Aeromonas bivalvium sp. nov., a novel species isolated from bivalve molluscs.

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Abbreviations:
AD, m-*Aeromonas* selective agar base Havelaar; ADH, arginine dehydrolase; BCCM™/LMG, Belgian Coordinated Collections of Microorganisms / Laboratorium voor Microbiologie from Universiteit Gent; bv., biovar; CECT, Spanish Type Culture Collection from Universitat de València; FAFLP, fluorescent amplified fragment length polymorphism; HG, hybridization group; LDC, lysine decarboxylase; MR, methyl red; O/129, 2,4-diamino-6,7-diisopropylpteridine; ODC, ornithine decarboxylase; TSA, tryptone soy agar; VP, Voges-Proskauer.

Footnote:
The GenBank accession numbers for the 16S rDNA sequences of strains 868E^T and 665N are DQ504429 and DQ504430, respectively.

SUMMARY

A polyphasic study was performed to determine the taxonomic position of two *Aeromonas* strains, 665N and 868E^T, isolated from bivalve molluscs that could not be identified at species level in a previous numerical taxonomy study. The DNA G+C content of these isolates ranged from 62·3 to 62·6 mol%. Sequence analysis of the 16S rRNA gene showed that both strains were closely related to members of the genus *Aeromonas*. Fluorescent Amplified Fragment Length Polymorphism (FAFLP) fingerprinting revealed that the isolates 665N and 868E^T clustered together with a similarity of 78 % but did not cluster with any of the *Aeromonas* genomospecies. DNA-DNA hybridization showed a high DNA-relatedness between both isolates (76 %) and a low DNA similarity with the phylogenetically more related *Aeromonas* genomospecies (30–44 %). Useful tests for the phenotypic differentiation of *A. bivalvium* from other mesophilic *Aeromonas* species included gas from glucose, lysine decarboxylase (LDC), Voges-Proskauer (VP), acid from L-arabinose, hydrolysis of aesculin and utilization of L-lactate. On the basis of genotypic and phenotypic evidences, the name *Aeromonas bivalvium* sp. nov. is proposed for these two isolates. The type strain is 868E^T (= CECT 7113^T = LMG 23376^T).

Members of the genus *Aeromonas*, belonging to the Class *Gammaproteobacteria*, are Gram-negative non-sporeforming bacilli or coccobacilli, facultatively anaerobic, chemoorganotrophic, oxidase and catalase positive, resistant to the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine), generally motile by a single polar flagellum and reduce nitrate to nitrite. Aeromonads are primarily aquatic, widespread in environmental habitats, frequently isolated from foods, often associated with aquatic animals and some species are primary or opportunistic pathogens in invertebrates and vertebrates including humans (Carnahan & Joseph, 2005).

At present, 17 *Aeromonas* species and 20 DNA-DNA hybridization groups (HG) have been described: *A. hydrophila* (HG1), *A. bestiarum* (HG2), *A. salmonicida* (HG3), *A. caviae* (HG4), *A. media* (HG5), *A. eucrenophila* (HG6), *A. sobria* (HG7), *A. veronii* bv. sobria (HG8/10), *A. jandaei* (HG9), *A. veronii* bv. veronii (HG10/8), *Aeromonas* sp. HG11, *A. schubertii* (HG12), *Aeromonas* sp. Group 501 (HG13), *A. trota* (HG14), *A. allosaccharophila* (HG15), *A. encheleia* (HG16), *A. popoffii* (HG17), *A. culicicola* (HG18), *A. simiae* (HG19) and *A. molluscorum* (HG20) (Pidiyar *et al*., 2002; Harf-Monteil *et al*., 2004; Miñana-Galbis *et al*., 2004; Carnahan & Joseph, 2005). In addition to continuous description of new species, the complexity of *Aeromonas* taxonomy is due to the isolation of motile and nonmotile,

mesophilic and psychrophilic, pigmented and non-pigmented strains within several *Aeromonas* species (Altwegg *et al*., 1990; Carnahan & Joseph, 2005), description of new subspecies of *A. hydrophila* (Huys *et al*., 2002; Huys *et al*., 2003) and *A. salmonicida* (Pavan *et al*., 2000), taxonomic rearrangement of *Aeromonas* sp. HG11 (Huys *et al*., 1997) and *A. culicicola* (Huys *et al*., 2005) and extended description of *A. eucrenophila* and *A. encheleia* (Huys *et al*., 1997) and *A. jandaei* (Esteve *et al*., 2003). In this context, some authors have proposed useful tables for the phenotypic differentiation of *Aeromonas* species by a limited number of key tests (Carson *et al*., 2001; Miñana-Galbis *et al*., 2002; Valera & Esteve, 2002; Abbott *et al*., 2003; Miñana-Galbis *et al*., 2004; Carnahan & Joseph, 2005). Genotypic classification of aeromonads at the genus level is currently recommended by DNA G+C content and 16S rRNA gene sequence analyses and DNA-DNA reassociation for species delineation, although other genomic methods, such as DNA profiling or *rpo*D and *gyr*B sequencing, are useful for *Aeromonas* species discrimination (Stackebrandt *et al*., 2002; Soler *et al*., 2004; Carnahan & Joseph, 2005; Morandi *et al*., 2005).

In the present study, a polyphasic approach was used in order to determine the taxonomic position of two *Aeromonas* strains isolated from bivalve molluscs that clustered together as a separate phenon (phenon VII) but could not be identified at species level in a previous phenotypic study of the genus *Aeromonas* (Miñana-Galbis *et al*., 2002). For this purpose, an extended phenotypic characterization, 16S rRNA gene sequencing, DNA G+C content, Fluorescent Amplified Fragment Length Polymorphism (FAFLP) analysis, and DNA-DNA hybridization were performed for both the isolates. Based on the results obtained, these strains represent a novel *Aeromonas* species, for which the name *Aeromonas bivalvium* sp. nov. is proposed.

The strains 868E^T (= CECT 7113^T = LMG 23376^T) and 665N (= CECT 7112 = LMG 23377) were isolated from cockles (*Cardium* sp.) and razor-shells (*Ensis* sp.), respectively, from retail markets in Barcelona (Spain). Isolation, growth and preservation of strains were performed as described previously (Miñana-Galbis *et al*., 2004).

Cell size, morphology and flagellar arrangement were determined by transmission electron microscopy (JEOL1010). Cells were grown on TSA supplemented with 0·5 % (w/v) NaCl for 24 h at 25 ºC and, after further suspension in MilliQ water, were examined by negative staining with 2 % (w/v) uranyl acetate.

Physiological and biochemical characterization, unless otherwise stated, was performed at 25–30 ºC and all media contained 1 % (w/v) NaCl. Gram-staining, motility, glucose oxidation-fermentation test, oxidase and catalase activity, nitrate reduction, indole production, susceptibility to vibriostatic agent O/129, swarming motility, production of a brown diffusible pigment, gas production from D-glucose, methyl red (MR) and Voges-Proskauer (VP) reactions, ONPG, hydrogen sulfide production from cysteine and thiosulfate, growth on MacConkey agar and m-*Aeromonas* selective agar base Havelaar (AD), salt tolerance, pH and temperature ranges for growth, acid production from carbohydrates, hydrolysis of aesculin, arbutin, DNA, elastin, erythrocytes, starch, urea and xanthine, utilization of substrates as sole carbon and energy sources and sensitivity to antibiotics were determined as described previously (Miñana-Galbis *et al*., 2002). Arginine dehydrolase (ADH), lysine decarboxylase (LDC) and ornithine decarboxylase (ODC) activity (Moeller's method) and gelatin hydrolysis by using tannic acid (1 %) as the gelatin-precipitating reagent were determined as described by Smibert & Krieg (1994).

For 16S rRNA gene sequencing and phylogenetic analysis, DNA was extracted by using a REALPURE genomic DNA extraction kit (RBMEG03; Durviz). Oligonucleotide primers used for PCR amplification and sequencing of the 16S rRNA gene were those described by Martínez-Murcia *et al*. (1999). DNA was subjected to PCR amplification in a total volume of 50 µl that contained 50 mM KCl, 15 mM Tris/HCl (pH 8·0), 1·5 mM $MgCl_2$, 0·2 mM each deoxyribonucleotide (dATP, dCTP, dGTP, dTTP; Amersham Biosciences), 1·25 U AmpliTaq Gold DNA polymerase (Applera) and 25 pmol each primer. The reaction mixtures were subjected to the following thermal cycling: an initial single step of 95 ºC for 9 min, 35 cycles of 94 ºC for 60 s, 51 ºC for 30 s and 72 ºC for 90 s, and a final single step of 72 ºC for 10 min. PCR products were purified by using Montage PCR centrifugal filter devices (Millipore) and prepared for sequencing by employing a BigDye Terminator v3·1 cycle sequencing kit (Applera). The amplified 16S rRNA genes were sequenced with an ABI PRISM 3730 DNA analyser by the Unitat de Genòmica of the Serveis Cientificotècnics of the Universitat de Barcelona.

The sequences obtained were aligned with 16S rRNA gene sequences of the type or reference strains of the *Aeromonas* DNA Hybridization Groups by using the DNASTAR Lasergene software (DNASTAR, Inc.). Distances and clustering with the neighbour-joining and maximum-parsimony methods (pairwise deletion and Kimura two-parameter model) were determined by using the MEGA program version 2·1 (Kumar *et al*., 2001). Stability of the relationships was assessed by bootstrapping (1000 replicates).

DNA fingerprinting using fluorescent amplified fragment length polymorphism (FAFLP), which included DNA extraction and purification (Gevers *et al*., 2001), FAFLP fingerprinting, data processing and

numerical analysis (Huys & Swings, 1999), was performed by the BCCM™/LMG (Belgian Coordinated Collections of Microorganisms / Laboratorium voor Microbiologie from Universiteit Gent) Identification Service.

For DNA-DNA hybridization and determination of DNA G+C content, genomic DNA of bacterial strains was prepared according to a modification of the procedure of Wilson (1987). The G+C content of each DNA sample was determined by three independent analyses using the HPLC technique (Mesbah *et al*., 1989). DNA-DNA hybridizations were performed in minimum four replicates at 47 ºC according to a modification of the method described by Ezaki *et al*. (1989). These analyses were also performed by the BCCM™/LMG Identification Service.

Both strains isolated from bivalve molluscs (868E^T and 665N) were identified as belonging to the genus *Aeromonas* as they were Gram-negative, rod-shaped, motile by one polar flagellum, oxidase-positive, facultatively anaerobic, glucose-fermentative and resistant to vibriostatic agent O/129, and they grew in the absence of NaCl but not at 7% (w/v) NaCl. Cell morphology of both isolates was from coccoid to rod (0·3–1·0 x 0·5–2 µm). Their antibiotic-resistance pattern was similar to that of most *Aeromonas* species (Kämpfer *et al*., 1999). Both strains were resistant to ampicillin and the strain 665N was also resistant to cephalothin in contrast to the strain 868E^T. These strains formed nonpigmented, circular colonies with a diameter of 3–4 mm on TSA when incubated at 25–30 ºC. The growth-temperature range was 4–37 ºC, although the strain 868E^T was able to grow at 40·5 ºC. Optimal growth occurred at 30–37 ºC. Our isolates showed several differentiating phenotypic features in relation to other mesophilic *Aeromonas* species (Table 1). Three or more tests allowed differentiation of strains 665N and 868E^T from all *Aeromonas* species except *A. caviae* and *A. media*. Positive reaction in the LDC test allowed the separation of these strains from *A. caviae* and *A. media*. In addition, they could be differentiated from *A. media* by the following tests: ONPG, brown pigment production, lactose and mannose fermentation and lactose, mannose and raffinose utilization. In contrast to *A. caviae*, the strains 665N and 868E^T were able to use glycerol and to produce acid from it. These results allowed the phenotypic discrimination of our strains from all *Aeromonas* taxa that have been described to date.

Comparative analysis of 16S rRNA gene sequences of the isolates 665N and 868E^T and those of all type or reference strains of *Aeromonas* species confirmed that our isolates belonged to the genus *Aeromonas*. 16S rRNA gene sequence similarity between both strains and rest of *Aeromonas* species ranged from 96·75 % (*A. simiae*) to 99·71 % (*A. popoffii*). Dendrograms generated by maximum-

parsimony and neighbour-joining methods were similar. The phylogenetic tree constructed by the neighbour-joining method is shown in Fig. 1. The isolates 665N and 868E^T showed two nucleotide differences between them and four and six nucleotides with regard to *A. popoffii*, respectively (Table 2).

The FAFLP banding patterns of our strains were compared with the laboratory database AEROLIB comprising well-characterized strains of all *Aeromonas* DNA HGs (Huys & Swings, 1999; Huys *et al*., 2003; Miñana-Galbis *et al*., 2004). This comparison revealed that the isolates 665N and 868E^T clustered together with a similarity of 78 % but did not cluster within one of the 20 *Aeromonas* DNA HGs currently described. Dendrogram of the cluster analysis of FAFLP fingerprints is shown in Fig. 2. These results indicated that our strains probably belonged to the same species and represented a new *Aeromonas* species.

The DNA G+C content of strains 665N and 868E^T was 62·3 and 62·6 mol%, respectively, which agrees with the range described for the genus *Aeromonas* (57–63 mol%; Carnahan & Joseph, 2005). DNA-relatedness was determined between the type strain of the presumptively novel *Aeromonas* sp (868E^T) and the type or reference strains of the phylogenetically more related *Aeromonas* hybridization groups (Fig. 1) and between 868E^T and the isolate 665N. Strains 868E^T and 665N showed 76 % DNA-DNA similarity, above the level of 70 % that is accepted as the limit for species delineation (Wayne *et al*., 1987). The type strain of the novel *Aeromonas* species clearly showed <70 % DNA reassociation (30–44 %) with any of the type or reference *Aeromonas* strains analysed (see Supplementary Table S1 in IJSEM Online). In conclusion, isolates 868E^T and 665N belong to the same species and should be treated as members of a novel species of the genus *Aeromonas*.

Based on the results of DNA-DNA hybridization, phenotypic characterization, FAFLP analysis and 16S rRNA gene sequencing, we propose that strains 868E^T and 665N represent a novel species within the genus *Aeromonas*, for which we propose the name *Aeromonas bivalvium* sp. nov.

Description of *Aeromonas bivalvium* sp. nov. *Aeromonas bivalvium* (N.L. pl. neut. n. Bivalvia (singular nominative Bivalve), scientific name of a class of molluscs; bi.val'vi.um. N.L. neut. gen. pl. n. bivalvium, of bivalves of the class *Bivalvia*).

Cells are Gram-negative, straight, motile coccoid / rods with a polar flagellum, 0·5–2·0 µm long and 0·3–1·0 µm wide. Colonies on TSA are 3–4 mm in diameter, opaque, circular and beige in colour after

48 h at 25 ºC. Optimal growth occurs at 30–37 ºC but growth occurs at 4–37 ºC and the strain 868E^T grows up to 40·5 ºC. Oxidase- and catalase-positive, reduces nitrate to nitrite, produces indole from tryptophan and is resistant to vibriostatic agent O/129 (150 µg). Positive for glucose oxidation-fermentation, LDC, MR and ONPG tests. Negative for ADH, ODC and VP tests. Brown diffusible pigment, swarming, gas from D-glucose and hydrogen sulfide from cysteine and thiosulfate are not produced. Grows on MacConkey and AD agar. Able to grow at pH 9·0 and 0–3% NaCl, grows weakly at 6 % NaCl but not at pH 4·5 or 7 % NaCl. Both strains hydrolyse arbutin, DNA, aesculin, gelatin and starch, but not elastin, erythrocytes, urea or xanthine. Both strains produce acid from L-arabinose, arbutin, dextrin, D-galactose, glycerol, D-mannitol, salicin, D-sucrose and D-trehalose, but not from lactose, D-mannose, D-melibiose, D-raffinose, L-rhamnose, sorbitol or D-xylose. The following substrates are used as sole carbon and energy sources: acetate, N-acetylglucosamine, aesculin, L-arabinose, ρ-arbutin, L-arginine, D-cellobiose, citrate, D-fructose, D-galactose, D-glucose, glycerol, L-histidine, L-lactate, maltose, D-mannitol, salicin, starch, D-sucrose and D-trehalose. None of the strains uses adonitol, dulcitol, inositol, lactose, D-mannose, D-melezitose, D-melibiose, D-raffinose, L-rhamnose, sorbitol, L-sorbose or D-xylose. Both strains are resistant to amoxicillin+clavulanic acid (30 µg), ampicillin (10 µg), erythromycin (15 µg) and penicillin G (10 µg), show intermediate sensitivity to cefuroxime (30 µg) and streptomycin (10 µg), and are sensitive to amikacin (30 µg), ceftriaxon (30 µg), ciprofloxacin (5 µg), colistin (50 µg), gentamicin (10 µg), imipenem (10 µg), polymyxin B (300 U), tetracycline (30 µg), tobramycin (10 µg) and trimethoprim+sulfamethoxazole (1·25 µg+23·75 µg). The strain 665N is resistant to cephalothin (30 µg), cefoxitin (30 µg) and ticarcillin (75 µg), in contrast with the type strain 868E^T. DNA G+C content is 62·3 mol% for 665N and 62·6 mol% for 868E^T.

The type strain, 868E^T (=CECT 7113^T =LMG 23376^T), was isolated from cockles (*Cardium* sp.) obtained from a retail market in Barcelona (Spain).

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Table 1. Key tests for the phenotypic differentiation of the strains 665N and 868E^T from other mesophilic *Aeromonas* species

Data were taken from Miñana-Galbis *et al*. (2004) and Carnahan & Joseph (2005) unless otherwise indicated. Taxa are identified as: 1, Strains 665N and 868E^T (data from this study); 2, *A. hydrophila*; 3, *A. bestiarum*; 4, *A. salmonicida*; 5, *A. caviae*; 6, *A. media*; 7, *A. eucrenophila*; 8, *A. sobria*; 9, *A. veronii* bv. sobria; 10, *A. jandaei*; 11, *A. veronii* bv. veronii; 12, *A. schubertii*; 13, *A. trota*; 14, *A. allosaccharophila*; 15, *A. encheleia*; 16, *A. popoffii*; 17, *A. culicicola*; 18, *A. simiae*; 19, *A. molluscorum*. Abbreviations: +, 85–100% of strains positive; –, 0–15% of strains positive; d, 16–84% of strains positive; ND, no data available. In brackets, results obtained in this study.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Production of:																			
Brown pigment	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Gas from D-glucose	–	+	+	+	–	–	+	+	+	+	+	–	+	+	+	+	+	–	–
H_2S from cysteine	–	+	+	+	–	–	+	+	+	+	d	–	+	+	+	(+)	(+)	(–)	–
Indole	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	(+)	+	–	–
LDC	+	+	+	+	–	–	–	+	+	+	+	+	+	+	–	–	+	+	–
ODC	–	–	–	–	–	–	–	–	–	–	+	–	–	d	–	–	–	–	–
VP	–	+	+	+	–	–	–	+	+	+	+	+	–	–	–	+	+	–	–
Acid from:																			
L-Arabinose	+	d	+	+	+	+	+	–	d	–	–	–	–	+	–	d	–	–	+
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–	+
Sorbitol	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D-Sucrose	+	+	+	+	+	+	d	+	+	d	+	–	–	+	d	–	+	+	+
Hydrolysis of:																			
Aesculin	+	+	+	+	+	+	+	–	–	–	+	–	–	+	+	(–)	(–)	(–)	+
Arbutin	+	+	+	+	+	+	+	–	–	–	+	–	d	–	+	(–)	(–)	(+)	+
Elastin	–	+	d	+	–	–	–	–	–	d	–	–	–	–	–	–	(–)	(–)	–
Starch	+	+	+	+	+	+	+	+	–	+	+	+	–	–	+	+	+	(+)	–
Utilization of L-lactate	+	+	–	–	d	+	–	–	–	–	–	+	+	d	–	+	ND	ND	d

Table 2. Base differences in the 16S rDNA sequences among the type strain of *A. popoffii* and the strains 665N and 868E^T

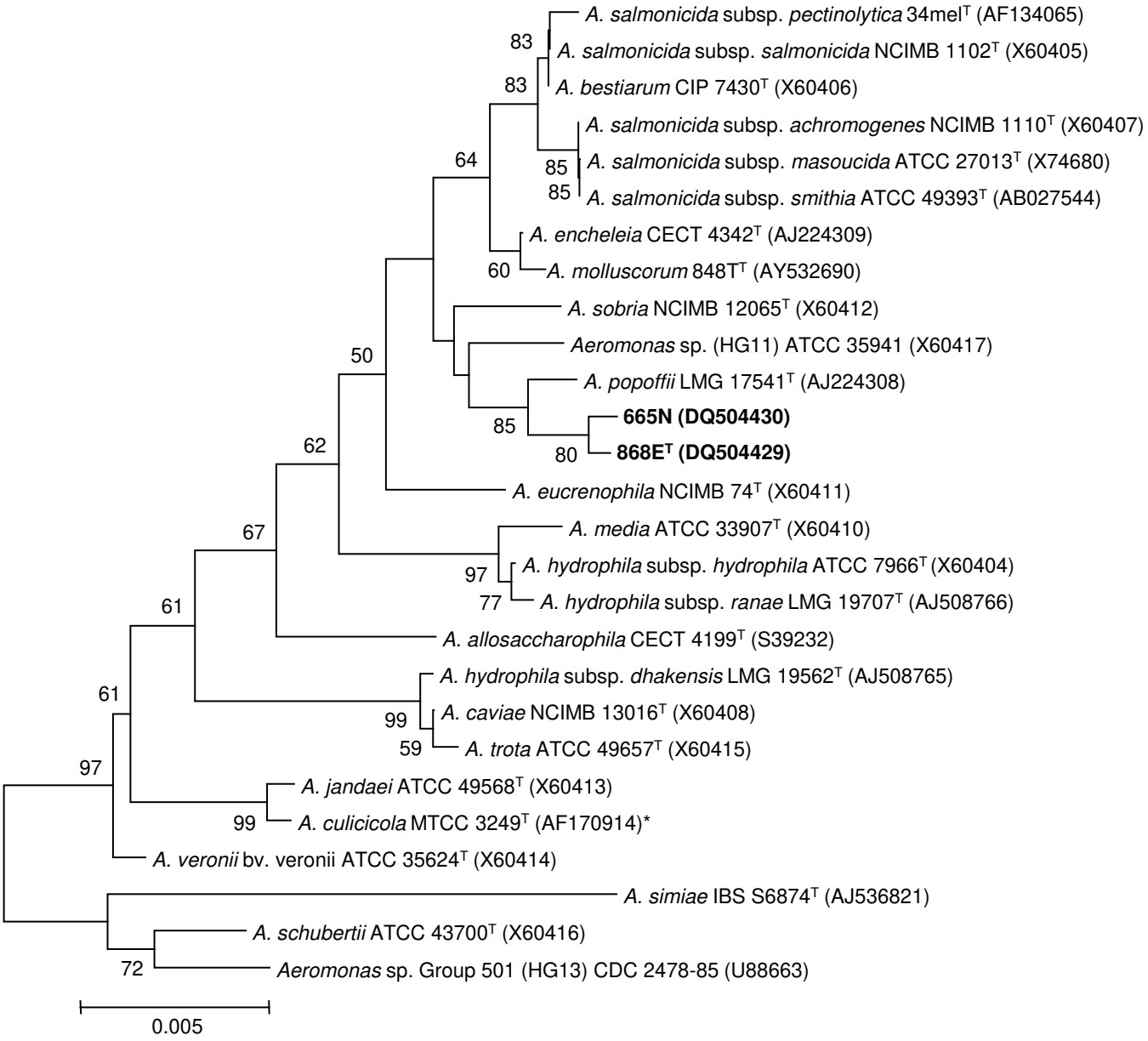
Strain	GenBank accession number	Positions*					
		456	459	469	476	1011	1018
A. popoffii LMG 17541^T	AJ224308	T	T	C	A	C	G
665N	DQ504430	A	C	T	T	C	G
868E^T	DQ504429	A	C	T	T	T	A

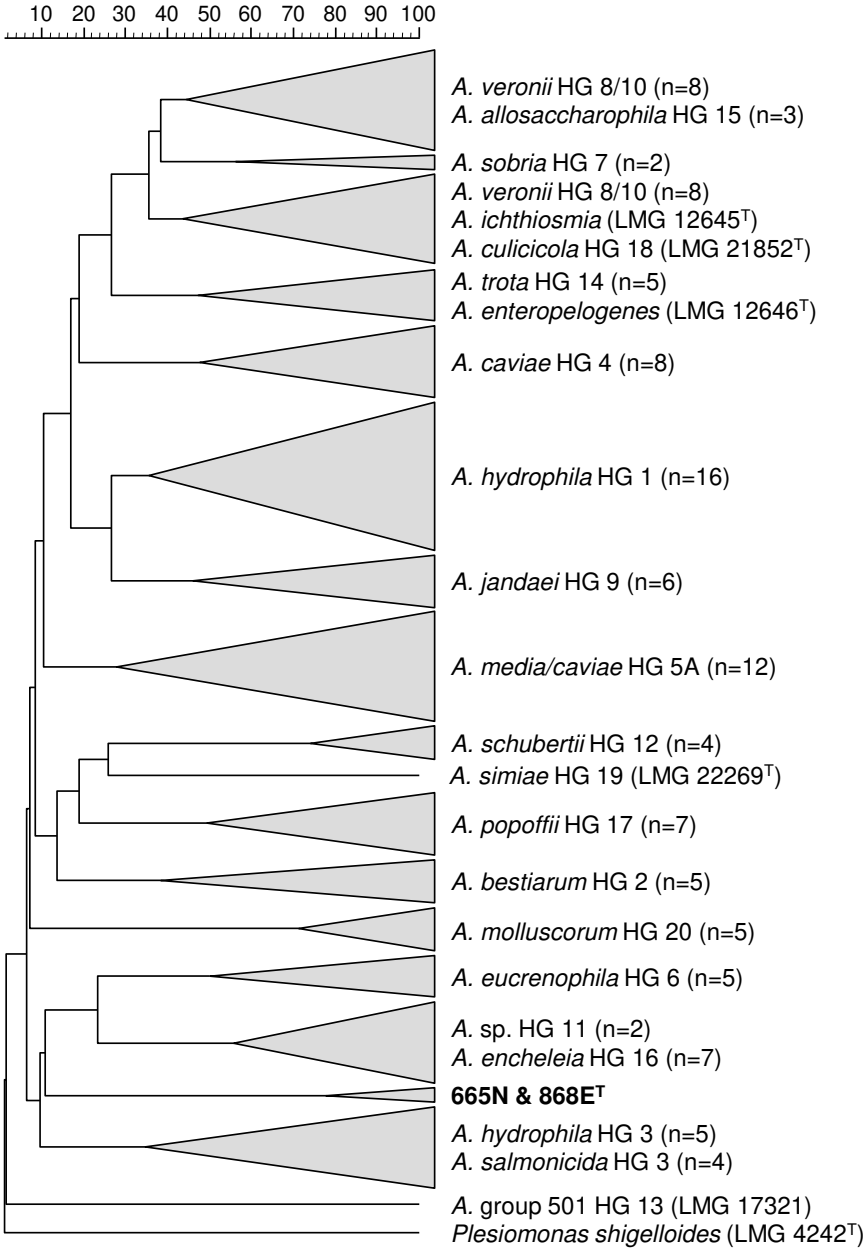
*Referred to the *E. coli* sequence numbering system described by Brosius *et al*. (1978).

FIGURE LEGENDS

Fig. 1. Phylogenetic relationships of the strains 665N and 868E^T to type or reference strains of the genus *Aeromonas*. The phylogenetic tree was constructed using 1544 nucleotides of 16S rDNA sequence by the neighbour-joining method in MEGA program version 2·1. The bar represents distance values calculated in MEGA and bootstrap values (> 50 %) after 1000 replicates are shown. **A. culicicola* is a later subjective synonym of *A. veronii* (Huys *et al*., 2005)

Fig. 2. Simplified dendrogram showing cluster analysis of the FAFLP fingerprints of the strains 665N and 868E^T and reference strains of the *Aeromonas* DNA hybridization groups. The *Aeromonas* reference strains from the AEROLIB database are listed in Huys & Swings (1999), Huys *et al*. (2003) and Miñana-Galbis *et al*. (2004), except for LMG 22269^T, 665N and 868E^T strains that were added to the database in the frame of the current study. The dendrogram was constructed with the UPGMA clustering method using the Pearson product-moment correlation coefficient (expressed as percentage value).


A. salmonicida subsp. pectinolytica 34melT (AF134065)
A. salmonicida subsp. salmonicida NCIMB 1102T (X60405)
A. bestiarum CIP 7430T (X60406)
A. salmonicida subsp. achromogenes NCIMB 1110T (X60407)
A. salmonicida subsp. masoucida ATCC 27013T (X74680)
A. salmonicida subsp. smithia ATCC 49393T (AB027544)
A. encheleia CECT 4342T (AJ224309)
A. molluscorum 848TT (AY532690)
A. sobria NCIMB 12065T (X60412)
Aeromonas sp. (HG11) ATCC 35941 (X60417)
A. popoffii LMG 17541T (AJ224308)
665N (DQ504430)
868ET (DQ504429)
A. eucrenophila NCIMB 74T (X60411)
A. media ATCC 33907T (X60410)
A. hydrophila subsp. hydrophila ATCC 7966T (X60404)
A. hydrophila subsp. ranae LMG 19707T (AJ508766)
A. allosaccharophila CECT 4199T (S39232)
A. hydrophila subsp. dhakensis LMG 19562T (AJ508765)
A. caviae NCIMB 13016T (X60408)
A. trota ATCC 49657T (X60415)
A. jandaei ATCC 49568T (X60413)
A. culicicola MTCC 3249T (AF170914)*
A. veronii bv. veronii ATCC 35624T (X60414)
A. simiae IBS S6874T (AJ536821)
A. schubertii ATCC 43700T (X60416)
Aeromonas sp. Group 501 (HG13) CDC 2478-85 (U88663)
83
83
64
85
85
60
50
85
80
62
67
97
77
61
61
99
59
97
99
72
0.005


10 20 30 40 50 60 70 80 90 100
A. veronii HG 8/10 (n=8)
A. allosaccharophila HG 15 (n=3)
A. sobria HG 7 (n=2)
A. veronii HG 8/10 (n=8)
A. ichthiosmia (LMG 12645T)
A. culicicola HG 18 (LMG 21852T)
A. trota HG 14 (n=5)
A. enteropelogenes (LMG 12646T)
A. caviae HG 4 (n=8)
A. hydrophila HG 1 (n=16)
A. jandaei HG 9 (n=6)
A. media/caviae HG 5A (n=12)
A. schubertii HG 12 (n=4)
A. simiae HG 19 (LMG 22269T)
A. popoffii HG 17 (n=7)
A. bestiarum HG 2 (n=5)
A. molluscorum HG 20 (n=5)
A. eucrenophila HG 6 (n=5)
A. sp. HG 11 (n=2)
A. encheleia HG 16 (n=7)
665N & 868ET
A. hydrophila HG 3 (n=5)
A. salmonicida HG 3 (n=4)
A. group 501 HG 13 (LMG 17321)
Plesiomonas shigelloides (LMG 4242T)