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Seasonal Variation of Bacterial Community in the Seawater of Gwangyang Bay Estimated by Amplified Ribosomal DNA Restriction Analysis

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To determine the seasonal variation of bacterial community in the seawater of Gwangyang Bay, three hundred thirty six bacterial strains were isolated on February, May, July and October 2011. Amplified Ribosomal DNA Restriction Analysis (ARDRA) was used to construct the phylotyes of the isolates using the restriction endonuclease, *Hae* III. Diversity indices of ARDRA patterns were calculated. One hundred and one phylotypes including 40 unique pylotypes were found at the 80% similarity level. Partial 16S rRNA genes of one hundred thirty nine strains representing each phylotypes were sequenced and compared. Bacterial community composed of 4 different phyla which include *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes*. *Proteobacteria* was the prevailing phylum in all seasons, followed by *Bacteroidetes* in winter, spring and autumn while *Actinobacteria* in summer. At the family level, *Flavobacteriaceae* dominated in winter and spring and *Pseudoalteromonadaceae* did in summer and autumn. Genera *Altererythrobacter*, *Loktanella*, *Pseudoalteromonas* and *Vibrio* were encountered in all seasons. The most diverse bacterial community was found in autumn followed by the order of spring, winter and summer.

Key words : Bacterial community, Amplified Ribosomal DNA Restriction Analysis (ARDRA), diversity, *Proteobacteria, Flavobacteriaceae*

Introduction

Microbial diversity can be seen in terms of variations in cell size and shape, motility, metabolic strategies, developmental biology, cell division, adaptation to environmental extremes and many other structural and functional aspects of the cell. Diversity may also be considered to be the amount and distribution of information, which is directly applicable to total genetic diversity or complexity in a community [29]. Microbial diversity describes complexity and variability at different levels of biological organization. It encompasses genetic variability within taxa (species), and the number (richness) and relative abundance (evenness) of taxa and functional groups in communities [29]. Molecular biological techniques used to identify microbes in environmental samples have recently provided new tools to study biodiversity. It is always observed that studies of microbial ecology, diversity and evolution were intimately together.

Amplified ribosomal DNA restriction analysis (ARDRA) was originally used for screening clone libraries and for strain typing to identify phylogenetic clusters within a microbial community and to examine the capacity of restriction based techniques for clone identification and the possibility of deriving phylogenetic information from ARDRA based dendrogram. In addition, it is commonly used as a tool for studying microbial diversity relying on DNA polymorphism [5, 26, 28].

Gwangyang bay which has a surface area of 230 km^2, average depth of 10m and tidal range of 2.4-3.7 m, is a closed inner bay located on the southern coast of Korea (34° 51′16″~34° 56′55″ N, 127° 37′23″~127° 50′86″E). This bay normally receives wastewater from the residential sewers and industrial facilities and might receive substantial freshwater input during heavy rainfall [21]. At present, huge ships can be seen around the bay, carrying different materials whether harmful or not to the marine environment. Also, large factories built along the shores might contribute pollution causing the water to be contaminated.

In recent years, this area has been designated as a special

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management area for coastal pollution, receiving great attention from the public due to its increasing environmental problems. There have been several investigations into the functions of environmental factors, water quality, and planktons and benthos [1, 3, 20], whereas studies on microorganisms are few. Therefore, we investigated the seasonal variation of bacterial community of Gwangyang bay seawater. To achieve the goal a number of bacteria were isolated and the phylotypes representing whole isolates was determined by ARDRA. Sequence comparison study based on 16S rRNA gene of each phylotype was carried out to determine the taxonomic positions of the isolates.

Materials and Methods

Sampling

The water samples were collected from the three selected sites (34° 54′ 18.7" N, 127° 47′ 94.9" E; 34° 51′ 20.3" N, 128° 47′ 29.7" E; 34° 57′ 20.5" N, 127° 44′ 03.9" E) of Gwangyang bay, Republic of Korea, at four different seasons; winter (Feb. 23. 2011), spring (May 06), summer (July 21) and autumn (Oct. 22). Water sample was collected 1 m below the surface of the seawater using electric pump. After the collection, three samples were mixed and stored in at 4°C for further use.

Isolation of bacteria

Isolation of bacteria was achieved by the standard dilution plating technique using marine agar 2216 (MA, Becton Dickinson) at 25°C for 7 days. Purified isolates were routinely cultured on MA and maintained as a glycerol suspension (20%, w/v) at -80°C.

Amplified rDNA restriction analysis (ARDRA) on isolates

Bacterial DNA preparation and PCR amplification were carried out as described previously [4]. Universal primers such as 27F (*E. coli* numbering 8~27; 5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (*E. coli* numbering 1492~1510; 5′-GGY TAC CTT GTT ACG ACT T-3′) were used for the amplification of 16S rRNA gene [16]. PCR amplification of nearly full-length of 16S rRNA gene was performed in 50 µl reaction mixtures. PCR products were detected after the electrophoresis using 1% agarose gel.

Amplified 16S rRNA genes were digested with *Hae* III (GG↓CC; TaKaRa, Japan) as described previously [2]. The resulting fragments were electrophoresed on a 4% NuSieve 3:1 Agarose (Cambrex, USA) gel. The gels were stained with EtBr and photographed with UV transillumination. Similarities were estimated using Gelcompar II program (Applied Maths, Belgium).

16S rRNA gene sequencing and phylogenetic analysis

Amplified PCR products containing 16S rRNA gene representing each phylotype were selected and purified using AccuPrep, PCR purification Kit (Bioneer, Korea) to remove salts, polymerase and excess primers and nucleotides following the manufacturer's instructions. Sequencing was performed with ABI PRISM ™ BigDye ™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) according to the manufacturer's instructions with the sequencing primer 27F [18]. The DNA sequences were edited using PHYDIT. Then, all sequences were analyzed for the presence of chimera by the CHIMERA CHECK program [http://decipher.cee.wisc.edu/; 30]. Partial 16S rRNA gene sequences were identified using EzTaxon-e database [http://eztaxon-e.ezbiocloud.net/; 14].

The 16S rRNA gene sequences were aligned with the PHYDIT program, version 3.1. An evolutionary tree was generated by the neighbor-joining method [27] and evolutionary distance matrices were generated according to Jukes and Cantor distance model [13].

Diversity indices

The diversity of isolates was calculated using diversity indices. Shannon-Weaver index (H; diversity characterization in a community), Species richness (d; number of species in a community), Evenness (e; the distribution of levels of abundance among the species), Simpson's dominance (c) and coverage (C) were calculated as previously described [10, 17, 25].

Results

Bacterial isolates

A total of 336 bacterial strains with different colony characteristics were isolated on MA after incubation at 25°C for 3-7 days. Seventy seven strains were obtained in winter, 87, 70 and 102 were done in spring, summer and autumn, respectively.

Table 1. Number of isolates and diversity indices based on the ARDRA of 16S rRNA genes of the isolates from Gwangyang bay seawater

Parameter	Winter	Spring	Summer	Autumn
Total strain (*N*)	77	87	70	102
No. of unique pattern (*n*)	7	14	4	15
No. of ARDRA patterns (*S*)	22	27	15	37
% Coverage (*C*)[a]	90.9	83.9	94.3	85.3
ARDRA pattern richness (*d*)[b]	4.8	5.8	3.3	7.8
Shannon-Weaver Diversity (*H*)[c]	2.8	2.890	2.383	3.273
Evenness (*e*)[d]	0.906	0.877	0.880	0.906
Equitability (*J*)[e]	0.820	0.699	0.841	0.772
Simpson's Dominance (*c*)[f]	0.073	0.075	0.115	0.052

[a] Coverage (C)=[1-(n/N)]×100, where n is the number of unique ARDRA patterns and N is the total number of isolates analyzed. [b] Calculated as d=(S-1)/(log$N$), where S is the total number of ARDRA patterns. [c] Shannon-Weaver diversity (H)=-$\sum$(pi)(log$_e$$pi$), where pi is the proportion for each ARDRA pattern. [d] Calculated from H as follows: e=H/ln$S$, where S is the total number of ARDRA patterns. [e] Equitability (J)=H/H_{max}, where H_{max} is the maximum Shannon-Weaver diversity index value in the community. [f] Calculated as c=$\sum$(pi)^2, where pi is the proportion for each ARDRA patterns.

Bacterial community diversity indices

The diversity of bacterial isolates collected from different seasons represented by different ARDRA patterns was evaluated and calculated using diversity indices such as Shannon-Weaver diversity (*H*), Richness (*d*), Evenness (*e*), Equitability (*J*) and Simpson's Dominance (*c*) (Table 1). Coverage (*C*) value is used to determine how well the sampling captured the total diversity. Coverage values calculated as 80 - 90% showed that the total diversity in bacterial community of the sampling sites was almost detected. The Shannon-Weaver diversity values obtained from winter, spring, summer and autumn bacterial isolates were as follows, respectively: 2.800, 2.890, 2.383 and 3.273. Simpson's Dominance values were 0.073, 0.075, 0.115 and 0.052 for winter, spring, summer and autumn bacterial isolates. Results from all the diversity indices revealed that autumn obtained the greatest diversity of bacterial community among four seasons followed by spring, winter and summer. In contrary, according to the data obtained in this study, summer season showed the least bacterial diversity among the seasons.

Bacterial community discovered by ARDRA with isolates

Using the universal primers 27F and 1492R, the 1.5 kb DNA fragments which corresponded to nearly complete 16S rRNA gene was amplified from all the isolates. One hundred and one phylotypes including 40 unique pylotypes were found at the 80% similarity level (Fig. 1). Twenty-two phylotypes (including 7 unique phylotypes), 27 (including 14 unique phylotypes), 15 (including 4 unique phylotypes), and 37 (including 15 unique phylotypes) were obtained in winter, spring, summer and autumn, respectively (Table 1). One or more representatives from each phylotype were selected for sequencing (Fig. 1). 16S rRNA genes of 35, 45, 19 and 40 isolates obtained in spring, summer and autumn, respectively, were sequenced.

Bacterial isolates were identified at the species level based on the partial sequencing analysis of 16S rRNA gene. After comparison of 16S rRNA gene sequences, bacterial composition was divided into 4 different phyla which include *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes*. The relative abundance of bacterial strains at the phylum level was as follows in the order of winter, spring, summer, autumn and winter: 42 strains (54.6%), 52 (59.8%), 59 (84.3%) and 60 (58.8%) for phylum *Proteobacteria*; 4 (5.2%), 1 (1.1%), 7 (10%) and 15 (14.7%) for *Actinobacteria*; 28 (36.4%), 31 (35.6%), 3 (4.3%) and 20 (19.6%) for *Bacteroidetes*; and 3 (3.9%), 3 (3.4%), 1 (1.4%) and 7 (6.9%) for *Firmicutes* (Table 2). Based on these results, *Proteobacteria* was the most prevailing phylum in all seasons followed by *Bacteroidetes* with an exception in summer because *Actinobacteria* was dominating next to *Proteobacteria*. Among the members of the phylum *Proteobacteria* class *Alphaproteobacteria* dominated in winter (31 isolates, 40.3%), spring (36, 41.4%) and summer (32, 45.7%) however *Gammaproteobacteria* did in autumn (45, 44.1%).

The isolated were affiliated with 24 families including one unclassified family. Eighteen families were detected in autumn while 13 were done in winter and each 10 were done in spring and summer. In the family level, in winter

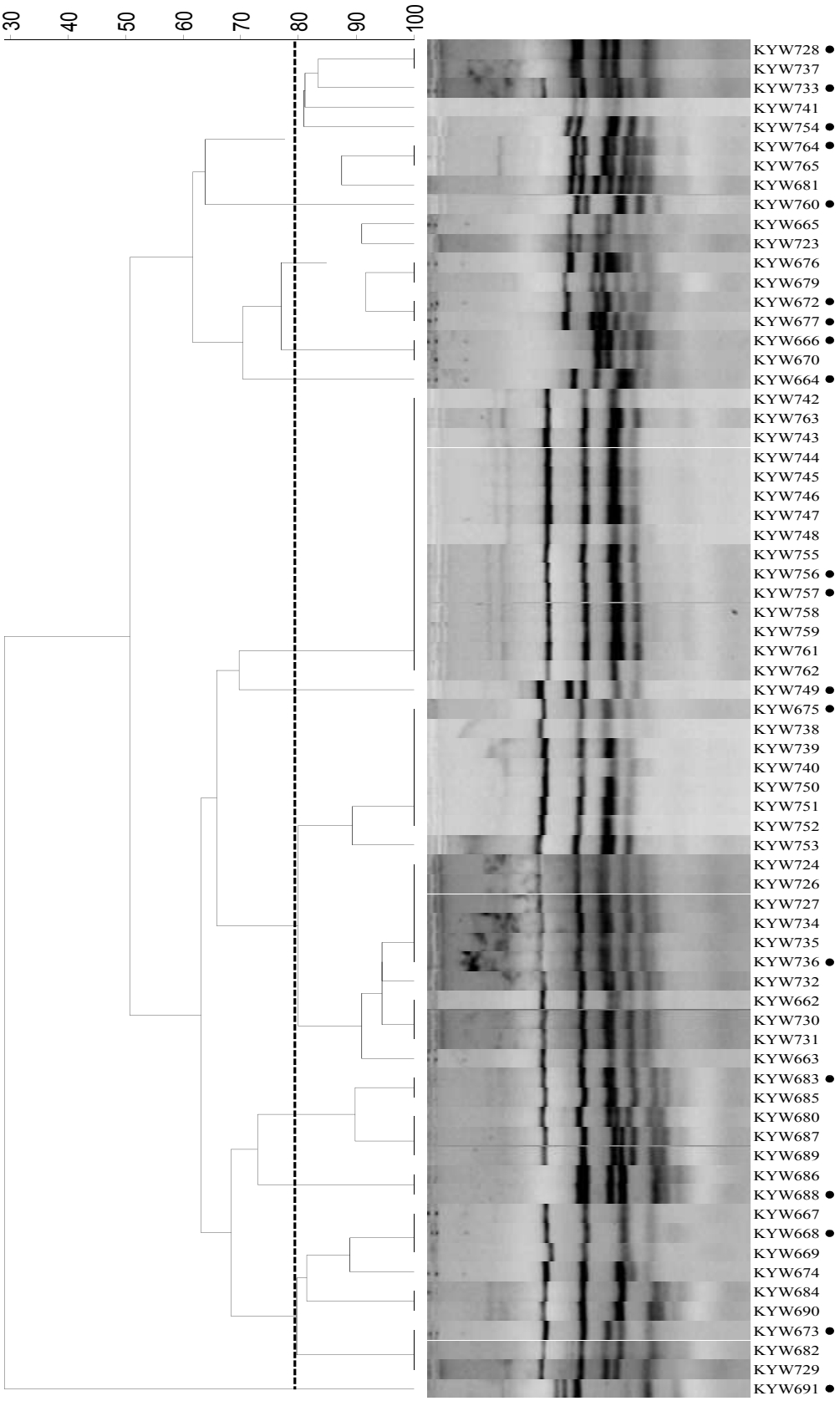

Fig. 1. Dendrogram constructed based on ARDRA pattern of bacterial isolates in autumn. Dots show the position of sequenced strains.

Table 2. Relative abundance of cultured bacteria isolated from the Gwangyang bay seawater

Phylum (Class)	Number of isolates (%)			
	Winter	Spring	Summer	Autumn
Proteobacteria	42 (54.6)	52 (59.8)	59 (84.3)	60 (58.8)
(*Alphaproteobacteria*)	31 (40.3)	36 (41.4)	32 (45.7)	15 (14.7)
(*Gammaproteobacteria*)	11 (14.3)	16 (18.4)	27 (38.6)	45 (44.1)
Actinobacteria	4 (5.2)	1 (1.1)	7 (10)	15 (14.7)
Bacteroidetes	28 (36.4)	31 (35.6)	3 (4.3)	20 (19.6)
Firmicutes	3 (3.9)	3 (3.4)	1 (1.4)	7 (6.9)
Total	77	87	70	102

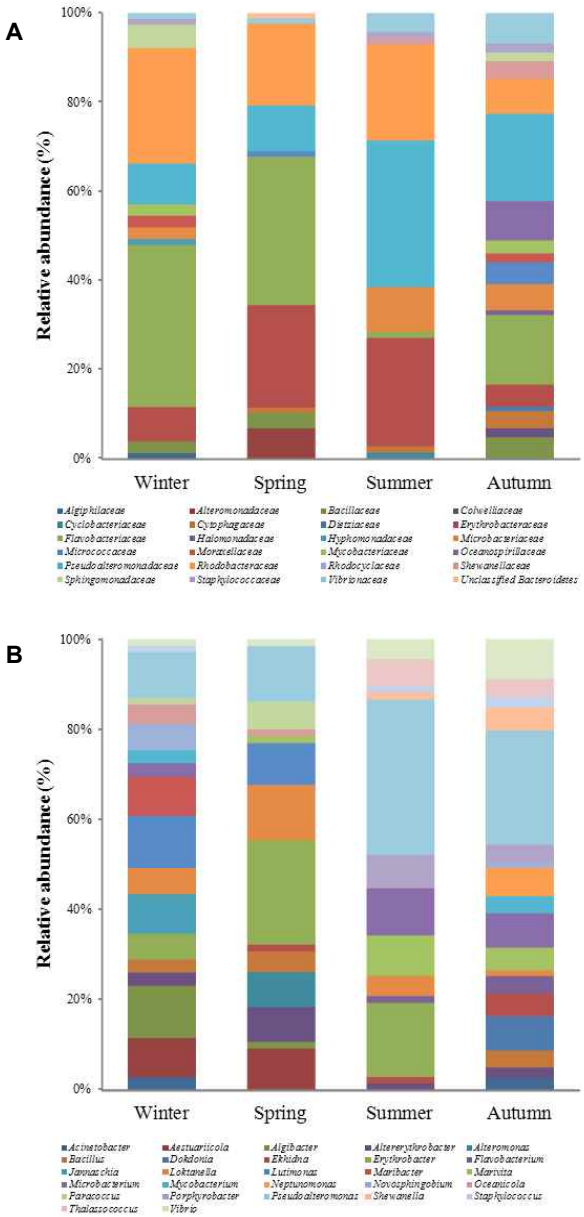

Fig. 2. Seasonal variation of bacterial community of Gwangyang bay seawater at the family (A) and genus (B) levels.

and spring bacterial community was dominated by *Flavobacteriaceae* followed by *Rhodobacteraceae* in winter while *Erythrobacteraceae* in spring. Meanwhile, summer and autumn revealed that *Pseudoalteromonadaceae* was the prevailing family followed by *Erythrobacteraceae* and *Rhodobacteraceae* in summer while *Flavobacteriaceae* in autumn (Fig. 2A).

The isolated were affiliated with 55 genera. Thirty, 24, 26 and 15 genera were detected in autumn, winter, spring and summer, respectively. Genera *Altererythrobacter*, *Loktanella*, *Pseudoalteromonas* and *Vibrio* were encountered in all seasons however 7 genera were found exclusively in winter, 9, 2, and 10 in spring, summer and autumn, respectively (Fig. 2B).

Phylogenetic position of isolates

Phylogenetic positions of 139 strains representing each phylotype retrieved from 336 isolates were analyzed. Phylogenetic analyses revealed that 24 genera with 26 different species, 26 genera with 31 species, 15 genera with 17 species and 30 genera with 35 species were obtained from winter, spring, summer and autumn, respectively (Fig. 3).

Novel strains

Based on the partial 16S rRNA gene sequence comparisons 20 isolates were found to be candidates for novel strains (Table 3). Twelve strains belonged to 9 genera in the phylum *Bacteroidetes* and 8 strains did to 6 genera in the phylum *Proteobacteria*.

Discussion

This study provides basic information about the diversity of bacterial community of Gwangyang bay sea water. Seasonal variation of cultivable bacterial community of

Table 3. List of novel strains isolated from Gwangyang bay seawater

Strain No.	Nearest Type Strain	Accession No.	Similarity (%)
Bacteroidetes			
KYW499	*Flaviramulus basaltis* H35^T	DQ361033	95.7
KYW576	*Tenacibaculum skagerrakense* D30^T	AF469612	96.6
KYW583	*Algibacter mikhailovii* LMG 23988^T	AM491809	95.7
KYW585	*Marinifilum fragile* JC2469^T	FJ394546	95.1
KYW589	*Algibacter mikhailovii* LMG 23988^T	AM491810	96.6
KYW614	*Olleya marilimosa* CAM030^T	JN175350	96.0
KYW630	*Winogradskyella damuponensis* F081-2^T	HQ336488	96.4
KYW635	*Ekhidna lutea* Alain BiosLi/39^T	AM746475	95.8
KYW643	*Algibacter mikhailovii* LMG 23988^T	AM491809	96.5
KYW652	*Polaribacter glomeratus* ATCC 43844^T	M58775	95.1
KYW884	*Flavobacterium ponti* GSW-R14^T	GQ370387	95.8
KYW612	*Polaribacter glomeratus* ATCC 43844^T	M58775	95.0
Proteobacteria			
KYW514	*Jannaschia donghaensis* DSW-17^T	EF202612	95.8
KYW537	*Algiphilus aromaticivorans* DG1253^T	DQ486493	93.1
KYW660	*Tropicibacter naphthalenivorans* C02^T	AB302370	95.7
KYW688	*Donghicola eburneus* SW-277^T	DQ667965	93.0
KYW728	*Donghicola eburneus* SW-277^T	DQ667965	95.4
KYW869	*Donghicola eburneus* SW-277^T	DQ667965	95.4
KYW894	*Sphingomicrobium lutaoense* CC-TBT-3^T	EU564841	95.4
KYW919	*Oleiphilus messinensis* ME102^T	AJ295154	91.9

Gwangyang bay sea water was examined by using the ARDRA method and 16S rRNA gene fragment sequencing. This study revealed that Gram-negative microorganisms were the prevailing bacteria in the Gwangyang bay sea water as was like the previous report [17]. Phylum *Proteobacteria* and class *Alphaproteobacteria* were most frequently encountered in all seasons, thereby concluding to be the most dominant among the isolates. This observation was also obtained from the earlier study on the estimation of bacterial diversity of seawater in Gwangyang bay by phylogenetic analysis conducted by Kim [15] except the predominance of *Firmicutes* in spring. A research on 16S rRNA-based bacterial diversity in the organic-rich sediments underlying oxygen deficient waters of eastern Arabian Sea also revealed that *Proteobacteria* was the most prevalent phylum represented by both cultivable and non-culturable classes [6]. The predominant presence of *Proteobacteria* was also observed in all sea water samples collected from four different locations or habitat along the coast of the Red Sea in Saudi Arabia [19] and Yellow Sea, Korea [23]. The phylogeny of prokaryotes states that phylum *Proteobacteria* is of biological significance as it is the most dominant and diverse group of the microbial assemblage [9]. It has been reported that occurrence of classes of *Proteobacteria* in sediments was dominant in certain regions of water [6, 16, 31]. Class *Alphaproteobacteria* are frequently encountered in the marine habitats especially in the pelagic zone [11] and *Gammaproteobacteria* has been reported among culturable and non-culturable bacteria of pelagic waters [7, 22]. In addition to the dominance of *Proteobacteria*, *Bacteroidetes* was the second predominant phylum in winter, spring and autumn, while *Actinobacteria* was in summer.

ARDRA with cultivated bacterial strains revealed only a small portion of the bacterial community, which 4 phyla were detected in this study. In addition, the members of phyla *Planctomycetes*, *Acidobacteria*, *Cyanobacteria*, *Verrucomicrobia*, which were detected previously in the pyrosequencing analysis in this area [15], were not discovered. This difference was caused by the reasons as follows: 1) most of the bacteria were uncultivable under single culture medium and incubation conditions, 2) slow-growing bacteria could be rejected in the selection process [8, 12]. Therefore, to overcome these weaknesses, it is needed to use the variety of culture conditions such as medium and incubation and apply the different methods such as cultivation-independent method, pyrosequencing. Despite the limitations of the culture-dependent analysis, 20 isolates were found to be a candidate for novel strains.

Bacterial community diversity of Gwangyang bay sea-

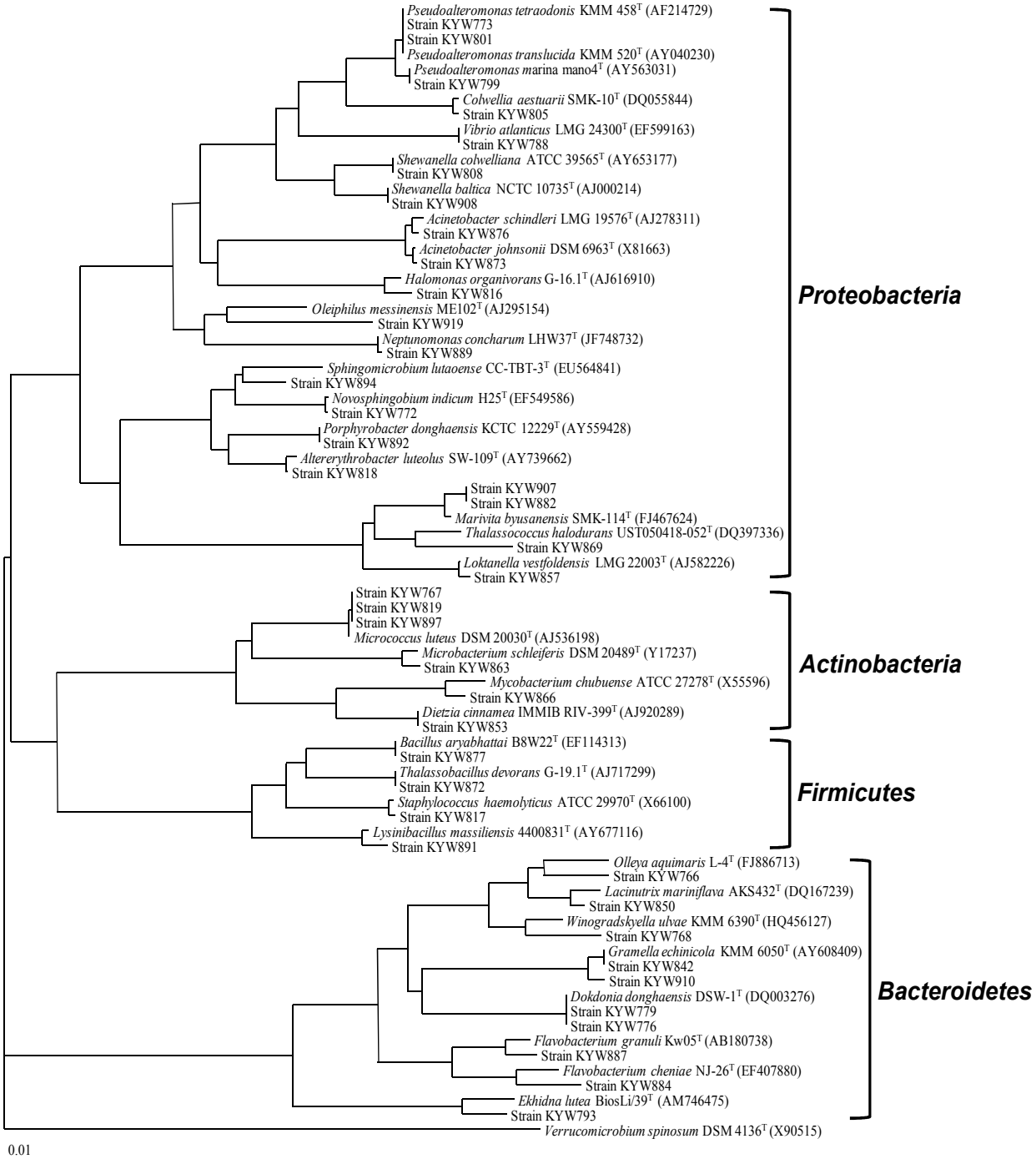

Fig. 3. Neighbor-joining tree based on the 16S rRNA gene sequence of the isolates from Gwangyang bay seawater in autumn. The 16S rRNA gene sequence of *Verrucomicrobium spinosum* DSM 4136^T (X90515) was included as a outgroup. Scale bar indicates 0.01 nucleotide substitution per nucleotide position. T, type strain.

water was high in autumn while low in summer. The diverse of bacterial community in autumn was also reported in the previous study [24]. And large population size of heterotrophic bacteria discovered in this season presumed to affect the diversity (Data not shown). This seasonal variation of diversity based on the ARDRA profile indices was accordance with that retrieved by the 16S rRNA gene sequence comparison study.

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초록 : Amplified Ribosomal DNA Restriction Analysis를 이용한 광양만 해수의 세균 군집의 계절적 변화

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본 연구에서는 광양만 해수의 세균군집 다양성의 계절적인 변화를 분석하기 위해 2011년 2월, 5월, 7월, 10월의 4계절에 총 336 균주를 분리하였다. 분리된 미생물의 16S rRNA를 제한효소 *Hae* III를 이용하여 Amplified Ribosomal DNA Restriction Analysis 를 실시하여 절편 양상을 군집화 시키고, 다양성 지수를 계산하였다. 80%의 유사도 수준에서 40개의 단일 계통형을 포함한 총 101개의 계통형을 얻을 수 있었다. 각 계통형을 대표할 수 있는 139개 균주를 선택하여 16S rRNA 염기서열을 결정한 후 유전자서열을 비교한 결과, 이들 균주는 *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* 및 *Firmicutes*를 포함한 4개의 문에 속하였다. 모든 계절에 *Proteobacteria* 문이 최 우점하였고, 겨울과 봄, 가을에는 *Bacteroidetes* 문, 여름에는 *Actinobacteria* 문이 차 우점하였다. 과(family) 수준에서는 겨울과 봄에는 *Flavobacteriaceae*가 우점하였고, 여름과 가을에는 *Pseudoalteromonadaceae*가 우점하였다. 모든 계절에 *Altererythrobacter*, *Loktanella*, *Pseudoalteromonas*, *Vibrio* 속(genus)이 관찰되었다. 미생물 군집의 다양성은 가을에 가장 높았으며, 다음으로 봄, 겨울, 여름 순서였다.