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ORIGINAL PAPER

Effect of restriction endonucleases on assessment of biodiversity of cultivable polar marine planktonic bacteria by amplified ribosomal DNA restriction analysis

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Abstract To choose a suitable restriction endonuclease for quick assessment of bacterial diversity in polar environments by ARDRA, we investigated the effect of restriction enzymes on ARDRA patterns of cultivable marine planktonic bacteria isolated from polar region. Thirty-three isolates were analyzed by ARDRA using five enzymes (*Hinf*I, *Hae*III, *Alu*I, and the mix *Afa*I/*Msp*I), respectively, resulting in different groups, each group corresponding to a particular genotype. A comparison of the ARDRA patterns was carried out, and phylogenetic position of all thirty-three bacteria was obtained by 16S rDNA sequencing. Consistent with phylogenetic analysis, ARDRA pattern comparison revealed that *Alu*I, being sensitive and reliable enough to generate species-specific patterns, was a suitable restriction enzyme used for evaluating bacterial diversity, suggesting a combination of ARDRA with *Alu*I and 16S rDNA sequencing can provide a simple, fast and reliable means for bacterial identification and diversity assessment in polar environments.

Keywords Restriction endonuclease · Amplified ribosomal DNA restriction analysis (ARDRA) · Biodiversity · Polar · Marine bacteria

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Introduction

Arctic and Antarctic regions remain among the least-characterized environments on earth, and thus offer a unique research opportunity concerning microbial diversity, evolution and ecology. Despite severe environmental conditions, investigations of microbial communities in polar oceans have shown that these permanently cold regions have been successfully colonized by numerous microorganisms exhibiting cold-adaptation strategies (Helmke and Weyland 1995; Priddle et al. 1996; Ruger et al. 2000; Purdy et al. 2003). As the dominant biomass component of polar marine ecosystems, microbial communities control many processes within the marine environment, including primary production, turnover of biogenic elements, degradation of organic matter and mineralization of xenobiotics and pollutants (Michaud et al. 2004). They are also essential components of polar pelagic marine food webs (Kirchman et al. 1993; Nichols 2003).

The development of molecular biology techniques, which are now widely applied to estimate microbial community composition and diversity in complex habitats, allows microbiologists to reveal the inhabitants of natural microbial communities. Compared with biochemical and physiological approaches that require cultivation of bacterial strains, molecular approaches are more sensitive and reliable and they are applicable to the uncultivable strains that represent the dominant portion of natural bacterial communities (Vandamme et al. 1996; Michaud et al. 2004). An approach using a combination of biochemical, physiological and molecular biological methods is needed to obtain objective information about the community composition, and also to evaluate its ecological and physiological function (Vandamme et al. 1996).

Of the various molecular biology techniques used to characterize microbial community structures in diverse environments such as marine ecosystems, the most useful is the determination of the sequences of 16S rRNA genes (Ward et al. 1990; Acinas et al. 1997; Cottrell and Kirchman 2000). These 16S rRNA molecules contain both highly conserved and variable regions (Woese 1987). However, the cloning and sequencing of all 16S rRNA gene fragments obtained in a given environments is time-consuming (Gich et al. 2000). Hybridization and probing are faster methods for determining which microorganisms are present, but require a sufficient knowledge of the community to choose the appropriate target sequences (Amann 1995; Gich et al. 2000). Even faster than hybridization and probing, requiring no sequence information about the amplified 16S rDNA fragments, the amplified ribosomal DNA restriction analysis (ARDRA) is a simple and fast method to determine bacterial diversity in different environments (Martínez-Murcia et al. 1995; Moyer et al. 1996; Vandamme et al. 1996; Kita-Tsukamoto et al. 2006). However, ARDRA gives little or no information about the type of microorganisms present in the sample (Gich et al. 2000). Therefore, an approach using a combination of ARDRA, cloning and sequencing of 16S rRNA gene fragments can be used for a quick assessment of genotypes in a given community and quick comparison of communities subjected to different environmental conditions.

ARDRA is a method based on restriction endonuclease digestion of the amplified bacterial 16S rDNA, and the type of restriction endonuclease chosen for digestion can inevitably affect the ARDRA patterns of the genotype and the objectivity and precision of the resulting estimate of microbial diversity in nature. The aim of this study was to investigate the effect of restriction endonucleases on ARDRA patterns of cultivable polar marine bacterioplankton to identify the most suitable restriction enzymes to be used for quick assessment of bacterial diversity in different polar environments by ARDRA. According to the strategy adopted (1) the 16S rDNA of Arctic and Antarctic marine isolates were amplified by polymerase chain reaction (PCR); (2) 17 isolates belonging to different genera based on 16S rDNA sequence were grouped into clusters by the analysis of ARDRA patterns using restriction endonucleases *Hinf*I, *Hae*III, *Alu*I, and the mix *Afa*I/*Msp*I, respectively (Eilers et al. 2000; Michaud et al. 2004; Delfederico et al. 2006); (3) 16 isolates of unknown genetic relatedness from the Antarctic were grouped into clusters by the analysis of ARDRA patterns using restriction endonucleases described above; (4) the 16S rDNA sequences of 16 Antarctic isolates were determined; (5) a comparison of ARDRA patterns by different restriction endonucleases was carried out to choose the restriction enzyme that best reflected microbial diversity.

Materials and methods

Bacterial strains and media

Seawater samples for this study were collected from several locations. During the Chinese national Antarctic research

Table 1 Planktonic bacterial isolates whose 16S rDNA sequences were determined prior to ARDRA

Isolate (accession number) and source	Nearest phylogenetic neighbor (accession number)	16S rDNA similarity (%)	Phylum or subphylum
BSw20306(DQ064606), Chukchi Sea	*Colwellia aestuarii* strain SMK-10 (DQ055844)	98.3	Gammaproteobacteria
BSw20308(AY646431), Chukchi Sea	*Pseudoalteromonas citrea* CIP105339 (AF529062)	99.5	Gammaproteobacteria
BSw20353(AY646432), Chukchi Sea	*Pseudoalteromonas tetraodonis* KMM458 (AF214729)	99.7	Gammaproteobacteria
BSw20587(DQ492787), Canadian Basin	*Pseudoalteromonas nigrifaciens* isolateS3-28 (AY771728)	99.7	Gammaproteobacteria
BSw20350(DQ064611), Bering Sea	*Pseudomonas anguilliseptica* BI (AF439803)	99.4	Gammaproteobacteria
BSw20352(DQ064612), Bering Sea	*Psychrobacter nivimaris* 88/2-7 (AJ313425)	99.7	Gammaproteobacteria
BSw20370(DQ064615), Chukchi Sea	*Psychrobacter okhotsukuensis* MD17 (AB094794)	99.9	Gammaproteobacteria
BSw20461(DQ064620), Chukchi Sea	*Psychrobacter pulmonis* strain KOPRI24933 (EF101551)	99.9	Gammaproteobacteria
BSw10170N(EF428448), Prydz Bay	*Psychrobacter alimentarius* strain JG-102 (AY513646)	99.7	Gammaproteobacteria
BSw10170W(DQ064630), Prydz Bay	*Psychrobacter alimentarius* strain JG-102 (AY513646)	99.9	Gammaproteobacteria
BSw20353D(DQ064613), Chukchi Sea	*Pseudoalteromonas haloplanktis* str. TAC125 (CR954246)	99.8	Gammaproteobacteria
BSw10166(DQ064627), Prydz Bay	*Shewanella frigidimarina* strain BSi20544 (DQ537513)	99.9	Gammaproteobacteria
BSw10167(DQ064628), Prydz Bay	*Shewanella frigidimarina* strain BSi20534 (DQ537511)	99.8	Gammaproteobacteria
BSw10168(DQ064629), Prydz Bay	*Shewanella frigidimarina* strain BSi20534 (DQ537511)	99.8	Gammaproteobacteria
BSw10175(DQ064632), Prydz Bay	*Leifsonia rubra* isolate S3-2 (AY771719)	99.5	Actinobacteria
BSw10179(DQ064634), Prydz Bay	*Marinobacter lipolyticus* (AY771710)	99.3	Gammaproteobacteria
BSw10005(AY646429), Prydz Bay	*Marinomonas pontica* strain 46-16 (AY539835)	97.5	Gammaproteobacteria

expeditions in the 1997/1998 and 2001/2002 Antarctic summer, samples were collected from a water depth of 5 m in Prydz Bay (62°00′–69°21′S, 68°30′–78°00′E). Samples were also collected during Chinese Arctic research expeditions in July–September of 1999 in the Bering Sea (55°59′31″–60°15′18.7″N, 175°53′56″W–173°20′31.6″E), Chukchi Sea (67°14′57.1″–75°25′17.4″N, 159°13′31.3″–175°01′41.8″W), and also in July–September of 2003 in the Canadian Basin (77°30′59″N, 152°52′04″W). Strains were isolated shipboard on marine agar 2216 medium (Difco) and incubated at 4–6°C for 20 days. They are currently maintained both on marine agar slopes at 4–6°C and in frozen glycerol cultures at –80°C. All media used in this study was sterilized at 121°C for 20 min.

DNA extraction and PCR amplification of 16S rDNA

Bacterial cells were grown for 5 days at 4–6°C in marine 2216 medium (Difco) and centrifuged at 2,600*g* for 10 min in 2-ml aliquots. The supernatant was discarded and DNA from the bacterial pellet was extracted using bacterial genomic DNA kit (TaKaRa, Dalian, China) according to the manufacturer's instructions.

PCR amplification of 16S rDNA was performed with an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) using the forward primer 8f (5′-AGAG TTTGATCCTGGCTCAG–3′) and the reverse primer 1492r (5′-GGTTACCTTGTTACGACTT–3′), designed from the conserved bacterial sequences at the 5′ and 3′ ends of the 16S rDNA gene (positions 8f and 1510r, respectively, on *Escherichia coli* rRNA) which enables the amplification of nearly the entire-gene (Bosshard et al. 2000). In a final volume of 50 µl, the PCR reaction mixture contained: 1.0 µl of template DNA, 5.0 µl of 10 × PCR buffer (TaKaRa, Dalian, China), 50 µM dNTPs, 0.2 µM of each described primers and 1U *Taq* DNA polymerase (TaKaRa, Dalian, China). The PCR reaction started with pre-denaturation at 95°C for 4 min followed by 25 cycles

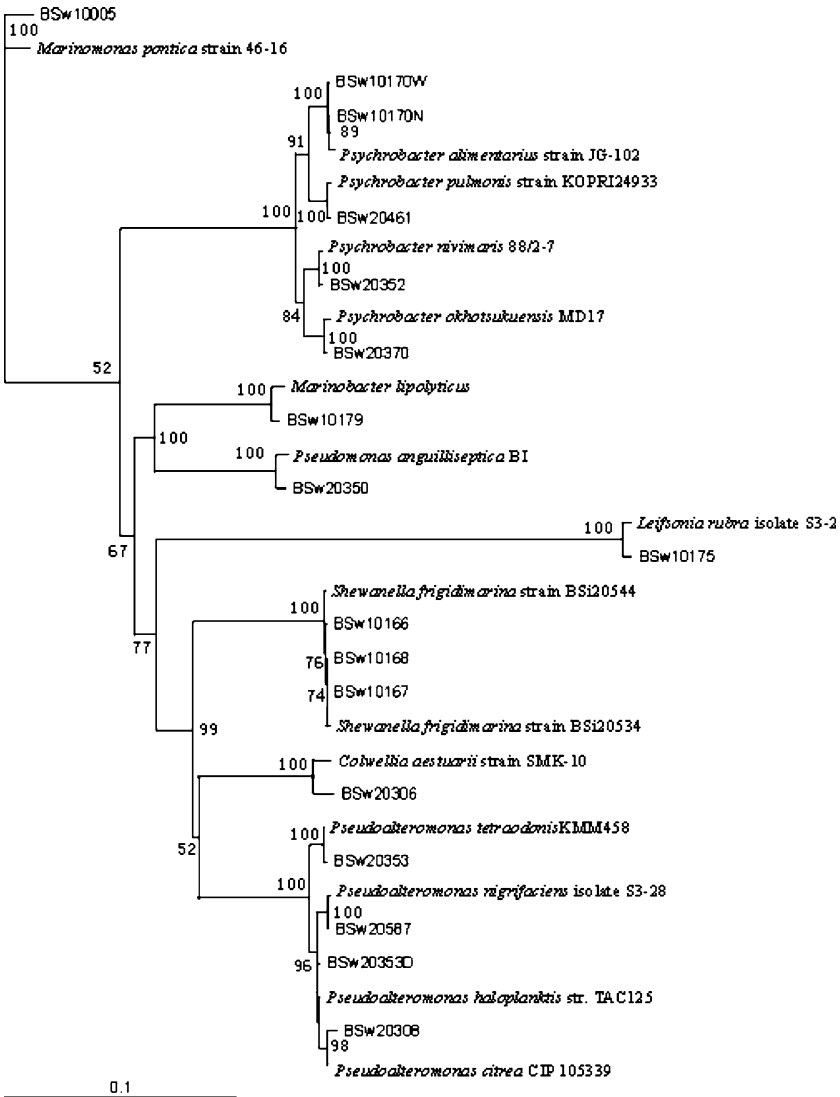

Fig. 1 Phylogenetic tree showing the relationships among the 16S rDNA sequences of 17 isolates belonging to different genera. The tree was constructed by using the neighbour-joining method for calculation. Bootstrap values based on 100 resamplings are shown at the branch points. Only bootstrap values greater than 50% are shown. *Bar* 0.1 substitutions per nucleotide position

of denaturation at 95°C for 45 s, annealing at 50°C for 45 s and extension at 72°C for 1.5 min, with a final extension at 72°C for 10 min.

Amplified 16S rDNA (4 µl) was analyzed by 0.8% (*w*/*v*) agarose (Oxoid, Hampshire, England) gel electrophoresis in TAE (Tris-Acetate-EDTA) buffer containing 1 µg ml^{-1} of ethidium bromide. Visualization was carried out on a Gel Doc XR documentation system (Bio-Rad, Hercules, CA, USA).

ARDRA

An 8-µl of aliquot of each PCR mixture containing amplified 16S rDNA was digested, respectively, with 10 U of the restriction endonuclease *Hinf*I, *Hae*III, *Alu*I, and the mix *Afa*I/*Msp*I (TaKaRa, Dalian, China) in a total volume of 20 µl at 37°C for 4 h. The restriction products were analyzed by agarose (2%, *w*/*v*) gel electrophoresis in TAE buffer containing 1 µg ml^{-1} of ethidium bromide. Gels were photographed under UV light.

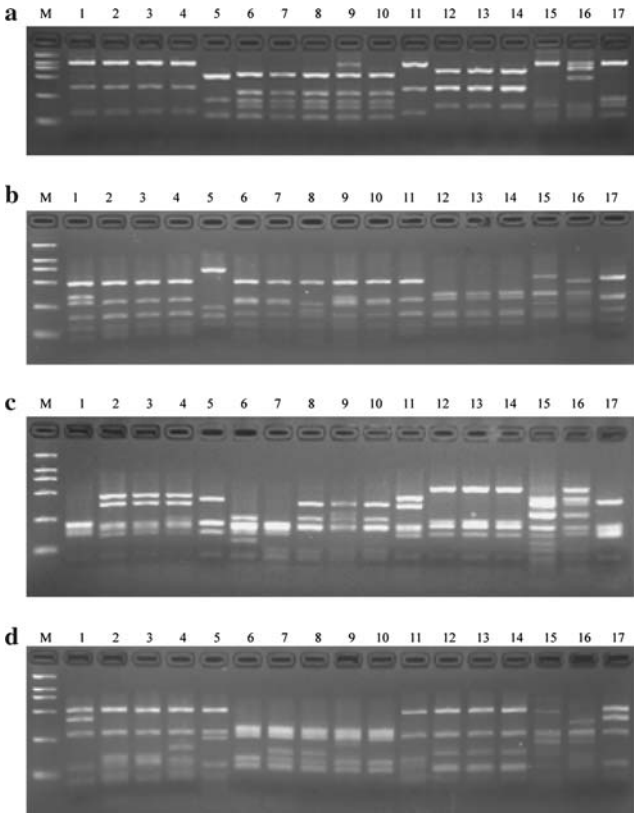

Fig. 2 ARDRA of 17 isolates from polar marine environments with **a** restriction endonuclease *Hinf*I, **b** restriction endonuclease *Hae*III, **c** restriction endonuclease *Alu*I and **d** double restriction endonucleases *Afa*I + *Msp*I. Lane *1* BSw20306, Lane *2* BSw20308, Lane *3* BSw20353, Lane *4* BSw20587, Lane *5* BSw20350, Lane *6* BSw20352, Lane *7* BSw20370, Lane *8* BSw20461, Lane *9* BSw10170N, Lane *10* BSw10170W, Lane *11* BSw20353D, Lane *12* BSw10166, Lane *13* BSw10167, Lane *14* BSw10168, Lane *15* BSw10175, Lane *16* BSw10179, Lane *17* BSw10005, Lane *M* DNA marker DL2000 (TaKaRa, Dalian, China)

Sequencing 16S rDNA

The amplified 16S rDNA was purified from agarose (0.8%, *w*/*v*) gel as follows. A small agarose slice containing the band of interest [observed under long-wave length (312 nm) UV light] was excised from the gel and purified using the gel extraction kit (TaKaRa, Dalian, China) according to the manufacturer's introductions. The PCR product was ligated into pMD18-T Vector (TaKaRa, Dalian, China) and transformed into competent *Escherichia coli* DH5α cells using standard protocols. The cloned 16S rDNAs were sequenced using M13 primers with an ABI Prism 3730 DNA analyzer (PE Applied Biosystems, Foster City, USA). The 16S rDNA sequences obtained were submitted to the GenBank where they were assigned the accession numbers.

Data analysis

The ARDRA patterns of each bacterial strain with different restriction endonuclease were compared, and 16S rDNA sequence analysis of isolates was carried out to examine their phylogenetic position. The 16S rDNA nucleotide sequence obtained was aligned with the most similar bacterial species in the GenBank by using BLAST search (http://www.ncbi.nlm.nih.gov). Sequences exhibiting close relatedness in a BLAST search were used for phylogenetic analysis. Multiple sequence alignment, calculation of nucleotide substitution rates (K_{nuc}) and construction of phylogenetic tree by the neighbour-joining method were performed by a combination of programs CLUSTAL X1.8 (Thompson et al. 1997) and PHYLIP v. 3.5c (Felsenstein 1993).

Results

ARDRA of bacterial isolates with 16S rDNA sequence determined

According to a previous study based on 16S rDNA sequencing (data not shown), seventeen isolates (Table 1) belonging to different genera, including the genera *Colwellia*, *Leifsonia*, *Marinobacter*, *Marinomonas*, *Pseudomonas*, *Pseudoalteromonas*, *Psychrobacter* and *Shewanella*, were chosen for ARDRA.

When the 16S rDNA region of each of the seventeen isolates was amplified by PCR, a major amplification band of about 1,500 bp was observed. The 16S rDNA sequences from the isolates were aligned to the most similar ones available in the GenBank Database and were used to construct the phylogenetic tree shown in Fig. 1.

Table 2 Planktonic bacterial isolates for ARDRA

Isolate (accession number) and source	Nearest phylogenetic neighbor (accession number)	16S rDNA similarity (%)	Phylum or subphylum
BSw10008(DQ789373), Prydz Bay	*Shewanella frigidimarina* ACAM 584 (U85902)	99.6	Gammaproteobacteria
BSw10009(EF191023), Prydz Bay	*Pseudoalteromonas elyakovii* strain BSi20429 (DQ537506)	99.6	Gammaproteobacteria
BSw10012(DQ789374), Prydz Bay	*Pseudoalteromonas haloplanktis* clone SE61 (AY771767)	98.7	Gammaproteobacteria
BSw10016(DQ789375), Prydz Bay	*Pseudoalteromonas haloplanktis* clone SE61 (AY771767)	98.8	Gammaproteobacteria
BSw10020(DQ789376), Prydz Bay	*Pseudoalteromonas haloplanktis* clone SE61 (AY771767)	98.7	Gammaproteobacteria
BSw10042(EF198113), Prydz Bay	*Idiomarina loihiensis* L2TR (AE017340)	99.8	Gammaproteobacteria
BSw10073(EF198114), Prydz Bay	*Idiomarina loihiensis* L2TR (AE017340)	99.5	Gammaproteobacteria
BSw10079(EF375561), Prydz Bay	*Pseudoalteromonas haloplanktis* str. TAC125 (CR954246)	99.6	Gammaproteobacteria
BSw10081(EF191024), Prydz Bay	*Idiomarina loihiensis* L2TR (AE017340)	99.8	Gammaproteobacteria
BSw10087(EF375562), Prydz Bay	*Pseudoalteromonas elyakovii* strain BSi20429 (DQ537506)	99.8	Gammaproteobacteria
BSw10088(EF375563), Prydz Bay	*Pseudoalteromonas elyakovii* strain BSi20539 (DQ537512)	99.7	Gammaproteobacteria
BSw10093(EF375564), Prydz Bay	*Pseudoalteromonas haloplanktis* str. TAC125 (CR954246)	99.6	Gammaproteobacteria
BSw10094(EF375565), Prydz Bay	*Pseudoalteromonas elyakovii* strain BSi20429 (DQ537506)	99.7	Gammaproteobacteria
BSw10097(EF375566), Prydz Bay	*Pseudoalteromonas haloplanktis* str. TAC125 (CR954246)	99.6	Gammaproteobacteria
BSw10098(EF191025), Prydz Bay	*Pseudoalteromonas haloplanktis* str. TAC125 (CR954246)	99.8	Gammaproteobacteria
BSw10100(EF375567), Prydz Bay	*Pseudoalteromonas haloplanktis* str. TAC125 (CR954246)	99.8	Gammaproteobacteria

ARDRA was carried out on the amplified 16S rDNA of each isolate with five-different restriction endonucleases. Digestion with *Hinf*I (Fig. 2a), *Hae*III (Fig. 2b), *Alu*I (Fig. 2c) and a combination of *Afa*I and *Msp*I (Fig. 2d) yielded 3–6, 4–6, 3–7 and 4–6 bands, respectively. On the basis of ARDRA with *Hinf*I, *Hae*III, *Alu*I and the mix *Afa*I/*Msp*I, all seventeen isolates were placed in eight, ten, twelve and eleven patterns, respectively (Fig. 2). Each pattern corresponded to a particular ARDRA genotype. *Hinf*I yielded the lowest number of genotypes, and *Hinf*I-generated ARDRA patterns did not distinguish *Colwellia* sp. strain BSw20306 from *Pseudoalteromonas* sp. strains including BSw20308, 20353, 20587 and 20353D (Fig. 2a). Compared with *Alu*I and a combination of *Afa*I and *Msp*I, *Hae*III did not distinguish *Pseudoalteromonas* sp. BSw20587 from other *Pseudoalteromonas* sp. strains BSw20308, 20353 and 20353D (Fig. 2b). Within the five restriction endonucleases, *Alu*I generated the largest number of different genotypes and most effectively detected differences within *Psychrobacter* strains at the species level. Polymorphism at the species level was thus reported. In contrast to four *Alu*I-generated ARDRA patterns within five *Psychrobacter* sp. strains, only two patterns within the same strains were detected by *Hinf*I, *Hae*III, and the double restriction endonuclease digestion of *Afa*I + *Msp*I.

Based on the criterion that the bacteria with 16S rDNA sequences that are 93–97% identical should be considered members of the same genus (Stackebrandt and Goebel 1994; Mullins et al. 1995), the phylogenetic analysis revealed that bacteria placed within the same genus, like *Psychrobacter* sp. strains BSw20352, 20370, 20461, 10170N and 10170W, each in close relation to a different species, could show different ARDRA patterns when *Alu*I was used (Fig. 2c). On the other hand, bacteria showing an identical ARDRA pattern usually joined the same genus, such as the genus *Pseudoalteromonas*, including strains BSw20308, 20353 and 20353D, and the genus *Shewanella*, including strains BSw10166, 10167 and 10168.

For bacterial strains showing a close phylogenetic relationship, different restriction endonucleases could generate different ARDRA groups, resulting in affecting the total number of different ARDRA patterns. For example, on the basis of ARDRA with *Hinf*I, *Hae*III, and *Alu*I, *Psychrobacter* sp. strains BSw10170N and 10170W, showing significant 16S rDNA sequence similarity of 99.8%, were placed in two patterns, respectively (Fig. 2a, b and c). However, on the basis of ARDRA with the mix *Afa*I/*Msp*I, these two strains showed an identical pattern (Fig. 2d).

ARDRA of bacterial isolates with undetermined 16S rDNA sequences

To test whether *Alu*I was the optimum restriction enzyme for ARDRA of polar marine planktonic bacteria whose genetic relatedness was unknown, sixteen Antarctic isolates (Table 2) and four reference strains, BSw10170W, 10175, 20308 and 20353 that belonged to different genera (Table 1), were chosen to amplify the 16S rDNA by PCR, then the PCR products were digested by five restriction endonucleases described above.

Digestion with *Hinf*I (Fig. 3a), *Hae*III (Fig. 3b), *Alu*I (Fig. 3c) and a combination of *Afa*I and *Msp*I (Fig. 3d) yielded 3–5, 4–5, 4–5 and 4 bands, respectively. On the basis of ARDRA with *Hinf*I, *Hae*III, *Alu*I and the mix *Afa*I/*Msp*I, all twenty isolates were placed in four, four, six and five patterns, respectively. Thus within the five restriction endonucleases, *Alu*I again generated the largest number of different ARDRA patterns.

Based on the identical ARDRA patterns with reference strains BSw20308 and 20353 (Fig. 3c), 11 bacterial isolates, including BSw10009, 10079, 10087, 20308, 20353, 10088, 10093, 10094, 10097, 10098 and 10100, were clustered in the genus *Pseudoalteromonas* (Table 1). According to *Alu*I-generated ARDRA patterns, isolates BSw10012, 10016 and 10020 were considered to represent a distinct genus, and isolates BSw10042, 10073 and 10081 were also grouped together.

Analysis of 16S rDNA of Antarctic bacterial isolates

To check the validity of analysis described above based on ARDRA patterns alone, bacterial 16S rDNA sequencing was performed on the 16 isolates. The 16S rDNA sequences obtained were aligned to the most similar ones available in GenBank Database (Table 2), and the alignments were used to construct the phylogenetic tree shown in Fig. 4. Sequence analysis of isolates showing an identical ARDRA pattern (Fig. 3c) confirmed that members of each ARDRA group were closely related to each other. As expected, consistent with ARDRA patterns, the phylogenetic analysis (Fig. 4) revealed that isolates BSw10042, 10073 and 10081, appearing closely related to strain *Idiomarina loihiensis* L2TR, were clustered in the genus *Idiomarina*. Isolates BSw10012, 10016 and 10020, closely related to strain *Pseudoalteromonas haloplanktis* clone SE61, joined a cluster of genus *Pseudoalteromonas*, which differed from another cluster of the same genus *Pseudoalteromonas*, including isolates BSw10009, 10079, 10087, 20308, 20353, 10088, 10093, 10094, 10097, 10098 and 10100. Results indicated that bacterial isolates with an identical *Alu*I-generated ARDRA pattern could consistently be placed in one genus, while sometimes isolates showing different ARDRA/*Alu*I patterns, such as *Psychrobacter* sp. strains BSw20352, 20370, 20461, 10170N and 10170W (Fig. 2c), also could be clustered in one genus.

Discussion

In this paper we report the effect of restriction endonucleases on assessment of biodiversity of marine planktonic bacteria isolated from polar region by comparing their ARDRA patterns and determining bacterial phylogenetic position based on 16S rDNA sequencing. These results indicate that suitable restriction endonuclease chosen for ARDRA can effectively reflect the microbial diversity in polar environments. Compared with *Hinf*I, *Hae*III and a combination of *Afa*I and *Msp*I, *Alu*I generated the largest number of different ARDRA patterns, indicating that it is the most suitable enzyme for assessment of genetic diversity of polar marine planktonic bacteria by ARDRA.

When the other restriction enzymes were used, many isolates with the same ARDRA pattern had sufficiently different 16S rDNA sequences that phylogenetic analyses placed them in different evolutionary lineages. For example, *Hinf*I- and *Hae*III-generated ARDRA patterns could not discriminate between the genera *Shewanella* and

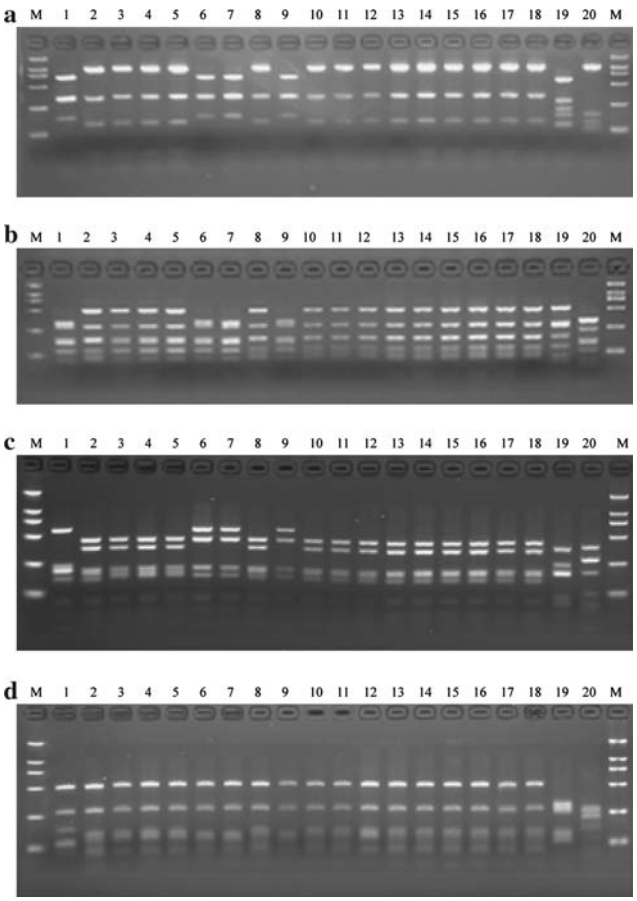

Fig. 3 ARDRA of 20 isolates from polar marine environments with **a** restriction endonuclease *Hinf*I, **b** restriction endonuclease *Hae*III, **c** restriction endonuclease *Alu*I and **d** double restriction endonucleases *Afa*I + *Msp*I. Lane *1* BSw10008, Lane *2* BSw10009, Lane *3* BSw10012, Lane *4* BSw10016, Lane *5* BSw10020, Lane *6* BSw10042, Lane *7* BSw10073, Lane *8* BSw10079, Lane *9* BSw10081, Lane *10* BSw10087, Lane *11* BSw20308, Lane *12* BSw20353, Lane *13* BSw10088, Lane *14* BSw10093, Lane *15* BSw10094, Lane *16* BSw10097, Lane *17* BSw10098, Lane *18* BSw10100, Lane *19* BSw10170W, Lane *20* BSw10175, Lane *M* DNA marker DL2000 (TaKaRa, Dalian, China)

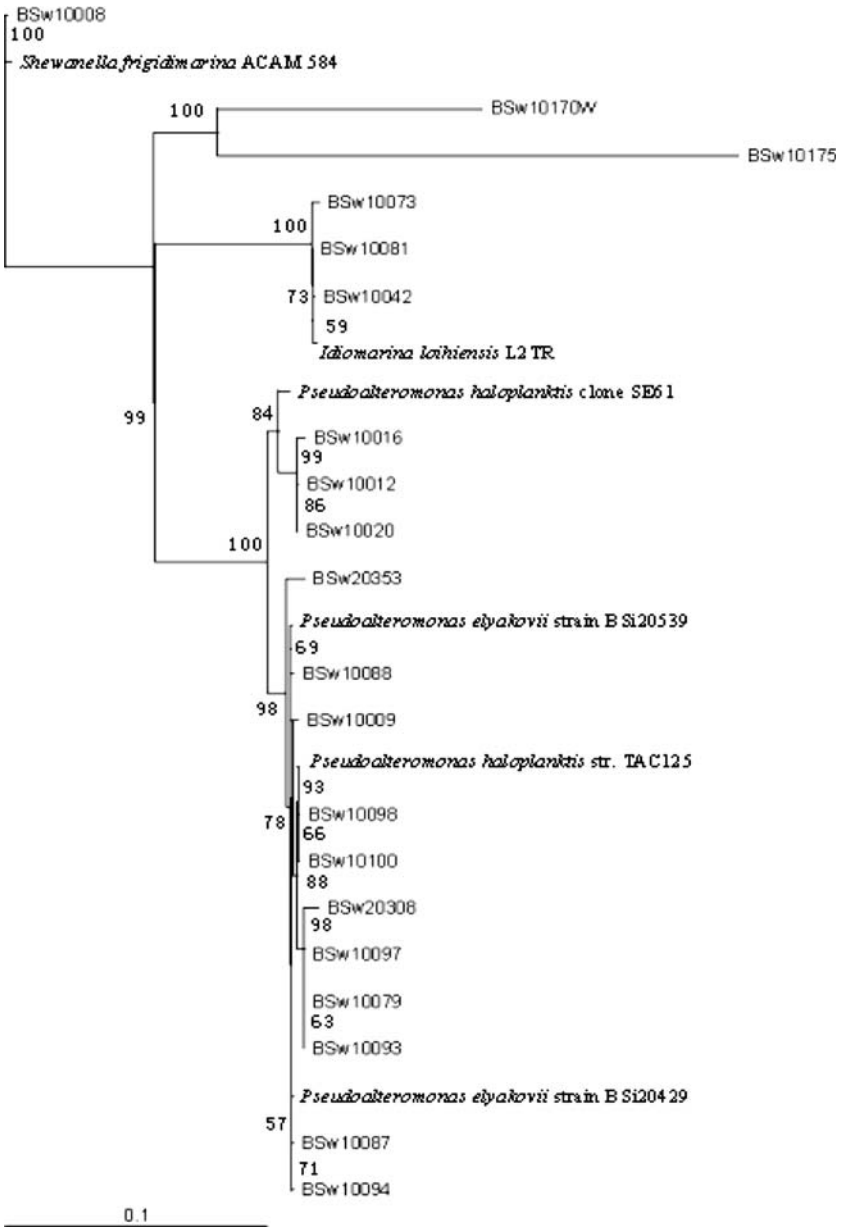

Fig. 4 Phylogenetic tree showing the relationships among the 16S rDNA sequences of 20 isolates from polar regions. The tree was constructed by using the neighbour-joining method for calculation. Bootstrap values based on 100 resamplings are shown at the branch points. Only bootstrap values greater than 50% are shown. *Bar* 0.1 substitutions per nucleotide position

Idiomarina (Fig. 3a, b). In addition, *Hinf*I-generated ARDRA pattern could not discriminate between the genera *Colwellia* and *Pseudoalteromonas* (Fig. 2a). Clustered into different families, the genera *Shewanella*, *Idiomarina*, *Colwellia*, and *Pseudoalteromonas* all belong to the order *Alteromonadales* in Gammaproteobacteria. Therefore, the choice of a suitable restriction enzyme used for ARDRA really affects the sensitivity of results revealing microbial diversity in polar habitats. Consistent with the phylogenetic position based on 16S rDNA sequencing, bacteria showing an identical *Alu*I -generated ARDRA pattern can be placed in one genus. Furthermore, ARDRA with *Alu*I can also generate species-specific patterns, consistent with previous reports (Heyndrickx et al. 1996). *Alu*I is also less expensive than some of the other restriction enzymes used in ARDRA.

It is worth mentioning that results expressed above relate to ARDRA patterns that can be visualized under UV light. Because concentration and purity of 16S rDNA fragment in PCR products can affect the visibility of ARDRA pattern, excluding the influence of restriction enzyme digestion, different ARDRA groups sometimes may belong to the same genotype.

Based on results of ARDRA, which can generate species-specific patterns and provide a simple and fairly robust means for rapid assessment of microbial diversity from

different environments, representative strains of each ARDRA pattern can be chosen for 16S rDNA sequencing to obtain information about the exact type of microorganisms present in the sample. Such an approach is helpful to obtain objective information about the community composition, and also to save time, money and energy. In this study, only five restriction endonucleases were chosen to investigate the effect of restriction enzymes on ARDRA patterns. More studies are required to understand the validity and efficiency of ARDRA with other restriction endonucleases for assessment of diversity of polar marine planktonic bacteria.

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