High-Resolution Genotypic Analysis of the Genus *Aeromonas* by AFLP Fingerprinting

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We investigated the ability of a recently developed genomic fingerprinting technique, named AFLP, to differentiate the 14 currently defined DNA hybridization groups (HGs) in the genus *Aeromonas*. We also determined the taxonomic positions of the phenospecies *Aeromonas allosaccharophila*, *Aeromonas encheleia*, *Aeromonas enteropelogenes*, and *Aeromonas ichthiosmia*, which have not been assigned to HGs yet. A total of 98 *Aeromonas* type and reference strains were included in this study. For the AFLP analysis, the total genomic DNA of each strain was digested with restriction endonucleases *Apa*I and *Taq*I. Subsequently, restriction fragments were selectively amplified under high-stringency PCR conditions. The amplification products were electrophoretically separated on a polyacrylamide gel and visualized by autoradiography. Following high-resolution densitometric scanning of the resulting band patterns, AFLP data were further processed for a computer-assisted comparison. A numerical analysis of the digitized fingerprints revealed 13 AFLP clusters which, in general, clearly supported the current *Aeromonas* taxonomy derived from DNA homology data. In addition, our results indicated that there is significant genotypic heterogeneity in *Aeromonas eucrenophila* (HG6), which may lead to a further subdivision of this species. *A. allosaccharophila* and *A. encheleia* did not represent a separate AFLP cluster but were found to be genotypically related to HG8/10 and HG6, respectively. In addition, the results of the AFLP analysis also confirmed the phylogenetic findings that *A. enteropelogenes* and *A. ichthiosmia* are in fact identical to *Aeromonas trota* (HG13) and *Aeromonas veronii* (HG8/10), respectively. The results of this study clearly show that the AFLP technique is a valuable new high-resolution genotypic tool for classification of *Aeromonas* species and also emphasize that this powerful DNA fingerprinting method is important for bacterial taxonomy in general.

Members of the genus *Aeromonas* are facultatively anaerobic, gram-negative rods which have been isolated from various aquatic (17, 39, 45) and terrestrial (36, 43) environments. Some *Aeromonas* species cause a broad range of extraintestinal infections in cold-blooded animals (18, 40), and members of the genus *Aeromonas* have recently received a great deal of attention because of their role as opportunistic pathogens in human gastroenteritis (22, 27, 30). Consequently, many microbiologists involved in hospital and public health research have concluded that new techniques for the identification and classification of unknown *Aeromonas* isolates should be developed. However, data obtained with methods such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins (34), serotyping (16), and pyrolysis mass spectrometry (31) are not consistent with the taxonomic data obtained in conventional biotyping and DNA-DNA homology studies, and it seems that these methods can be used only to discriminate among *Aeromonas* strains in epidemiological studies. On the other hand, multilocus enzyme electrophoresis and gas-liquid chromatographic analysis of cellular fatty acid methyl esters appear to be more valuable tools for identification and classification of unknown aeromonads, and these methods have already proven to be useful in routine analyses (5, 25, 26). Nevertheless, it has also been shown that the results obtained with these two chemotaxonomic techniques may not always be in perfect agreement with DNA-DNA hybridization data (5, 26). Moreover, since an essential part of current *Aeromonas* taxonomy is based on determining the levels of DNA relatedness within and between phenotypically defined groups (phe-

nospecies), which has resulted in the description of at least 14 DNA hybridization groups (HGs), there is a tendency to look for additional genomic methods that are less laborious and require only small amounts of purified DNA.

Over the past 15 years, a significant number of DNA-based techniques have been introduced into the field of bacterial characterization and taxonomy. These genomic fingerprinting methods were developed to detect DNA sequence polymorphisms by using general principles, such as restriction endonuclease analysis, molecular hybridization, and PCR amplification. In a comparative study of different typing methods, Altwegg et al. (4) demonstrated that restriction fragment length polymorphism analysis and ribosomal DNA typing (ribotyping) were very successful in differentiating epidemiologically unrelated *Aeromonas* isolates. In a later paper Martinetti Lucchini and Altwegg (32) reported that ribotyping patterns can also be used as taxonomic markers and can be used to identify most *Aeromonas* strains at the HG level; unfortunately, in this study the ribotyping analysis relied on comparing band patterns by eye, which is not only time consuming but also often inaccurate. More recently, the randomly amplified polymorphic DNA technique was used to genetically differentiate *Aeromonas* strains that were isolated mainly from fish (35). Because only a limited number of species (*Aeromonas hydrophila* and *Aeromonas salmonicida* subsp. *salmonicida*) were included, additional fingerprinting data are needed to assess the usefulness of the randomly amplified polymorphic DNA technique for reliable identification of aeromonads.

Previously, we evaluated the use of the AFLP technique for identification and classification of bacteria at the subgeneric level (28). The concept of the AFLP technique was originally developed for plant-breeding purposes, but this technique is now considered a universal method for fingerprinting DNA from any source or DNA of any complexity, including human, animal, and plant DNAs (44, 47). Essentially, the AFLP method

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combines the principle of restriction fragment length polymorphism analysis with highly specific PCR amplification. Despite its obvious resemblance to restriction fragment length polymorphism, the name AFLP should in no way be considered an acronym because AFLP analysis in fact detects only the presence or absence of restriction fragments rather than length polymorphisms (44).

In this study, our aim was to investigate the taxonomic potential of the AFLP technique for discriminating the various HGs delineated in the genus *Aeromonas*. We therefore analyzed the AFLP patterns of 98 *Aeromonas* strains, representing all currently described genomic species in this genus, as well as the phenospecies *Aeromonas allosaccharophila*, *Aeromonas encheleia*, *Aeromonas enteropelogenes*, and *Aeromonas ichthiosmia*, which have not yet been allocated to particular HGs.

MATERIALS AND METHODS

Strains and culture conditions. All of the bacterial cultures used in this study are listed in Table 1. The strains used were either obtained from the Culture Collection of the Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium, or were kind gifts from M. Altwegg (Institute of Medical Microbiology, University of Zürich, Zürich, Switzerland), E. Falsen (Culture Collection of the University of Göteborg, Göteborg, Sweden), or B. Austin (Department of Biological Sciences, Heriot-Watt University, Riccarton, Edinburgh, Scotland). Most *Aeromonas* strains were grown aerobically on Trypticase soy agar containing 3% (wt/vol) Trypticase soy broth (BBL) and 1.5% (wt/vol) bacteriological agar no. 1 (Oxoid) overnight at 28°C; the only exception was the psychrophilic species *A. salmonicida*, which was incubated at 22°C. *Plesiomonas shigelloides* strains were grown at 37°C.

Isolation and purification of DNA. Approximately 25 mg (wet weight) of cells grown on Trypticase soy agar was washed with 500 µl of resuspension solution (150 mM NaCl, 10 mM EDTA; pH 8.0). The resulting cell suspension was centrifuged for 2 min at 14,926 × g with a Heraeus Biofuge 13 centrifuge and resuspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 7.6). The cells were lysed with 500 µl of GES reagent (5 M guanidium thiocyanate–100 mM EDTA–0.5% [vol/vol] sarcosyl), and the DNA was extracted with chloroform-isoamyl alcohol (24:1, vol/vol) essentially as described by Pitcher et al. (37). Total genomic DNA was precipitated with 0.54 volume of isopropanol, washed twice with 150 µl of 70% (vol/vol) ethanol, and vacuum dried. Finally, DNA pellets were redissolved in 100 µl of TE buffer and treated with 25 µl of DNase-free RNase (0.25 mg ml⁻¹) for 1.5 h at 37°C. The DNA concentrations were determined by measuring the *A*₂₆₀ with a UVikon model 940 spectrophotometer. The integrities and molecular weights of the DNA preparations were checked on a 1% agarose gel containing ethidium bromide (0.5 µg ml⁻¹) by using TAE (40 mM Tris-acetate, 1 mM EDTA; pH 8.0) as the electrophoresis buffer.

Preparation of template DNA for PCR. All protocols relating to the preparation of AFLP templates were performed essentially as described previously by Janssen et al. (28). Briefly, purified intact chromosomal DNA (1 µg) was digested with *ApaI* and *TaqI* (Pharmacia Biotech, Uppsala, Sweden). Subsequently, double-stranded restriction-halfsite-specific adaptors were ligated to the restriction fragments by using T4 ligase (Pharmacia Biotech, Uppsala, Sweden). *ApaI* and *TaqI* adaptors were prepared by mixing equimolar amounts of the partially complementary oligonucleotide sequences 5'-TCGTAGACTGCGTACAGG CC-3' and 5'-TGTACGAGCTCTAC-3' (for *ApaI*) and 5'-GACGATGAGTC-CTGAC-3' and 5'-CGGTCAGGACTCAT-3' (for *TaqI*). Completed *ApaI*-*TaqI* templates were precipitated with 1.25 M ammonium acetate and 50% (vol/vol), isopropanol and washed with 70% ethanol before they were resuspended in 100 µl of T0.1E buffer (10 mM Tris, 0.1 mM EDTA; pH 8.0).

AFLP reactions. *ApaI*-*TaqI* restriction fragments tagged with specific adaptors were used as template DNAs for selective PCR amplification directed by primers A01 (5'-GACTGCGTACAGGCCCA-3') and T01 (5'-CGATGATCCTGAC CGAA-3') (selective bases at the 3' ends of the primers are underlined). The primer notations and amplification procedures used have been described previously (28). Primer A01 was labelled at its 5' end with a T4 kinase (Pharmacia Biotech) assay by using [³²P]ATP (Amersham International, Buckinghamshire, England) as described by Zabeau and Vos (47).

Electrophoresis and visualization of PCR products. Selectively amplified fragments were separated electrophoretically in a denaturing 5% polyacrylamide–8.3 M urea matrix (SequaGel; National Diagnostics) by using TBE (100 mM Tris, 100 mM boric acid, 2 mM EDTA; pH 8.3) as the electrophoresis buffer (28). The gels were vacuum dried with a gel dryer (model 583; Bio-Rad) for 55 min at 80°C. The AFLP fingerprints were visualized autoradiographically by exposing the dried gels to Hyperfilm-MP (Amersham International) for 5 to 24 h depending on the amount of radiation determined with a regular Geiger-Müller counter. Following exposure, the film was developed with a model RGII X-ray film processor (Fuji, Tokyo, Japan).

Densitometric scanning and data processing. Autoradiograms were scanned by using a high-resolution densitoscanner (model RayVen RSU1; X-Ray Scanner Corp.). Transmission image data were stored in TIFF files and were further processed by using the GelCompar software, version 3.1 (Applied Maths, Kortrijk, Belgium). Following conversion, normalization, and background subtraction by using mathematical algorithms, levels of similarity between AFLP fingerprints were calculated by using the Pearson product-moment correlation coefficient applied to all points falling in the active zones of the band patterns. A cluster analysis was performed by using the unweighted pair group method with arithmetic averages. The GelCompar library manager was used to generate an AFLP database from representative band patterns.

RESULTS AND DISCUSSION

Complexity of AFLP patterns. *ApaI*-*TaqI*-digested genomic DNAs from 102 strains were used as templates for selective PCR amplification with primers A01 and T01, both of which have an adenine as a selective base at the 3' end (see Materials and Methods). Our choice of restriction enzymes and primer sequences was based on the results of preliminary research (24, 28), from which we concluded that this specific endonuclease-primer combination resulted in the visualization of about 40 to 60 PCR products per lane for *Aeromonas* genomes. We found that within this range, bands were evenly distributed along the length of the gel lane. This is important, since we have found that a good band distribution is critical for optimal normalization and a high level of discrimination in cluster analyses (28). In addition, whereas most of the ribotyping patterns obtained with *Aeromonas* strains (32) had only 10 to 15 bands per lane, the AFLP patterns obtained in this study are far more complex. Clearly, it is conceivable that a larger number of DNA polymorphisms favors more accurate comparative analysis results and thus strongly facilitates strain-to-strain discrimination.

The AFLP patterns of *P. shigelloides*, a taxon which has historically been closely associated with members of the genus *Aeromonas* (14), contained only 15 to 25 bands (data not shown). The explanation for this low number of bands lies in the fact that *Plesiomonas* genomes, which have a mean G+C content of 51 mol%, are not as G+C rich as the average *Aeromonas* genome (G+C content, 57 to 63 mol%) (14). Since the G+C content of the *ApaI* recognition sequence is very high (GGGCC/C), it is predictable that G+C-rich genomes like the *Aeromonas* genomes will exhibit higher restriction rates and thus yield more bands under the PCR conditions used. Conversely, it has been shown that the frequency of *TaqI* cleavage (T/CGA) is not significantly affected in the G+C content range from 40 to 70 mol% (28).

Reproducibility of AFLP patterns. It has been reported previously (28) that AFLP fingerprinting of bacterial genomes can be considered a highly reproducible technique that results in the generation of new databases for identification purposes. In this study, the level of reproducibility was determined by using the AFLP pattern of strain LMG 13459 as an internal reference during each electrophoresis run. Following normalization and subtraction of background values, we found that the intragel-specific correlation levels for the reference samples were between 95.0 and 98.5%. Minimal differences in the correlation values between reference lanes were usually introduced by inevitable variations in background intensities and electrophoresis times. On the whole, these empirical parameters did not significantly affect the results derived from the cluster analysis.

Cluster analysis of AFLP data. Using the unweighted pair group method with arithmetic averages for clustering, we identified a total of 13 AFLP clusters (clusters I through XIII) when taxa were delineated at a correlation level of 43% (Fig. 1). Each of the clusters except clusters II, III, VIII, and IX,

TABLE 1. Strains used

Taxon	HG ^a	Strain ^b	Designation as received (other designation[s]) ^b	Source of isolation
<i>A. hydrophila</i>	HG1	LMG 2844 ^T	ATCC 7966 ^T (A 306 ^T)	Milk with fishy odor
		LMG 13439	A 17	Unknown
		LMG 13442	A 52	Feces
		LMG 13443	A 229	Feces
		LMG 13656	A 82	Feces
		LMG 13658	A 162	Feces
		LMG 13660	A 188	Bactec vial
<i>A. hydrophila</i>	HG2	LMG 13444 ^c	A 307	Diseased fish
		LMG 13446	A 13	Environmental
		LMG 13447	A 39	Water
		LMG 13448	A 169	Feces
		LMG 13662	A 295	Feces
<i>A. hydrophila</i>	HG3	LMG 13449	A 99	Feces
		LMG 13450	A 63	Feces
		LMG 13451 ^c	A 308	Freshwater
		LMG 13453	A 8	Environmental
		LMG 13674	A 14	Environmental
		LMG 13675	A 15	Environmental
<i>A. salmonicida</i> subsp. <i>salmonicida</i>	HG3	LMG 3780 ^T	NCMB 1102 ^T (ATCC 33658 ^T)	Atlantic salmon
<i>A. salmonicida</i> subsp. <i>masoucida</i>	HG3	LMG 3782 ^T	ATCC 27013 ^T (NCMB 2020 ^T)	<i>Oncorhynchus masou</i>
<i>A. salmonicida</i> subsp. <i>achromogenes</i>	HG3	LMG 14900 ^T	ATCC 33659 ^T (NCMB 1110 ^T)	Brown trout
<i>A. salmonicida</i> subsp. <i>smithia</i>	HG3	LMG 16264 ^T	CCM 4103 ^T (ATCC 49393 ^T)	Ulcer on <i>Rutilus rutilus</i>
<i>A. caviae</i>	HG4	LMG 3775 ^T	ATCC 15468 ^T (A 309 ^T)	Epizootic guinea pig
		LMG 13454	A 1	Human
		LMG 13456	A 41	Feces
		LMG 13457	A 102	Feces
		LMG 13676	A 12	Environmental
		LMG 13678	A 26	Blood culture
		LMG 13680	A 61	Feces
<i>A. caviae</i>	HG5A	LMG 13459 ^c	A 310	Fish
		LMG 13460	A 6	Environmental
		LMG 13461	A 75	Feces
		LMG 13681	A 163	Feces
		LMG 13683	A 208	Feces
		LMG 13684	A 225	Feces
<i>A. caviae</i>	HG5B	LMG 13465	A 117	Feces
		LMG 13467	A 242	Feces
		LMG 13468	A 284	Feces
<i>A. media</i>	HG5B	LMG 9073 ^T	ATCC 33907 ^T (A 912 ^T)	Water
		LMG 14687	BA 91	Fish farm, England
		LMG 14688	BA 92	Fish farm, England
		LMG 14689	BA 93	Fish farm, England
<i>A. eucrenophila</i>	HG6	LMG 3774 ^T	NCMB 74 ^T (A 311 ^T)	Freshwater fish
		LMG 13057	CCUG 30341 (A 914)	Unknown
		LMG 13058	CCUG 30342 (A 1650)	Carp
		LMG 13060	CCUG 30344 (A 1652)	Water
		LMG 13061	CCUG 30345 (A 1653)	Water
		LMG 13062	CCUG 30346 (A 1654)	Groundwater
		LMG 13687	CCUG 30343 (A 1651)	Water
		LMG 13691	CCUG 30347 (A 1655)	Groundwater
<i>A. sobria</i>	HG7	LMG 3783 ^T	CIP 74.33 ^T (A 312 ^T)	Fish
		LMG 13469	A 915	Fish
<i>A. veronii</i> biogroup <i>sobria</i>	HG8	LMG 13067	CCUG 30356 (A 10)	Environmental
		LMG 13068	CCUG 30357 (A 64)	Feces
		LMG 13070	CCUG 30359 (A 68)	Feces
		LMG 13071	CCUG 30360 (A 132)	Feces
		LMG 13072	CCUG 30361 (A 151)	Feces
		LMG 13073	CCUG 30362 (A 155)	Feces
		LMG 13074	CCUG 30363 (A 916)	Feces
		LMG 13693	A 11	Environmental
		LMG 13694	A 27	Unknown
		LMG 13695	A 28	Feces
		LMG 13700	A 168	Feces
<i>A. jandaei</i>	HG9	LMG 12221 ^T	ATCC 49568 ^T (A 1642 ^T)	Feces
		LMG 13064	CCUG 30348 (A 179)	Feces
		LMG 13065	CCUG 30350 (A 919)	Feces
		LMG 13066	CCUG 30351 (A 950)	Unknown

Continued on following page

TABLE 1—Continued

Taxon	HG ^a	Strain ^b	Designation as received (other designation[s]) ^b	Source of isolation
<i>A. veronii</i> biogroup <i>veronii</i>	HG10	LMG 13077	CCUG 30352 (A 1641, ATCC 49570)	Wound below left eye (human)
		LMG 13079	CCUG 30355 (A 1644, ATCC 49571)	Leg wound, diver
		LMG 9075 ^T	ATCC 35624 ^T (A 901 ^T)	Near-drowning victim
		LMG 16183	A 920 (CDC 1067-84)	Stool, New Mexico
		LMG 16332	ATCC 35622 (CDC 0140-84)	Stool, Connecticut
<i>A. veronii</i> -like	HG11	LMG 16333	ATCC 35623 (CDC 0964-83)	Stool, Pennsylvania
		LMG 16334	ATCC 35625 (CDC 1170-83)	Wound, Michigan
		LMG 13075	CCUG 30364 (A 902, ATCC 35941)	Ankle fracture
		LMG 13076	CCUG 30365 (A 926)	Water, Mohawk River
		LMG 9074 ^T	ATCC 43700 ^T (A 903 ^T)	Abscess on forehead
<i>A. schubertii</i>	HG12	LMG 12655	CCUG 25582	Human leg wound
		LMG 12668	CCUG 25583	Human leg wound
		LMG 13473	A 922 (ATCC 43701)	Human skin
		LMG 12223 ^T	ATCC 49657 ^T (A 1646 ^T)	Human stool, India
		LMG 13080	CCUG 30366 (A 1645, ATCC 49659)	Appendix, California
<i>A. trota</i>	HG13	LMG 13081	CCUG 30368 (A 1647, ATCC 49660)	Human stool, Thailand
		LMG 13082	CCUG 30369 (A 1648, ATCC 49658)	Human stool, Bangladesh
		LMG 13083	CCUG 30370 (A 1649, ATCC 49661)	Human stool, Indonesia
		LMG 12646 ^T	DSM 6394 ^T	Human stool, India
		LMG 12645 ^T	DSM 6393 ^T	Surface water
<i>A. enteropelogenes</i>	ND ^d	LMG 14021	ATCC 35942	Human stool, South Carolina
<i>A. ichthiosmia</i>	ND	LMG 14058	CECT 4200	Diseased elver, Valencia, Spain
<i>A. allosaccharophila</i>	ND	LMG 14059 ^T	CECT 4199 ^T	Diseased elver, Valencia, Spain
		LMG 16328	CECT 4340 (S 177)	European eel, Valencia, Spain
		LMG 16329	CECT 4341 (S 176)	European eel, Valencia, Spain
		LMG 16330 ^T	CECT 4342 ^T (S 181 ^T)	European eel, Valencia, Spain
		LMG 16331	CECT 4343 (S 191)	European eel, Valencia, Spain
<i>Aeromonas</i> sp.	ND	LMG 13470	A 925	Kidney of tarbot
		LMG 13471	A 923	Kidney of brook trout
		LMG 13472	A 924	Kidney of rainbow trout
		LMG 16184	A 921 (CDC 0715-84)	Stool, South Carolina
		LMG 4242 ^T	NCTC 10360 ^T (ATCC 14029 ^T)	Dog feces
<i>P. shigelloides</i>	ND	LMG 12657	CCUG 12881B	Human feces
		LMG 12658	CCUG 12890	Human cholecystitis
		LMG 12674	CCUG 10616D	Aquarium sludge

^a HG, as defined at the Centers for Disease Control (19).

^b A, Institute of Medical Microbiology, Zürich, Switzerland; ATCC, American Type Culture Collection, Rockville, Md.; BA, Heriot-Watt University, Riccarton, Edinburgh, Scotland; CCM, Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic; CCUG, Culture Collection of the University Göteborg, Göteborg, Sweden; CDC, Centers for Disease Control, United States Public Health Service, Atlanta, Ga.; CECT, Colección Española de Cultivos Tipo, Universidad de Valencia, Valencia, Spain; CIP, Collection bactérienne de l'Institut Pasteur, Paris, France; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; LMG, Culture Collection of the Laboratorium voor Microbiologie Gent, Universiteit Gent, Ghent, Belgium; NCMB, National Collection of Marine Bacteria, Aberdeen, Scotland; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom; S, Departamento de Microbiología y Ecología, Universitat de València, Valencia, Spain.

^c HG reference strain.

^d ND, not defined.

which are discussed below, contained strains belonging to one HG. Within these genotypically defined groups, each strain usually produced a unique band pattern which could be used as a genomic fingerprint for bacterial identification and typing at the strain level. In some cases, however, the AFLP patterns of strains belonging to an HG appeared to be very similar, if not identical. For instance, strains LMG 14688 and LMG 14689 (cluster V) grouped together at a correlation level of 98.1% on the basis of their very similar band patterns (Fig. 1). Since these two isolates originated from the same site (Table 1) and were probably isolated at the same time, it is possible that these strains represent the same bacterial clone. Clearly, additional AFLP-based research (e.g., research in which other primer combinations are used) can be performed to determine the true clonal identity of strains which produced very similar patterns in this study. Below, all AFLP clusters shown in Fig. 1 are discussed with regard to their taxonomic significance and their relationship to DNA-DNA hybridization results and other data.

Together, clusters I and II comprise eight strains belonging to *Aeromonas eucrenophila* (HG6), four *A. encheleia* strains, and two isolates allocated to genomic species 11 (*Aeromonas veronii*-like); the latter taxon was mentioned for the first time by Hickman-Brenner et al. (21) in the original description of the ornithine decarboxylase-positive (ODC⁺) species *Aeromonas veronii* (now *A. veronii* biogroup *veronii* HG10). Hickman-Brenner et al. found that human ODC⁺ isolate LMG 13075 does not belong to *A. veronii* because its level of relatedness to the type strain of HG10 (strain LMG 9075) was much less than 70% at 60°C as determined by DNA hybridization. Ever since, this strain has been placed in HG11 together with environmental isolate LMG 13076, and these strains may represent an unnamed species belonging to the genus *Aeromonas*. More recently, phenotypic (10) and chemotaxonomic (26) evidence has shown that HG11 does not belong to *A. veronii* biogroup *veronii* but belongs to the *Aeromonas caviae* complex (which comprises HG4, HG5A, HG5B, and HG6). Our AFLP data confirmed these findings because strains LMG 13075 and

10 20 30 40 50 60 70 80 90 100

Cluster no

A. eucrenophila HG6 LMG 13057
A. eucrenophila HG6 LMG 3774¹
A. eucrenophila HG6 LMG 13058
A. eucrenophila HG6 LMG 13687
A. eucrenophila HG6 LMG 13060

I

A. veronii HG11 LMG 13075
A. veronii HG11 LMG 13076
A. eucrenophila HG6 LMG 13061
A. eucrenophila HG6 LMG 13691
A. eucrenophila HG6 LMG 13062
A. encheleia LMG 16330¹
A. encheleia LMG 16331
A. encheleia LMG 16329
A. encheleia LMG 16328

II

A. trota HG13 LMG 12223¹
A. trota HG13 LMG 13080
A. trota HG13 LMG 13081
A. trota HG13 LMG 13082
A. enteropelogenes LMG 12646¹
A. trota HG13 LMG 13083

III

A. caviae HG5A LMG 13683
A. caviae HG5A LMG 13459⁸
A. caviae HG5A LMG 13681
A. caviae HG5A LMG 13684
A. caviae HG5A LMG 13461
A. caviae HG5A LMG 13460

IV

A. caviar HG5B LMG 13465
A. caviar HG5B LMG 13467
A. caviar HG5B LMG 13468
A. media HG5B LMG 14689
A. media HG5B LMG 14688
A. media HG5B LMG 14687
A. media HG5B LMG 9073¹

V

A. caviae HG4 LMG 13457
A. caviar HG4 LMG 13678
A. caviar HG4 LMG 13680
A. caviar HG4 LMG 3775¹
A. caviar HG4 LMG 13454
A. caviar HG4 LMG 13676
A. caviar HG4 LMG 13456

VI

A. hydrophila HG2 LMG 13446
A. hydrophila HG2 LMG 13448
A. hydrophila HG2 LMG 13662
A. hydrophila HG2 LMG 13444⁸
A. hydrophila HG2 LMG 13447

VII

A. veronii biogroup sobria HG8 LMG 13071
A. veronii biogroup sobria HG8 LMG 13072
A. veronii biogroup sobria HG8 LMG 13073
A. veronii biogroup sobria HG8 LMG 13700
A. veronii biogroup sobria HG8 LMG 13070
A. allosaccharophila LMG 14058
A. allosaccharophila LMG 14059¹
A. sp. LMG 16184
A. allosaccharophila LMG 14021
A. veronii biogroup sobria HG8 LMG 13074

VIIIa

A. veronii biogroup sobria HG8 LMG 13068
A. veronii biogroup sobria HG8 LMG 13693
A. veronii biogroup sobria HG8 LMG 13695
A. ichthiosmia LMG 12645¹
A. veronii biogroup sobria HG8 LMG 13694
A. veronii biogroup sobria HG8 LMG 13067
A. veronii biogroup veronii HG10 LMG 16334
A. veronii biogroup veronii HG10 LMG 16333
A. veronii biogroup veronii HG10 LMG 16332
A. veronii biogroup veronii HG10 LMG 9075¹
A. veronii biogroup veronii HG10 LMG 16183

VIIIb

A. sobria HG7 LMG 13469
A. sp. LMG 13470
A. sp. LMG 13471
A. sp. LMG 13472
A. sobria HG7 LMG 3783¹

IX

A. jandaei HG9 LMG 12221¹
A. jandaei HG9 LMG 13064
A. jandaei HG9 LMG 13066
A. jandaei HG9 LMG 13065
A. jandaei HG9 LMG 13079
A. jandaei HG9 LMG 13077

X

A. hydrophila HG1 LMG 13660
A. hydrophila HG1 LMG 13658
A. hydrophila HG1 LMG 2844¹
A. hydrophila HG1 LMG 13439
A. hydrophila HG1 LMG 13443
A. hydrophila HG1 LMG 13656
A. hydrophila HG1 LMG 13442

XI

A. hydrophila HG3 LMG 13449
A. hydrophila HG3 LMG 13451⁸
A. hydrophila HG3 LMG 13674
A. hydrophila HG3 LMG 13675
A. hydrophila HG3 LMG 13453
A. hydrophila HG3 LMG 13450
A. salmonicida subsp. *salmonicida* HG3 LMG 3780¹
A. salmonicida subsp. *achromogenes* HG3 LMG 14900¹
A. salmonicida subsp. *maconculda* HG3 LMG 3782¹
A. salmonicida subsp. *smithii* HG3 LMG 16264¹

XII

A. schubertii HG12 LMG 12668
A. schubertii HG12 LMG 13473
A. schubertii HG12 LMG 9074¹
A. schubertii HG12 LMG 12655

XIII

LMG 13076 were found in cluster II and clearly grouped together with three representatives of HG6. In addition, cluster II also contains the four isolates recovered from European eels (strains LMG 16328, LMG 16329, LMG 16330^T [T = type strain], and LMG 16331) which were recently found to belong to a new *Aeromonas* species named *A. encheleia* (13). We found that in the AFLP analysis *A. encheleia* LMG 16329, LMG 16330^T, and LMG 16331 produced very similar band patterns which had a correlation level of 95.3%, as determined by cluster analysis. Strain LMG 16328, whose level of DNA homology with labeled LMG 16330^T DNA was only 70% (13), differed somewhat from strains LMG 16329, LMG 16330^T, and LMG 16331, as shown in Fig. 1. However, all four *A. encheleia* strains were clearly members of the same AFLP cluster (cluster II), which also includes members of HG6 and HG11. Thus, the results described above are not consistent with the genotypic and phenotypic data from which Esteve et al. (13) concluded that strains LMG 16328, LMG 16329, LMG 16330^T, and LMG 16331 belong to a separate *Aeromonas* species. To further examine the observed discordance between DNA homology results and AFLP data, it would be worthwhile to perform additional DNA-DNA hybridization experiments with *A. encheleia* and *A. eucrenophila* in which well-characterized HG6 reference strains other than type strain LMG 3774 are used (13). In fact, our AFLP data indicated that LMG 3774^T was a member of cluster I, which also includes four other *A. eucrenophila* strains. The level of correlation for clusters I and II is only 35.2%, which is about 8% below the delineation level, as shown in Fig. 1. From the results described above, it could be suggested that the phenospecies *A. eucrenophila* actually consists of two subgroups which, at the moment, can be separated only genotypically by AFLP fingerprinting. A polyphasic taxonomic study is under way to further examine the possible subdivision of HG6, as well as to clarify the taxonomic positions of genomic species 11 and *A. encheleia* in the genus *Aeromonas*.

Cluster III contains five strains belonging to the ampicillin-susceptible (Amp^s) species *Aeromonas trota* (HG13) and the type strain of *A. enteropelogenes* (LMG 12646). The latter species was originally proposed by Schubert et al. (41) for a group of *Aeromonas sobria*-like psychrotrophic aeromonads which can be phenotypically and genotypically separated from all other *Aeromonas* species that have been described. However, Schubert et al. provided DNA-DNA homology data for only three other species (*A. hydrophila*, *A. sobria*, and *A. veronii*) and thus did not include all members of the genus *Aeromonas* in their study. Moreover, phylogenetic evidence (11) has brought into serious question the species status of *A. enteropelogenes* by revealing that the 16S rRNA sequence of LMG 12646^T exhibits 100% similarity with the 16S rRNA sequence of the type strain of *A. trota* (strain LMG 12223). Consequently, Collins et al. (11) suggested that additional DNA-DNA hybridization studies should be performed in order to determine the true genomic relatedness of the two taxa. To some extent, we addressed this problem by placing the type strain of *A. enteropelogenes* in cluster III on the basis of its AFLP pattern. In addition, Carnahan (8) mentioned that the phenotypic profile of *A. enteropelogenes* (41) is consistent with the biochemical properties of *A. trota* (9). However, no data

concerning the susceptibility of *A. enteropelogenes* strains to ampicillin have been published.

Clusters IV and V contain HG5A (phenotypically *A. caviae*) and HG5B (phenotypically *Aeromonas media* or *A. caviae*), respectively; the correlation level for these taxa is 35.2%. This relatively high level of linkage reflects the fact that these two groups are more closely related as determined by DNA-DNA hybridization than any other *Aeromonas* HGs (3). In fact, it has been suggested (6) that HG5A and HG5B should be considered two subspecies of *A. media*, since HG5B contains the type strain of *A. media* (strain LMG 9073). To examine this proposal, the AFLP technique should prove to be a valuable tool for discriminating HG5A and HG5B at a high level of resolution. In addition, we determined that the striking biochemical and physiological differences between the nonmotile *A. media* strains (which do not utilize sucrose as a sole carbon source and produce a brown diffusible pigment) and the motile *A. caviae* isolates obtained from clinical sources (which utilize sucrose as a sole carbon source and do not produce a brown diffusible pigment) belonging to HG5B are consistent with the fact that all four *A. media* strains included in our study (Table 1) belong to a separate subgroup within cluster V (Fig. 1). Moreover, these results may indicate that HG5B should be subdivided into two biogroups (6), analogous to the taxonomic position of *A. veronii* in cluster VIII (see below).

Cluster VI comprises seven strains, mainly from clinical sources, which have been classified as members of *A. caviae* HG4. As described above, members of the phenospecies *A. caviae* exhibit a wide range of genomic relatedness values and, as a result, have been placed in HG4, HG5A, and HG5B. However, the type strain of *A. caviae* (strain LMG 3775), is a member of HG4 and can also be differentiated from HG5 by phenotypic (29) and chemotaxonomic (26) characteristics.

Clusters VII, XI, and XII are composed of strains belonging to HG2, HG1, and HG3, respectively, of the *A. hydrophila* complex. Ever since Popoff et al. (38) delineated three DNA homology groups (now HG1, HG2, and HG3) in the phenospecies *A. hydrophila*, workers have questioned whether these groups can also be differentiated by phenotypic characteristics. Over the years, several studies (1, 6, 15) have indeed shown that a limited set of biochemical tests can be used to distinguish HG1, HG2, and HG3. As a result, strains belonging to HG1 (cluster XI) have been placed in the type species of the genus *Aeromonas*, *A. hydrophila*, since HG1 includes the type strain of this phenospecies (strain LMG 2844). Along with the DNA-DNA hybridization results (38), the currently available phenotypic data indicate that HG2 (cluster VII) also deserves recognition as a separate phenospecies of the genus *Aeromonas* rather than recognition as an unnamed genomic subgroup of *A. hydrophila*. In fact, Ali et al. (2) proposed the name "*Aeromonas bestiarum*" at the Fourth International Symposium on *Aeromonas* and *Plesiomonas*; the specific epithet of this taxon refers to the fact that most HG2 strains originate from environmental and veterinary sources. Moreover, these authors suggested that a cutoff DNA-DNA homology value of 85% at 60°C allowed them to separate "*A. bestiarum*" from all other *Aeromonas* species. In close agreement with these findings, Hänninen (15) reported that the levels of DNA relatedness between a collection of mainly environmental HG2 strains and

FIG. 1. Dendrogram derived from an unweighted average pair group cluster analysis of the AFLP patterns of all 98 *Aeromonas* strains included in this study. The tracks show the processed band patterns after conversion, normalization, and subtraction of background. Levels of linkage are expressed as the Pearson product-moment similarity coefficients. The level of cluster delineation (43%) is indicated by an arrow. As indicated in the text, HG11 is phenotypically similar to *A. veronii*, but represents an unnamed *Aeromonas* species.

the reference strain of *A. hydrophila* HG3 (strain LMG 13451) were 45 to 87% at the optimal temperature (60°C). Clearly, these data suggest that HG2 is genotypically more closely related to HG1 and HG3 than Popoff et al. (38) realized. Moreover, since the DNA homology values for members of the HGs belonging to the *A. hydrophila* complex (2, 15) do not strictly support the phylogenetic definition of a bacterial species (46), perhaps subspecies rank should be considered for HG2. However, the AFLP results in Fig. 1 leave no doubt about the genotypic separation of the HG2 strains in cluster VII from HG1 and HG3, which belong in clusters XI and XII, respectively. On the other hand, the taxonomic position of HG3 is complicated by the fact that two easily separable *Aeromonas* phenospecies apparently exhibit enough DNA relatedness to be classified in the same DNA homology group. It could be suggested that HG3 should correspond to the name *A. salmonicida* because this HG contains the type strains of *A. salmonicida* subsp. *salmonicida* (LMG 3780), *A. salmonicida* subsp. *achromogenes* (LMG 14900), *A. salmonicida* subsp. *masoucida* (LMG 3782), and *A. salmonicida* subsp. *smithia* (LMG 16264), which represent a group of psychrophilic, nonmotile, fish-associated aeromonads. Altwegg et al. (6) suggested that *A. hydrophila* HG3 should be classified in a new subspecies of *A. salmonicida*, although it has never been reported that motile HG3 strains are strictly fish associated or might represent a potential danger to fish farm industries. Still, it is clear that each proposal for the naming of HG3 should take into account both the phenotypic diversity and the genetic similarity of the constituent subgroups without introducing any confusion by nomenclatural changes.

Cluster VIII is defined by the AFLP patterns of 21 strains that belong to the phenospecies *A. veronii* biogroup *sobria* (HG8), *A. veronii* biogroup *veronii* (HG10), *A. ichthiosmia*, and *A. allosaccharophila*. It has been reported previously (6) that *A. veronii* biogroup *sobria* and *A. veronii* biogroup *veronii* exhibit a very high level of genomic relatedness, which results in the placement of both taxa in the same hybridization group (HG8/10). On the other hand, the striking phenotypic diversity within HG8/10 has led to the conclusion that *A. veronii* should be split into two biogroups corresponding to HG8 and HG10. Consistent with the DNA-DNA hybridization results, the AFLP patterns of cluster VIII strains (Fig. 1) do not permit clear-cut differentiation between the two biogroups. A similar phenomenon has been reported by Altwegg et al. (5, 6), who noticed that many of the HG8/10 strains used in our study were actually spread over several phenons when they performed a cluster analysis with data derived from multilocus enzyme electrophoresis. In fact, we determined that phenon 3 and 4 revealed by multilocus enzyme electrophoresis (5, 6) correspond to some extent to clusters VIIIA and VIIIB, respectively (Fig. 1). For instance, cluster VIIIA strains LMG 13073, LMG 13074, and LMG 16184 were also members of phenon 3. However, strain LMG 16184 has never been unequivocally assigned to HG8/10 because it exhibited only 69% DNA relatedness with the type strain of HG10 (strain LMG 9075) under optimal reassociation conditions (21). Therefore, it has been suggested that strain LMG 16184 has diverged from strain LMG 9075^T, which belongs to cluster VIIIB. This latter cluster also includes the type strain of *A. ichthiosmia* (strain LMG 12645), a psychrotrophic species that consists of *A. sobria*-like aeromonads (42). Like the species rank of *A. enteropelogenes* (see above), the species rank of *A. ichthiosmia* is still open to question because the 16S rRNA gene sequence of LMG 12645^T is identical to that of the type strain of *A. veronii* (11). Our fingerprinting data again support the phylogenetic results, despite the inability of the AFLP technique to distinguish be-

tween the two *A. veronii* biogroups. Since all strains designated *A. ichthiosmia* lack the potential for ornithine decarboxylation and esculin hydrolysis (42), two key tests which are uniformly positive for *A. veronii* biogroup *veronii* isolates (21), it seems reasonable to classify *A. ichthiosmia* in *A. veronii* biogroup *sobria* on the basis of phenotypic characteristics. Still, additional DNA homology studies are needed to verify this classification. Likewise, DNA-DNA hybridizations have also been used in an attempt to clarify the taxonomic position of *A. allosaccharophila*, a recently described mesophilic member of the genus *Aeromonas* (12) which belongs to cluster VIIIA. In the original description of this species (33), Martinez-Murcia et al. concluded on the basis of phenotypic and phylogenetic data that three biochemically atypical *Aeromonas* strains (LMG 14021, LMG 14058, and LMG 14059^T), which have identical 16S rRNA sequences, in fact represent a new genomic species closely related to *A. sobria* and *A. veronii*. On the basis of DNA homology data (12), *A. allosaccharophila* was found to be a very homogeneous taxon, although unlabelled DNA from strain LMG 14021 exhibited only 70% homology under optimal reassociation conditions with labelled DNA from the type strain (strain LMG 14059). Strains LMG 14058 and LMG 14059^T, on the other hand, exhibited a level of relative genomic relatedness of 100%. In close agreement with these findings, the latter two strains also produced very similar AFLP patterns which were tightly linked at a correlation level of 96.2% (Fig. 1). Strain LMG 14021, however, was more closely related to *A. veronii* LMG 13074 and LMG 16184. Solely on the basis of AFLP data, it appears that *A. allosaccharophila* does not represent an additional genotypic group in the genus *Aeromonas*, as would be expected from DNA-DNA hybridization data (12), but rather should be considered a new phenospecies which seems genetically similar to HG8 and HG10. In light of these findings, it is our opinion that more DNA homology data are required to justify definition of *A. allosaccharophila* as HG14 (12).

Cluster IX joins two representatives of *A. sobria* HG7 with *Aeromonas* sp. strains LMG 13470, LMG 13471, and LMG 13472 at a correlation level of 45%. According to the phenotypic data of Kämpfer and Altwegg (29), strains LMG 13470, LMG 13471, and LMG 13472 are closely related to the type strain of *A. veronii* biogroup *veronii* HG10 (strain LMG 9075) and constitute a separate phenon in the genus *Aeromonas*. Since all of the strains in this phenon were biochemically distinct from HG8, Kämpfer and Altwegg considered them representatives of HG10. Consequently, strains LMG 13470, LMG 13471, and LMG 13472 were listed as members of *A. veronii* HG10 in our recent chemotaxonomic study of the use of fatty acid methyl ester profiles for discriminating between phenospecies and HGs in the genus *Aeromonas* (26); together with LMG 9075^T, these three strains were recovered in a separate fatty acid methyl ester cluster which allowed clear differentiation from HG8 isolates. However, it should be noted that this cluster was obtained by using mean fatty acid methyl ester data instead of individual fatty acid profiles. In fact, a cluster analysis of the individual fatty acid methyl ester profiles revealed that the type strain of HG10 (strain LMG 9075) is in no way similar to strains LMG 13470, LMG 13471, and LMG 13472 (23). This finding was readily confirmed by the results of our AFLP study (Fig. 1). In addition, it has been reported previously (5) that the five strains in AFLP cluster IX (Fig. 1) also constitute a distinct phenon in the genus *Aeromonas* on the basis of the results of a multilocus enzyme electrophoresis analysis. From both the genotypic point of view and the chemotaxonomic point of view it thus appears that strains LMG 13470, LMG 13471, and LMG 13472 may represent a new and

currently undefined *Aeromonas* taxon which is phenotypically similar to HG10 (29) but seems genotypically more closely related to HG7. Notwithstanding this, proper taxonomic allocation of these strains in the genus *Aeromonas* will require intensive DNA homology studies and additional phenotypic research.

Clusters X and XIII comprise representative strains of the phenospecies *Aeromonas jandaei* (HG9) and *Aeromonas schubertii* (HG12), respectively. The majority of these strains originated from human specimens (including feces, blood, skin, and wounds [Table 1]), illustrating the clinical significance of HG9 and HG12. In the original descriptions of *A. jandaei* (7) and *A. schubertii* (20), it was stated that both of these taxa have unique biochemical profiles which provide a limited number of simple tests for differentiation from other *Aeromonas* sp. On genotypic grounds, HG9 and HG12 indeed constitute two homogeneous AFLP clusters (Fig. 1), which confirms their taxonomic standing in the genus *Aeromonas*.

In conclusion, in this investigation we clearly elucidated the taxonomic significance and discriminative power of the AFLP technique for differentiating the current members of the genus *Aeromonas*. Moreover, the high overall level of correlation between our fingerprinting results and the previously published DNA hybridization data indicates that the AFLP concept can be considered a relatively fast, reproducible, high-resolution alternative to conventional DNA-DNA hybridization at the subgeneric level. In addition, some of the AFLP data presented in this paper strongly suggest that this technique will prove to be a valuable tool for refining the existing *Aeromonas* taxonomy. Most of the AFLP patterns produced in this study were used as representative library entries to generate a new HG-specific genotypic database named AERO94. At this time, we are evaluating the usefulness of the AERO94 library for identifying unknown *Aeromonas* isolates that originate from a wide range of environmental and clinical habitats.

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