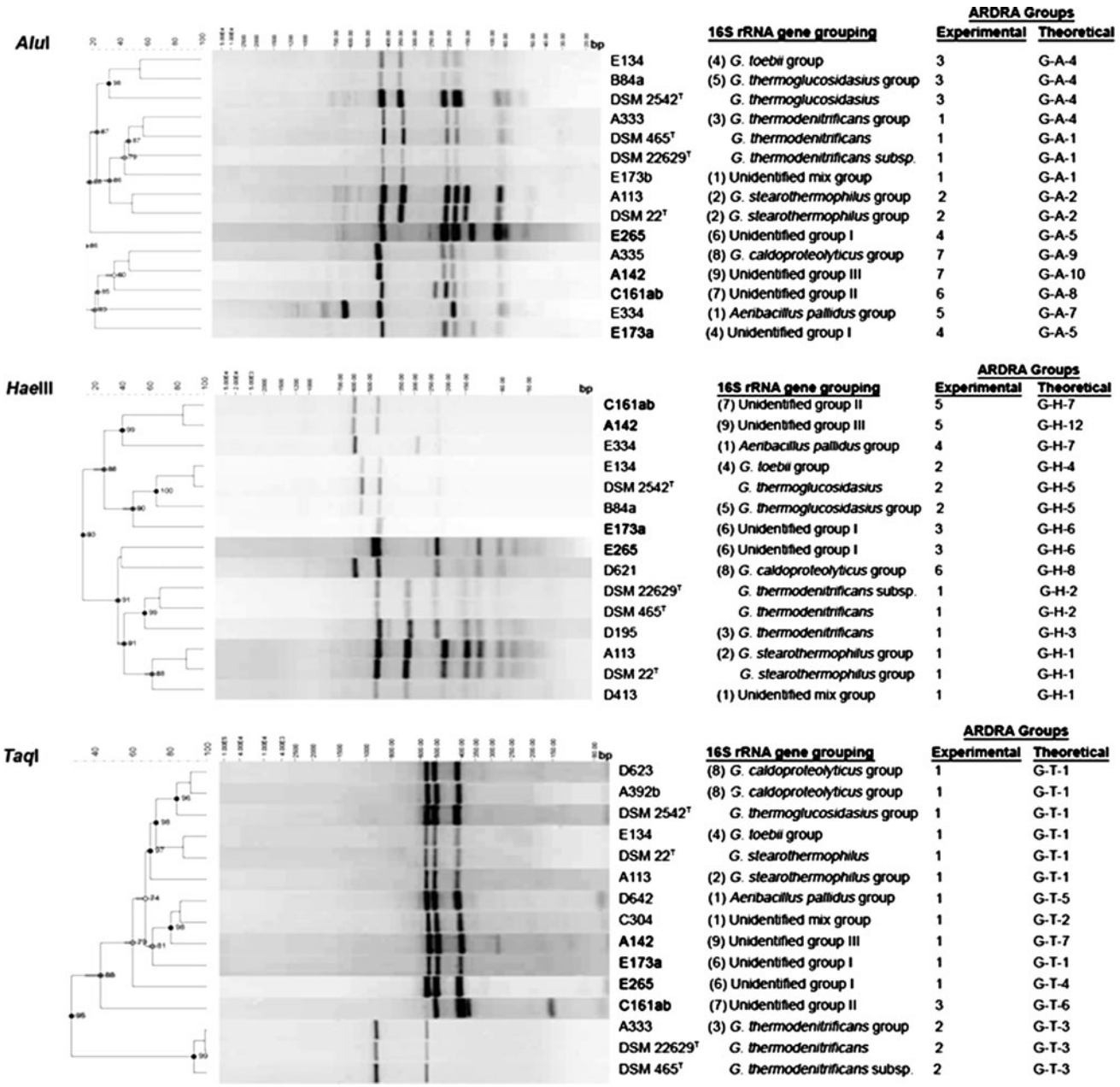

Fig. 2 Cluster analysis of some representative digitized banding patterns, generated by restriction digestions with *Alu*I, *Hae*III and *Taq*I enzymes of the amplified 16S rRNA genes of isolates from genera *Geobacillus* and *Aeribacillus*. The *dendrogram* was constructed by using UPGMA, with correlation levels expressed as percentage values of the Dice coefficient. The 16S rRNA gene and the ARDRA groups derived from both experimental and theoretical restriction digestions were also indicated beside the designation of the isolate. The novel strains were written in *bold character*

be differentiated in the experimental analysis. As can be seen in the similarity dendrogram in Fig. 2, the members of these genera formed 6 clusters. Of those clusters, cluster 1 was composed of species from *G. thermodenitrificans* (G-H-2 and G-H-3), *G. stearothermophilus* (G-H-1) and unidentified mix group (G-H-1). The presence of the second restriction fragment of 326 bp was the distinctive property of this cluster. Cluster 2 was diverged into two species groups: *G. toebii* (G-H-4) and *G. thermoglucosidasius* (G-H-5), whose first restriction fragment (581 bp) differed this group from other *Geobacillus* species. Cluster 3 was comprised from unidentified group I isolates of E173a and E265. The unique theoretical G-H-6 profile and the double 466 bp (1st) restriction fragments were the most

Table 4 Some representative theoretical and experimental 16S rRNA gene *Alu*I restriction fragments of isolates belonging to genera *Geobacillus* and *Aeribacillus*

16S rRNA gene sequence grouping	Bacteria	Experimental *Alu*I								Grouping	Theoretical *Alu*I							Grouping
		Fragments (bp)									Fragments (bp)							
		1	2	3	4	5	6	7	8		1	2	3	4	5	6	7	
1-Unidentified mix group	E173b	426	346	207	185	91	**75**			1	426	349	210	185	88	85		G-A-1[b]
2-*G. stearothermophilus* group	A113	426	346	210	187	**165**	95			2	427	367	210	185	103[a]	89		G-A-2[b]
3-*G. thermodenitrificans* group	A333	421	342	204	183	89	**77**			1	429	349	210	185	87	85		G-A-1[b]
	D195	426	346	210	187	93	**70**			1	349	336	210	189	91	87	85	G-A-3
4-*G. toebii* group	E134	420	341	207	190	**180**	90	82	70	3	428	349	210	185	173	106		G-A-4[b]
5-*G. thermoglucosidasius* group	B84a	420	341	207	190	**177**	90	82	70	3	416	349	209	185	173			G-A-4[b]
	DSM 2542^T	415	337	205	190	**175**	90	82	70	3	638	349	185	172	75[a]			X
6-Unidentified group I	**E173a**	433	213	186	68	**150**	97	70		4	426	214	208	182	142	87	85	G-A-5[b]
	E265	436	256	216	188	**152**	92			4	433	215	210	186	143	88	88	G-A-5[b]
7-Unidentified group II	**C161ab**	433	**239**	210	188	169	93	70		6	420	208	206	185	175	87	85	G-A-8[b]
8-*G. caldoproteolyticus* group	A335	458	223	199	**165**	100	70			7	426	383	208	185	87	84		G-A-9[b]
9-Unidentified group III	**A142**	443	216	192	**165**	95	70			7	383	250	208	185	179	88	84	G-A-10[b]
1-*Aeribacillus pallidus* group	A364	**648**	448	188	89	80				5	641	386	274	85				G-A-6
	E334	**648**	448	190	89	80				5	635	382	185	84				G-A-7[b]

[a] The 5′ fragment of the 16S rRNA gene sequence

[b] The dominant theoretical profile among the distinct 16S rRNA gene groups

*G-A-# Geobacillus-Alu*I-#nd theoretical group

Bold fragment the distinctive *Alu*I restriction fragment

Underlined fragment the 3′ fragment of the 16S rRNA gene sequence

The designation of the novel isolates were showed in bold character

distinctive characters of this group. Cluster 4 contained isolates belonging to *A. pallidus* due to the presence of an unmatched 320 bp (2nd) restriction fragment and G-H-7 theoretical-ARDRA profile. The fifth cluster included isolates of C161ab and A142 from unidentified group I and II, respectively. Although both of these isolates had a common 90 bp fifth restriction fragment, they displayed different theoretical profiles such as G-H-8 (C161ab) and G-H-12 (A142). The cluster 6 was only compost of *G. caldoproteolyticus* isolates with a dominant G-H-8 theoretical-ARDRA profile with exceptions of G-H-9 from D494, G-H-10 from A394 and G-H-11 from A412b, but theoretical groups from G-H-9 to G-H-11 were indistinguishable from each other in the experimental analysis. Isolates belonging to this cluster differed from the others by the presence of a 145 bp (5th) fragment.

*Taq*I-ARDRA groups of the isolates from genera *Geobacillus* and *Aeribacillus*

Totally nine 16S rRNA species group from *Geobacillus* and 1 from *Aeribacillus* comprised only 3 experimental and 7 theoretical *Taq*I-ARDRA groups (Table 6). Most of the isolates and reference strains were branched within cluster 1 with a dominant G-T-1 theoretical group as presented in a similarity dendrogram in Fig. 2. Cluster 1 contained isolates and species from unidentified mix group (G-T-1, G-T-2), *G. stearothermophilus* (G-T-1), *G. toebii* (G-T-1), *G. thermoglucosidasius* (G-T-1), E173a (G-T-1) and E265 (G-T-4) from unidentified group I, *G. caldoproteolyticus* (G-T-1), A142 (G-T-7) isolate from unidentified group III, and finally *A. pallidus* (G-T-1, G-T-5). All of these groups of bacteria in cluster 1 included a unique 504 bp (2nd) restriction fragment, but the distinct species in this cluster could not be differentiated from each other. Cluster 2 solely comprised of *G. thermodenitrificans* isolates having a unique *Taq*I restriction profile from the other *Geobacillus* species. The 929 bp first restriction fragment and the G-T-3 theoretical-ARDRA profile were only peculiar to *G. thermodenitrificans* species. The last cluster was only comprised from the isolate C161ab from unidentified group II. Not only the specific 520 bp (1st) and 393 bp (3th) fragments, but also the G-T-6 theoretical profiles differed isolate C161ab in all the other species.

Table 5 Some representative theoretical and experimental 16S rRNA gene *Hae*III restriction fragments of isolates belonging to genera *Geobacillus* and *Aeribacillus*

16S rRNA gene sequence grouping	Bacteria	Experimental *Hae*III								Grouping	Theoretical *Hae*III						Grouping
		Fragments (bp)									Fragments (bp)						
		1	2	3	4	5	6	7	8		1	2	3	4	5	6	
1-Unidentified mix group	D413	457	**326**	214	162	135	89	80	70	1	460	325	151	150[a]	84		G-H-1[b]
2-*G. stearothermophilus* group	A113	453	**326**	210	158	131	86	80	70	1	461	324	152	152[a]	84		G-H-1[b]
3-*G. thermodenitrificans* group	A333	453	**325**	228	157	127	95	77	70	1	460	325	176[a]	153	84		G-H-2[b]
	D195	447	**325**	229	157	130	98	80	70	1	460	325	175[a]	153	84		G-H-3
4-*G. toebii* group	E134	**581**	469	230						2	563	460	213[a]	138			G-H-4[b]
5-*G. thermoglucosidasius* group	B84a	**586**	475	236						2	584	460	175[a]				G-H-5[b]
6-Unidentified group I	**E173a**	**469**	221	160	130	85	70			3	467	342	174[a]	133	81		G-H-6[b]
	E265	**466**	236	160	133	87	70			3	468	348	183[a]	135	85		G-H-6[b]
7-Unidentified group II	**C161ab**	631	481	236	171	**90**	70			5	589	440	171[a]	82	77		G-H-8[b]
8-*G. caldoproteolyticus* group	D621	621	467	241	176	**140**	91	75		6	594	458	170[a]	77			G-H-8[b]
	D494	630	473	245	179	**143**	94	70		6	458	397	201	179[a]	77		G-H-9
	A394	636	481	236	180	**145**	95	70		6	458	360	235	170[a]	77		G-H-10
	A412b	656	496	257	188	**151**	100	70		6	458	167					G-H-11
9-Unidentified group III	**A142**	641	486	232	174	**90**	70			5	458	361	174[a]	138	98		G-H-12[b]
1-*Aeribacillus pallidus* group	E334	631	**320**	230						4	562	300	174[a]	155	109	77	G-H-7[b]

[a] The 5′ fragment of the 16S rRNA gene sequence

[b] The dominant theoretical profile among the distinct 16S rRNA gene groups

*G-H-# Geobacillus-Hae*III-#nd theoretical group

Double underlined fragment double restriction fragment

Bold fragment the distinctive *Hae*III restriction fragment

Underlined fragment the 3′ fragment of the 16S rRNA gene sequence

The designation of the novel isolates were showed in bold character

Discussion

16S rRNA gene sequencing is a widely used standard technique in bacterial taxonomy and it is also routinely used in 'polyphasic approach' when new descriptions of bacterial species or higher taxa are made (Ludwig and Schleifer 1999; Rosselló-Mora 2005). There are also some limitations when comparing the 16S rRNA gene sequences of phylogenetically homogeneous groups of bacteria as the structurally conserved sequences found in 16S rRNA gene might not allow strains to identify up to species level in closely related microorganisms. Moreover, it was accepted that species having 70% or greater DNA similarity usually have more than 97% sequence identity. Thus, the Ad Hoc Committee strongly recommended the DNA-DNA hybridizations in cases of species descriptions when 16S rRNA gene sequences of the novel strains show 97% or more similarity with its closest relatives (Stackebrandt and Goebel 1994; Stackebrandt et al. 2002; Logan et al. 2009).

The 16S rRNA gene sequence analyses of the 31 *Geobacillus* and *Aeribacillus* isolates carried out in this study revealed that genus *Geobacillus* was more predominant on genus *Aeribacillus* with its 27 members in the mentioned geothermal regions of Turkey. The phylogenetic analyses based on the individual 16S rRNA gene sequence homologies to their closest relatives showed that all the isolates fell into *Bacillus* genetic group 5 along with other thermophilic species. The comparative sequence analyses also revealed that the sequence similarity values between isolates from genus *Geobacillus* and the reference type strains were determined as 88.9–99.8%. In addition, *Geobacillus* isolates demonstrated 16S rRNA gene sequence similarities from 89.0 to 99.8% to each other. Based on this phylogenetic analysis, isolates from genus *Geobacillus* clustered into nine distinct lineages and 19 of the *Geobacillus* isolates could be identified within some of the described type species. The most abundant species was *G. caldoproteolyticus* with 13 isolates, followed by *G. thermodenitrificans* with 3 isolates, and *G. stearothermophilus*, *G. thermoglucosidasius, G. toebii* all of which contained one isolate.

Moreover, eight of the isolates could not be belonged to any of the described type species due to their high or low

Table 6 Some representative theoretical and experimental 16S rRNA gene *Taq*I restriction fragments of isolates belonging to genera *Geobacillus* and *Aeribacillus*

16S rRNA gene sequence grouping	Bacteria	Experimental *Taq*I					Grouping	Theoretical *Taq*I				Grouping
		Fragments (bp)						Fragments (bp)				
		1	2	3	4	5		1	2	3	4	
1-Unidentified mix group	D413	555	**503**	406			1	497	490	406[a]		G-T-1[b]
	C304	562	**504**	406			1					G-T-2
2-*G. stearothermophilus* group	A113	555	**504**	406			1	499	497	409[a]		G-T-1[b]
3-*G. thermodenitrificans* group	A333	**929**	567				2	907[a]	492			G-T-3[b]
	DSM 465[T]	**929**	560				2	590	500	409		Y
4-*G. toebii* group	E134	569	**513**	417			1	501	498	408		G-T-1[b]
5-*G. thermoglucosidasius* group	B84a	575	**513**	422			1	498	484	406[a]		G-T-1[b]
6-Unidentified group I	**E173a**	569	**506**	412			1	500	497	401[a]		G-T-1
	E265	563	**507**	410	75		1	515	503	319	92	G-T-4
8-Unidentified group II	**C161ab**	**520**	417	**393**	172	82	3	489	402[a]	334	157	G-T-6[b]
9-*G. caldoproteolyticus* group	A392b	555	**503**	406			1	496	493	401[a]		G-T-1[b]
	D623	564	**506**	402			1	495	493	401[a]		G-T-1[b]
10-Unidentified group III	**A142**	555	**507**	408	75		1	492	406[a]	269	230	G-T-7[b]
1-*Aeribacillus pallidus* group	A364	552	**505**	400	91		1	501	496	405[a]		G-T-1[b]
	D642	544	**504**	399	88		1	988	404[a]			G-T-5

[a] The 5′ fragment of the 16S rRNA gene sequence, *G-T-# Geobacillus-Taq*I-#nd theoretical group

[b] The dominant theoretical profile among the distinct 16S rRNA gene groups

Bold fragment the distinctive *Taq*I restriction fragment

Underlined fragment the 3′ fragment of the 16S rRNA gene sequence

The designation of the novel isolates were showed in bold character

level 16S rRNA gene sequence similarities. Among these unidentified isolates, 4 of them demonstrated sequence similarity values from 98.6 to 99.7% to *G. thermoleovorans*, *G. vulcani*, *G. lituanicus*, *G. kaustophilus*, *G. caldovelox*, *G. caldotenax* and to *G. uralicus* type species. As these isolates showed more than 99.0% sequence similarity to at least three of these closest relatives and as they could not be identified without DNA hybridization analyses, they were named as unidentified mix group. Furthermore, 4 of the unidentified isolates showed sequence similarity values lower than 97.0%, thus they were represented as potentially novel *Geobacillus* species. Of those from novel isolates, E173a and E265 formed a distinct cluster from all the *Geobacillus* isolates and the type species, displayed similarity values of 94.3 and 95.6% to *G. toebii* type strain, respectively and also showed 97.5% identity to each other. In addition, C161ab and A142 isolates separated and formed distinct branches from each other and from their closest relative *G. caldoproteolyticus,* therefore represented as novel *Geobacillus* strains as they showed low level sequence identity (<96.7%) to this type species. As a consequence, the sequence comparisons of E173a, E265, C161ab and A142 isolates revealed that they represented novel species among genus *Geobacillus* and these data will to their further genotypic and phenotypic analysis.

The polyphasic approach has been taking attention not only in microbial ecological researches and in taxonomic studies (Mora et al. 1998). This approach clusters a great number of similar bacteria belonging to the same genus and includes obtaining information about these clusters with definitive phenotypic and DNA-directed genotypic fingerprinting methods such as amplified ribosomal DNA restriction analysis (Vaneechoutte et al. 1992; White et al. 1993; Mora et al. 1998). ARDRA profiles of the genus *Bacillus* and *Geobacillus* have been well-studied among the thermophilic, spore-forming bacteria, and shown to be a valuable, easy and accurate technique for the identification of genus *Geobacillus* (Mora et al. 1998; Caccamo et al. 2001; Kuisiene et al. 2002, 2007). In these previous studies, restriction endonucleases of *Alu*I, *Cfo*I, *Hae*III, *Hinf*I, *Mse*I, *Rsa*I, *Taq*I were used when genotyping the genus *Geobacillus.* Of those from enzymes, *Alu*I and *Hae*III are the most frequently used enzymes for ARDRA in the genus *Geobacillus*, as they produced the highest number of differentiating bands (Kuisiene et al. 2007). It is also known that rDNA genes are organized as a multigene family and

expressed with a copy number from 1 to 15 (Klappenbach et al. 2001). As there might be sequence heterogeneity among multiple 16S rRNA genes, this will probably affect the recognition sites of the restriction endonucleases. Consequently, it was recommended that the theoretically and experimentally obtained digestion profiles should be compared (Caccamo et al. 2001; Kuisiene et al. 2007).

For this reasons, the amplified 16S rRNA gene products of the isolates from genera *Geobacillus* and *Aeribacillus* were also subjected to both experimental and theoretical digestions with *Alu*I, *Hae*III and *Taq*I restriction enzymes. The gel electrophoresis of *Alu*I, *Hae*III and *Taq*I profiles allowed us to distinguish most of the *Geobacillus* and *Aeribacillus* isolates and the reference strains within 7, 6 and 3 experimental groups, respectively (Fig. 2). According to these results, *Alu*I ARDRA patterns of *G. stearothermophilus*, *G. caldoproteolyticus*, *A. pallidus*, E173, E265 and C161ab; *Hae*III ARDRA patterns of *G. caldoproteolyticus*, *A. pallidus*, E173, E265, C161ab and A142 and finally *Taq*I ARDRA patterns of *G. thermodentirificans* and C161ab were all unique. However, isolates from the *G. thermoglucosidasus*, *G. toebii* groups, and isolates from unidentified mix group could not be differentiated from their closest relatives as they displayed high intergenic sequence similarities within genus *Geobacillus*. It is obvious that the potential of proposed *Alu*I and *Hae*III ARDRA techniques are superior on ARDRA profiles obtained using *Taq*I due to the number of restriction fragments, but species from *G. thermodentirificans* could only be distinguished from other *Geobacillus* species by its unique *Taq*I profile. In addition, some differences in the theoretical and experimental ARDRA profiles can be explained by the size of the 16S rRNA genes sequenced by us and the ones published in databases. The other reason may also be some technical and functional errors in sequences, which might contain the disappearance or appearance of one or more nucleotides (Rodas et al. 2003). This kind of ARDRA techniques was not always found useful when identifying genetically polymorphic groups of strains such as *G. toebii* and *G. thermoglucosidasius* or *G. thermoleovorans*, *G. vulcani*, *G. lituanicus*, *G. kaustophilus*, *G. caldovelox*, *G. caldotenax* and *G. uralicus,* for which DNA hybridization remains the needed method for identifying these closely related taxa at the species level (Wayne et al. 1987). As a consequence, although there were some limitations, such as ARDRA analyses were carried out on conserved 16S rRNA genes, we were able to differentiate and cluster our isolates by using their ARDRA patterns. The ARDRA results also showed resemblance with the 16S rRNA gene sequence analyses. By ARDRA results, not only the discriminative restriction fragments of these isolates and type species were determined, but also the novelty of our E173, E265, C161ab and A142 isolates could be demonstrated.

Conclusions

The phylogenetic diversity of a large number of thermophilic isolates from genera *Geobacillus* and *Aeribacillus* in geothermal areas of Turkey were presented, some of which are phylogenetically novel by this study. Certain differentiating phenotypic characters and the ability of amylase, glucosidase and protease production of these isolates were studied and biotechnologically valuable thermostable enzyme producing isolates were determined in order to use in further studies. The reliability of species identification scheme of proposed ARDRA techniques were also proved in congruence with the phylogenetic analyses of the 16S rRNA gene sequences.

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