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Distribution of *Aeromonas* spp. as identified by 16S rDNA restriction fragment length polymorphism analysis in a trout farm

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Aims: This study used restriction fragment length polymorphism (RFLP) with *Aeromonas*-specific primers to identify species of *Aeromonas* and to investigate their distribution in a trout farm and stream.

Methods and Results: In January, May, August and November 2000, presumptive *Aeromonas* species were recovered from a farm and a sedimentation pond in a fish farm and stream, and identified by PCR-RFLP analysis with *Aeromonas*-specific primers. The specificity of *Aeromonas*-specific primers and the suitability of PCR-RFLP analysis for identifying *Aeromonas* spp. were confirmed with fatty acid methyl esters (FAMEs) and 16S rDNA sequencing analyses, respectively. Levels of *Aeromonas* spp. sampled in May and August were higher than in January and November at all sampling sites. *Aeromonas salmonicida* was the dominant species in January and November, and the proportion of pathogenic species (*Aer. hydrophila*, *Aer. caviae* and *Aer. veronii*) increased in May and August.

Conclusions: PCR-RFLP analysis with *Aeromonas*-specific primers is a rapid and reliable method for identifying widely distributed *Aeromonas* spp. from environmental samples.

Significance and Impact of the Study: To minimize human health risk, monitoring the levels and species composition of *Aeromonas* in fish farm is advisable.

INTRODUCTION

Aeromonas spp. are mesophilic motile or psychrophilic nonmotile Gram-negative bacteria. They are ubiquitous and have been widely isolated from clinical, environmental and food samples (Halda-Alija and Johnston 1999; Kunene *et al*. 1999; Ko *et al*. 2000). These bacteria can cause furunculosis and fin rot in fishes and septicaemia and gastrointestinal infections in humans (Noonan and Trust 1995; Merino *et al*. 1995; Janda and Abbott 1998). They are commonly found among the microflora of trout (Horsely 1973; Spanggaard *et al*. 2000), and consequently trout farms can be contaminated with *Aeromonas* spp. Since trout farms are a potential reservoir of these bacteria and can affect nearby environments through the release of *Aeromonas* spp., it is necessary to monitor seasonal variations in species distribution to prevent potential health risks.

The genus *Aeromonas* currently includes 14 named species (*Aer. hydrophila*, *Aer. bestiarum*, *Aer. salmonicida*, *Aer. caviae*, *Aer. media*, *Aer. eucrenophila*, *Aer. sobria*, *Aer. jandaei*, *Aer. veronii*, *Aer. schubertii*, *Aer. trota*, *Aer. allosaccharophila*, *Aer. encheleia* and *Aer. popoffii*) including two biogroups of *Aer. veronii* and two unnamed species (*Aeromonas* Group 501 and *Aeromonas* HG11) recognized by DNA–DNA hybridization (Huys *et al*. 1997a, b; Martínez-Murcia 1999). *Aeromonas ichthiosmia* and *Aer. enteropelogenes* appear to be synonymous with *Aer. veronii* and *Aer. trota*, respectively (Collins *et al*. 1993). *Aeromonas hydrophila*, *Aer. caviae*, *Aer. jandaei*, *Aer. veronii* and *Aer. schubertii* are considered as human pathogenic species, but other species have also been recovered from clinical samples (Janda and Abbott 1998; Figueras *et al*. 2000a).

One of the most complex problems concerning the identification of *Aeromonas* spp. is their phenotypic hetero-

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geneity (Janda *et al.* 1996). Since conventional biochemical tests are time-consuming and not always reliable, several approaches have been attempted for characterizing *Aeromonas* spp., including the use of multilocus electrophoresis, fatty acid methyl methyl esters (FAMEs) analysis, and amplified fragment length polymorphism (AFLP) analysis (Altwegg *et al.* 1988; Huys *et al.* 1995; Janssen *et al.* 1996). However, problems remain with the identification of environmental isolates, because discrimination is not always clear-cut, methods are time-consuming, and instruments are often very costly.

The 16S rDNA sequences of *Aeromonas* have been published, and have proven to be valuable in the identification of *Aeromonas* spp. (Martínez-Murcia *et al.* 1992, 1993; Huys *et al.* 1997b; Dermata *et al.* 1999). Overall, sequence similarity between *Aeromonas* species is very high (ca 98–100%), but shows enough variability to discriminate among species. Recently, a protocol was proposed based on RFLP patterns of a PCR-amplified 16S rDNA sequence that enabled the identification of all *Aeromonas* spp. (Borrell *et al.* 1997; Figueras *et al.* 2000b). All *Aeromonas* type strains and almost all environmental *Aeromonas* isolates were identified reliably with this protocol. Nevertheless, some biochemical tests were still required to confirm presumptive *Aeromonas* spp. Consequently, a modified PCR-RFLP method with *Aeromonas*-specific primers is needed to identify presumptive *Aeromonas* spp. from environmental sources rapidly and reliably.

Several reports describe the distribution of *Aeromonas* spp. and the relationships among *Aeromonas* spp., indicator bacteria and physical parameters in various environments (Burke *et al.* 1984; Monfort and Baleux 1990; Ortega *et al.* 1996; Pettibone 1998). However, few data are available on the monitoring of the distribution of *Aeromonas* species identified with reliable methods in fish farms. Therefore, the aims of this study were: (i) to apply PCR-RFLP analysis with a genus-specific primers to identify presumptive *Aeromonas* spp. isolated in the fish farm and stream, and to evaluate the specificity of genus-specific primers by comparing the PCR-amplified presumptive *Aeromonas* spp. with FAMEs analysis and the reliability of this method by comparing PCR-RFLP results with sequencing results; and (ii) to compare the seasonal variation of *Aeromonas* levels in a trout farm and nearby stream, to determine the impact of fish farm effluents on the stream.

MATERIALS AND METHODS

Study area, sampling and temperature measurement

In January, May, August and November 2000, water samples were taken from four different sites in Jeongseon, Kangwon Province, Korea. The site maps and locations are shown in the previous study (Cho *et al.* 1995). Briefly, the four sites were a farm (Site 1), a sedimentation pond (Site 2) in the trout farm, and 30 m upstream (Site 3) and 30 m downstream (Site 4) from the junction of the stream and an effluent outlet from the trout farm. All samples were collected aseptically in sterile polypropylene bottles to avoid any contamination. Samples were stored on ice, immediately transported to the laboratory and processed within 8 h after collection. The water temperature was measured *in situ* using portable electrode-carrying devices (Checkmate 90, Corning, NY, USA). In addition, four diseased trout fry were caught at the farm, wrapped in a plastic wrap and stored in an ice chamber, immediately transported to the laboratory and processed within 4 h after collection in August 2000.

Heterotrophic plate counts (HPC)

The numbers of heterotrophic bacteria were determined using the plate count technique on R2A agar (Difco Laboratories, MI, USA). Samples were serially diluted up to 10^{-4} and appropriate dilutions were spread on R2A agar plate in triplicate as described by American Public Health Association (1995). Plates were incubated at 20 °C and colonies were enumerated after 14 d.

Isolation of presumptive *Aeromonas* spp. from water samples and trout fry intestines

Water samples were filtered in 0·1 and 1·0 ml volumes through 0·45 μm pore membrane filters (Nucleopore, MA, USA). The filters were transferred to Glutamate Starch Phenol Red (GSP) agar plates (Merck, Darmstadt, Germany) with 100 000 U l^{-1} of penicillin G (Sigma Chemical Co., St Louis, MO, USA) in triplicate and incubated aerobically at 25 °C for 48–72 h. Yellow colonies were considered presumptive *Aeromonas* spp. and were counted. Yellow colonies that were easily determined from others were picked from each plate and incubated on tryptic soy broth agar (Difco Laboratories, MI, USA) at 30 °C for 24 h. The surfaces of four trout fry were washed for 30 s with ethanol (70%, v/v). The intestines were obtained aseptically, washed for 1 min with 10 ml sterilized phosphate-buffered saline (PBS: 130 mmol l^{-1} NaCl, 10 mmol l^{-1} NaH_2PO_4, pH 7·2) and then homogenized for 1 min with Omni EZ Connect Homogenizer (Omni International, Inc., Warrenton, USA) after addition of 100 ml sterilized PBS. Presumptive *Aeromonas* spp. in the homogenate were isolated as described above.

Primers design

Aeromonas genus-specific primers (Table 1) were designed from conserved regions of the *Aeromonas* 16S rDNA gene

Table 1 The primers used in this study

Primer name	Position*	Sequence (5′–3′)
AERF	87–105	CTA CTT TTG CCG GCG AGC GG
AERR	1041–1022	TGA TTC CCG AAG GCA CTC CC

*Position of primers corresponding to the base numbers in the *Escherichia coli* 16S rRNA.

sequences available in the GenBank database. The apparent specificity of each primer was determined by matching it to the Ribosomal Database Project II (RDP II) microbial ribosomal small-subunit data set, using the CHECK-PROBE function (Maidak *et al.* 2000) to confirm their adequacy. The forward and reverse primers were AERF and AERR, and the product of these two primers is expected to be 953 bp.

DNA extraction and PCR

A total of 814 isolates were examined for identification by PCR. DNA was extracted from each sample using lysozymes, freeze–thawing and phenol–chloroform as described by Lee *et al.* (1996). The resulting DNA was stored at − 20 °C until use. PCR was performed with a thermal cycler (PE2400; Perkin-Elmer Co., Norwalk, CT, USA) using the following program: 94 °C for 4 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 68 °C for 30 s, and extension at 72 °C for 45 s; and 72 °C for 10 min. Reaction mixtures (final volume, 50 μl) contained 1·5 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} Tris-HCl (pH 8·8 at 25 °C), 50 mmol l^{-1} KCl, 0·1% Triton X-100, 200 ng of bovine serum albumin μl^{-1}, 200 μmol l^{-1} each deoxynucleoside triphosphate, 0·2 μmol l^{-1} each oligonucleotide primer, and 2 U of *Taq* polymerase (Promega Co. Madison, WI, USA). Approximately 50 ng of DNA template were added to each PCR tube. The PCR products were analysed by electrophoresis on 1·5% agarose gels and stained with ethidium bromide.

Fatty acid methyl esters (FAMEs) analysis

FAMEs analysis was performed using an HP 6890 gas chromatograph (Hewlett Packard, Rolling Meadows, IL, USA) to confirm the specificity of the PCR products. Isolates from water samples collected in January, May and August ($n = 342$) and from trout fry ($n = 37$) were cultured and harvested according to Manufacturer's instructions (MIDI 1999). FAMEs were extracted and identified using Sherlock software (TSBA library, version 4·10; Microbial ID, Newark, NJ, USA).

Computer analysis of *Aeromonas* 16S rDNA gene sequences

For restriction endonuclease selection and species discrimination, the amplified regions within published 16S rDNA gene sequences (Martínez-Murcia *et al.* 1992, 1993; Dermata *et al.* 1999) were analysed using DNASIS software (Hitachi) on a personal computer. An analysis of the theoretical banding patterns for various restriction enzymes was performed, and *Alu*I, *Cfo*I, *Pvu*II and *Xho*II were selected for species discrimination.

RFLP pattern analysis

In total, 238 amplified PCR products were digested for 3·5 h at 37 °C with 2 U of each enzyme (*Alu*I, *Cfo*I, *Pvu*II and *Xho*II; Promega Co.). The resulting products with *Alu*I and *Cfo*I were separated by gel electrophoresis in 4% NuSieve 3 : 1 agarose (FMC BioProducts, Rockland, ME, USA) at 100 V for 1 h. Products digested with *Pvu*II and *Xho*II were electrophoretically separated on 2% SeaKem LE agarose (FMC BioProducts) at 100 V for 40 min.

Sequencing

For the validation of *Aeromonas* identification by RFLP pattern analysis, two or three randomly chosen strains of each species identified by RFLP pattern analysis were selected for sequencing. In total, the PCR products of 18 strains were sequenced using the chain termination method on an ALF express DNA autosequencer (Pharmacia Biotech, Uppsala, Sweden) with primer AERF and the Cy5 AutoRead sequencing kit. Species identification and similarity calculations were performed, comparing sequences of approximately 500 bases (except *Aer. caviae* strains) with sequences available in GenBank using BLAST network services (Altschul *et al.* 1997). Two strains identified as *Aer. caviae* were sequenced using primers AERF and AERR, and sequences of 950 bases were compared as described above. The 16S rDNA sequences in this study were submitted to the GenBank database under nucleotide accession numbers AF472489 to AF472506.

RESULTS

Variation in temperature, pH, HPC and counts of presumptive *Aeromonas* spp.

The temperatures of water samples collected in May and August were higher than those in January and November (Table 2). HPC of samples collected from each site in the

Table 2 Variation in temperature, pH, heterotrophic plate counts and counts of presumptive *Aeromonas* spp. at each sampled site from January to November 2000

	Site 1				Site 2				Site 3				Site 4			
	Jan	May	Aug	Nov	Jan	May	Aug	Nov	Jan	May	Aug	Nov	Jan	May	Aug	Nov
Temperature (°C)	8·4	14·2	15·1	12·9	7·9	14·7	15·5	13·1	3·7	15·4	18·1	12·5	4·5	15·2	18·4	12·5
Heterotrophic plate counts (cfu ml^{-1})	$1{\cdot}7 \times 10^{4}$*	$3{\cdot}8 \times 10^{5}$	$1{\cdot}1 \times 10^{5}$	$6{\cdot}0 \times 10^{3}$	$2{\cdot}0 \times 10^{5}$	$7{\cdot}3 \times 10^{5}$	$3{\cdot}5 \times 10^{5}$	$1{\cdot}0 \times 10^{4}$	$1{\cdot}9 \times 10^{4}$	$2{\cdot}1 \times 10^{5}$	$6{\cdot}5 \times 10^{4}$	$4{\cdot}8 \times 10^{3}$	$5{\cdot}8 \times 10^{4}$	$8{\cdot}1 \times 10^{4}$	$1{\cdot}4 \times 10^{5}$	$2{\cdot}5 \times 10^{3}$
Presumed *Aeromonas* spp. (cfu 100 ml^{-1})	$1{\cdot}9 \times 10^{3}$	$4{\cdot}5 \times 10^{3}$	$1{\cdot}3 \times 10^{3}$	$6{\cdot}4 \times 10^{2}$	$6{\cdot}7 \times 10^{2}$	$2{\cdot}4 \times 10^{3}$	$1{\cdot}1 \times 10^{3}$	$1{\cdot}5 \times 10^{2}$	$3{\cdot}7 \times 10^{2}$	$1{\cdot}3 \times 10^{3}$	$1{\cdot}0 \times 10^{3}$	$4{\cdot}8 \times 10^{1}$	$4{\cdot}0 \times 10^{2}$	$1{\cdot}1 \times 10^{3}$	$1{\cdot}0 \times 10^{3}$	$2{\cdot}7 \times 10^{1}$

*Mean values of triplicate samples.

warmer months (May and August) appeared to be higher than those collected in the colder months (January and November). In each sampled month, HPC were higher at Site 2 than at the other sites. Presumptive *Aeromonas* spp. were detected in all water samples. They appeared to have patterns of variation similar to HPC but their levels were always high at Site 1 in each sampled month.

Screening of *Aeromonas* by PCR amplification

Of the 595 isolates from water samples and diseased trout, 40% were PCR-positive strains (Table 3). Isolates from Site 3 and 4 collected in January were all PCR-negative strains, but the percentage of PCR-positive strains from the other water samples ranged from 21 to 72%. Percentages of PCR-positive strains from diseased trout (78%) were somewhat higher than those from water samples. To validate the specificity of the PCR products, we performed FAMEs analysis on 379 strains isolated from the trout farm, stream and trout fry. This analysis yielded the same results as the PCR amplification (data not shown). All PCR-positive strains were identified as *Aeromonas* species by FAMEs analysis. PCR-negative strains were identified as other Gram-negative species. However, the seasonal patterns of confirmed *Aeromonas* spp. were similar to those of presumptive *Aeromonas* spp.

Species identification by RFLP pattern analysis

For species identification of confirmed *Aeromonas* strains, four different restriction endonucleases (*Alu*I, *Cfo*I, *Pvu*II and *Xho*II) were selected after a computer analysis of the PCR-amplified regions within published 16S rDNA gene sequences of the type strains (Table 4). Six species (*Aer. sobria*, *Aer. veronii*, *Aer. jandaei*, *Aer. schubertii*, *Aer. trota* and *Aeromonas* Group 501) were identified by their specific patterns using endonuclease *Alu*I. The other species required endonucleases *Cfo*I, *Pvu*II or *Xho*II for species identification. Three pairs (*Aer. bestiarum–Aer. salmonicida*, two biogroups of *Aer. veronii*, and *Aer. encheleia–Aeromonas* HG11) were not distinguishable with these four endonucleases. *Aer. encheleia* and *Aeromonas* HG11 could be distinguished with other endonucleases, e.g. *Hae*III. However, *Aer. bestiarum* and *Aer. salmonicida* subspecies could not be discriminated, because they had only two base-pair differences near the terminal region of the PCR-amplified product. Two biogroups of *Aer. veronii* were also not distinguishable due to their identical nucleotide sequences (Martínez-Murcia *et al.* 1992, 1993). RFLP patterns from the PCR-RFLP analysis of 238 strains are shown in Fig. 1. Since it was difficult to examine the bands below 100 bp on agarose gels, only restriction fragments above 100 bp were considered. All RFLP patterns shown in Fig. 1 agree with

Table 3 Results of PCR amplification of presumptive *Aeromonas* spp.

	Site 1			Site 2			Site 3			Site 4				
	Jan	May	Aug	Nov	Jan	May	Aug	Nov	Jan	May	Aug	Nov	Trout	Total
PCR-positive strains/ Presumptive *Aeromonas* spp.	12/19 (63)*	13/32 (41)	11/35 (31)	23/82 (28)	5/20 (25)	12/24 (50)	6/29 (21)	18/72 (25)	0/11 (0)	5/13 (38)	11/32 (34)	28/52 (54)	0/12 (0)	23/32 (72)	12/31 (38)	30/62 (48)	29/37 (78)	238/595 (40)
Confirmed *Aeromonas* spp. counts (cfu 100 ml^{-1})	$1{\cdot}2 \times 10^3$	$1{\cdot}8 \times 10^3$	$4{\cdot}1 \times 10^2$	$1{\cdot}8 \times 10^2$	$1{\cdot}7 \times 10^2$	$1{\cdot}2 \times 10^3$	$2{\cdot}3 \times 10^2$	$3{\cdot}8 \times 10^1$	NA†	$5{\cdot}0 \times 10^2$	$3{\cdot}4 \times 10^2$	$2{\cdot}6 \times 10^1$	NA	$7{\cdot}9 \times 10^2$	$3{\cdot}8 \times 10^2$	$1{\cdot}3 \times 10^1$	–	–

*Figures in parentheses indicate percentage values.
†All tested strains were negative for PCR amplification.

Table 4 Computer analysis of amplified regions within published *Aeromonas* 16S rDNA gene sequences (a) RFLP patterns obtained from sequence analysis with restriction endonucleases *Alu*I, *Cfo*I, *Pvu*II and *Xho*II

Restriction endonuclease	Pattern	Restriction fragments (bp)
AluI	A1	42, 162, 177, 211, 361
	A2*	42, 162, 173, 177, 188, 211
		42, 162, 174, 177, 187, 211
	A3	14, 42, 162, 173, 174, 177, 211
	A4	14, 42, 79, 84, 173, 174, 177, 211
	A5	42, 79, 84, 173, 177, 188, 211
	A6	42, 79, 84, 177, 187, 385
	A7	42, 162, 173, 177, 399
	A8	42, 162, 177, 361
*Cfo*I	C1	135, 357, 461
	C2	116, 376, 461
	C3	461, 492
*Pvu*II	P1	953
	P2	391, 562
*Xho*II	X1	334, 619
	X2	44, 334, 575
	X3	44, 910

*Two different sets of restriction fragments were included in the same pattern because they could not be distinguished by agarose gel electrophoresis.

(b) Species identification from characteristic RFLP patterns

Species	RFLP pattern(s)
Aer. hydrophila	A1/C1
Aer. bestiarum	A1/C2
*Aer. salmonicida**	A1/C2
Aer. caviae	A2/P1
Aer. media	A2/P2/X1
Aer. eucrenophila	A2/P2/X3
Aer. sobria	A3
Aer. veronii†	A4
Aer. jandaei	A5
Aer. encheleia	A1/C3/X2
Aeromonas HG11‡	A1/C3/X2
Aer. schubertii	A6
Aer. trota	A7
Aer. alloscharophila	A2/P2/X2
Aeromonas Group 501	A8
Aer. popoffii	A1/C3/X1

*Identical RFLP patterns for *Aer. bestiarum* and *Aer. salmonicida*.
†Identical RFLP patterns for *Aer. veronii* biogroup *sobria* and *Aer. veronii* biogroup *veronii*.
‡Identical RFLP patterns for *Aeromonas* HG11 and *Aer. encheleia*.

Table 4a, although RFLP patterns A5, A6, A7, A8 and X3 were not found. All strains could be identified using the scheme given in Table 4b, and no other characteristics of the patterns were detected.

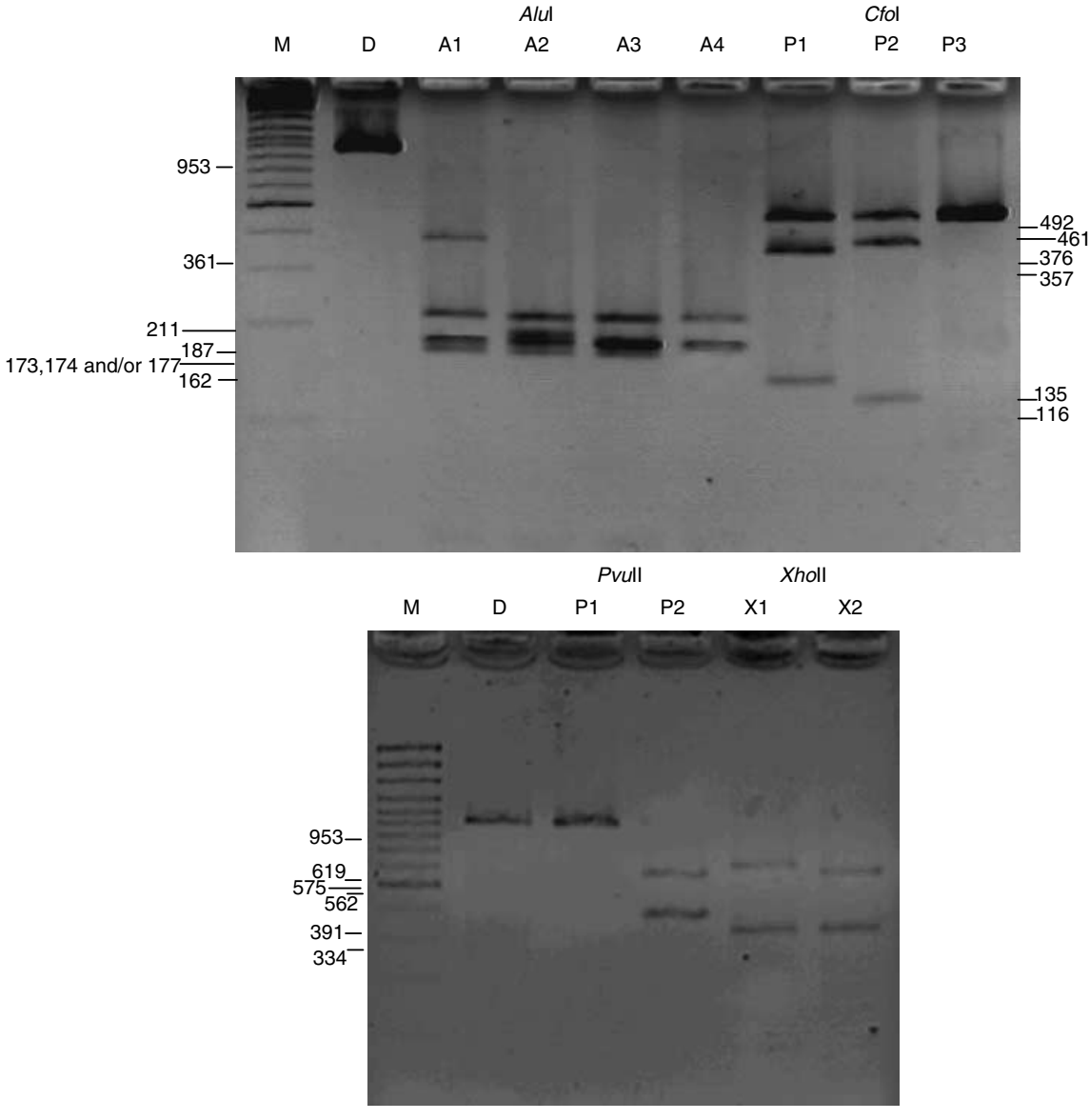

Fig. 1 RFLP patterns of PCR products using the restriction endonucleases *Alu*I, *Cfo*I, *Pvu*II and *Xho*II. Lane M: molecular weight marker; lane D: DNAs amplified by PCR; lanes A1–A4: RFLP patterns observed for *Alu*I digests; lanes C1–C3: RFLP patterns observed for *Cfo*I digests; lanes P1 and P2: RFLP patterns observed for *Pvu*II digests; lanes X1 and X2: RFLP patterns observed for *Xho*II digests. These patterns correspond to the patterns listed in Table 4a

Distribution of species

Five species (*Aer. hydrophila*, *Aer. caviae*, *Aer. media*, *Aer. sobria* and *Aer. popoffii*) and two different pairs (*Aer. bestiarum–Aer. salmonicida*, two biogroups of *Aer. veronii*) were identified from the PCR-RFLP analysis of 238 strains (Table 5). The *Aer. bestiarum–Aer. salmonicida* pair (50%) was dominant and found at all sites, although it was more abundant in trout fry and in water samples collected during the colder months. *Aer. hydrophila* (14%), *Aer. caviae* (8%) and *Aer. media* (18%) were also found at all sites and in trout fry. However, *Aer. hydrophila* and *Aer. caviae* were frequently isolated from samples collected in the warmer months, whereas *Aer. media* did not show any characteristic distribution differences. *Aeromonas sobria* (8%) was isolated from many water samples, but was not found in samples collected in August. *Aeromonas veronii* (1%) was found only in stream water samples collected in May, while *Aer. popoffii* (1%) was found only in the farm during the colder months.

Table 5 Distribution of *Aeromonas* species identified by PCR-RFLP analysis

	Site 1				Site 2				Site 3			Site 4				
	Jan	May	Aug	Nov	Jan	May	Aug	Nov	May	Aug	Nov	May	Aug	Nov	Trout fry	Total
Aer. hydrophila		1	3			4	2		2	1	1	9	5		5	33 (14)†
*Aer. bes/sal**	8	2	1	13	5	2	2	7		5	21	8		23	21	118 (50)
Aer. caviae			4	1		2		1		5		1	3		1	18 (8)
Aer. media		8	3	3		3	2	5	2		6	3	4	2	2	43 (18)
Aer. sobria	3	2		4		1		5						5		20 (8)
Aer. veronii									1			2				3 (1)
Aer. popoffii	1			2												3 (1)
Total	12	13	11	23	5	12	6	18	5	11	28	23	12	30	29	238

**Aer. bestiarum–Aer. salmonicida* pair.
†Percentage value of strains identified as each species/total strains.

Table 6 Sequencing results of *Aeromonas* species identified by PCR-RFLP analysis

Strain	Identification by PCR-RFLP	Identification by sequencing	Similarity‡
S2M9*	*Aer. hydrophila*	*Aer. hydrophila*	1·000
S2M19	*Aer. hydrophila*	*Aer. hydrophila*	1·000
S4A5	*Aer. hydrophila*	*Aer. hydrophila*	0·998
S2M5	*Aer. bestiarum–Aer. salmonicida*	*Aer. bestiarum–Aer. salmonicida*	1·000
S2A32	*Aer. bestiarum–Aer. salmonicida*	*Aer. bestiarum–Aer. salmonicida*	1·000
T5†	*Aer. bestiarum–Aer. salmonicida*	*Aer. bestiarum–Aer. salmonicida*	1·000
S1A19	*Aer. caviae*	*Aer. caviae*	1·000
S4A16	*Aer. caviae*	*Aer. caviae*	1·000
S1M4	*Aer. media*	*Aer. media*	1·000
S3A6	*Aer. media*	*Aer. media*	1·000
S4A17	*Aer. media*	*Aer. media*	1·000
S1J6	*Aer. sobria*	*Aer. sobria*	1·000
S1J10	*Aer. sobria*	*Aer. sobria*	1·000
S2M10	*Aer. sobria*	*Aer. sobria*	1·000
S3M31	*Aer. veronii*	*Aer. veronii*	0·993
S4M13	*Aer. veronii*	*Aer. veronii*	1·000
S1N5	*Aer. popoffii*	*Aer. popoffii*	1·000
S1N17	*Aer. popoffii*	*Aer. popoffii*	1·000

*Strains isolated in water samples. S1, S2, S3, S4 denote Sites 1, 2, 3 and 4, respectively. J, M, A, N denote January, May, August, November, respectively, followed by isolate numbers.
†Strain isolated in trout fry. T denotes trout, followed by isolate numbers.
‡Similarity were determined as described in Materials and Methods.

Comparison of PCR-RFLP results with sequence data

Eighteen strains identified by PCR-RFLP analysis were sequenced using primer AERF. Two strains identified with *Aer. caviae* were further sequenced using primer AERR, because the sequence of *Aer. caviae* was identical with that of *Aer. trota* in the region of 16S rDNA amplified by primer AERF. Species identification and the similarity values of 18 strains are shown in Table 6. The identification by sequence analysis corresponded to the identification by PCR-RFLP analysis. The sequenced regions of 16 strains were identical to the regions in the 16S rDNA gene of their type strains. The sequences of two strains, identified with *Aer. hydrophila* and *Aer. veronii*, differed from the type strain by only one and three bases, respectively. However, each sequence was closely related to the *Aer. hydrophila* or *Aer. veronii* type strain, and showed the highest similarity value in the available *Aeromonas* sequences of the GenBank.

DISCUSSION

Aeromonas species exhibited very high levels of overall 16S rDNA sequence similarity to each other (*ca* 98–100%; Martínez-Murcia *et al*. 1992). This enabled us to design specific primers from their common regions and to select AERF (forward) and AERR (reverse) primers. Kämpfer *et al*. (1994) reported that fatty acid patterns are very useful for improving characterization at the genus level; therefore, FAMEs analysis was performed according to MIDI references (MIDI 1999) to confirm the specificity of the PCR primers for *Aeromonas* 16S rDNA amplification. The results from the PCR amplification of all isolates were the same as the FAMEs analysis, thereby confirming the specificity of the PCR primers. Although the ratios of confirmed *Aeromonas* spp. to presumptive *Aeromonas* spp. varied with sampled sites and times, the seasonal patterns in the levels of confirmed *Aeromonas* spp. were similar to those of presumptive *Aeromonas* spp. Actual *Aeromonas* levels from stream water samples may have been overestimated in January, because no presumptive *Aeromonas* spp. were confirmed by PCR amplification and FAMEs results. However, *Aeromonas* spp. still persisted, because *Aeromonas* 16S rDNA was amplified with direct DNA extraction from stream water samples (data not shown).

The RFLP patterns of all confirmed *Aeromonas* strains showed the characteristic patterns expected from computer analysis of all published 16S rDNA sequences (Martínez-Murcia *et al*. 1992, 1993; Dermata *et al*. 1999). Borrell *et al*. (1997) reported that reference strains of all *Aeromonas* species and 76 biochemically determined clinical isolates of *Aeromonas* were almost in full agreement with RFLP analysis. Figueras *et al*. (2000b) extended this method with additional endonucleases to separate the species *Aer. salmonicida*, *Aer. bestiarum* and the recently described *Aer. popoffii*, which were not discriminated by the earlier method, and reported that all tested strains could be identified with this method. We modified their protocol by using genus-specific primers in addition to the restriction endonucleases *Alu*I, *Cfo*I, *Pvu*II and *Xho*II instead of universal primers and *Alu*I–*Mbo*I combination, *Nar*I–*Hae*III combination, and *Pst*I. This modification resulted in a better resolution by agarose gel electrophoresis than the earlier methods described by Borrell *et al*. (1997) and Figueras *et al*. (2000b); we obtained fewer bands because we used only one restriction endonuclease for each experiment rather than a combination of two. In addition, initial identification using biochemical tests was not required, because genus-specific primers were used for species isolation and identification; thus our method should be useful for the rapid identification of *Aeromonas* spp. from environmental samples. However, some problems arose from our modifications. *Aeromonas bestiarum* and *Aer. salmonicida* were not discriminated because there was only two base-pair difference in the near terminal region of the PCR-amplified products (Martínez-Murcia *et al*. 1992; Pavan *et al*. 2000). Although *Aer. encheleia* and *Aeromonas* HG11 were also not discriminated by our method, they could be distinguished with other endonucleases, such as *Hae*III.

Sequencing results for 18 strains identified by PCR-RFLP analysis validated the PCR-RFLP analysis for *Aeromonas* identification based on the 16S rDNA sequence. Sixteen strains had the same 16S rDNA sequences as their reference strains, and only two strains identified by PCR-RFLP analysis as *Aer. hydrophila* and *Aer. veronii* had one and three base differences, respectively. These differences did not affect the identification by 16S rDNA sequencing and PCR-RFLP analysis. Consequently, our method can reliably identify *Aeromonas* spp. from environmental samples.

Aeromonas spp. were isolated from water samples collected from a trout farm, stream and trout fry in Korea. In accordance with earlier studies (Huys *et al*. 1996; Hänninen *et al*. 1997; Halda-Alija and Johnston 1999; Ivanova *et al*. 2001), our results confirm the ubiquity of *Aeromonas* spp.

The distributions of *Aeromonas* species showed a close relationship between sampling time and dominant species. Although *Aer. bestiarum–Aer. salmonicida* pair was widely distributed regardless of sampling season and site, this pair was dominant at all sites in the colder months, especially. Pathogenic species (*Aer. hydrophila*, *Aer. caviae* and *Aer. veronii*; Figueras *et al*. 2000b) were dominant at all sites during the warmer months. To precisely discriminate the *Aer. bestiarum–Aer. salmonicida* pair, biochemical tests (Janda *et al*. 1996) were conducted for all isolates identified as belonging to the pair, which were identified as *Aer. salmonicida* (data not shown). From these results, *Aer. salmonicida* was found to be widely distributed,and was especially dominant at lower temperature, while levels of pathogenic species increased with increasing temperature. Consequently, species composition and dynamics may be affected by temperature, which agrees with earlier investigation (Monfort and Baleux 1991).

The same species composition was found in the intestines of four trout fry and in water samples collected from the farm in August, although the dominant species were different (see Table 5). As described earlier, the microflora in trout also may be an important factor for relative increases in *Aeromonas* levels, and thus may affect the species composition of the farm. Inversely, the species densities and composition in the farm may reflect those in the microflora of trout, suggesting that the trout contaminated with *Aeromonas* could lead to human disease, which is supported by previously reported outbreaks of food poisoning involved *Aeromonas* (Zeng-Shan *et al*. 1988; Krovacek *et al*. 1995). Due to the pathogenic nature of *Aeromonas* spp., monitoring of the levels and species distribution of

Aeromonas in fish farms should be required to prevent risk to human health.

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