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Signature region within the 16S rDNA sequences of *Aeromonas popoffii*

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Abstract

To identify a group of eight *Aeromonas* strains of our collection showing ribotyping patterns similar to those described for the species *Aeromonas popoffii*, 16S rRNA gene sequence analysis was performed. Results were in agreement with the DNA binding values, and allowed the identification of a 'signature region' differentiating the *A. popoffii* strains from all other members of the genus *Aeromonas*.

Keywords: *Aeromonas popoffii*; 16S rDNA; Signature region

1. Introduction

The genus *Aeromonas* comprises bacteria that are autochthonous inhabitants of aquatic environments [1], and have been increasingly recognised as pathogenic for both animals and human beings [2,3].

Currently, 16 species of *Aeromonas* are identified on the basis of DNA–DNA hybridisation, but many of the taxa remain difficult to distinguish from one another through phenotypic tests because of the limited number of discriminating characteristics [4].

Besides DNA–DNA hybridisation, several approaches have been proposed in order to differentiate between aeromonad hybridisation groups (HGs) including ribotyping and multilocus enzyme electrophoresis [5], restriction fragment length polymorphism (RFLP) [6], and amplified fragment length polymorphism (AFLP) [7]. The complete 16S rDNA sequences of members of the genus *Aeromonas* were published [8–10], and subsequently used to design PCR-primers and hybridisation probes for the specific detection of some species based on 16S rDNA regions which show enough variability to discriminate, with few exceptions, between species [11–13].

Recently, Huys and coworkers [14] have described the new species *Aeromonas popoffii*. Strains belonging to this species were isolated from Flemish drinking water production plants and Scottish drinking water supplies. In our laboratory, we performed ribotyping of 286 *Aeromonas* strains isolated from children suffering from diarrhoea, as well as from their domestic environments, including drinking and pipe system waters. By clustering the riboprofiles, we found a group of eight strains of environ-

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mental origin which could not be classed with certitude in any *Aeromonas* species.

The aim of this work was to classify our unidentified strains by comparing their low-molecular-eight bands patterns with those of reference strains of *A. popoffii* and by 16S rDNA sequencing, methods which have both been demonstrated to be valuable for the characterisation of *Aeromonas* genospecies [13,15].

2. Materials and methods

2.1. Bacterial strains

The strains of our collection (Table 1) were collected in the period between August 1992 and September 1993 from drinking water samples and a fountain swab taken during a study on the diffusion of *Aeromonas* strains in domestic environments of diarrhoeic children in our region (Canton Ticino, Switzerland).

Water samples were filtered through 0.45-μm nitrocellulose membranes and incubated on m-*Aeromonas* Selective Agar (Biolife, Milan, Italy) with 10 mg l^{-1} ampicillin at 30°C for 24 h. Columbia Agar Base with 5% sheep erythrocytes supplemented with 10 mg l^{-1} ampicillin was used for the direct plating of the swabs, for plating after enrichment in alkaline Peptone Water at pH 8.6 (incubated at room temperature for 24 h), and for subculturing colonies showing the typical characteristics of the genus after growing on m-*Aeromonas* Selective Agar. The strains were further analysed using classical tests to confirm the genus *Aeromonas*: Gram stain, oxidase, catalase, oxidation and fermentation of glucose, resistance to the vibriostatic compound 0/129.

Seven strains from the LMG collection (Table 1) were also analysed, and the following reference strains were included for the ribotyping: *A. hydrophila* ATCC 7966 (HG 1), *A. bestiarum* CDC 9533-76 (HG 2), and *A. hydrophila* A909 (HG 3).

The strains used in filter hybridisation assays were the following: *A. popoffii* strains LMG 17541T, LMG 17542, LMG 17543, F498B and F479E; *A. hydrophila* ATCC 7966 (HG 1); *A. bestiarum* CDC 9533-76 (HG 2); *A. salmonicida subsp. salmonicida* ATCC 33658 (HG 3); *A. caviae* ATCC 15468 (HG 4); *A. media* ATCC 33907 (HG 5B); *A. eucrenophila* NCMB 74 (HG 6); *A. sobria* CIP 7433 (HG 7); *A. veronii* ATCC 35624 (HG 8/10); *A. jandaei* ATCC 49568 (HG 9); *A. encheleia* ATCC 35941 (HG 11); *A. schubertii* ATCC 43700 (HG 12); *A. trota* ATCC 49657 (HG 13); *A. ichthiosmia* DSM 6393; *A. enteropelogenes* DSM 6394; *A. allosaccharophila* CECT 4199; *A. encheleia* DSM 11577.

2.2. Ribosomal DNA gene restriction fragment analysis

Purified genomic DNA samples were prepared according to the method described by Ausubel and coworkers [16] and successively digested with restriction endonuclease *Sma*I. Fragments were separated by electrophoresis on a 0.8% agarose gel in TBE buffer 1×(Tris-Borate-EDTA, pH 8.0) at 25 V for 14 h. The fragments were transferred to a positively charged nylon membrane as described in Maniatis et al. [17], fixed to the membrane for 30 min at room temperature and for 15 min at 80°C.

Plasmid pKK3535 [18], labelled with digoxigenin (DIG DNA labelling Kit, Boehringer Mannheim), was used as probe. Prehybridisation, hybridisation (both at 68°C), and the immunological detection of the fragments were performed according to the protocol of the DIG Nucleic Acid Detection Kit (Boehringer Mannheim).

Reconstructed images of the visualised ribotyping band patterns in the low-molecular-weight zone (0.8–4.0 kb) were scanned with Sharp JX-330 densitoscanner. Transmission image data were stored in TIFF files and were further processed by using the GelCompare software, version 3.1 (applied Maths, Kortrijk, Belgium). Levels of similarity between ribotyping profiles were calculated by using the band-matching Dice coefficient, and cluster analysis was performed by using the unweighted pair group method with arithmetic averages.

2.3. Sequencing methods

Genomic DNA was isolated as described above. The 16S rDNA was selectively amplified by PCR using the forward primer corresponding to position 8–26 of *Escherichia coli* rDNA (5′- AGAGTTT-

GATCATGGCTCA-3′), and the reverse primer corresponding to the complement of position 1411–1391 (5′- GTGTGACGGGCGGTGTGTA-3′). From 0.1 to 0.75 μg of the extracted DNA was mixed with 1 U of *Taq* DNA polymerase (Boerhinger Mannheim), 10×PCR Buffer (Boerhinger Mannheim), 10 mM of each dNTP (Boerhinger Mannheim), and 0.5 μM of each primer in a final volume of 50 μl and amplified by PCR using the GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer). After an initial denaturation at 94°C for 5 min, the mixture was subjected to 35 cycles, with each cycle consisting of denaturation for 30 s at 94°C, primer annealing at 52°C for 30 s, and chain extension at 72°C for 1 min (final extension 7 min).

The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen), and prepared for sequencing employing the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer), according to the respective manufacturer's instructions. Excess terminators were removed by spin-column purification (Centri-Sep spin columns, Princeton Separations) before preparing and loading the samples onto the ABI PRISM 310 Genetic Analyzer (Perkin-Elmer) as specified on the manufacturer's protocol.

Sequencing primers were the following (numbers in brackets indicate the relative position on *E. coli* rDNA): forward primers, 5′-GCTGGTCTGAGAGGATGA-3′ (289–334), 5′-GATAAGTTAGATGTGAA-3′ (592–624), 5′-CAAGCGGTGGAGCATGTG-3′ (936–953); and reverse primers, 5′-GCACCTGTGTTCTGATTCC-3′ (510–492), 5′-CTCTACAAGACTCTAGCTGG-3′ (896–877), 5′-GCTGATCATCCTCTCAGAC-3′ (1250–1232).

2.4. *A. popoffii* probe design, labelling and hybridisation conditions

From the 16S rDNA sequences of the *A. popoffii* strains, the oligonucleotide probe 5′-GTTGCTGGRTATTAGCCAA-3′ (corresponding to the complement of position 477–459 of *E. coli* rDNA) was designed, and purchased from Pharmacia Biotech. The *A. popoffii* oligonucleotide was labelled with fluorescein-11-dUTP by using the ECL 3′-end labelling kit, and detection was carried out as indicate in the manufacturer's protocol (Amersham). The stringency of the hybridisation was achieved in the post-hybridisation washes using as stringency wash buffer 0.2×SSC, 0.1% (w/v) SDS at 44°C.

2.5. Filter hybridisation assays

Four microlitres of purified nucleic acid [16] were dot-blotted on a nylon membrane, fixed and hybridised with the labelled *A. popoffii* probe following the conditions described above.

Table 1
A. popoffii strains used in the study

Strain[a]	Source of isolation	Geographical origin
LMG 17541[T]	Drinking water production plant	Oelegem (Belgium)
LMG 17542	Drinking water production plant	De Blankaart (Belgium)
LMG 17543	Drinking water production plant	Snellegem (Belgium)
LMG 17544	Drinking water production plant	Eeklo (Belgium)
LMG 17545	Drinking water production plant	Snellegem (Belgium)
LMG 17546	Drinking water service reservoir	Udny Station (Scotland)
LMG 17547	Drinking water treatment plant	Turriff (Scotland)
F479E	Fountain swab	Vezio (Switzerland)
F498B	Tap water	Cadro (Switzerland)
F533E	Tap water	Pregassona (Switzerland)
F539A	Tap water	Viganello (Switzerland)
F548B	Tap water	Viganello (Switzerland)
F548C	Tap water	Viganello (Switzerland)
F600B	Tap water	Viganello (Switzerland)
F600C	Tap water	Viganello (Switzerland)

[a]LMG, Culture Collection of the Laboratorium voor Microbiologie Gent, Universiteit Gent, Ghent, Belgium; F, culture collection of the Istituto Cantonale Batteriosierologico, Lugano, Switzerland.

For Southern blot assays, 10 μl of the 16S rDNA samples obtained by PCR were electrophoresed on 0.8% agarose gel and blotted onto a nylon membrane following the same procedure as for ribotyping. Hybridisation of the fragments with the labelled *A. popoffii* probe and detection were performed at the conditions described. The autoradiography film (Hyperfilm-ECL; Amersham) was exposed for 3 h.

3. Results and discussion

In 1997, Huys et al. [14] proposed the new species *A. popoffii* to accommodate mesophilic *Aeromonas* strains recovered from Flemish and Scottish drinking water production plants and reservoirs. The strains showed AFLP fingerprints constituting a genotypic cluster unidentified against their AFLP-based identification library, unique phenotypic profiles and ribotyping patterns, and DNA binding values to other *Aeromonas spp*. comprised between 22 and 63%.

In order to verify if eight strains of our collection, isolated from drinking water of our region, could belong to the species *A. popoffii*, we performed the ribotyping of our strains together with those of the LMG collection, and the reference strains of the hybridisation groups which were indicated by Huys et al. as the most closely related (HG 1, HG 2 and HG 3).

Clustering analysis of ribotyping patterns generated from the strains analysed (Fig. 1) clearly demonstrate that our strains are closely related to the *A. popoffii* reference strains. The strains formed a group that was distinct from the related taxa HG 1, HG 2 and HG 3. As already found by Huys et al. [14], no bands were present in the molecular weight region between 0.8 and 1.6 kb, whereas the band at 1.8 kb was present in all *A. popoffi* strains, except LMG 17542. The *A. popoffii* strains shared at least three bands with the reference strains of HG 1, HG 2 and HG 3 analysed. Besides the two bands in positions 0.8 and 1.6, described by Huys et al. [14], we found an additional band at 3.7 kb.

The 16S rDNA sequences (from position 8 to 1411 in the *E. coli* sequence) of all of our strains, as well as of those of strains LMG 17541^{T}, LMG 17542, LMG 17543 and LMG 17546, were determined by PCR-DNA sequencing.

The sequences of all the strains were nearly identical, and consisted of a continuous stretch of 1407

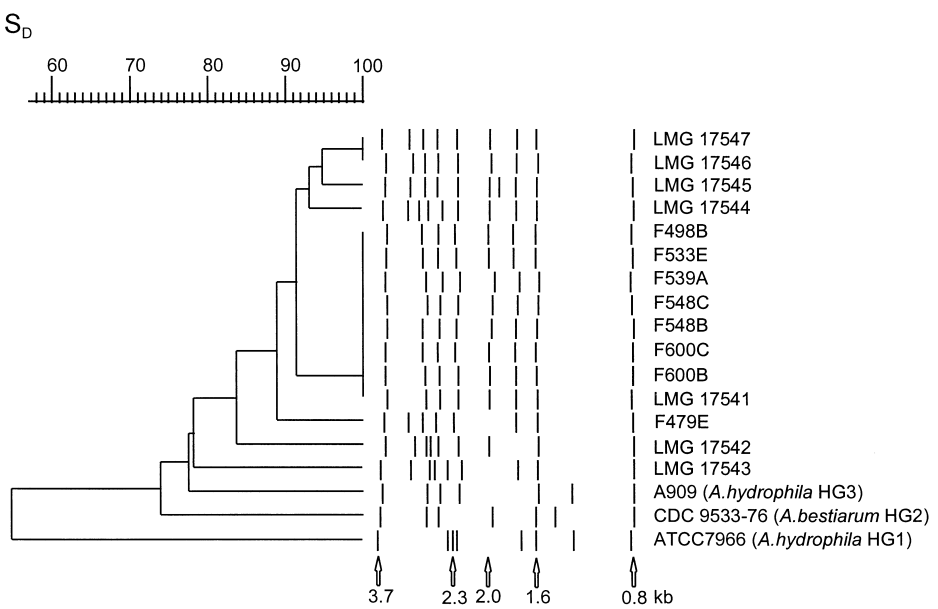

Fig. 1. Clustering analysis (unweighted pair-group method using arithmetic averages) of ribotyping patterns of 15 *A. popoffii* strains, and type and/or reference strains for *Aeromonas* HG 1, HG 2 and HG 3. Similarities are expressed as the band-matching Dice coefficient (S_D). Positions of the markers are indicated by arrows.

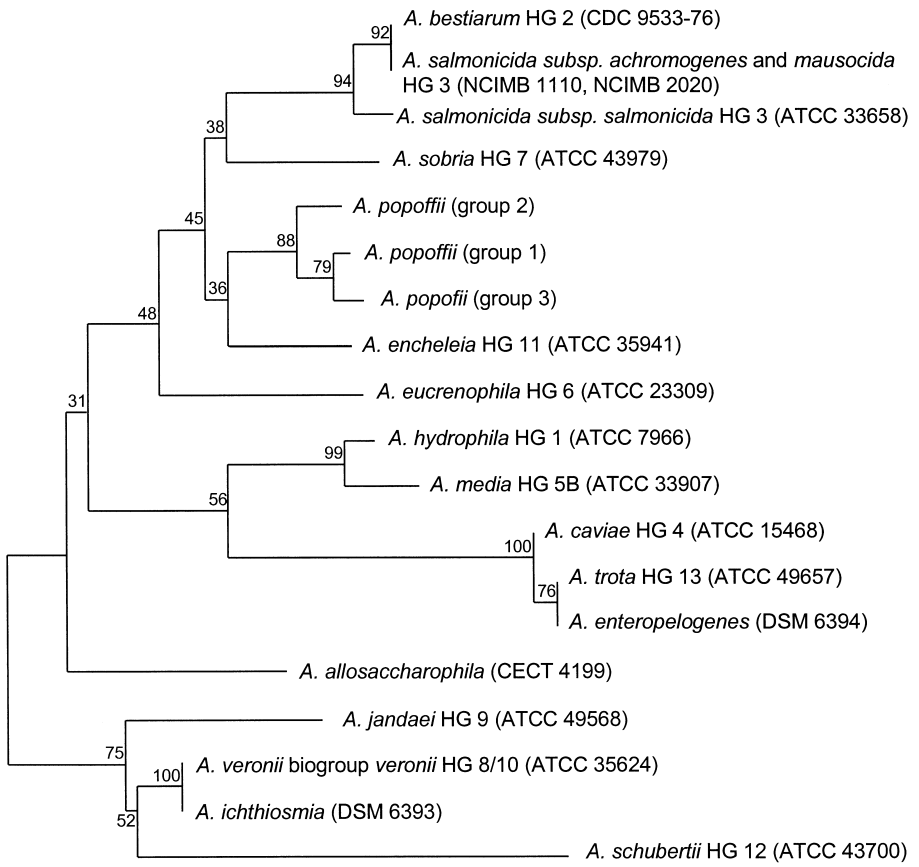

Fig. 2. Phylogenetic interrelationships in the genus *Aeromonas*. The tree was obtained using the Jukes–Cantor distance and the Neighbor-joining method [19] to compare 16S rDNA sequences of 1407 bp derived from our own or from published data (see Table 3). Numbers indicate the bootstrap confidence level (100 replications).

nucleotides representing more than 90% of the total 16S rRNA molecule. Although among the 12 strains analysed we found variations in five nucleotide positions, allowing for clustering the strains into three groups (Table 2), the analysis of the 12 *A. popoffii* sequences showed that they formed a distinct genetic line within the genus *Aeromonas* (Fig. 2).

The relationship between *Aeromonas* species derived by 16S rDNA sequencing generally correlates well with the results of DNA–DNA reassociation [8,9], although several discrepancies between the two approaches have been described [10]. Our study demonstrated that in the case of *A. popoffii*, the 16S rDNA sequencing results agree with the DNA binding values. The phylogenetic tree constructed on the basis of 1407 bp placed the new species in the group formed by *A. bestiarum* (HG 2), *A. salmonicida* (HG 3), *A. eucrenophila* (HG 6), *A. encheleia* (HG 11), and *A. sobria* (HG 7), which are all mainly from environmental origin.

The *A. popoffii* 16S rDNA sequences differed from those of other aeromonads at least in seven nucleotides. The sequence in position 154–167 (Table 3) was identical to that determined for *A. hydrophila* HG 1, *A. bestiarum* HG 2, *A. salmonicida* subsp. *salmonicida* HG 3, *A. caviae* HG 4, *A. media* HG 5B, *A. sobria* HG 7, *A. encheleia* HG 11, *A. trota* HG 13, *A. enteropelogenes*, and *A. allosaccharophila*, whereas the sequence in position 457–476 allowed the differentiation of all the *A. popoffii* strains from the other species. In fact, the nine *A. popoffii* strains showed a T instead of G or A in position 459 and a G instead of C or T in position 473 (Table 3).

An oligonucleotide probe was thus designed and

Table 2
Base differences in the 16S rDNA sequences among the *A. popoffii* strains analysed

Group	Strain	Positions[a]				
		469	559	782	1011	1018
1	LMG 17541^T, LMG 17546[b], F498B, F533E, F539A, F548B, F548C, F600B, F600C	C	A	A	C	G
2	LMG 17543, F479E	C	A	A	T	A
3	LMG 17542	T	R	R	C	G

The following sequences have been assigned EMBL accession numbers AJ223179 (F498B), AJ223180 (LMG 17542), AJ223181 (LMG 17543), and AJ224308 (LMG 17541^T).
[a]Referred to *E. coli* system.
[b]Strain LMG 17546 differs from the other strains of this group only in position 865 were we could not confirm the presence of the A observed in all *A. popoffii* strains (forward sequence giving A, but the reverse a T).

used in filter hybridisation assays in order to recognise this variable region. These methods of bacterial identification do not require the sequencing of fragments and therefore represent less expensive and simpler tools approachable by most laboratories for the identification of bacterial species. The probe permitted the specific identification of strains belonging to the species *A. popoffii* in dot blot (data not shown) and Southern blot of 16S rDNA fragments tests (Fig. 3).

In conclusion, the species *A. popoffii*, which was proposed by Huys et al. [14] on the basis of AFLP,

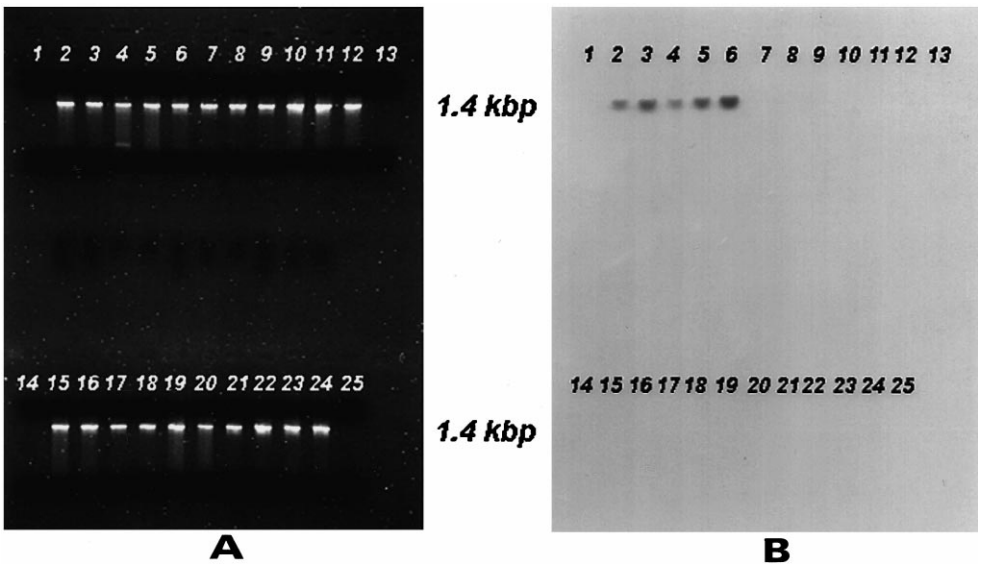

Fig. 3. Southern blot assay. (A) PCR performed with genomic DNA of *Aeromonas* strains and universal primers for 16S rRNA genes (see Section 2). (B) Southern blot hybridisation of the amplified 16S rRNA genes using labelled *A. popoffii* oligonucleotide as probe. Lane 1, negative control (no DNA); lane 2, *A. popoffii* LMG 17541^T; lane 3, *A. popoffii* LMG 17542; lane 4, *A. popoffii* LMG 17543, lane 5, *A. popoffii* F498B; lane 6, *A. popoffii* F479E; lane 7, *A. eucrenophila* NCMB 74 (HG 6); lane 8, *A. media* ATCC 33907 (HG 5B); lane 9, *A. schubertii* ATCC 43700 (HG 12); lane 10, *A. veronii* ATCC 35624 (HG 8/10); lane 11, *A. hydrophila* ATCC 7966 (HG 1); lane 12, *A. caviae* ATCC 15468 (HG 4); lane 13, negative control (no DNA); lane 14, negative control (no DNA); lane 15, *A. sobria* CIP 7433 (HG 7); lane 16, *A. bestiarum* CDC 9533-76 (HG 2); lane 17, *A. salmonicida subsp. salmonicida* ATCC 33658 (HG 3); lane 18, *A. jandaei* ATCC 49568 (HG 9); lane 19, *A. encheleia* ATCC 35941 (HG 11); lane 20, *A. trota* ATCC 49657 (HG 13); lane 21, *A. ichthiosmia* DSM 6393; lane 22, *A. enteropelogenes* DSM 6394; lane 23, *A. allosaccharophila* CECT 4199; lane 24, *A. encheleia* DSM 11577; lane 25, negative control (no DNA).

Table 3
16S rDNA sequences of *Aeromonas* strains at two variable regions corresponding to positions 154–167 and 457–476 of the *E. coli* 16S rDNA sequence

Taxon	DNA group	Strain[a]	Region 1 (position 154–167)	Region 2 (position 457–476)
A. hydrophila[b]	1	ATCC 7966	AGTTGGAAACGACTGCT	TGATGCCTAATACGTATCAA
A. bestiarum[c]	2	CDC 9533-76	AGTTGGAAACGACTGCT	TGGCGCCTAATACGTGTCAA
A. salmonicida subsp. salmonicida[b]	3	ATCC 33658	AGTTGGAAACGACTGCT	TGGCGCCTAATACGTGTCAA
A. caviae[b]	4	ATCC 15467	AGTTGGAAACGACTGCT	CAGTAGCTAATATCTGCTGG
A. media[b]	5B	ATCC 33907	AGTTGGAAACGACTGCT	TGATGCCTAATACGCATCAG
A. eucrenophila[b]	6	ATCC 23309	AGTTGGAAACGGCTGCT	TGATGCCTAATACGCATCAG
A. sobria[b]	7	ATCC 43979	AGTTGGAAACGACTGCT	TGGCAGCTAATATCTGTCAG
A. veronii biogroup veronii[b]	8/10	ATCC 35624	TACTGGAAACGGTAGCT	TGGTAGCTAATAACTGCCAG
A. jandaei[b]	9	ATCC 49568	TACTGGAAACGGTAGCT	CAGTAGCTAATATCTGCTGG
A. encheleia[b]	11	ATCC 35941	AGTTGGAAACGACTGCT	TGGTCGCTAATAACGGCCAA
A. schubertii[b]	12	ATCC 43700	TACTGGAAACGGTAGCT	TGGTGGTTAATACCTGCCAG
A. trota[b]	13	ATCC 49657	AGTTGGAAACGACTGCT	CAGTAGCTAATATCTGCTGG
A. enteropelogenes[d]	ND[f]	DSM 6394	AGTTGGAAACGACTGCT	CAGTAGCTAATATCTGCTGG
A. ichthiosmia[d]	ND	DSM 6393	TACTGGAAACGGTAGCT	TGGTAGCTAATAACTGCCAG
A. allosaccharophila[e]	ND	CECT 4199	AGTTGGAAACGACTGCT	TGGTAGCGAATAACTGCCAG
A. popoffii[c]	ND	LMG 17541[T]	AGTTGGAAACGACTGCT	TGTTGGCTAATACCCAGCAA

[a]A, Institute of Medical Microbiology, Zürich, Switzerland,; ATCC, American Type Culture Collection, Rockville, MD; CDC, Centers for Disease Control, United States Public Health Service, Atlanta, GA, USA; CECT, Colección Española de Cultivos Tipo, Universidad de Valéncia, Valéncia, Spain; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; LMG, Culture Collection of the Laboratorium voor Microbiologie Gent, Universiteit Gent, Ghent, Belgium.
[b]Data from [8].
[c]Our data.
[d]Data from [10].
[e]Data from [9].
[f]ND, not defined.

ribotyping and fatty acid profiles, phenotypic characteristics, and DNA–DNA hybridisation data, could also be identified by a unique primary structure of the 16S rRNA genes, and by a 'specific signature' in the variable region comprised between positions 457 and 476, which is the most highly diagnostic region for delineating *Aeromonas* genospecies. This sequence could also be recognised in filter hybridisation assays, showing that this zone represents effectively a signature region for this species.

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