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Bacterial Diversity and Biogeochemical Analysis of Sediments in Eastern Mediterranean Sea

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Abstract. Phylogenetic diversity of isolated bacteria and biogeochemical variability of sediments in different regions of Eastern Mediterranean Sea were studied. Totally 185 strains isolated from this one of the most oligotrophic environments in the world were found as affiliated to the *Firmicutes*, *Actinobacteria* and *Gammaproteobacteria*. As determined by 16S rRNA gene sequence analysis, the *Firmicutes* with *Bacillus* the most frequent genus were dominant in each sediment samples of Eastern Mediterranean Sea. In addition to the isolates representing new taxa, the influence of environmental parameters was observed as the variability of bacterial community composition such that phylogenetic diversity was much higher in shallower regions, especially in North Aegean Sea as compared to deep basins of Eastern Mediterranean Sea. The present study provided highly diverse bacterial strains especially with probability of representing new taxa and also demonstrated the species-area relationships of isolated bacteria by giving biogeochemical patterns of Eastern Mediterranean Sea sediments.

Keywords: Phylogenetic diversity, 16S rRNA genes, sediments, Eastern Mediterranean Sea.

1. Introduction

Bacteria associated to particles as well as free-livings are key-players for both benthic and pelagic ecosystems in terms of their phylogenetic diversity and roles in biogeochemical cycles. On the other hand, environmental parameters and geographical differences play roles on the bacterial community composition in marine sediments.

Cultivation-independent methods are mostly used to study bacterial diversity in deep sediments due to the largest part of the microbial community formed by non-cultivable ones [1], [2]. In recent studies based on the comparative analysis of 16S rRNA clone libraries, it was reported that the dominance in deep sediments was constitute the *Gammaproteobacteria* in Northeastern Pacific Ocean [3] and eastern South Atlantic Ocean [4], *Acidobacteria* in Eastern Mediterranean Sea (EMS) [3], [5].

Cultivation-based studies, on the other hand, are generally preferred for further studies such as production of secondary metabolites and determination of physiological characteristics needed in especially identification of new species [6], [7]. According to 16S rRNA gene sequences, the *Gammaproteobacteria* with *Halomonas* the most frequent genus and then *Firmicutes* were mainly identified from deep sediments of eastern South Atlantic Ocean [8] whereas mainly *Firmicutes* with *Bacillus* the most frequent genus and then *Actinobacteria* from deep sediments of the EMS [9].

Since there is a limited number of studies on the relationship between environmental parameters together with geographical differences and the bacterial community composition based on bacterial biomass, nucleic acid concentration, cultivation-independent studies in the sediments of EMS which is one of the most oligotrophic regions in the world [3], [5], [10]-[12], much more research including the phylogenetic diversity are needed to understand the biogeochemical variability in EMS. Therefore, the purpose of the present study

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is to isolate and phyogenetically analyze bacteria from sediments of EMS with cultivation-based methods and also to analyze biogeochemical parameters of EMS with regional variability.

2. Material and Methods

2.1. Sediment Sampling and Analysis

The Eastern Mediterranean Sea is one of the most oligotrophic regions in the world [13], [14], in addition to the increase of oligotrophy with longitudinal gradient in Mediterranean Sea. One of EMS basins is the Aegean Sea (AS) separated by the Cyclades plateau into the North Aegean (NA) and the South Aegean (SA) which display different hydrographic and trophic characteristics due to the influence of Black Sea (BS) and Levantine Sea (LS), respectively. While relatively shallow NA receives the nutrient-rich surface waters from the BS through Marmara Sea, SA is nutrient depleted.

In the present study, sediment samples were taken from stations A, B and C in EMS for both bacterial isolation and sediment analysis (Fig. 1). The sampling was performed from stations A (5 stations in the inner, 2 in the middle and 2 in the outer bays) at intertidal zone of Izmir Bay which is one of the largest bays and under stress of anthropogenic activities such as tourism-derived, industrial, agricultural and nautical activities in AS. On the other hand, the research vessel RV/K Piri Reis (Dokuz Eylul University, Turkey) was used for sampling at stations B (totally 5 stations) between Lesvos Island and Karaburun of NA and also at stations C1–C10 (Fig. 1). While the stations C1–C3 were located at the upper most NA, especially C1 near to the mouth of Dardanelle Strait carrying BS water passing through Marmara Sea to AS, the stations C6–C8 were at the lower most SA where influenced by LS waters. Then, the sediment samples were collected into sterile plastic bags, 40 ml glass containers and sterile plastic cores for different processes and kept at –20 ℃ till the analysis.

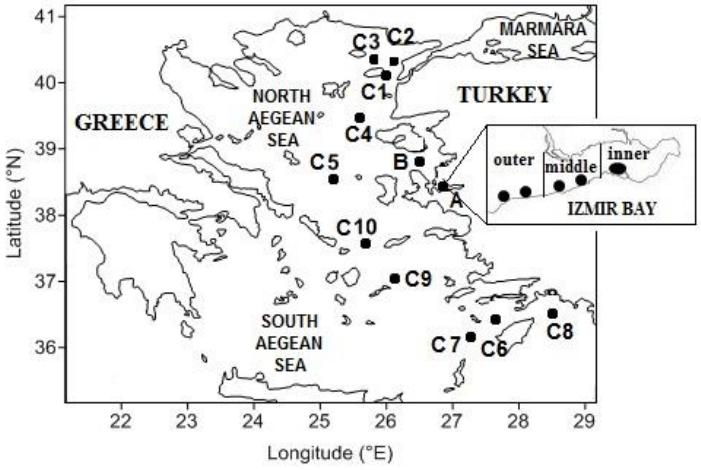

Fig. 1: Stations A (9 stations), B (5 stations) and C1–C10 in Eastern Mediterranean Sea.

The particle size of the sediments was determined by the sieve analysis and the hydrometer method for the larger and the finer particles, respectively according to standard test method for particle size analysis of soils D 422-63 issued by American Society for Testing and Materials [15].

Total and organic carbon and nitrogen contents (TC and TOC, TN and TON, respectively) were obtained using Carlo Erba NC2500 model CHN analyzer, on the other hand, total and organic phosphorus contents (TP and TOP, respectively) were measured spectrophotometrically [16].

2.2. Bacterial Isolation

Isolation of bacteria was achieved using seven different sediment processing methods and seven isolation media prepared with sterile seawater. The isolation media consisted of the following: M1, 18 g agar, 10 g starch, 4 g yeast extract, 2 g peptone, 1 liter sterile seawater; M2, 18 g agar, 1 g starch, 0.4 g yeast extract, 0.2 g peptone, 1 liter sterile seawater; M3, 18 g agar, 2.5 g starch, 1 g yeast extract, 0.5 g peptone, 750 ml sterile seawater, 250 ml distilled water; M4, 18 g agar, 1 liter sterile seawater; M5, 18 g agar, 750 ml sterile seawater, 250 ml distilled water; M6 (Difco^{TM} marine agar), 55 gr medium, 1 liter distilled water; M7

(Difco™ actinomycete isolation agar, modified), 22 g medium, 5 ml glycerol, 500 ml sterile sea water and 500 ml distilled water. The isolation media M1 and M7 were used with or without six different antibiotics as cycloheximide (100 µg/ml), nystatin (50 µg/ml), polymixin B sulfate (5 µg/ml), rifampin (5 µg/ml), kanamycin sulfate (5 µg/ml), novobiocin (25 µg/ml).

Seven different sediment processing methods were performed. In the first processing method (a) [17], 10 ml wet sediment sample were dried overnight and then 0.5 g dry sediment was aseptically spread in circular fashion onto the agar media. In the dry spot method (b) dry sediment was taken with sterile sponge and put clockwise on the agar media. In the third method (c) [18], 1 ml wet sediment was diluted with sterile seawater (1:4) and then heated for 6 min at 55 ℃. After vortexing for 30 s, 75–100 µl was spread aseptically onto agar-based isolation media. In the fourth method (d) [19], wet sediment was heated for 15 min at 70 ℃ and then spread aseptically on the agar surface in a circular fashion. In the fifth method (e) [20], wet sediment was kept for 30 sec under UV and then spread aseptically in a circular fashion onto the agar media. In the sixth method (f) 1 ml wet sediment sample was diluted with sterile seawater (1:1, 1:10 and 1:100) and then vortexed for 30 s. Then, 75–100 µl was spread aseptically onto agar-based isolation media. In the seventh method (g) without processing, wet sediment sample was aseptically spread onto agar-based isolation media.

The plates containing M6 medium were incubated at 20–22 ℃ for 2-3 days and the rest at 26–28 ℃ upto 2 months. The colonies selected according to colony morphology were subcultured on M1, M6 or M7 media. Then, the isolates were cryopreserved with 50% glycerol at –20 ℃.

2.3. Nuclear DNA Extraction and 16S rRNA Amplification

Genomic DNA of isolated bacteria was extracted with a commercial kit (Invitrogen, Carlsbad, CA) according to the user's manual for both Gram-negative and Gram-positive bacterial cell lysate.

The 16S rRNA genes were amplified from genomic DNA by PCR using the universal primer pairs of FC27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and RC1492 (5′-TACGGCTACCTTGTTACGACTT-3′) and also the pairs of 63f (5′-CAGGCCTAACACATGCAAGTC-3′) and 1387r (5′-GGGCGGWGTGTACAAGGC-3′). The 50 µl PCR mixture contained 20 to 50 ng of DNA, One Taq Quik-Load 2X Master mix (New England Biolabs, Inc. Beverly, MA), 10 pmol of each primer (Fermentas, Thermo Fisher Scientific, Waltham, MA), and 10 mM deoxynucleoside triphosphate mixture (Fermentas, Thermo Fisher Scientific, Waltham, MA). The PCR program consisted of 30 cycles of 94 ℃ for 1 min, 55 ℃ for 1 min, and 72 ℃ for 1 min followed by a final extension step at 72 ℃ for 7 min. Amplification products were examined by agarose gel electrophoresis.

2.4. Sequencing and Phylogenetic Analysis

Sequencing service was taken from Gene Research and Technology (RefGen, Turkey). For the phylogeny, all nucleotide sequences were analyzed using Geneious (version 6.1; Biomatters Ltd., NZ) and compared within the NCBI database (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST). Phylogenetic analysis was performed with 1000 bootstrap neighbor-joining method [21].

All those partial 16S rRNA gene sequences have been deposited into GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under the accession numbers KC815705–KC815847 and KF366670–KF366711.

2.5. Statistical Analysis

Hierarchical cluster analysis was performed using PRIMER (version 5; Plymouth Marine Laboratory, UK) to compare bacterial community composition among the environmental samples.

3. Results

3.1. Sediment Parameters

According to sediment analysis results, hierarchical cluster analysis was indicated that the inner bay stations grouped together as Ai and the middle-outer bay stations as Aj for stations A in Izmir Bay with 91%

similarity whereas the shallower stations (100–152 m) as Bi and the deepest stations (198–202 m) as Bj for stations B in Aegean Sea with 95% similarity.

Grain size analysis showed that the largest particles were at stations A as expected, and the finest particles were at stations C. Sediment types of stations C were generally clayey silt at depths changing from 416 to 1235 m, except the stations C1 and C4 which were sand at 72 m and silty sand at 207 m depth, respectively (Table I). On the other hand, stations A located at intertidal zone of Izmir Bay were sand, gravelly sand or sandy gravel and stations B with approximately 100, 150 and 200 m depths were sand or sand-silt-clay.

In contrast to total and organic phosphorus content, carbon and nitrogen contents of sediments showed variation among stations A, B and C. For TC and TOC, the stations C1 and C2 had the lowest values whereas the station C3 had the highest values (Table I). High TC percentages were also obtained from stations Ai (10.24–20.94), in contrast to low values of TC and TOC in stations Aj (2.97–7.78 and 0.12–0.90, respectively) and Bj (5.2–5.9 and 1.0–3.4, respectively). On the other hand, the lowest TN percent was seen in station C2 and the highest in station C1 which in fact generally had the lowest values for the rest of the contents (Table I). Low TN values were also taken from stations Ai (0.11–0.22%) and B (0.02–0.25%) unlike stations Aj (0.29–0.69%). TON percent ranges were 0.01–0.09, BDL–0.18 and BDL–0.21 for stations A, B and C, respectively. Moreover, TP percentages were the highest in stations Ai (0.032–0.046) and Bi (0.037–0.053) than C (0.011–0.035) whereas Bj (0.030–0.038) approximately doubled Aj (0.018–0.022). On the other hand, TOP percents were ranging 0.004–0.022 for stations C, 0.006–0.017 and 0.007–0.010 for Ai and Aj, respectively and 0.001–0.015 and 0.010–0.017 for Bi and Bj, respectively.

Table I: The sediment parameters for stations A, B and C in Eastern Mediterranean Sea. Stations A and B were grouped as Ai, Aj and Bi, Bj given with the averaged parameters and ranged depths.

Stations	Depth (m)	TC (%)	TOC (%)	TN (%)	TON (%)	TP (%)	TOP (%)
C1	72	2.9	0.6	0.94	BDL	0.011	0.004
C2	522	5.0	2.8	0.02	BDL	0.033	0.016
C3	899	22.7	11.6	0.68	BDL	0.035	0.022
C4	207	9.3	8.1	0.47	0.17	0.027	0.013
C5	708	12.6	0.6	0.59	BDL	0.032	0.016
C6	703	15.3	9.0	0.57	0.21	0.026	0.011
C7	1035	9.6	5.3	0.11	BDL	0.030	0.013
C8	1235	17.9	1.6	0.38	0.01	0.028	0.011
C9	416	14.5	5.9	0.27	0.09	0.030	0.018
C10	661	4.4	1.9	0.30	0.12	0.030	0.016
Ai	0	15.2	4.8	0.15	0.06	0.038	0.010
Aj	0	5.6	0.4	0.50	0.05	0.021	0.008
Bi	100-152	6.9	1.6	0.10	0.03	0.043	0.008
Bj	198-202	5.5	2.3	0.18	0.04	0.034	0.013

TC: total carbon; TOC: total organic carbon; TN: total nitrogen; TON: total organic nitrogen; TP: total phosphorus; TOP: total organic phosphorus; BDL: below detection limit.

3.2. Bacterial Phylogeny

Bacterial isolates were obtained from mostly M1 medium and then M6 and M7 media but the rest of isolation media was not successful. Among seven sediment processing methods, the methods (a) and (f) which were overnight drying wet sediment and diluting wet sediment with sterile seawater were highly efficient. Additionally, 20% of isolates required NaCl to grow.

From stations A, B and C, totally 185 strains having different spatial distribution and morphological features including antimicrobial activity (data not shown) were phylogenetically analyzed. Three classes as

Bacilli, *Actinobacteria* and *Gammaproteobacteria* were obtained and within those classes, there were 14 families as *Alteromonadaceae*, *Bacillaceae*, *Exiguobacteriaceae*, *Halomonadaceae*, *Moraxellaceae*, *Nocardiopsaceae*, *Planococcaceae*, *Pseudoalteromonadaceae*, *Pseudomonadaceae*, *Pseudonocardiaceae*, *Staphylococcaceae*, *Streptomycetaceae*, *Sporolactobacillaceae* and *Vibrionaceae*. When the phylogenetic tree was constructed using nearly full 16S rRNA gene sequence of representative isolate for each nearest type strain, it was seen that the tree clearly supported those three classes forming separate clades (Fig. 2 and 3).

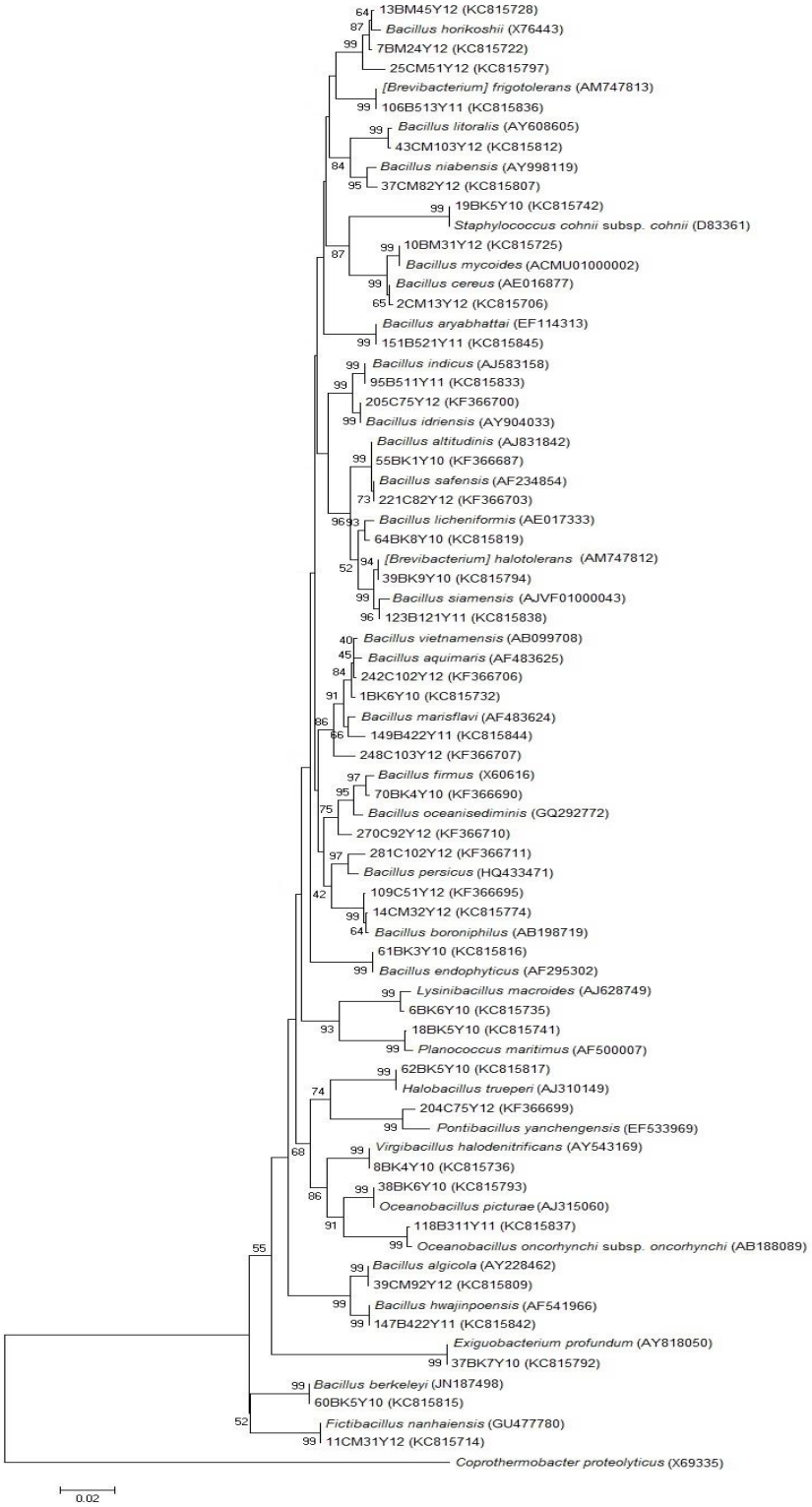

Fig. 2: For the order *Bacillales*, neighbor-joining distance tree constructed using nearly full 16S rRNA gene sequences. GenBank accession numbers were given in parentheses. Bootstrap values calculated from 1000 re-samplings were in percentage. *Coprothermobacter proteolyticus* was used to position the root.

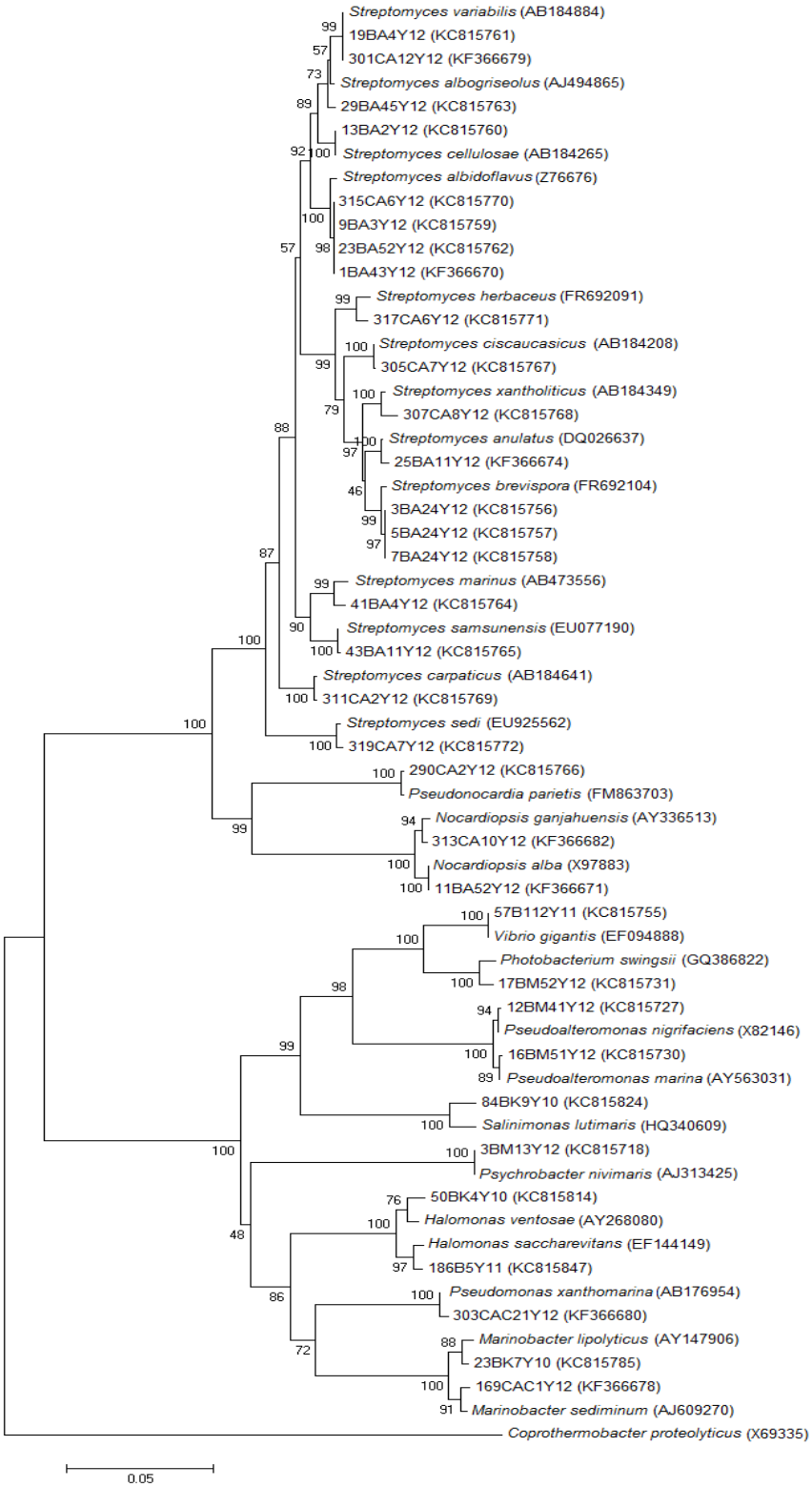

Fig. 3: For the classes *Actinobacteria* and *Gammaproteobacteria*, neighbor-joining distance tree constructed using nearly full 16S rRNA gene sequences. GenBank accession numbers were given in parentheses. Bootstrap values calculated from 1000 re-samplings were in percentage. *Coprothermobacter proteolyticus* was used to position the root.

The majority of the strains formed a highly diverse clade with members belonging to the order *Bacillales* of the phylum *Firmicutes* (Fig. 2). They shared a high phylogenetic affiliation with *Bacillus* species. While approximately half of the *Bacillus* strains were closely related to *Bacillus aryabhattai*, *[Brevibacterium] frigoritolerans*, *Bacillus subtilis* group and *Bacillus hwajinpoensis* (16%, 14%, 9% and 7%, respectively), the quarter were to *[Brevibacterium] halotolerans*, *Bacillus algicola*, *Bacillus aquimaris*, *Bacillus cereus* group and *Bacillus vietnamensis* (5% for each). Moreover, the other genera within this order *Bacillales* were

Exiguobacterium, *Fictibacillus*, *Halobacillus*, *Lysinibacillus*, *Oceanobacillus*, *Planococcus*, *Pontibacillus*, *Staphylococcus* and *Virgibacillus*.

Other two clades had 5 different orders as *Actinomycetales*, *Alteromonadales*, *Oceanospirillales*, *Pseudomonadales* and *Vibrionales* (Fig. 3). Within the order *Actinomycetales* forming a seperate clade, the majority of the strains belonged to the genus *Streptomyces* and there were also the genera *Nocardiopsis* and *Pseudonocardia*. On the other hand, the second clade included 8 different genera as *Halomonas*, *Marinobacter*, *Psychrobacter*, *Pseudoalteromonas*, *Pseudomonas*, *Photobacterium*, *Salinimonas* and *Vibrio* falling within 6 separate family level groupings.

The *Actinobacteria* and *Gammaproteobacteria* classes and the genera (belonging to the order *Bacillales*) other than the genus *Bacillus* were mainly isolated from stations A and B, additionally few from stations C (near to coastal area; C1–C3, C6–C8). The isolation and diversity of the *Gammaproteobacteria* were higher in Bi and *Actinobacteria* in Bj stations. Furthermore, the *Bacillus* strains, except *[Brevibacterium] frigoritolerans* and *Bacillus algicola* were mainly isolated from stations B. On the other hand, from stations A, the strains of *[Brevibacterium] halotolerans*, *Bacillus aryabhattai*, *Bacillus subtilis* group and *Bacillus algicola* were obtained. While the strains of *[Brevibacterium] frigoritolerans* and *Bacillus hwajinpoensis* were isolated from stations C, *Bacillus algicola* and *Bacillus cereus* group were from shallow stations C as C1, C2, C4 and C9.

Approximately half of the halophile *Bacillus* strains (12% of total *Bacillus* strains) were obtained from stations C and the rest from stations A and B. In addition to *Halomonas*, *Photobacterium*, *Planococcus*, *Pontibacillus* and *Pseudoalteromonas* species, all strains closely related to *Bacillus algicola*, *Bacillus hwajinpoensis* and *Bacillus boroniphilus* require NaCl to grow.

The *Halomonas* strain 50BK4Y10 and the *Salinimonas* strain 84BK9Y10 isolated from the stations A (Fig. 3) and the *Pontibacillus* strain 204C75Y12 from station C7 (Fig. 2) have a high probability of representing new taxa due to pairwise similarity < 98% with their nearest type strains. When a limit of 98.5 to 99% was considered, as revised and proposed by [22], the number of new taxa increases such as the *Bacillus*, *Halomonas*, *Photobacterium* and *Streptomyces* strains (10CM31Y12, 149B422Y11, 17BM52Y12, 17CM41Y12, 186B5Y11, 235C101Y12, 248C103Y12, 270C92Y12, 317CA6Y12, 37CM82Y12, 41BA4Y12, 43CM103Y12 and 8BM24Y12) isolated from stations B, C3, C4, C6, C8–C10.

3.3. Biogeochemical Comparison

Latitudinal separation in bacterial diversity was obviously seen in hierarchical cluster analysis based on similarity matrices for bacterial community comparison at the genus level (Fig. 4). Although mainly the phylum *Firmicutes* with *Bacillus* the most frequent genus was obtained from geographically close stations C1–C3 in the NA, cluster analysis revealed their phylogenetic differences supporting their geochemical variations as given in Table I. Similarly, stations A and B in NA showed those differences in the dendogram. Compared to stations C, they had the highest phylogenetic diversity and the separation even within those two stations was seen in the dendogram. On the other hand, like the stations C4, C5, C9 (nearer to C10), the stations C6–C8 in SA formed a closer group including the families *Bacillaceae* and *Streptomycetaceae* in the dendogram underlying their geographical closeness. Moreover, the similar latitudinal separation in phylogenetic diversity was also obtained in the hierarchical cluster analysis for the higher taxa.

4. Discussion

The geochemical composition of the study area was generally in the range of Aegean Sea. In addition to the decrease in grain sizes with depth, the chemical contents of the sediments reflected the regional variability. For stations A in Izmir Bay, geochemical separation of the inner bay (Ai) highly influenced by industrial and domestic sewage discharge and river input was observed. Similarly, for stations B closer to the coastal area as Lesvos Island, Izmir and Candarli Bays in NA, the shallower Bi stations distinguished from Bj stations. On the other hand, the stations C1–C3 located in the upper most NA having complex bottom topography and highly dynamic hydrographic conditions influenced by AS and nutrient-rich BS and also the stations C6–C8 influenced by AS and oxygen-depleted and oligotrophic LS also had geochemical variations. Furthermore, the stations C3 in NA and C6–C8 in SA had higher carbon contents than the previous results as

0.33–15.63% and 1.30–13.10% for TC, 0.22–6.76% and 0.42–0.99% for TOC in NA and SA, respectively [5], [12], [23], [24], [25], [26], [27]. However, for the nitrogen contents, the station C2 had lower TN values than the previous 0.02–0.07% in NA [24] and the station C6 had higher TON than the previous 0.04–0.10% in SA [5].

Like the frequent isolation of the *Firmicutes* and *Actinobacteria* from marine sediments [28], [29], [30], [31], the present study indicated that Gram-positive bacteria including mainly the *Firmicutes* with *Bacillus* the most frequent genus and then *Actinobacteria* were clearly dominant among isolates from sediments of the EMS. Although they accounted minor part in 16S rRNA gene libraries of Mediterranean sediments [32], especially for EMS sediments as 3% *Firmicutes* and 4–28% *Actinobacteria* of total sequence [5], they formed the majority in culture-dependent studies of EMS sediments [9] together with *Gammaproteobacteria* as in diverse marine sediments [8], [33], [34], [35]. Even if the *Gammaproteobacteria* became dominant under hydrostatic pressure incubation of EMS sediments [9], few isolation of this phylum mostly from shallow sediments of the present study supported the frequent 16S rRNA gene clones of the phylum compared to the deeper sediments of EMS [12]. Moreover, much diverse genera belonging to phyla *Firmicutes* and *Gammaproteobacteria* were obtained especially from shallower stations A and B of the present study rather than the phylum *Actinobacteria* from previous study of EMS sediments [9].

In this study, it was underlined that environmental parameters together with geographical differences influenced the bacterial community composition in sediments of EMS [3], [5], [10], [11], [12]. For instance, in a previous study, cluster analysis of bacterial compositions supported the environmental and geographical differences and especially gave the north-south latitudinal separation for bacterial diversity in sediments of Eastern Mediterranean Sea [12]. Furthermore, in another study, when 16S rRNA clone compositions for North East Pacific and Eastern Mediterranean sediments were compared, hierarchical cluster analysis again provided the geographical separation [3]. Similarly, in the present study, the geochemical differences in addition to the variations of the geomorphologic, hydrographic and trophic states reflected on the phylogenetic diversity as seen in hierarchical cluster analysis (Fig. 4). The shallowest sediments belonging to stations A had the highest phylogenetic diversity in higher taxa due to the dynamic environmental factors as continuous terrestrial and anthropogenic effect i.e. river and sewage input increasing the eutrophication. Even higher influence on the inner bay (stations Ai) was observed as biogeochemical separation from the middle and outer bays (stations Aj). In contrast, deep and oligotrophic stations C9, C10 in NA and C6, C7 in SA had higher diversity in lower taxa due to high hydrostatic pressure, depletion of oxygen and nutrients so that bacterial community composition shifted according to hard environmental conditions. However, compared to those stations, diversity increased at the family level for especially station C1 in NA due to lower depth and higher nutrient input from BS. On the other hand, stations B showed the highest bacterial diversity in both lower and higher taxa indicating the optimum environmental conditions. In addition, the difference in bacterial community composition was seen even between the deepest stations (Bj) and shallower ones (Bi).

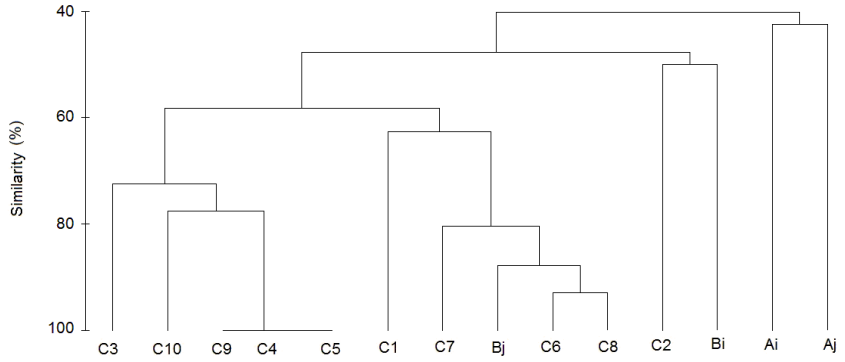

Fig. 4: Cluster analysis dendogram based on the comparison of bacterial community at the genus level from different sediment samples.

The results of the present study demonstrated the species-area relationships of isolated bacteria by giving biogeochemical patterns of EMS sediments. The importance of the cultivation-based techniques in

phylogenetic diversity was underlined as obtaining frequent isolates of the *Firmicutes* with high diversity in the genus *Bacillus* in contrast to culture-independent methods. For further studies i.e. identification of new species and production of secondary metabolites, the present study provided morphologically different bacterial strains especially with probability of representing new taxa forming 9% of total isolates.

5. Acknowledgements

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