Aeromonas aquariorum sp. nov., isolated from aquaria of ornamental fish

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During a survey to determine the prevalence of *Aeromonas* strains in water and skin of imported ornamental fish, 48 strains presumptively identified as *Aeromonas* were isolated but they could not be identified as members of any previously described *Aeromonas* species. These strains were subjected to a polyphasic approach including phylogenetic analysis derived from *gyrB*, *rpoD* and 16S rRNA gene sequencing, DNA–DNA hybridization, MALDI-TOF MS analysis, genotyping by RAPD and extensive biochemical and antibiotic susceptibility tests in order to determine their taxonomic position. Based on the results of the phylogenetic analyses and DNA–DNA hybridization data, we describe a novel species of the genus *Aeromonas*, for which the name *Aeromonas aquariorum* sp. nov. is proposed, with strain MDC47^T (=DSM 18362^T =CECT 7289^T) as the type strain. This is the first *Aeromonas* species description based on isolations from ornamental fish.

Species of *Aeromonas* are common inhabitants of aquatic environments and have been described in connection with fish and human diseases (Altwegg, 1999; Austin & Adams, 1996; Saavedra *et al.*, 2004; Figueras, 2005). Apart from the psychrophilic suspected fish pathogen *Aeromonas salmonicida*, many other mesophilic aeromonads are considered to be opportunistic pathogens, capable of producing infections in weakened fish or as secondary invaders in fish populations suffering from other diseases (Camus *et al.*, 1998). Given the large numbers of ornamental fish imported from areas of the world where sanitation is often inadequate and where numerous diseases of man are endemic, it is surprising that little consideration has been given to the role of these aquarium species as vectors of potential pathogens for man. When the occurrence of bacterial pathogens in ornamental fish has been investigated, *Aeromonas* strains were found in more than 50 % of fish disease cases examined by Kuo & Chung (1994) and were involved in 18 of 23 bacterial disease outbreaks investigated by Hettiarachchi & Cheong (1994). Moreover, noticeable antibiotic resistance has been detected in *Aeromonas* strains isolated from ornamental fish (Dixon & Issvoran, 1992).

The genus *Aeromonas* belongs to the family *Aeromonadaceae* (Colwell *et al.*, 1986; Martínez-Murcia *et al.*, 1992a; Yáñez *et al.*, 2003). According to the last edition of *Bergey's Manual of Systematic Bacteriology* (Martin-Carnahan & Joseph, 2005), the genus comprises the species *Aeromonas hydrophila*, *A. bestiarum*, *A. salmonicida*, *A. caviae*, *A. media*, *A. eucrenophila*, *A. sobria*, *A. veronii* (biovars Sobria and Veronii), *A. jandaei*, *A. schubertii*, *A. trota*, *A. allosaccharophila*, *A. encheleia* and *A. popoffii* and two DNA homology groups, *Aeromonas* sp. HG11 and *Aeromonas* sp. HG13 (formerly enteric group 501), which remain without a species name. Furthermore, *Aeromonas ichthiosmia* (Schubert *et al.*, 1990b) and *Aeromonas enteropelogenes* (Schubert *et al.*, 1990a) are now considered synonyms of *A. veronii* and *A. trota*, respectively (Carnahan, 1993; Collins *et al.*, 1993; Huys *et al.*, 2001, 2002; Martin-Carnahan & Joseph, 2005). Four additional species, *Aeromonas culicicola* (Pidiyar *et al.*, 2002), *A. simiae* (Harf-Monteil *et al.*, 2004), *A. molluscorum*

Abbreviation: RAPD, randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MDC47^T is EU085557. Strains MDC259, MDC302 and MDC442 show an identical sequence.

Details of strain origins and sequence accession numbers, the antibiotic resistance profile of the novel group of strains and a MALDI-TOF MS-based dendrogram are available as supplementary material with the online version of this paper.

(Miñana-Galbis *et al.*, 2004) and *A. bivalvium* (Miñana-Galbis *et al.*, 2007), have recently been described, and the species *A. culicicola* has been proposed as a synonym of *A. veronii* (Huys *et al.*, 2005; Saavedra *et al.*, 2006). The recently proposed species *Aeromonas sharmana* (Saha & Chakrabarti, 2006) was not considered a member of the genus *Aeromonas* by Martínez-Murcia *et al.* (2007). Although 16S rRNA gene sequencing has contributed notably to the elucidation of the phylogenetic interrelationships between *Aeromonas* species (Martínez-Murcia *et al.*, 1992a, b; Martínez-Murcia, 1999), the resolution of this molecular 'clock' has now been superseded by those of some protein-encoding housekeeping genes such as *gyrB* and *rpoD* (Yáñez *et al.*, 2003; Soler *et al.*, 2004; Martínez-Murcia *et al.*, 2005; Saavedra *et al.*, 2006, 2007). In the present study, 48 *Aeromonas* isolates recovered from the water of aquaria and the skin of imported ornamental fish were subjected to a polyphasic approach including phylogenetic analysis derived from *gyrB*, *rpoD* and 16S rRNA gene sequencing, DNA–DNA hybridization, genotyping by RAPD, MALDI-TOF MS analysis and extensive biochemical and antibiotic susceptibility tests in order to determine their taxonomic position. Based on the reported data, we describe a novel species of the genus *Aeromonas*.

Samples were collected monthly between November 2004 and February 2005 from aquaria of ornamental fish shops located in Porto, Portugal (see Supplementary Table S1, available in IJSEM Online). Randomly selected fish showing symptoms of weakness were placed into ordinary plastic bags and transported to the laboratory. Water samples were taken from the same aquaria by using sterile polypropylene bottles and examined within 2 h of collection. Water samples (100 ml) filtered onto nitrocellulose membranes and samples from fish skin were incubated at 28 °C for 24 h on glutamate starch penicillin (GSP) medium (Oxoid). From each sample, typical colonies (i.e. yellow on GSP medium) were isolated, subcultured into TSA plates (Oxoid) and, following standard procedures, were first subjected to the cytochrome oxidase, catalase activity, nitrate reduction and vibriostatic agent O/129 (150 μg) tests to identify *Aeromonas* at the genus level, and further key biochemical tests that allow differentiation among *Aeromonas* species (listed in Table 1) were performed as described by Miñana-Galbis *et al.* (2002). Forty-nine carbohydrates for substrate fermentation were tested by using API 50CH (bioMérieux) at 30 °C for 48 h, following the manufacturer's instructions. For MALDI-TOF MS analysis, the starting material was a single colony of the type strains of *Aeromonas* species.

Table 1. Presumptive key tests for the phenotypic differentiation of *Aeromonas aquariorum* sp. nov. from other *Aeromonas* species

Data were taken from Harf-Monteil *et al.* (2004), Martin-Carnahan & Joseph (2005) and Miñana-Galbis *et al.* (2007) unless indicated otherwise. Tests: 1–6, production of brown pigment (1), gas from D-glucose (2), H_2S from cysteine (3), indole (4), lysine decarboxylase (5) and ornithine decarboxylase (6); 7, Voges–Proskauer test; 8–13, acid from L-arabinose (8), D-mannitol (9), sorbitol (10), sucrose (11), cellobiose (12) and salicin (13); 14, hydrolysis of aesculin; 15, hydrolysis of arbutin; 16, utilization of L-lactate. +, 85–100 % of strains positive; d, 16–84 % of strains positive; −, 0–15 % of strains positive; ND, no data available. Results obtained in the present study for type strains are given in parentheses.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
A. aquariorum sp. nov.	−	+	+	+	+	−	+	−	+	−	+	−	+	+	+	−
A. hydrophila	−	+	+	+	+	−	+	d	+	−	+	−	+	+	+	+
A. bestiarum	−	+	+	+	+	−	+	+	+	−	+	−	d	+	+	−
A. salmonicida	−	+	+	+	+	−	+	+	+	+	+	(−)	(−)	+	+	−
A. caviae	−	−	−	+	−	−	−	+	+	−	+	+	+	+	+	d
A. media	+	−	−	+	−	−	−	+	+	−	+	+	d	+	+	+
A. eucrenophila	−	+	+	+	−	−	−	+	+	−	d	+	+	+	+	−
A. sobria	−	+	+	+	+	−	+	−	+	−	+	+	−	−	−	−
A. veronii bv. Sobria	−	+	+	+	+	−	+	d	+	−	+	d	−	−	−	−
A. jandaei	−	+	+	+	+	−	+	−	+	−	d	−	−	−	−	−
A. veronii bv. Veronii	−	+	d	+	+	+	+	−	+	−	+	d	+	+	+	−
A. schubertii	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	+
A. trota	−	+	+	+	+	−	−	−	+	−	−	+	−	−	d	+
A. allosaccharophila	−	+	+	+	+	d	−	+	+	−	+	+	−	+	−	d
A. encheleia	−	+	+	+	−	−	−	−	+	−	d	−	+	+	+	−
A. popoffii	−	+	+	+	−	−	+	d	+	−	−	−	−	−	−	+
A. culicicola	−	+	+	+	+	−	+	−	+	−	+	−	−	−	−	ND
A. simiae	−	−	−	−	+	−	−	−	−	−	+	+	−	−	+	ND
A. molluscorum	−	−	−	−	−	−	−	+	+	−	+	(−)	(−)	+	+	d
A. bivalvium	−	−	−	+	+	−	−	+	+	−	+	ND	ND	+	+	+

A thin microbial film was overlaid with matrix (alpha-cyano-4-hydroxycinnamic acid; HCCA). Mass spectra were acquired using a MALDI-TOF Autoflex III mass spectrometer (Bruker Daltronics) in linear positive mode at maximum frequency. The measured mass range of spectra was 2000–20 000 Da. Peak recognition and comparison of spectra was done with the BioTyper software as described by Maier *et al.* (2006).

Type and reference strains of all described *Aeromonas* species, together with two aquarium strains, MDC47^T and MDC310, were selected for DNA hybridization studies. DNA–DNA reassociation was determined according to the spectrophotometric method. DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by hydroxyapatite chromatography (Cashion *et al.*, 1977). DNA–DNA hybridization was carried out at 66 °C according to the method of De Ley *et al.* (1970), with some modifications (Escara & Hutton, 1980; Huß *et al.*, 1983), by using a model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian). Hybridization values were determined at least twice for any given strain pair. For PCR amplification, a single colony from a fresh culture was resuspended in 100 µl TE buffer and 200 µl Chelex (Bio-Rad) was added. The tube was vortexed at high speed for 1 min, incubated at 96 °C for 10 min and then kept at −20 °C for 10 min, and this process was repeated three times. The tube was again vortexed and centrifuged for 5 min at 12 000 ***g***. The supernatant was transferred to a fresh tube and stored at –20 °C. Procedures and characteristics of oligonucleotide primers for amplification and PCR-based sequencing of the *gyrB*, *rpoD* and 16S rRNA genes were described previously (Martínez-Murcia *et al.*, 1992a; Yáñez *et al.*, 2003; Soler *et al.*, 2004), except that the BigDye Terminator v3.1 cycle sequencing kit was used with an ABI 3100-Avant Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions, by the Molecular Diagnostics Center (MDC), Orihuela, Spain. Nucleotide sequences were aligned by the CLUSTAL_X program version 1.8 (Thompson *et al.*, 1997). For alignments, previously published reference sequences (Martínez-Murcia *et al.*, 1992a; Martínez-Murcia, 1999; Saavedra *et al.*, 2006, 2007) were used. Genetic distances were obtained by Kimura's two-parameter model (Kimura, 1980) and evolutionary trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) with the MEGA program (Kumar *et al.*, 2001). Randomly amplified polymorphic DNA (RAPD) analysis was performed by using the oligonucleotide OPA16 (5′-AGCCAGCGAA-3′). Approximately 50 ng extracted DNA was subjected to PCR amplification in a total volume of 50 µl following the protocol described previously (Martínez-Murcia *et al.*, 1995). The reaction mixtures were subjected to 40 cycles on an MJ Research PTC100 thermal cycler, with a thermal cycling program of 94 °C for 60 s, 36 °C for 60 s and 72 °C for 120 s. Following amplification, 10 µl PCR product was electrophoresed on a 1.2 % agarose gel in TBE buffer. A 1 kb DNA ladder was used as a molecular size marker. Agarose gels were stained with ethidium bromide, visualized with a UV lamp and photographed in a GelDocMega cabinet.

Antibiotic susceptibility tests were achieved by the disc method of Kirby and Bauer on Mueller–Hinton agar plates (Oxoid) with inocula adjusted to an optical density of 0.5 McFarland standard units. After 24 h incubation at 28 °C, organisms were classified as sensitive (S), intermediately resistant (I) or resistant (R) according to the guidelines of the NCCLS (2004). The following antibiotic-containing discs were obtained from Oxoid: ampicillin (AMP_{10}), carbenicillin (CAR_{100}), amoxicillin (AML_{10}), amoxicillin/clavulanic acid (AMC_{30}), piperacillin (PRL_{100}), piperacillin/tazobactam (TZP_{110}), ticarcillin (TIC_{75}), ticarcillin/clavulanic acid (TIM_{85}), cephalothin (KF_{30}), cefoxitin (FOX_{30}), cefotaxime (CTX_{30}), cefoperazone (CFP_{30}), ceftazidime (CAZ_{30}), ceftriaxone (CRO_{30}), cefepime (FEP_{30}), aztreonam (ATM_{30}), imipenem (IMP_{10}), gentamicin (CN_{10}), kanamycin (K_{30}), tobramycin (TOB_{10}), amikacin (AK_{30}), netilmicin (NET_{30}), tetracycline (TE_{30}), ciprofloxacin (CIP_5), norfloxacin (NOR_{10}), erythromycin (E_{15}), trimethoprim/sulfamethoxazole (SXT_{25}) and chloramphenicol (C_{30}).

During the course of a bacterial isolation study, hundreds of *Aeromonas* isolates obtained from water and skin of ornamental fish were subjected to partial sequencing (approx. 500 bp) of the *gyrB* gene for species identification (data not shown), and the corresponding *gyrB* phylogenetic analysis including representative strains from all known *Aeromonas* species indicated that some isolates represent a hitherto unknown phylogenetic line within the genus *Aeromonas*. This new cluster first comprised 36 isolates; however, only 13 *gyrB* sequences showed nucleotide differences from each other, indicating that they represent different clones of this as-yet undescribed species. Representative strains of these 13 clones were selected for *gyrB* sequencing of approx. 1040 bp (Supplementary Table S1), and the corresponding phylogeny (Fig. 1) confirmed the preliminary results derived with the shorter fragment. EMBL accession numbers for the sequences used as references have been published (Saavedra *et al.*, 2006). As recommended in the description of novel species (Figueras *et al.*, 2006), these strains, together with 23 isolates that showed sequences identical to some of these clones and other colonies isolated from the same sample (a total of 48 isolates), were subjected to genotyping by using RAPD analysis in order to ascertain overall genomic similarities, and 19 different RAPD patterns were obtained (Supplementary Table S1). Strains with identical *gyrB* sequences may show identical RAPD profiles, as in the case of strains MDC318, MDC319, MDC320 and MDC321, or, in other instances, they showed different RAPD patterns, as obtained for strains MDC310 and MDC444 or MDC442 and MDC474. Strains with different *gyrB* sequences never showed identical RAPD patterns. From different samples,

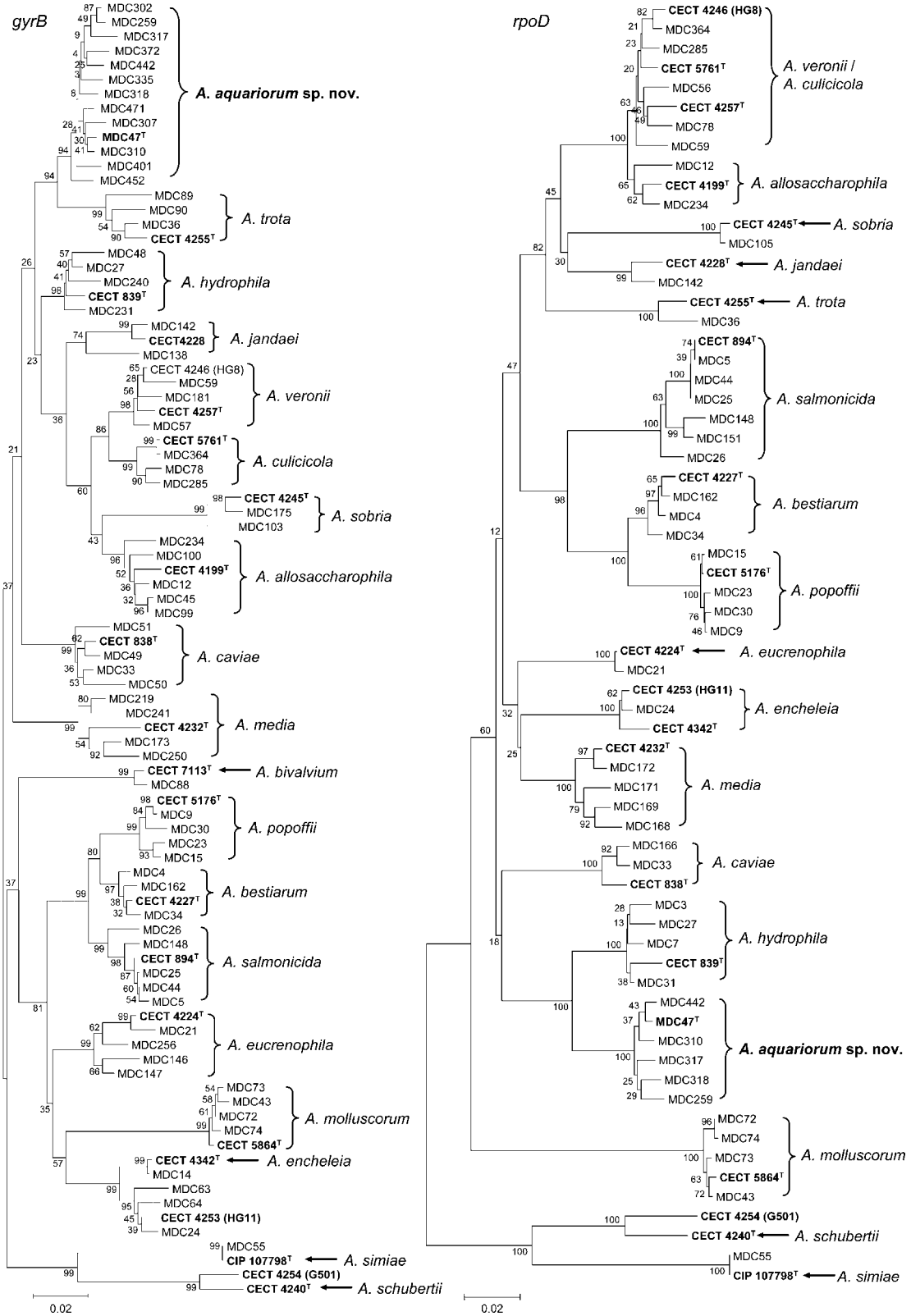

Fig. 1. Unrooted neighbour-joining phylogenetic trees derived from *gyrB* (left) and *rpoD* (right) gene sequences showing the corresponding relationship of strains of *Aeromonas aquariorum* sp. nov. to known species of *Aeromonas*. Numbers next to each node indicate bootstrap values (percentage of 1000 replicates). Corresponding type strains and reference strains of homology groups are in bold. Accession numbers for reference sequences are given in Figs 1 (*gyrB*) and 2 (*rpoD*) of Saavedra *et al.* (2006). Bars, 0.02 substitutions per nucleotide position.

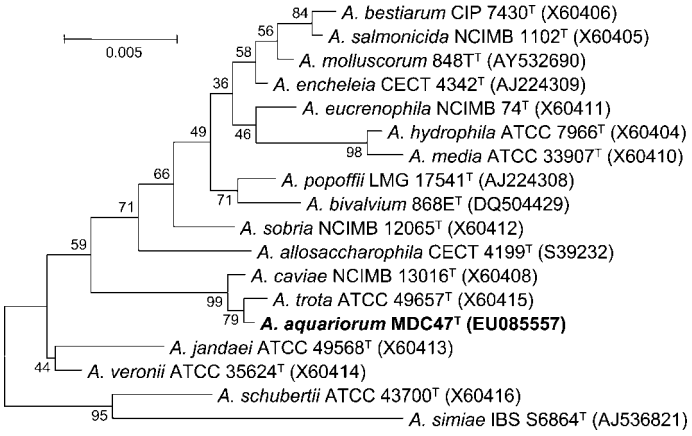

Fig. 2. Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships of type strains of all recognized *Aeromonas* species. Numbers next to each node indicate bootstrap values (percentage of 1000 replicates). Bar, 0.005 substitutions per nucleotide position.

fish and water, of the same date, isolates with identical *gyrB* and RAPD were collected, e.g. MDC444 from fish and MDC476 from water, both isolated on 4 January 2005. Moreover, the phylogenetic clone represented by strain MDC310 was recovered several times from samples collected 2 months apart (strains MDC332, MDC333, MDC444, MDC476, MDC498, MDC509, MDC599 and MDC645).

The phylogenetic neighbour of this novel *Aeromonas* cluster in the *gyrB* tree was found to be *A. trota*; however, it was related as distantly as some other species of the genus, for instance *A. salmonicida*, *A. bestiarum* and *A. popoffii* (Fig. 1). The 16S rRNA gene sequences of strains MDC47^T, MDC259, MDC302 and MDC442 were determined, and identical sequences were obtained from the four strains. The obtained sequence was aligned with those of the type strains of all recognized *Aeromonas* species and the derived phylogenetic tree (Fig. 2) also showed a close relationship with the species *A. trota*, with only two nucleotide differences. Some representative strains of this new *gyrB* cluster were also subjected to *rpoD* sequencing, and the resulting phylogenetic tree confirmed that these strains all belong to an undescribed species of the genus *Aeromonas* (Fig. 1). However, the closest relative in the *rpoD* tree is *A. hydrophila*, with *A. trota* being remotely related.

Levels of DNA–DNA relatedness were determined between the type strain of the presumptively novel species (MDC47^T) and the type strains of all recognized *Aeromonas* species and between strains MDC47^T and MDC310 (Table 2). These two strains MDC47^T and MDC310 showed a level of DNA–DNA relatedness of 83.5 %, notably above the suggested limit (i.e. 70 %) for species delineation (Wayne *et al.*, 1987). Proposed type strain MDC47^T showed levels of DNA–DNA hybridization (20.4–55.7 %) that were clearly below the aforementioned limit. In particular, DNA reassociation values of 44.4 and 46.0 %, respectively, were determined with the type strains of the phylogenetically closely related species *A. trota* and *A. hydrophila*.

MALDI-TOF MS analysis of *Aeromonas* type strains indicated the high resolution power of this method, as the majority of type strains can be well separated on the basis of protein masses emerging from the TOF analysis (Supplementary Fig. S1). Only the pairs of type strains *A. veronii* CECT 4247^T and *A. culicicola* CECT 5761^T and *A. salmonicida* CECT 894^T and *A. popoffii* CECT 5167^T were less well resolved. Strain MDC47^T stands isolated, which supports the novel species status of this group of strains.

The strains of this undescribed *Aeromonas* species showed several differential phenotypic characteristics with regard to the other *Aeromonas* species (Table 1). Three to ten tests allowed differentiation from all known *Aeromonas* species,

Table 2. DNA–DNA relatedness between strains MDC47^T and MDC310 and type strains of known *Aeromonas* species

Results are expressed as means of two determinations.

Source of unlabelled DNA	DNA–DNA hybridization (%) with labelled DNA from strain MDC47^T
Strain 310	83.5
A. trota CECT 4255^T	44.4
A. hydrophila CECT 389^T	46.0
A. caviae CECT 838^T	47.5
A. encheleia CECT 4342^T	37.4
A. bestiarum CECT 4227^T	55.2
A. salmonicida CECT 894^T	50.9
A. media CECT 4232^T	20.4
A. molluscorum DSM 17090^T	48.7
A. schubertii CECT 4240^T	27.7
A. sobria CECT 4245^T	29.8
A. veronii CECT 4257^T	24.3
A. jandaei CECT 4228^T	39.8
A. eucrenophila CECT 4224^T	55.7
A. allosaccharophila CECT 4199^T	53.0
A. popoffii CECT 5176^T	49.9
A. simiae DSM 16559^T	41.5
A. bivalvium CECT 7113^T	45.5

except for *A. encheleia* and *A. bestiarum*. The first was only distinguishable by the lysine decarboxylase test and *A. bestiarum* could be differentiated on the bases of the Voges–Proskauer test and acid production from L-arabinose. All of the isolates tested showed resistance to ampicillin, carbenicillin and amoxicillin (Supplementary Table S2); however, they were sensitive to ceftriaxone, ceftazidime, cefotaxime, cefepime and imipenem. Resistance to first- and second-generation cephalosporins (cephalothin and cefoxitin, respectively) was measured in 88 and 98 % of the isolates. Notably, 50 % of the isolates showed resistance to tobramycin. Isolate MDC372 was resistant to aztreonam, a monobactam antibiotic. At the time of the initial isolation of MDC47^T, 3 years ago, it was resistant to imipenem. Recent tests, however, have indicated that the strain is now more sensitive.

Based on the results of phylogenetic analyses using *gyrB*, *rpoD* and 16S rRNA genes, DNA–DNA hybridization, MALDI-TOF MS analysis, genotyping and phenotypic characterization, it is evident that the strains isolated from ornamental fish and water from aquaria represent a single novel species of the genus *Aeromonas*, for which the name *Aeromonas aquariorum* sp. nov. is proposed.

Description of *Aeromonas aquariorum* sp. nov.

Aeromonas aquariorum (a.qua.ri.o′rum. L. gen. neut. pl. n. *aquariorum* from/of aquaria).

Cells are Gram-negative, motile rods with a polar flagellum. Oxidase- and catalase-positive, reduces nitrate to nitrite and is resistant to 2,4-di-amino-6,7-diisopropylpteridine (vibriostatic agent O/129; 150 μg). No brown diffusible pigment is produced. Chemo-organotrophic, with both oxidative and fermentative metabolism. Growth occurs in 0–3 % (w/v) NaCl. Positive for lysine decarboxylase, arginine dihydrolase, gelatinase and ONPG tests. Negative for ornithine decarboxylase, tryptophan deaminase, urease and Voges–Proskauer tests. Produces hydrogen sulfide from cysteine and thiosulfate, gas from D-glucose and indole from tryptophan and utilizes L-lactate. Grows on MacConkey agar; optimal growth occurs at 30–37 °C. Hydrolyses arbutin, aesculin, gelatin, and starch. Acid is produced from D-fructose, D-galactose, D-glucose, D-glycerol, glycogen, maltose, D-mannitol, D-mannose, *N*-acetylglucosamine, sucrose, D-ribose, aesculin, salicin, arbutin, trehalose, gluconate and methyl α-D-glucopyranoside. Does not produce acid from melibiose, adonitol, amygdalin, D-arabinose, L-arabinose, D- or L-arabitol, gentiobiose, cellobiose, dulcitol, erythritol, D- or L-fucose, D-lyxose, inositol, inulin, 2-ketogluconate, 5-ketogluconate, lactose, melezitose, raffinose, L-rhamnose, D-sorbitol, L-sorbose, D-tagatose, turanose, xylitol, D- or L-xylose, methyl *β*-D-xylopyranoside or methyl α-D-mannopyranoside. The type strain is resistant to ampicillin (10 μg), carbenicillin (100 μg), amoxicillin (10 μg), amoxicillin/clavulanic acid (30 μg), ticarcillin (75 μg), piperacillin (100 μg), piperacillin/tazobactan (110 μg), cephalothin (30 μg), cefoxitin (30 μg), tobramycin (10 μg) and erythromycin (15 μg), shows intermediate resistance to ticarcillin/clavulanic acid (85 μg) and kanamycin (30 μg) and is sensitive to cefotaxime (30 μg), cefoperazone (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cefepime (30 μg), aztreonam (30 μg), imipenem (10 μg), gentamicin (10 μg), amikacin (30 μg), netilmicin (30 μg), tetracycline (30 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), trimethoprim/sulfamethoxazole (25 μg) and chloramphenicol (30 μg).

The type strain is MDC47^T (=DSM 18362^T =CECT 7289^T).

Acknowledgements

This work has been supported by grant IMIDTA/2004/369 from IMPIVA, Generalitat Valenciana. M. J. S. was the recipient of a grant (SFRH/BSAB/439/2004) from the Fundação para a Ciência e Tecnologia. Invaluable advice was received from Professor Hans G. Trüper when the Latin epithet was assigned for this species. In memoriam to F. Martínez and B. Murcia, who died in May and August 2007, respectively.

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