

Characterization of Environmental *Bacillus* Isolates by Protein Fingerprinting and Random Amplified Polymorphic DNA Profiles

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Abstract The aim of the present study was to investigate antibiotic resistance, plasmid, random amplified polymorphic DNA (RAPD) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of the *Bacillus* spp. isolated from soil of Sinop environs, Turkey. A total of fifty-seven isolates were identified as *Bacillus* spp. based on morphological and physiological properties. All isolates were found resistant to bacitracin. Susceptibility patterns of the isolates to other tested antibiotics varied from 100 to 31.5 %. The plasmid analysis results indicated that isolates harbored 2 to 7 plasmids. The numerical analysis of whole cell and extracellular protein profiles of the isolates displayed similarity levels between 78–100 and 67–100 % respectively reflecting the less discriminative power of SDS-PAGE at subspecies level. However, RAPD analysis of genomic DNA using four different universal primers revealed 100 % polymorphism indicating the strength of RAPD in the differentiation of *Bacillus* isolates.

Keywords *Bacillus* · Antibiotic resistance · Random amplified polymorphic DNA · Molecular characterization · Protein fingerprinting

Introduction

The genus *Bacillus* is a large and heterogeneous group of aerobic and facultatively anaerobic, rod-shaped, gram-positive and endospore forming bacteria [1]. The genus includes species with different properties such as thermophilic, psychrophilic, acidophilic, alkalophilic and halophilic [2]. In addition to phenotypic incoherency, the genus also offers phylogenetic heterogeneity [3]. The identification of microbial species by phenotypic methods can sometimes be uncertain, complicated and time-consuming. The use of molecular methods has revolutionised their identification by quality improvement. According to the molecular analyses of 16S rRNA sequences, genus *Bacillus* was clustered into at least six phylogenetically distinct groups [4].

Several methods have been used for the molecular identification of bacterial species involving random amplified polymorphic DNA (RAPD), 16S-23S rRNA internal transcribed spacer-PCR (ITS-PCR), repetitive sequence element (REP-PCR) and amplified ribosomal DNA restriction analysis (ARDRA) [5]. The superior discriminative power of RAPD-PCR at the strain level compared with other PCR-based methods was emphasized by Weiss et al. [6]. In the analysis of microbial communities RAPD fingerprinting is a very useful technique with the elimination advantage of culture dependent procedures for the representation of the entire microbial community [7]. Another important advantage is the use of arbitrary primers to generate polymorphic genomic fingerprints with no prior knowledge of the specific sequence information [8, 9].

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A number of studies including whole-cell and extracellular protein profiles have been used in the characterization and the discrimination of bacterial genome investigations providing a second level information for a cell [10–19]. The investigators proposed that whole-cell and extracellular protein profiles were sufficient for the differentiation at species level, but the differences were inadequate at the strain level [12, 20]. In addition, Berber et al. [20] revealed that extracellular protein profiles provided better distinction than whole-cell protein profiles. The finding that protein profiles of whole cell extracts correlated with DNA hybridization results offered the use of SDS-PAGE for bacterial identification [21, 22].

In the present study, Sinop was chosen as the isolation location of bacteria since there was no study of soil bacilli in this province. Physico-chemical properties of soil samples in Sinop can be defined as sandy, pH values ranging between 4.25 and 7.24 on average, organic material content between 0.19–0.95 % and CaCO_3 content between 0.65–1.22 % [23]. Investigation of *Bacillus* spp. is preferred because of its potential industrial importance. *Bacillus* species are attractive organisms due to their rapid growth rates and capacity to secrete important proteins and enzymes which are estimated to constitute about 50 % of the total enzyme market [24]. The isolated strains were identified based on phenotypic characteristics and their antibiotic susceptibility profiles. The total plasmid contents were also determined. The heterogeneity relationships were assessed with the combination RAPD-PCR and SDS-PAGE analysis of protein profiles.

Material and Methods

Isolation and Identification of Bacteria from Soil Samples

Soil samples ($n = 104$) from different towns and villages of Sinop province of Turkey were collected and 57 bacteria were isolated (Fig. 1). The soil samples (10 g) used in the experiments were collected from the 2 to 5 cm deep layers.

One gram of the samples collected from different provinces of Sinop were transferred to tubes harboring 9 ml sterile physiological saline solution and mixed strongly for 3 min. Then the soil suspensions were serially diluted from 10^{-1} to 10^{-7} and the dilutions from 10^{-4} to 10^{-7} (0.1 ml) were plated on Nutrient Agar (NA) medium and let to the incubation at 30 °C for 24 h. At the end of the incubation period, colonies morphologically resembling to *Bacillus* were selected [25]. Pure cultures of the selected colonies were obtained by inoculating into NA. The isolates were identified on the basis of various morphological characteristics (colony size, shape, color, gram's nature, spore staining and motility), physiological tests (growth at 4, 30, 55, 65 °C, growth in NaCl at 2, 5, 7, and 10 % concentration). In addition, following biochemical identification tests were carried out: production of catalase, hydrolysis of starch, gelatin, casein, and urea, H_2S production, citrate utilization, Voges Proskauer test, carbohydrate fermentation tests (D-glucose, L-arabinose, D-xylose, D-mannitol, fructose, galactose, maltose, lactose, and sucrose). All tests were performed according to Bergey's manual of systematic bacteriology [26].

Fig. 1 Satellite map of sampling stations, Sinop, Turkey



Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed using the disk diffusion method on nutrient agar using ten antibiotic discs. Microorganisms were grown overnight at 30 °C and 1 ml of the fresh cultures were spread on nutrient agar plates using sterile glass rod. The plates were dried at room temperature for 15–20 min before placing the antibiotic discs at equidistance (6 discs per plate). The following antibiotics were used (µg/disk): amikacin (30 µg), gentamicin (10 µg), clindamycin (10 µg), ofloxacin (5 µg), penicillin G (10 µg), ampicillin (10 µg), ceftazidime (30 µg), novobiocin (5 µg), tetracycline (30 µg), imipenem (10 µg), bacitracin (10 µg). The plates were incubated at 30 °C for 24 h and the zone of inhibition was measured in terms of diameter (mm).

Plasmid Isolation

Plasmid DNA of the strains was extracted as described by the method of Sambrook et al. [27] with some modifications. *Bacillus* spp. samples were inoculated into 5 ml Nutrient Broth (NB) medium from a single colony and incubated at 30 °C for 24 h. Then, 1.5 ml culture was transferred into an Eppendorf tube and centrifuged at 10,000 rpm for 1 min. The upper phase was discarded and bacteria in the pellet were resuspended in 100 µl cold solution I (50 mM glucose, 25 mM Tris–HCl pH 8.0, 10 mM EDTA pH: 8.0) and vortexed. Samples were then treated with 200 µl solution II (1 % SDS, 0.2 N NaOH), mixed thoroughly by inverting and incubated on ice for 15 min. Afterwards, samples were centrifuged at 10,000 rpm for 7 min and the upper phases were transferred into sterile Eppendorf tubes. The application of phenol/chloroform at equal volumes was followed with vortexing and centrifugation at 10,000 rpm for 5 min and the supernatant was transferred into a clean Eppendorf tube. This application was repeated twice in order to eliminate the protein residues from plasmid DNA. The supernatant was mixed with cold pure ethanol as double volume of the sample and kept on ice for 15 min. Plasmid DNA was precipitated by centrifugation at 10,000 rpm for 10 min followed by washing with 70 % (v/v) cold ethanol, dried, and resuspended in 50 µl TE buffer containing RNase. The plasmids were separated by agarose gel electrophoresis and size determination was carried out with a molecular weight marker (O' Gene Ruler 1 kb DNA Ladder, ready-to-use, Thermo Scientific, USA).

Chromosomal DNA Isolation

Bacillus spp. samples were activated with incubation at 30 °C for 24 h. Recovered bacteria were centrifuged at

3,000 rpm for 5 min and cell pellets were resuspended in 500 µl TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA). Pellets were then incubated at 55 °C for 30 min following the addition of 50 µl SDS (10 %) and 25 µl proteinase K (20 mg/ml). Total DNA was recovered by sequential extractions with 575 µl phenol/chloroform (1:1). Then, tubes were inverted and incubated on ice for a few minutes and centrifuged at 14,000 rpm for 10 min. Upper layer (500 µl volume) was transferred into a new Eppendorf tube and treated with 50 µl sodium acetate (3 M, pH 5.2) and 330 µl isopropanol (100 %). Tubes were inverted and DNA was precipitated by centrifugation at 14,000 rpm for 10 min followed by washing with 70 % (v/v) ethanol, dried, and resuspended in 100 µl TE buffer containing 2 µl RNase.

Random Amplification of Polymorphic DNA (RAPD) Analysis

RAPD-PCR analysis was performed using four different oligonucleotide primers (Table 1). PCR was carried out in a reaction volume of 50 µl containing 1X PCR buffer, 1.5 mM MgCl₂, 200 µM each deoxynucleotide triphosphates (dNTPs), 100 pmol primer, 1.25 U of Taq DNA polymerase (Thermo Scientific, USA) and 3 µl genomic DNA. Thermal cycler (Techne TC-5000, California, USA) conditions included an initial denaturation step at 94 °C for 3 min, 34 °C for 1 min and 72 °C for 1 min during one cycle. This initial denaturation step was followed by denaturation at 94 °C for 30 s, primer annealing (34 °C for OPL-3 and OPL-12 and 54 °C for M13-10 and M13-14) for 30 s, extension at 72 °C for 30 s during 30 cycles and final extension at 72 °C for 5 min. RAPD-PCR products were run by agarose gel (1 %) electrophoresis with a molecular size marker (O' Gene Ruler, 1 kb DNA Ladder, ready-to-use, Thermo Scientific, USA) and analyzed under UV transilluminator (Cleaver-MicroDOC, UK).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

Extraction of whole cell proteins was carried out with a modified method of Laemmli [28] developed by Berber [21]. *Bacillus* strains isolated from soil were activated on

Table 1 Primer sequences used for RAPD analysis

Primers	Oligonucleotide sequence
M13-10	5'-CCGCAGCCAA-3'
M13-14	5'-GAGGGTGGCGGTTC-3'
OPL3	5'-CCAGCAGCTT-3'
OPL12	5'-GGGCGGTACT-3'

Nutrient Agar plates at 30 °C for 24 h. A loopful of bacteria from overnight cultures were suspended in 10 ml nutrient broth and incubated at 30 °C, 150 rpm for 24 h. Then 1.5 ml of the samples were transferred into clean Eppendorf tubes and centrifuged at 12,000 rpm for 5 min. Supernatants were discarded and pellets were washed with 100 µl physiological saline solution. Washed pellets were treated with 20 µl SDS-sample buffer (0.06 M Tris-HCl, 2.5 % glycerol, 0.5 % SDS, 1.25 % β-mercaptoethanol). The soluble proteins were extracted with boiling at 80 °C for 5 min.

Extraction of extracellular proteins was carried out with the method described by Wessel and Flugge [29]. Activated bacteria strains on plates were incubated into nutrient broth. Some amount of grown cultures was centrifuged at 12,000 rpm for 5 min. A volume of 500 µl upper phase was passed through 0.45 µm diameter cellulose acetate membrane filter (Sartorius) and transferred into sterile Eppendorf tubes. These culture supernatants were preserved at −70 °C until the following extraction step. Then, 400 µl methanol, 200 µl chloroform, and 300 µl dH₂O were added to these supernatants and tubes were centrifuged at 10,700 rpm for 5 min. Following centrifugation, supernatant was discarded and pellets were treated with 300 µl methanol and centrifuged again at 10,700 rpm. The supernatants were removed and the precipitated proteins were dried at room temperature. The extraction of extracellular proteins was completed with the treatment of samples with 25 µl SDS-sample buffer and protein denaturation in boiling water at 80 °C for 5 min.

Protein analysis was carried out with SDS-PAGE in gel slabs of 1 mm thickness (4 % stacking gel both for total and extracellular proteins; 10 and 12 % resolving gels for total proteins and extracellular proteins respectively) as described by Laemmli [28]. Electrophoresis was performed with a discontinuous buffer system in a vertical electrophoresis (Hoefer SE400, USA) at approximately 30 mA and protein molecular masses were calculated with the comparison of a standard marker (PageRuler™ Protein Ladder, Fermentas, 10–200 kDa molecular weight range). After electrophoresis, gels were stained overnight with Coomassie brilliant blue R-250 and destained in a mixture of methanol-acetic acid–water (3:1:6) until clearly visible protein bands obtained.

Statistical Analysis

The molecular weights of the bands were calculated with Total Lab 1D Manual R11.1, UK programme according to their pixel positions and molecular size marker based scoring. RAPD-PCR and protein patterns were subjected to

cluster analysis by using unweighted pair group method using arithmetic averages (UPGMA) based on Jaccard's similarity coefficient with the Phoretix 1D-Pro (Total lab, UK) programme.

Results and Discussion

Molecular diversity studies on bacteria have been conducted with different molecular techniques. Conserved 16S rRNA sequence analysis offers advantage of using this taxonomic marker for classification studies but on the other hand, sequence analysis may not be appropriate enough for the measurement of intraspecies relationships [30]. In the present paper, the authors focused on a combined approach of RAPD-PCR and SDS-PAGE analysis for molecular typing of *Bacillus* isolates.

The present data show that all of the isolates were gram-positive, rod-shaped, and motile and most of them had spores including central, terminal, and lateral forms. According to the carbohydrate fermentation tests it was determined that all of the strains utilized glucose, sucrose, arabinose, lactose, fructose, galactose, maltose, mannose, xylose and mannitol. All isolates were catalase-positive, whereas their urease and hydrogen sulfide kinetics were negative. The isolates were positive in terms of gelatinase activity, except for the strain numbered as 34. The results of Voges-Proskauer, casein, citrate, and amylase tests were found to be variable. It was confirmed by biochemical test results that 57 isolates belonged to the genus *Bacillus* (Table 2).

Antibiotic susceptibility testing results are presented in Table 3. Bacterial species of soil, water and sewage may have resistance to natural antibiotics or may acquire these features through genetic exchange from other bacteria [31]. Antibiotic susceptibility pattern of the isolates showed that all (100 %) isolates were sensitive to amikacin, ofloxacin, and tetracycline. Susceptibility patterns for gentamicin, clindamycin, penicillin G, ampicillin, ceftazidime, novobiocin, and imipenem were observed in 98.2 %, 75.4, 31.5, 43.8, 61.4, 45.6, and 98.2 % of the isolates respectively. Moreover, all (100 %) isolates were resistant to bacitracin.

Plasmid analysis may also be helpful if plasmid carriage is known to be frequent. The isolates were first characterized with their plasmid profiles and demonstrated to harbor 2 to 7 plasmids with molecular weights ranging between 22,200 bp to 600 bp. The plasmid profiles of the tested *Bacillus* strains are shown in Fig. 2. Agarose gel electrophoresis analysis results showed that the strains showed variation in plasmid number ranging from 2 to 7 plasmids and 2, 3, 4, 5, 6, and 7 plasmids were detected in 2.6, 63.2, 23.7, 0, 7.9 and 2.6 % of the isolates respectively.

Table 2 Morphological and phenotypical properties of *Bacillus* isolates

Characteristics	Isolate numbers																													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	
Gram staining	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Amylase	+	+	+	+	+	+	–	+	+	+	–	+	+	+	+	+	+	+	–	+	+	+	+	–	–	+	+	+	+	
Gelatinase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
H ₂ S production	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
Hydrolysis of urea	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
Citrate utilization	+	+	–	–	+	+	+	–	+	+	+	+	–	–	–	+	+	+	–	+	+	–	–	–	–	–	+	+	–	+
Hydrolysis of casein	+	+	+	–	+	+	+	–	+	+	–	–	+	+	+	+	–	+	–	–	+	–	–	–	–	–	+	+	+	+
Voges-Proskauer	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Growth in 2 % NaCl	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in 5 % NaCl	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	–	–	+	–	–	+
Growth in 7 % NaCl	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	+
Growth in 10 % NaCl	–	–	+	–	+	+	+	+	+	+	–	+	–	–	+	+	+	–	+	+	+	+	–	–	+	+	+	+	+	+
Growth at 4 °C	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Growth at 30 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 55 °C	–	–	–	–	–	+	+	+	–	+	+	–	–	–	–	+	+	+	+	–	+	+	–	–	–	–	–	+	–	–
Growth at 65 °C	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	+	+	+	–	–	+	–	–	–	–	–
Characteristics	Isolate numbers																													
	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57		
Gram staining	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Amylase	+	–	+	–	+	–	+	–	–	–	–	–	–	–	+	+	–	–	+	+	+	+	–	–	–	+	–	–	–	
Gelatinase	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
H ₂ S production	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
Hydrolysis of urea	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
Citrate utilization	–	+	–	+	+	+	+	–	–	–	–	–	–	+	+	+	–	+	+	+	+	+	+	–	–	–	–	–	+	+
Hydrolysis of casein	+	–	+	–	+	–	+	+	–	–	–	–	–	–	+	+	–	–	–	–	+	–	+	–	–	+	–	–	–	
Voges – Proskauer	+	–	–	–	+	–	–	–	–	–	–	+	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Growth in 2 % NaCl	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	
Growth in 5 % NaCl	+	+	–	–	+	–	+	–	+	–	+	+	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	
Growth in 7 % NaCl	+	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	+	
Growth in 10 % NaCl	+	+	–	–	+	+	+	+	+	–	–	+	+	+	+	–	+	–	+	–	–	–	–	–	–	–	–	+	–	
Growth at 4 °C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
Growth at 30 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Growth at 55 °C	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
Growth at 65 °C	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	

Table 3 Antibiotic susceptibility patterns of isolates

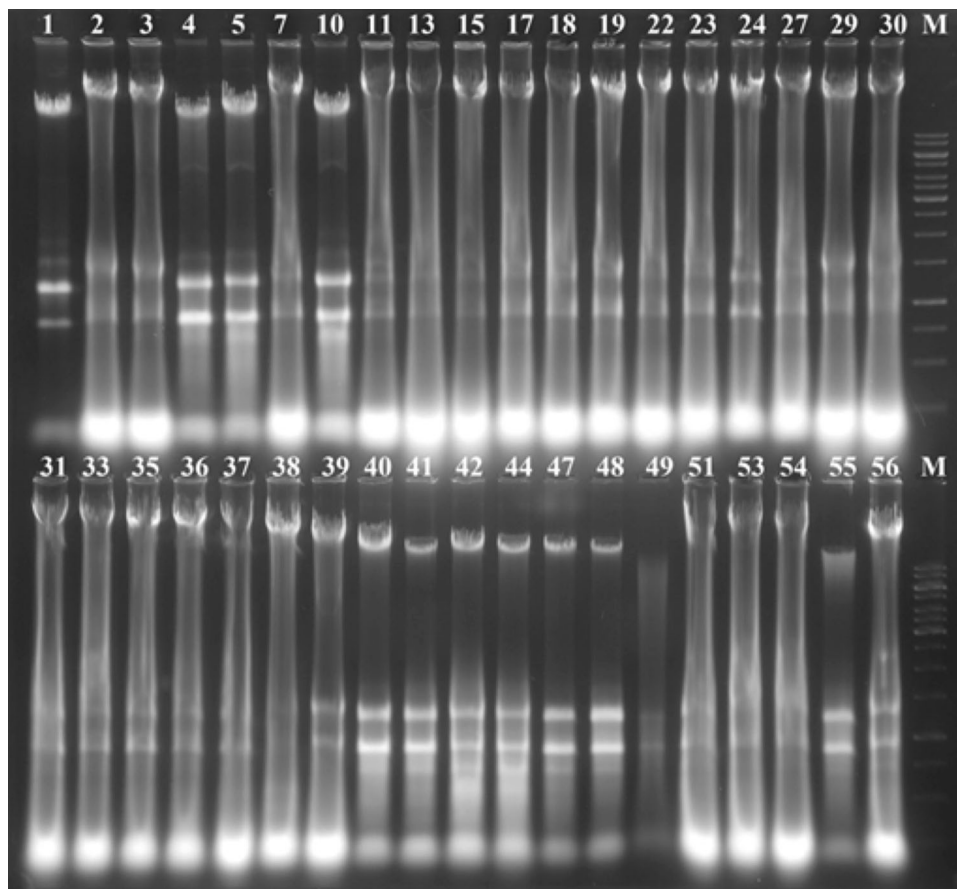
Isolates	The diameter inhibition zone (mm) of tested antibiotics										
	Amikacin	Gentamicin	Clindamycin	Ofloxacin	Penicillin G	Ampicillin	Ceftazidime	Novobiocin	Tetracycline	Imipenem	Bacitracin
1	18	16	10	14	–	–	27	–	40	32	–
2	21	16	7	7	9	–	19	26	20	30	–
3	28	22	12	18	10	–	24	–	15	33	–
4	25	20	–	20	–	30	25	33	25	33	–
5	27	25	34	15	–	21	21	30	24	30	–
6	12	17	–	13	–	–	–	–	29	35	–
7	16	13	–	15	24	18	–	–	31	30	–
8	30	22	31	33	–	–	14	19	23	40	–
9	27	20	30	30	10	13	–	19	32	36	–
10	18	16	24	26	10	–	–	–	16	26	–
11	30	22	43	32	–	24	11	18	32	32	–
12	32	16	–	18	–	–	40	–	32	36	–
13	25	24	33	32	–	15	–	16	25	25	–
14	20	17	15	28	–	–	–	12	24	24	–
15	28	21	36	12	–	14	–	14	27	27	–
16	18	12	20	32	–	–	–	–	16	16	–
17	22	17	30	24	32	–	8	11	31	31	–
18	22	18	30	34	14	–	–	14	32	26	–
19	20	16	32	22	–	24	9	23	32	26	–
20	24	22	34	32	–	12	–	15	21	39	–
21	32	32	40	40	10	11	6	17	21	31	–
22	24	24	40	33	25	12	7	15	22	32	–
23	24	22	36	34	27	16	–	18	30	40	–
24	15	20	32	28	10	21	20	–	27	40	–
25	10	–	35	13	–	–	15	–	20	35	–
26	25	21	15	31	–	–	28	–	10	–	–
27	22	18	18	32	23	–	18	–	15	28	–
28	20	18	32	32	–	–	–	14	30	22	–
29	34	30	34	36	26	–	–	16	32	28	–
30	28	22	34	26	–	–	–	14	32	30	–
31	18	16	34	26	15	16	8	18	32	32	–
32	25	16	28	30	–	–	12	–	30	30	–
33	18	14	–	26	–	12	36	10	15	33	–
34	18	12	–	28	–	9	7	15	30	28	–
35	36	30	36	34	30	12	10	17	30	30	–
36	16	14	34	27	22	15	10	–	17	31	–
37	15	10	–	23	–	–	27	–	12	32	–
38	16	10	–	24	–	15	30	–	11	34	–
39	20	16	–	28	–	–	27	–	24	35	–
40	26	20	32	22	20	30	30	32	34	32	–
41	20	16	32	26	22	35	–	12	38	36	–
42	18	12	–	25	–	–	7	–	20	32	–
43	18	15	–	24	–	17	30	–	22	27	–
44	20	12	–	26	–	–	12	–	20	30	–
45	12	10	–	28	–	–	–	–	14	33	–
46	18	12	15	23	–	–	–	–	24	26	–
47	16	13	20	28	–	–	–	–	27	27	–
48	18	14	22	36	–	–	–	–	24	25	–
49	16	13	–	22	–	–	27	–	14	27	–
50	18	14	20	20	–	–	22	–	14	15	–
51	22	17	22	40	–	–	22	–	25	25	–

Table 3 continued

Isolates	The diameter inhibition zone (mm) of tested antibiotics										
	Amikacin	Gentamicin	Clindamycin	Ofloxacin	Penicillin G	Ampicillin	Ceftazidime	Novobiocin	Tetracycline	Imipenem	Bacitracin
52	20	14	20	36	–	–	8	–	19	29	–
53	22	16	20	38	–	9	–	–	20	27	–
54	20	15	20	38	–	16	–	14	16	30	–
55	20	16	20	24	–	15	–	–	22	27	–
56	20	15	20	30	–	–	18	–	23	23	–
57	18	16	20	28	–	–	20	–	22	30	–

–: no activity

Fig. 2 Plasmid patterns of *Bacillus* spp. isolates. *Upper lanes* 1 isolate number 1, 2 isolate number 2, 3 isolate number 3, 4 isolate number 4, 5 isolate number 5, 6 isolate number 7, 7 isolate number 10, 8 isolate number 11, 9 isolate number 13, 10 isolate number 15, 11 isolate number 17, 12 isolate number 18, 13 isolate number 19, 14 isolate number 22, 15 isolate number 23, 16 isolate number 24, 17 isolate number 27, 18 isolate number 29, 19 isolate number 30, 20 molecular weight marker. *Lower lanes* 1 isolate number 31, 2 isolate number 33, 3 isolate number 35, 4 isolate number 36, 5 isolate number 37, 6 isolate number 38, 7 isolate number 39, 8 isolate number 40, 9 isolate number 41, 10 isolate number 42, 11 isolate number 44, 12 isolate number 47, 13 isolate number 48, 14 isolate number 49, 15 isolate number 51, 16 isolate number 53, 17 isolate number 54, 18 isolate number 55, 19 isolate number 56, 20 molecular weight marker

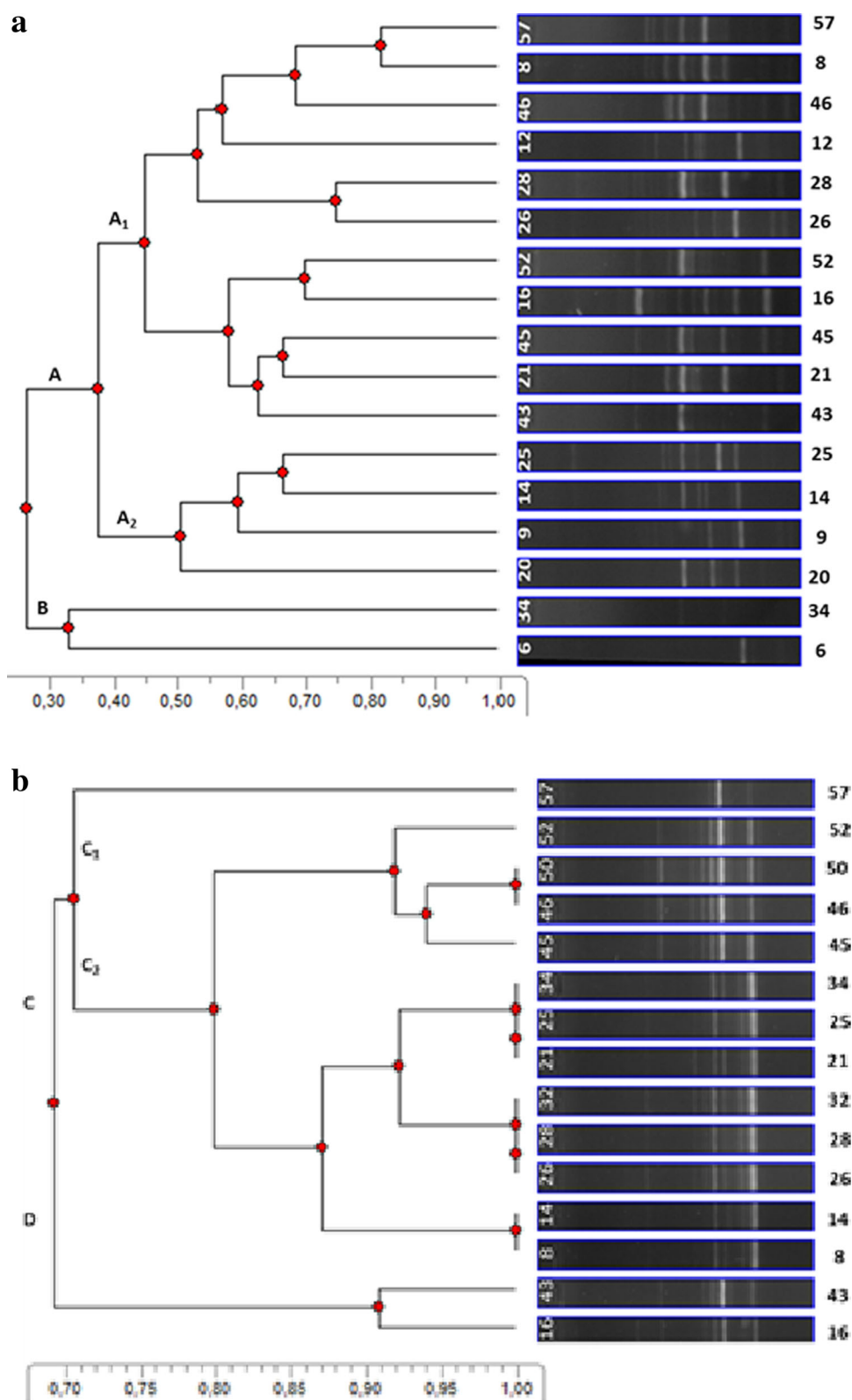


Interspecific genomic polymorphism among isolates was analyzed with RAPD-PCR using 4 different universal primers. The four primers used produced a total of 516 scorable bands. The greatest number of bands (162) was produced with OPL3 primer, M13-10 primer followed this with a less number of bands (149), OPL-12 primer produced 107 bands, and the lowest number of bands (98) was produced with M13-14 primer. All the primers tested gave 100 % polymorphic results.

A total of 17 strains generated DNA fragments with primer M13-10. The molecular weights of the DNA bands produced with M13-10 primer ranged from 3.300 to 200 bp

for the isolates (Fig. 3a). In the dendrogram in Fig. 3a, the isolates were separated into two major clusters (A and B) at similarity levels between 27 and 82 %. The cluster A consists of two sub-clusters, namely A₁ and A₂ at similarity levels of 38 % or above. The cluster B had only two strains numbering 6 and 34. A total of 15 strains generated DNA fragments with primer M13-14. The molecular weights of the DNA bands produced with M13-14 primer ranged from 2.500 bp to 200 bp for the isolates (Fig. 3b). In the dendrogram in Fig. 3b, the isolates were separated into two major clusters (C and D) at similarity levels between 69 and 100 %. The cluster C consists of two sub-clusters,

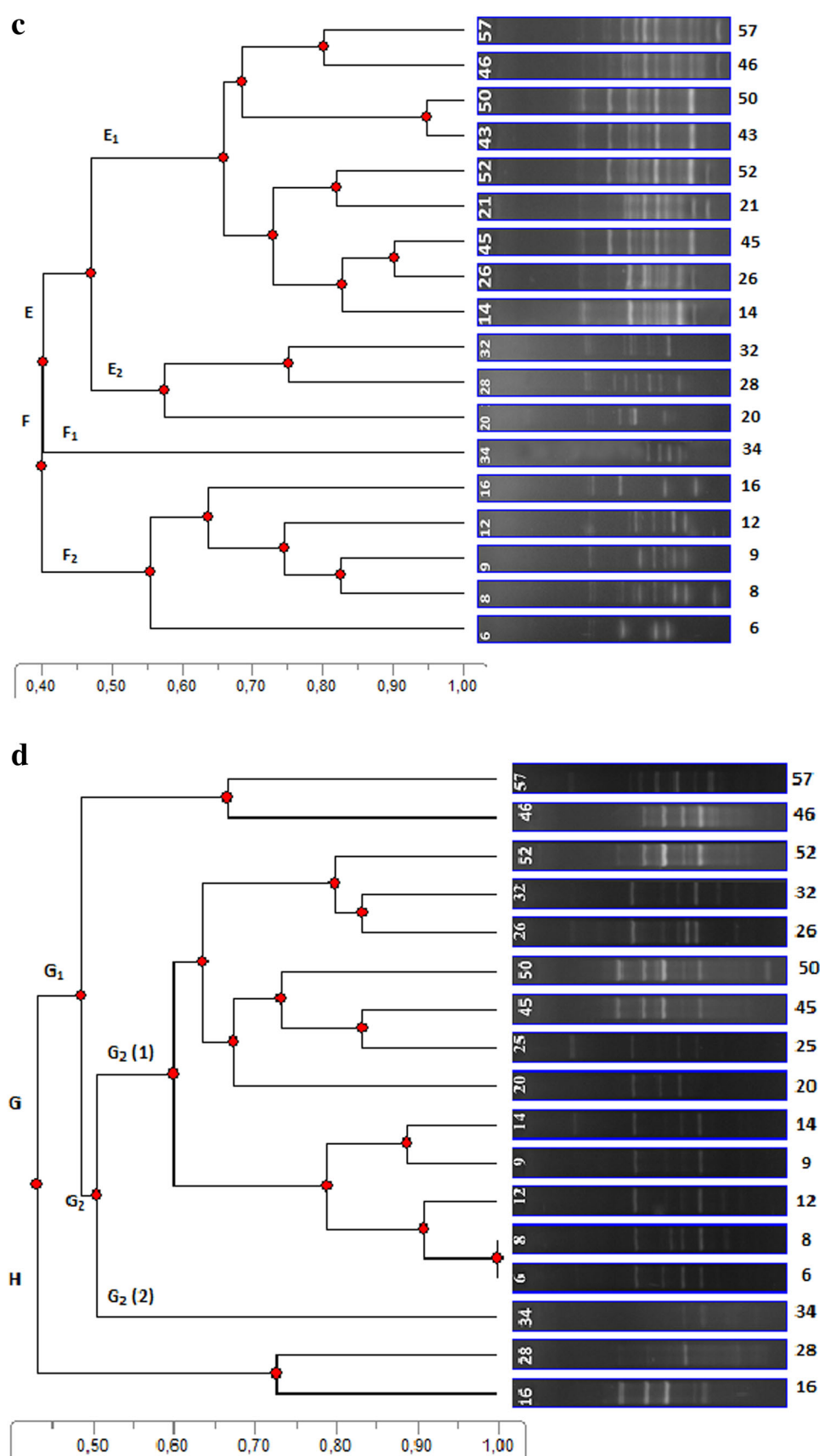
Fig. 3 Dendrograms based on RAPD-PCR analysis of M13-10 (a), M13-14 (b), OPL3 (c), OPL12 (d)



namely C₁ and C₂ at similarity levels of 71 % or above. Sub-cluster C₁ had only one strain group numbered as 57 while sub-cluster C₂ formed the major group of cluster C with 12

isolates. Cluster D had only two strains numbered as 16 and 43. A total of 18 strains generated DNA fragments with primer OPL3. The molecular weights of the DNA bands

Fig. 3 continued



produced with OPL3 primer ranged from 3.400 to 300 bp for the isolates (Fig. 3c). In the dendrogram in Fig. 3c, the isolates were scattered into two major clusters (E and F) at similarity levels between 42 and 96 %. The cluster E consists of two sub-clusters, namely E₁ and E₂ at similarity levels of 47 % or above. The cluster F also consists of two sub-clusters, namely F₁ and F₂, while sub-cluster F₂ was the major group of cluster F with 5 members, sub-cluster F₁ had only one strain numbered as 34. A total of 17 strains generated DNA fragments with primer OPL12. The molecular weight of the DNA bands produced with OPL12 primer ranged from 16.000 to 300 bp for the isolates (Fig. 3d). In the dendrogram in Fig. 3d, the isolates were scattered into two major clusters (G and H) at similarity levels between 43 and 100 %. Cluster H had two strains numbered as 16 and 28. Cluster G was first divided into two sub-clusters (G₁ and G₂) at similarity level of 49 % or above. Sub-cluster G₂ was further divided into two sub-clusters [G₂(1) and G₂(2)] at similarity level of 51 % or above. Sub-cluster G₂(2) had only one strain numbered as 34 while sub-cluster G₂(1) was the major group of the cluster with 12 members.

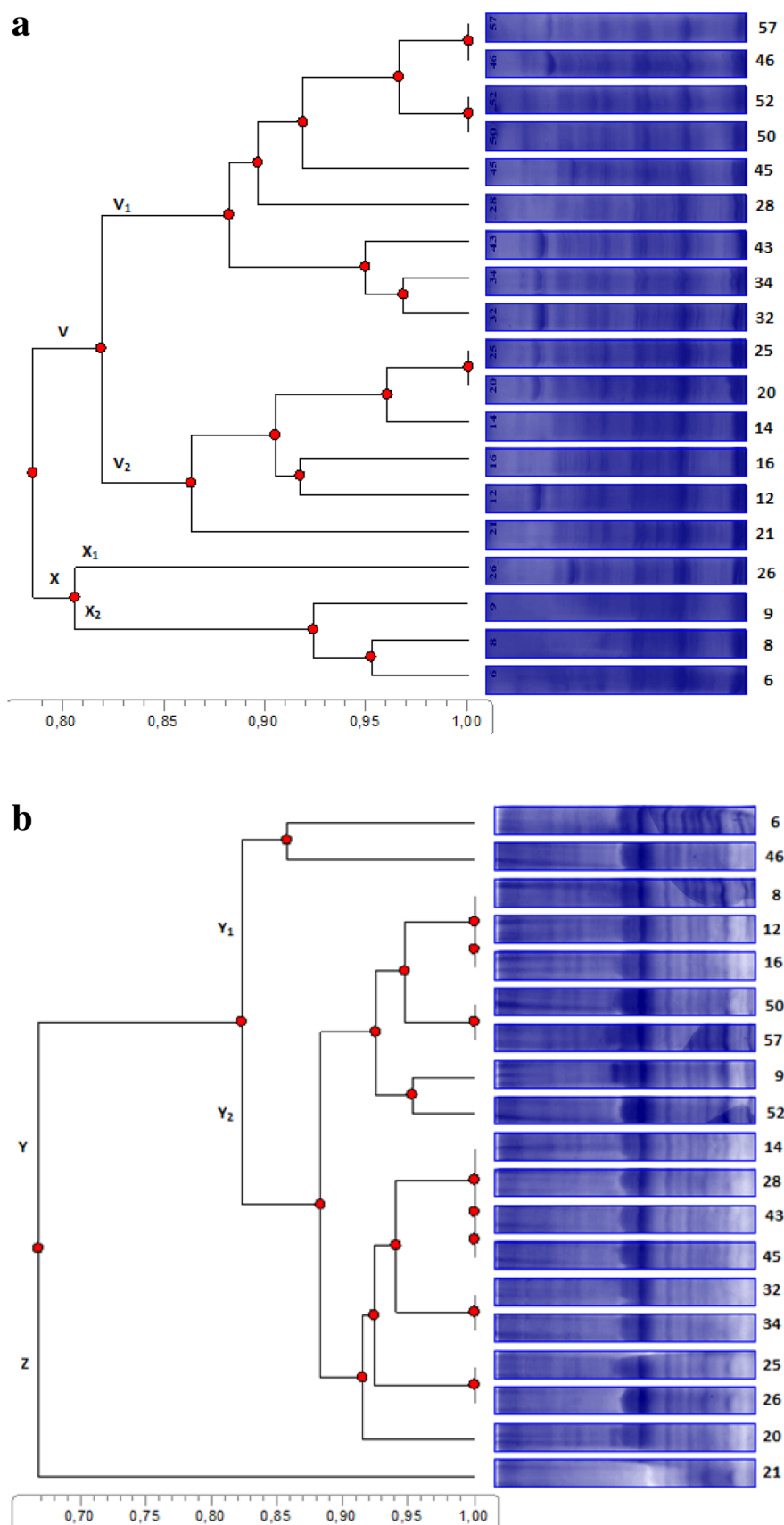
Gupta et al. [32] described RAPD-PCR as the best means for molecular typing of *Bacillus*. They also drew attention to the fact that *Bacillus* sp. isolated from different types of soils were phylogenetically identical probably due to the stability of *Bacillus* in different environments attributed to the endospore. On the other hand this issue needs to be further clarified since the authors used two generic primers for 16S rRNA amplification of *Bacillus*. The authors demonstrated 100 % diversity for the soil isolates with all of the four primers tested. In the light of this comparison, the presence of protective endospore seems regardless of affecting the molecular identity and the primers of choice and the target region seems to be more determinative for analyzing phylogenetic relationships among bacteria in different ecosystems. A recent study focusing on genetic variability of alkaline protease producing bacteria from Indian soils took advantage of RAPD and 16S rDNA analysis methods. Five of 20 isolates in total were identified as *Bacillus* spp. based on the similarity of 16S rRNA gene sequences and their RAPD amplification profiles displayed 100 % polymorphism in accordance with the present study [33]. The sum of these two studies is convincing in terms of reflecting RAPD assay as an advantageous molecular tool for differentiation of *Bacilli* though more supportive studies are desired. RAPD assay has also been successfully used for the identification of thermophilic bacilli in milk powders because of their commercial importance in dairy industry [5, 34, 35].

Interspecific genomic polymorphism among isolates was also assessed with SDS-PAGE profiles of whole cell

proteins (Fig. 4a) and extracellular proteins (Fig. 4b). Depending upon the different ambient conditions (salt, temperature, etc.) different proteins can be produced in different media. However, SDS-PAGE is a standardization-based method and thus quite efficient in the characterization of the microorganisms upon the standardization of the conditions. Whole cell protein bands and extracellular protein bands ranged from 171 to 30 kDa and 264 to 22 kDa, respectively. In the dendrogram in Fig. 4a, the isolates were separated into two major clusters (V and X) at similarity levels between 78 and 100 %. These major clusters were further separated into sub-clusters. Cluster V consists of two sub-clusters, namely V₁ and V₂ at similarity level of 82 % or above. The cluster X consists of two sub-clusters, namely X₁ and X₂ at similarity level of 91 % or above. The sub-cluster X₁ had only one member numbered as 26. In the dendrogram in Fig. 4b, the isolates were separated into two major clusters (Y and Z) at similarity levels between 67 and 100 %. Cluster Y was scattered into two sub-clusters, namely Y₁ and Y₂ at similarity level of 88 % or above. Cluster Z was a unique cluster with only one member numbered as 21.

One of the efficient methods for bacterial fingerprinting is the investigation of protein profiles. Profiles of whole-cell and extracellular proteins are beneficial methods to discriminate bacteria at species level [36]. On the other hand, protein profiling may not be the best tool in any case to differentiate bacteria and this may be dependent upon the species under investigation. For instance, the limits of SDS-PAGE technique for differentiation were pointed out for *Staphylococcus aureus* [11], *Salmonella* strains [37] and *Aeromonas* species [38]. Despite the high specificity at the species level, the observation of low specificity at the strain level has been proposed as a general concern. It has already been reported that both whole-cell and extracellular protein profiles seem insufficient to differentiate *S. aureus* and other *Staphylococcus* strains at either the species or the subspecies level [20]. So, whole-cell and extracellular protein profiles may offer adequate specificity for most bacterial genera at species level, but not at subspecies level. In coherence with the previous study regarding facultative alkaliphilic *Bacillus* species [39], *Bacillus* strains used in this study could not be adequately differentiated at subspecies level depending on neither the whole cell nor the extracellular protein profiles since they displayed similarity levels between 78–100 % and 67–100 % respectively. The members of both V and X clusters in whole-cell SDS-PAGE dendrogram were compared with the RAPD profiles acquired with four different primers. A great majority of the members in V and X clusters also fell into the same clusters in RAPD dendrogram reflecting the favorable correlation between the two methods used.

Fig. 4 Dendrograms based on SDS-PAGE analysis of whole cell proteins (**a**), extracellular proteins (**b**)



Conclusion

The study allowed the display of the advantages of RAPD-PCR technique in terms of detecting genetic heterogeneity and also enhanced the conception of assessing this technique either alone or in combination with SDS-PAGE protein profiles in terms of evaluating identification quality and noticing the determinative limits. One can say that SDS-PAGE method may provide discrimination at species level, however surveys at subspecies level demand molecular approaches such as RAPD. In terms of strengthening the benefits and handicaps of both techniques, more studies concentrated on the same topic in different habitats may help to increase the determinative effect.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interest.

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