Vol. 109: 127–137, 2014
doi: 10.3354/dao02733

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published May 13

Characterization and antimicrobial susceptibility of motile aeromonads isolated from freshwater ornamental fish showing signs of septicaemia

S. S. S. de S. Jagoda[1,2], T. G. Wijewardana[2,4], A. Arulkanthan[2], Y. Igarashi[1], E. Tan[1], S. Kinoshita[1], S. Watabe[3], S. Asakawa[1,*]

[1]Laboratory of Aquatic Molecular Biology and Biotechnology, Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

[2]Center for Aquatic Animal Disease Diagnosis and Research, Department of Veterinary Pathobiology, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Peradeniya 20400, Sri Lanka

[3]School of Marine Biosciences, Kitasato University, Minami Ku, Sagamihara, Kanagawa 252-0373, Japan

[4]*Present address:* No. 408/35, Mount Pleasant Garden II, Bowelawatte, Kandy 20000, Sri Lanka

ABSTRACT: A total of 74 phenotypically identified presumptive motile *Aeromonas* isolates recovered from septicaemic freshwater ornamental fish in Sri Lanka were genetically characterized by sequencing of *rpoD* and *gyrB* genes. *rpoD*/*gyrB* phylogeny confirmed only 53 isolates as *Aeromonas*, among which *A. veronii* was the predominant species (79.2%), followed by *A. hydrophila* (7.5%), *A. caviae* (5.7%), *A. jandaei* (1.9%), *A. dhakensis* (3.8%) and *A. entero pelogenes* (1.9%). The aeromonads confirmed by sequencing were further subjected to 16S rDNA PCR-RFLP which substantiated sequencing results for 83% of isolates. Fingerprinting of *A. enteropelogenes* (n = 42) using ERIC-PCR revealed no dominant clones, and the majority were genetically distinct. All isolates were screened by PCR for 7 virulence determinant genes (*aer*, *act*, *ast*, *alt*, *fla*, *ser*, *exu*) and 2 integrase encoding genes (*intI1*, *intI2*). Each isolate contained ≥3 of the virulence genes tested for, with a heterogeneous distribution. Of the isolates, 77% harboured the *intI1* gene, while none had *intI2*. *In vitro* antimicrobial susceptibility testing showed highest resistances towards tetracycline (58.5%) and erythromycin (54.7%). Our results indicate the diverse range of aeromonads that could potentially be associated with motile aeromonad septicaemia in ornamental fish. This is the first isolation of *A. dhakensis* from a septicaemic ornamental fish since its original description from the same host.

KEY WORDS: *Aeromonas veronii* · Antimicrobial resistance · Motile aeromonad septicaemia · Ornamental fish · Phylogenetic identification · Virulence genes

INTRODUCTION

The ornamental fish industry provides numerous economic and social benefits. Export of live aquarium fish has emerged as a valuable foreign exchange earner, particularly in developing countries. Sri Lanka supplies about 2.7% of the world's demand for ornamental fish, generating an annual income of ~10 million US$ (as of 2011; Sri Lanka EDB 2014). The current trend towards intensification and commercialization of ornamental fish culture increases the risk of fish diseases.

Aeromonads are Gram-negative rods ubiquitous in aquatic environments (Janda & Abbott 2010, Beaz-Hidalgo & Figueras 2012). Motile aeromonad septicaemia (MAS), with mesophilic, motile *Aeromonas* species implicated in its development, is probably the most common bacterial disease in freshwater aquarium fish (Lewbart 2001). It is often an opportunistic infection, characterized by non-specific signs

*Corresponding author: asakawa@mail.ecc.u-tokyo.ac.jp

Publisher: Inter-Research · www.int-res.com

such as fin rot, ulceration, haemorrhages, exophthalmia and dropsy. Historically, *Aeromonas hydrophila* has gained much attention as the most common fish pathogenic motile *Aeromonas* species, but other *Aeromonas* species may also play an important role in fish pathology that needs to be explored (Beaz-Hidalgo & Figueras 2012). Well-documented discrepancies in phenotypic identification of *Aeromonas* at the species level (Kozińska 2007, Beaz-Hidalgo et al. 2010) may have impacted the species designations of the fish pathogenic aeromonads identified to date (Austin 2011), but the use of molecular approaches, such as the sequencing of *rpoD* and *gyrB* genes (Yáñez et al. 2003, Soler et al. 2004, Martínez-Murcia et al. 2011), has led to increased accuracy of *Aeromonas* species identification.

In this context, the present study aimed to characterize a collection of *Aeromonas* isolates, from ornamental fish with clinical signs of MAS, at the species level using a polyphasic approach; our focus was to determine the relative occurrence of different mesophilic *Aeromonas* species as ornamental fish pathogens. This is an area that remains largely unexplored despite the common occurrence of MAS in ornamental fish. We also investigated the antimicrobial susceptibility patterns and the frequency of occurrence of some virulence genes in the study isolates. Molecular fingerprinting of *A. veronii*, which was the predominant species isolated in the present study, was also performed using enterobacterial repetitive intergenic consensus (ERIC-)PCR to determine the presence of any dominant clones associated with septicaemia in fish.

MATERIALS AND METHODS

Sampling of freshwater ornamental fish

Moribund freshwater ornamental fish presenting at least one or more of the clinical signs of septicaemia (focal ulcerations on skin, haemorrhages on skin and fins, fin rot, exophthalmia, abdominal distension and scale protrusion) were collected from commercial aquaria and breeding farms located in the northwestern, central and western, north central provinces of Sri Lanka during the period from May 2007 to June 2008. Fish were transported to the laboratory in well-aerated water. Selection of aquaria from which to collect fish was based on the availability of diseased fish at the time of sampling, accessibility and convenience. In addition, septicaemic fish samples submitted for disease investigation to the Center for Aquatic Animal Disease Diagnosis and Research (University of Peradeniya) were also included.

Bacterial isolation and phenotypic characterization

All the diseased fish (Table 1) were subjected to a detailed preliminary laboratory examination and humanely euthanized using an overdose of MS 222. The kidneys and/or liver of affected fish were cultured aseptically on trypticase soy agar (TSA; Oxoid) for bacterial isolation and on *Aeromonas* starch DNA agar (Himedia) and Rimler Shotts agar (Himedia) for the preferential selection of aeromonads, and cultures were incubated in duplicate at 28°C and 35°C for 24 h. On few occasions where the fish were not sacrificed for sample collection, swabs from external lesions (ulcers) were used. Cultures in all plates were examined for colony morphology and Gram-staining reactions. Colonies were picked in order to represent all types of colonies in pure cultures and mixed cultures, and were subcultured on TSA. All Gram-negative isolates were subjected to a series of classical phenotypic tests including a cytochrome oxidase test, a motility test, an oxidation fermentation test and a catalase test. Furthermore, the ability to grow at 0% NaCl and the susceptibility to novobicin were tested. Accordingly, the isolates that were Gram negative, cytochrome oxidase positive, motile, fermentative, catalase positive, able to grow at 0% NaCl and resistant to novobicin were presumed to be motile *Aeromonas* and were used in further analysis. In instances where more than 1 fish was sampled from the same disease incidence, only 1 presumptive aeromonad isolate was analysed. Stock cultures were maintained for short periods at room temperature on TSA slants, and, for long-term storage, they were maintained at −20°C in tryptic soy broth medium supplemented with 10% glycerol (v/v). Details of the *Aeromonas* cultures used in this study are included in Table 1.

DNA extraction and molecular identification

Genomic bacterial DNA was extracted from cultures using the DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's instructions and stored at −20°C for further use. All presumptive *Aeromonas* isolates were first screened using *Aeromonas*-genus-specific primers (Chacón et al. 2002) targeting the glycerophospholipid cholesterol

Table 1. Origin, laboratory reference numbers and the phylogenetic identification through *rpoD*/*gyrB* sequencing of *Aeromonas* isolates (n = 53) recovered from diseased freshwater ornamental fish

Sample source		Isolates recovered		Phylogenetic identification
Host	Tissue	n	Lab. ref. no(s).	
Poecillia reticulata	Kidney and/or liver	15	Ae 1, Ae 3, Ae 13, Ae 15, Ae 20, Ae 22, Ae 31, Ae 39, Ae 44, Ae 46, Ae 47, Ae 49–Ae 51, Ae 53	15 *A. veronii*
Carassius auratus	Kidney	9	Ae 4, Ae 6, Ae 10, Ae 11, Ae 21, Ae 32, Ae 35, Ae 45, Ae 52	3 *A. hydrophila*, 4 *A. veronii*, 1 *A. caviae*, 1 *A. jandaei*
	Ulcer	3	Ae 8, Ae 27, Ae 42	2 *A. veronii*, 1 *A. caviae*
Cyprinus carpio (*koi*)	Kidney and/or liver	5	Ae 23, Ae 25, Ae 26, Ae 34, Ae 41	3 *A. veronii*, 1 *A. hydrophila*, 1 *A. enteropelogenes*
	Ulcer	1	Ae 43	1 *A. dhakensis*
Betta splendens	Kidney and/or liver	5	Ae 2, Ae 19, Ae 29, Ae 33, Ae 38	5 *A. veronii*
Pterophyllum spp.	Kidney and/or liver	6	Ae 9, Ae 14, Ae 18, Ae 28, Ae 36, Ae 40	5 *A. veronii*, 1 *A. caviae*
Helostoma temminckii	Kidney and/or liver	3	Ae 5, Ae 17, Ae 30	3 *A. veronii*
Poecilia sphenops	Kidney and/or liver	3	Ae 12, Ae 37, Ae 48	3 *A. veronii*
Osphronemus goramy	Ulcer	1	Ae 24	1 *A. dhakensis*
Symphysodon spp.	Liver	1	Ae 7	1 *A. veronii*
Xiphophorus helleri	Liver	1	Ae 16	1 *A. veronii*

acyltransferase gene (*gcat*) using the reaction and cycling conditions described by Soler et al. (2002); they were then identified at the species level based on the sequencing analysis of 2 housekeeping genes, *rpoD* and *gyrB.* An approximately 1100 bp fragment of *gyrB* and a 820 bp fragment of *rpoD* were amplified from the template DNA, purified using the FastGene Gel/PCR extraction kit (Nippon Genetics) and sequenced using the BigDye Terminator V3.1 Cycle Sequencing Kit on the 3130*xl* Genetic Analyzer (Applied Biosystems). The primers and conditions used for PCR amplifications and DNA sequencing were those described by Soler et al. (2004) and Yáñez et al. (2003). Resulting *rpoD* and *gyrB* contigs were assembled separately (DNA baser V.3.5.3, Heracle BioSoft), and a BLAST search was carried out to compare the sequences with those held in the National Centre for Biotechnology Information (NCBI, Bethesda, MD).

The aeromonads confirmed by nucleotide sequencing were re-identified using RFLP (restriction fragment length polymorphism) analysis of the PCR-amplified 16S rRNA gene. The objective of this step was to evaluate the concordance between *rpoD*/*gyrB* gene sequencing and 16S rDNA-RFLP, since the latter can easily be adopted in ordinary fish disease diagnostic laboratories with no access to gene sequencing facilities. Primers, amplification conditions and the endonuclease digestion conditions for 16S rDNA-RFLP were those described by Borrell et al. (1997) and Figueras et al. (2000). The 16S rDNA digestion products were electrophoresed in 18% v/v polyacrylamide gels at 155 V for 4 h.

Phylogenetic analysis

A 667 bp sequence of *rpoD* and a 926 bp sequence of *gyrB* were used in the final analysis (Martínez-Murcia et al. 2011). Each nucleotide sequence was determined at least twice to resolve ambiguous areas. These partial gene sequences were aligned (both independently and as a concatenated sequence of 1593 bp) using Clustal W in MEGA Version 5 (Tamura et al. 2011) with those from *Aeromonas* reference strains representing all published species to date. Genetic distances and clustering were determined using Kimura's 2-parameter model, and evolutionary trees were constructed by the neighbour-joining method with MEGA 5, with bootstrapping determined for 1000 replicates. The nucleotide sequences determined in this study have been

deposited in DDBJ/EMBL/GenBank databases with the following accession numbers: *rpoD* AB828727–AB828779 and *gyrB* AB829112–AB829164 (Table S1 in the Supplement at www.int-res.com/articles/suppl/d109p127_supp.pdf). The GenBank accession numbers for the *rpoD* and *gyrB* gene sequences of reference strains used in alignments are listed in Table S2 in the Supplement.

Molecular fingerprinting of *Aeromonas veronii* using ERIC-PCR

The genetic diversity of *Aeromonas veronii* isolates (n = 42) was assessed by ERIC-PCR using the primers ERIC 1 (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) and ERIC 2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′) (Versalovic et al. 1991) and the amplification conditions described by Fontes et al. (2011). Reactions were carried out 2 times for each isolate to confirm reproducibility. The amplification products were electrophoresed in a 1.2% agarose gel in Tris-borate buffer, and the fragment sizes were analysed using PyElph (Pavel & Vasile 2012). Banding patterns of each isolate were converted into a binary matrix, based on the presence and absence of the DNA fragments. To construct the dendrogram, levels of similarity between the profiles were calculated using the Dice coefficient, and cluster analysis of similarity matrices was calculated with the unweighted pair group method with arithmetic averages (UPGMA) (Garcia-Vallvé et al. 1999, Garcia-Vallvé & Puigloo 2002).

Determination of the susceptibility of isolates to antimicrobial agents

Susceptibility of all isolates to 8 different antimicrobial agents was determined by the disk diffusion (Kirby-Bauer) method on Mueller Hinton agar (Oxoid) according to the protocols established by the Clinical and Laboratory Standard Institute (CLSI 2008). The antibiotics and concentration (per disc) tested were as follows: amoxicillin (10 µg), neomycin (10 µg), sulphamethoxasole-trimethoprim (25 µg), chloramphenicol (30 µg), tetracyclin (30 µg), enrofloxacin (0.5 µg), erythromycin (15 µg) and nitrofurantoin (50 µg) (Oxoid). The antimicrobial agents used for susceptibility testing were chosen to cover different antibiotic groups that are used in ornamental fish aquaculture. Plates were incubated at 28°C for 24 h. Based on the size of the inhibition zones, isolates were characterised as sensitive, intermediate, or resistant.

Detection of virulence-related genes by PCR

All the isolates were screened by PCR for the presence of 7 genomic markers potentially linked to virulence—aerolysin (*aerA*), cytotoxic enterotoxin (*act*), the heat-stable and heat-labile cytotonic enterotoxins (*ast* and *alt*, respectively), serine protease (*ser*), DNase (*exu*) and flagellin (*fla*)—using the primers and conditions described by Nawaz et al. (2010) and Soler et al. (2002). The sequence of each primer used to amplify the target genes, the expected size of the PCR products and their references are listed in Table S3 in the Supplement.

PCR amplification of integrons

To determine whether the *Aeromonas* isolates carry integrons, we used PCR amplification to detect Class 1 and Class 2 integrase genes, *intI1* and *intI2*, respectively (Mazel et al. 2000). The primers used for the amplification of these genes and the predicted sizes of the amplification products are listed in Table S3 in the Supplement.

RESULTS

Characteristics of diseased fish and phenotypic identification of isolates

A total of 173 moribund freshwater fish (guppy *Poecillia reticulata*, n = 42; goldfish *Carassius auratus*, n = 56; koi carp *Cyprinus carpio*, n = 19; fighter *Betta splendens*, n = 27; kissing gourami *Helostoma temminckii*, n = 9; giant gourami *Osphronemus goramy*, n = 1; platy *Xiphophorus maculatus*, n = 2; angel *Pterophyllum* spp., n = 12; swordtail *Xiphophorus* spp., n = 1; molly *Poecilia sphenops*, n = 3; and discus *Symphysodon* spp., n = 1) originating from 34 aquaria (commercial level, n = 29; small-scale, household-based, n = 5) and ornamental fish breeding farms (n = 7) were examined in this study. All the aquaria/farms from which the diseased fish were collected had many species of fish. Fin rot, haemorrhages, dermal ulceration, ascites, scale protrusion and exophthalmia were the most commonly observed gross signs associated with infections.

Of the bacterial isolates selected from cultures grown on TSA and *Aeromonas*-selective media, a total of 74 isolates were presumptive *Aeromonas* (Table 2), as identified by the phenotypic methods, while the rest of the isolates consisted of *Citrobacter*

Table 2. Comparison of results obtained through different identification approaches for the clinical isolates of *Aeromonas* from freshwater ornamental fish. *gcat*: *Aeromonas*-specific glycerophospholipid cholesterol acyl transferase gene

Phenotypic identification	Screening for *gcat*	*rpoD*/*gyrB* identification	16S rDNA-RFLP identification
74 *Aeromonas* spp.	62 *Aeromonas* spp. (*gcat*+)	41 *A. veronii*	34 *A. veronii*, 7 atypical
		4 *A. hydrophila*	4 *A. hydrophila*
		3 *A. caviae*	3 *A. caviae*
		2 *A. dhakensis*	2 atypical
		1 *A. jandaei*	1 *A. jandaei*
		1 *A. enteropelogenes*	1 *A. enteropelogenes*
		10 non-*Aeromonas*	Not done
	12 non-*Aeromonas* (*gcat*–)	1 *A. veronii*	1 *A. veronii*
		11 non-*Aeromonas*	Not done

spp., *Pseudomonas* spp., *Flavobacterium* spp., *Enterobacter* spp., *Vibrio* spp. and few other Gram-negative isolates that could not be identified using phenotypic methods (data not shown).

Molecular identification

Amplification of *gcat* gene

Out of 74 isolates presumptively identified as *Aeromonas* spp. by phenotypic tests, only 62 isolates (62/74 = 84%) showed amplification of the *gcat* gene, suggesting that 12 isolates do not belong to the genus *Aeromonas* (Table 2). However, when the identities of all 74 isolates were confirmed with housekeeping gene sequencing (see the following subsection), only 52 *gcat*-positive isolates and one *gcat*-negative isolate were confirmed as aeromonads. Thus, through amplification of the *gcat* gene, correct identification at the genus level occurred in 98% (52/53) of the *Aeromonas* isolates characterized in the present study, corroborating the findings of Chacón et al. (2002, 2003) and Beaz-Hidalgo et al. (2010). The rest of the *gcat*-negative isolates were found by nucleotide sequencing to belong to other genera (data not shown). However, a false positive amplification was observed for 10 isolates which were subsequently identified as *Vibrio* spp.

Phylogenetic analysis using *rpoD* and *gyrB*

Of the 74 presumptive aeromonads, phylogenetic analysis of the *rpoD* and *gyrB* gene sequences resulted in a definitive identification of 53 isolates (72%) as belonging to *Aeromonas* species. Hence, subsequent investigations were carried out only for those 53 isolates of *Aeromonas* for which speciation was confirmed by *rpoD*/*gyrB* sequencing.

The unrooted neighbour-joining phylogenetic tree, constructed using the concatenated *rpoD* and *gyrB* gene sequences (1593 bp; Fig. 1) showed a clear clustering of all isolates with the reference strains of the respective species with high bootstrap values. Phylogenetic identification results revealed that *Aeromonas veronii* was the predominant species with 42 isolates (79.2%), followed by 4 *A. hydrophila* (7.5%), 3 *A. caviae* (5.7%), 2 *A. dhakensis* (formerly *A. aquariorum*) (3.8%), 1 *A. jandaei* (1.9%) and 1 *A. enteropelogenes* (*formerly A. trota*) (1.9%) (Table 2).

Identification by 16S rDNA PCR-RFLP

Out of 53 isolates subjected to 16S rDNA RFLP, 44 isolates (83%) exhibited 'typical' restriction patterns enabling an accurate speciation comparable with identification obtained through housekeeping gene sequencing. The remaining 9 isolates (7 *Aeromonas veronii* and 2 *A. dhakensis*) (17%) could not be assigned to a known species by 16S rDNA RFLP. Those 7 *A. veronii* isolates exhibited atypical restriction patterns (i.e. different to the pattern published for this species; Borrell et al. 1997, Figueras et al. 2000) (Table 2, Fig. S1 in the Supplement). The 2 *A. dhakensis* isolates shared an atypical pattern that is closely related to *A. caviae*. Figueras et al. (2009) reported that *A. dhakensis* produce either the *A. caviae* RFLP pattern or a somewhat similar pattern with extra bands.

Molecular fingerprinting of *Aeromonas veronii* by ERIC-PCR

ERIC-PCR fingerprints of *Aeromonas* isolates (Fig. S2 in the Supplement) consisted of 2 to 12 fragments ranging from 125 to 5015 bp. The dendrogram obtained from the ERIC-PCR analysis (Fig. 2) revealed 7 clusters at the 90% similarity level. Isolates within these clusters were considered to be genetically related. Six of these clusters were 2-isolate

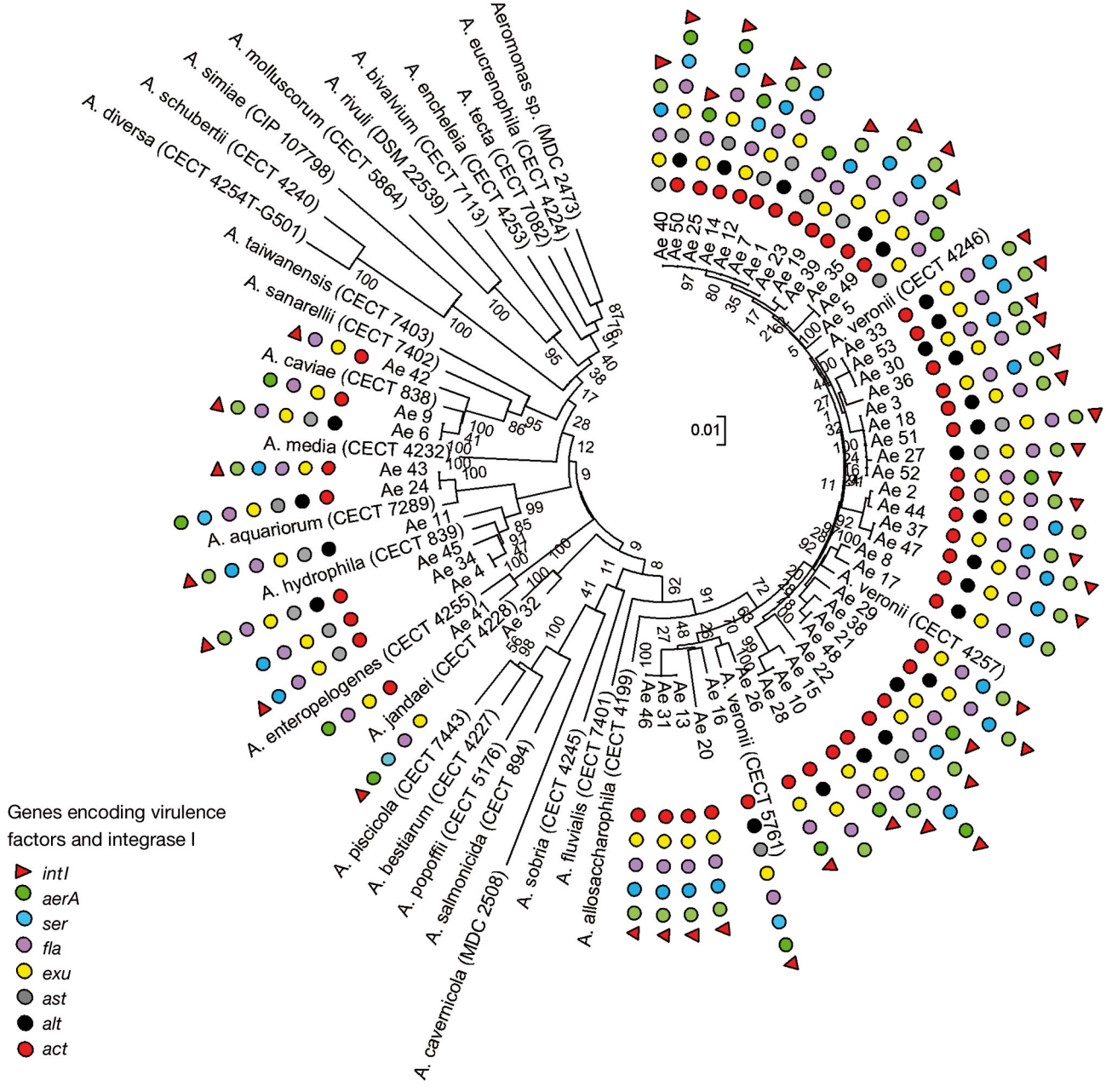

Fig. 1. Unrooted neighbour-joining phylogenetic tree constructed from the concatenated partial *rpoD* and *gyrB* gene sequences showing the relationships of the 53 *Aeromonas* isolates isolated in this study with reference strains. Numbers shown next to each node indicate bootstrap values (percentages of 1000 replicates). Coloured circles indicate the presence of the virulence factor genes analysed in this study for each isolate; the coloured triangle indicates the presence of the integrase 1 gene (*intI*). *aerA*: aerolysin; *ser*: serine protease; *fla*: flagellin; *exu*: DNase; *ast*: heat-stable cytotonic enterotoxin; *alt*: heat-labile cytotonic enterotoxin; *act*: cytotoxic enterotoxin

clusters and had 100% similarity. In the remaining cluster, 3 isolates grouped with a similarity level of 100% and grouped with a similarity of 90.9% with the fourth isolate. The ERIC patterns of all remaining isolates (n = 26) were diverse and hence considered as genetically distinct and unrelated, with similarities below 90%. Among these genetically unrelated members, the highest similarity between 2 isolates was 83.3%, as indicated by the Dice coefficient and observed for the isolates Ae 13 and Ae 46, which

were both isolated from the same fish species (guppy).

It was of interest to see that isolates with identical ERIC-PCR patterns (100% similarity) were isolated from different species of fish, indicating that genetically related isolates could infect different species of fish.

Antimicrobial susceptibility testing

The susceptibility levels of the 53 *Aeromonas* isolates against 8 antimicrobial agents are shown in Table 3. Besides the classical resistance of aeromonads to amoxicillin (beta lactam antibiotics) (98.1%), the highest resistances encountered were 58.5% to tetracycline and 54.7% to erythromycin. In contrast, the majority of isolates were susceptible to enrofloxacin (84.9%), followed by chloramphenicol (81.1%), neomycin (77.3%) and sulphamethoxasole-trimethoprim (71.7%). Multi-resistance to the tested antibiotics was found in 26 isolates (49%).

Detection of virulence-related genes and integrons

The distribution of the 7 virulence genes among motile *Aeromonas* isolates is shown in Table 4. All the isolates harboured at least 3 of the virulence genes tested, while all the virulence genes were present in 6/53 (11%) of isolates that included 5 isolates of *A. veronii* and 1 isolate of *A. dhakensis*. Overall, the genomic marker for flagellin (*fla*, 100%) was the most prevalent, followed by that for DNase (*exu*, 98%), aerolysin/hemolysin (*aerA*, 94%), cytotoxic enterotoxin (*act*, 83%), serine protease (*ser*, 62%), heat-labile cytotonic enterotoxin (*alt*, 49%) and heat-stable cytotonic enterotoxin (*ast*, 38%) in the isolates analysed. The most common combination of putative virulence genes was *aer*$^+$*ser*$^+$*fla*$^+$*exu*$^+$*act*$^+$*alt*$^-$*ast*$^-$, which was present in 19% (10/53) of isolates. Results of the amplification of integrase genes revealed that *intI1*-carrying bacteria corresponded to 77% (41) of the isolates, whereas the *intI2* gene was not detected in any of the isolates.

Table 3. Numbers of *Aeromonas* isolates (n = 53) susceptible/resistant to antimicrobial agents (CLSI 2008). Percent of isolates in parentheses

Antimicrobial agent	Susceptibility level		
	Susceptible	Intermediate	Resistant
Amoxicillin	1 (1.9)	0 (0)	52 (98.1)
Neomycin	41 (77.3)	7 (13.2)	5 (9.4)
Trimethoprim-sulphamethoxazole	38 (71.7)	1 (1.9)	14 (26.4)
Chloramphenicol	43 (81.1)	6 (11.3)	4 (7.5)
Tetracycline	15 (28.3)	7 (13.2)	31 (58.5)
Enrofloxacin	45 (84.9)	4 (7.5)	4 (7.5)
Erythromycin	4 (7.5)	20 (37.7)	29 (54.7)
Nitrofurantoin	34 (64.1)	7 (13.2)	12 (22.6)

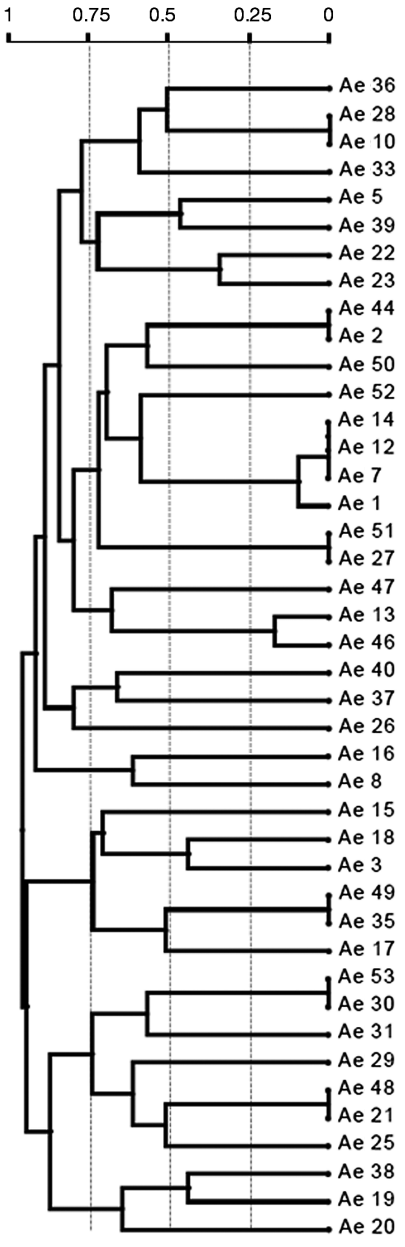

Fig. 2. Relatedness of ERIC-PCR fingerprint patterns from 42 *Aeromonas veronii* isolates. Genetic distance was calculated by the UPGMA cluster method based on the Dice coefficient

Table 4. Numbers of putative virulence genes in a total of 53 *Aeromonas* isolates recovered from ornamental fish. Percent of isolates in parentheses. *aerA*: aerolysin; *act*: cytotoxic enterotoxin; *alt*: heat-labile cytotonic enterotoxin; *ast*: heat-stable cytotonic enterotoxin; *ser*: serine protease; *exu*: DNase; *fla*: flagellin

Aeromonas species	n	Virulence gene						
		aerA	*act*	*alt*	*ast*	*ser*	*exu*	*fla*
A. veronii	42	42 (100)	36 (86)	22 (52)	14 (33)	27 (64)	41 (98)	42 (100)
A. hydrophila	4	2 (50)	3 (75)	2 (50)	4 (100)	3 (75)	4 (100)	4 (100)
A. caviae	3	2 (67)	2 (67)	1 (33)	1 (33)	0 (0)	3 (100)	3 (100)
A. dhakensis	2	2 (100)	2 (100)	1 (50)	1 (50)	2 (100)	2 (100)	2 (100)
A. jandaei	1	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)
A. enteropelogenes	1	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)
Total	53	50 (94)	44 (83)	26 (49)	20 (38)	33 (62)	52 (98)	53 (100)

DISCUSSION

Isolation of 6 different species of motile aeromonads from 10 diverse species of ornamental fish during the current study adds more evidence for the wide host range and geographical distribution of this bacterium. Motile aeromonads often act as secondary pathogens in fish; therefore, their isolation from septicaemic fish is not so surprising in view of the intensive culture practices in ornamental fish farming that might favour opportunistic infections. The role of other bacteria such as *Citrobacter* spp., *Pseudomonas* spp., *Flavobacterium* spp., *Enterobacter* spp., or *Vibrio* spp., isolated either as sole pathogens or co-pathogens with aeromonads, should not be overlooked since a certain role of these genera as fish pathogens has been recognized (Lewbart 2001, Musa et al. 2008). Moreover, mixed infections are probably not uncommon in ornamental fish that live together with highly diverse microbial communities. However, incidences of mixed infections were not examined in the current study, which focused preferentially on aeromonads and characterized only a single isolate from each infection incidence.

Aeromonas hydrophila is the most cited motile *Aeromonas* species classically linked to MAS in freshwater fish (Hettiarachchi & Cheong 1994, Nielsen et al. 2001, Austin 2011). In contrast, we found *A. veronii* (79.2%) to be the predominant species which is in agreement with others who reported the dominance of *A. veronii* among fish pathogenic aeromonad isolates following molecular identification (Sreedharan et al. 2011, 2013, Hu et al. 2012, Yi et al. 2013). This difference could possibly be related to the host species selected and the geographical location, but it may have been due, at least in part, to the methods of bacterial identification employed. In a study done to investigate the causative organisms behind incidences of bacterial disease (n = 23) among a number of different species of ornamental fish in Sri Lanka, Hettiarachchi & Cheong (1994) found *A. hydrophila* to be the most dominant species, associated with 18 incidences (78.26%). In contrast, Sreedharan et al. (2013) investigated 3 incidences of disease (1 each in gourami, goldfish and oscar), in Kerala, India, and isolated *A. veronii* as the causative organism in all 3 cases. A comparatively higher occurrence of *A. veronii* over *A. hydrophila* has been reported among aeromonads isolated from diseased eels in the Republic of Korea (Yi et al. 2013) and diseased freshwater aquaculture fish from China (Hu et al. 2012).

The identification of aeromonads is fraught with numerous difficulties due to the phenotypic, serological and genotypic heterogeneity existing within the genus. Discrepancies in identification of *Aeromonas* resulting from poor correlation between phenotypic and genetic identification schemes have been well documented (Kozińska 2007, Beaz-Hidalgo et al. 2010). This problem also occurred in the present study, where identification using specific but a limited number of phenotypic properties resulted in the apparent misidentification of 21 isolates as aeromonads. *gcat*, a gene that is used to identify *Aeromonas* at the genus level (Chacón et al. 2002), was useful in the majority (98%) of isolates, with slight deviations in the rest. *gcat* is often considered a virulence-related gene but aeromonads for which the *gcat* gene cannot be amplified have been reported (Nawaz et al. 2010), as we observed for 1 of our isolates. This could be the result of a possible mismatch of primers, as noted by Chacón et al. (2002). However, false positive amplification of this gene resulted in the misidentification of 10 isolates as belonging to the genus *Aeromonas* even though they actually belonged to the genus *Vibrio*. This could have been due to the low annealing temperature (56°C) during amplification. Weak amplification of the same size fragment at

low annealing temperatures was observed in some *Vibrio* isolates by Chacón et al. (2002); this problem was overcome by increasing the temperature to 65°C.

The *rpoD* and *gyrB* genes have already been used successfully as accurate, unequivocal molecular chronometers for identification of the genus *Aeromonas* (Yáñez et al. 2003, Soler et al. 2004, Martínez-Murcia et al. 2011). Combined analysis of the *rpoD* and *gyrB* genes improved resolution and enabled unambiguous speciation of all isolates used in the present study. In comparison, 16S rRNA PCR-RFLP (Borrell et al. 1997, Figueras et al. 2000), while being discriminatory for the majority of isolates (83%), produced either atypical restriction patterns or patterns that were very similar to those of other species in the rest of the isolates. This could be due to the high sequence similarity and the occurrence of microheterogeneities in the 16S rRNA genes (Alperi et al. 2008). Closely related species (i.e. those that have an identical or almost identical 16S rRNA gene sequence) produce the same RFLP pattern, as in the case of *A. piscicola* which has the same pattern as *A. salmonicida* and *A. bestiarum* (Beaz-Hidalgo et al. 2010) and *A. dhakensis* which has the same pattern as *A. caviae* (Figueras et al. 2009). According to these observations, a need for the incorporation of polyphasic molecular approaches in precise species identification of aeromonads becomes clearly evident. While the exact taxonomic position of fish-pathogenic *Aeromonas* species might not be of interest to all fish pathologists, its use in epidemiological studies and in recognizing new pathogenic species and subspecies should not be overlooked. However, for ordinary fish disease diagnostic laboratories, with no access to gene sequencing, other techniques such as those mentioned above are still of use, even though the known limitations could result in misidentification/underestimation of different pathogenic species.

A. jandaei and *A. enteropelogenes* have rarely been isolated from ornamental fish (John & Hatha 2012), and little information is available regarding their association with clinical disease in ornamental fish. To the best of our knowledge, this is the first time that *A. dhakensis* has been recovered from clinically diseased ornamental fish since the original description of this species from the aquarium water and skin of ornamental fish (Martínez-Murcia et al. 2008, Beaz-Hidalgo et al. 2013). Indeed, it is the first time this species has been isolated in Sri Lanka. Isolation of this seemingly globally distributed species (Aravena-Román et al. 2011, Yi et al. 2013) from diseased ornamental fish provides additional evidence that a diverse range of motile aeromonads could potentially be associated with septicaemia in ornamental fish. The occurrence and pathogenesis of these rarely isolated motile aeromonads in tropical aquarium fish are not well understood and deserve further study.

ERIC-PCR, a low cost, rapid, reproducible strain typing method with high discriminatory power (Soler et al. 2003) revealed the intraspecific diversity that exists within the *A. veronii* isolated in the present study. The majority of isolates were genetically distinct (62% of the isolates had similarities below 90%), with no dominant clones of *A. veronii* associated with MAS in the fish population investigated. This kind of high genetic diversity had been observed among clinical and environmental isolates of aeromonads from different sources typed by the above technique (Davin-Regli et al. 1998, Szczuka & Kaznowski 2004).

High levels of antimicrobial resistance in bacteria isolated from ornamental fish and their environment is not a novel observation (Verner-Jeffreys et al. 2009, Cízek et al. 2010, Dias et al. 2012). In agreement, surprisingly numerous multi-antibiotic-resistant bacteria (49%) were observed among our isolates, apart from their classical resistance to beta lactam antibiotics (Janda & Abbott 2010). Tolerance to tetracycline and erythromycin was particularly widespread (>50%), a finding that was in common with other comparable investigations of motile aeromonads from ornamental fish (Dias et al. 2012, Sreedharan et al. 2012). While tolerance of these antibiotics has likely resulted from their use in the aquarium fish industry, resistance can also arise from gene mutations or by acquisition of transferable genetic elements such as integrons (Jacobs & Chenia 2007). The observed levels of multi-resistance could be attributed to the horizontal spread of resistance genes, which is further supported by the presence of Class 1 integrons in 77% of the isolates.

Screening for the presence of virulence genes as a method to evaluate the potential virulence of aeromonads could be speculative, since virulence is a complex process, and empirical testing with a disease challenge is often necessary for conclusive results. However, expression of the putative virulence-associated factors in *Aeromonas* appears to be affected by environmental conditions (Tso & Dooley 1995, Merino et al. 1998), making the detection of true virulent strains difficult. Nevertheless, screening for virulence genes has been used in many studies as a practical approach for evaluating the genetic potential of aeromonads to express virulence factors (Puthucheary et al. 2012). In agreement with previ-

ous studies (Nawaz et al. 2010, Hu et al. 2012, Yi et al. 2013), we found high heterogeneity in the distribution of toxin genes among the tested isolates, with a heterogeneous distribution forming 18 different virulence gene combinations. High prevalences of *gcat*, *exu*, *fla*, *act* and *aerA* genes are consistent with the results of Yi et al. (2013) and Nawas et al. (2010) from clinical isolates of fish.

In conclusion, the present study highlights the diversity of mesophilic *Aeromonas* species that could potentially be associated with MAS in ornamental fish. The ability of rare aeromonad species to act as fish pathogens needs to be explored further, in order to clarify their role in disease. The relatively high prevalence of antimicrobial-resistant bacteria harbouring multiple virulence genes raises concerns about possible treatment failures in fish disease outbreaks and the public health threats they may pose, given the importance of aeromonads as emerging human pathogens (Janda & Abbott 2010). Overall, the results obtained highlight the need to promote responsible ornamental fish ownership, good husbandry practices and prudent use of antimicrobials in the ornamental fish industry.

Acknowledgements. The authors thank the Council for Agricultural Research Policy (CARP), Sri Lanka, for the financial support (Grant No: CARP 12/689/517) during the initial part of this study and the National Aquaculture Development Authority of Sri Lanka (NAQDA) for assistance with sample collection. We also thank Dr. D. R. A. Dissanayake for her constructive comments, Dr. Temdoung Somisiri for providing reference cultures and Ms. R. E. Edirisinghe for her valuable technical support. This work was also supported by JSPS KAKENHI Grant Number 24248034.

LITERATURE CITED

Alperi A, Figueras MJ, Inza I, Martínez-Murcia AJ (2008) Analysis of 16S rRNA gene mutations in a subset of *Aeromonas* strains and their impact in species delineation. Int Microbiol 11:185–194

Aravena-Román M, Harnett GB, Riley TV, Inglis TJ, Chang BJ (2011) *Aeromonas aquariorum* is widely distributed in clinical and environmental specimens and can be misidentified as *Aeromonas hydrophila.* J Clin Microbiol 49:3006–3008

Austin B (2011) Taxonomy of bacterial fish pathogens. Vet Res 42:20

Beaz-Hidalgo R, Figueras MJ (2012) Molecular detection and characterization of furunculosis and other *Aeromonas* fish infections. In: Carvalho ED, David GS, Silva RJ (eds) Health and environment in aquaculture. InTech, Rijeka, p 97–132

Beaz-Hidalgo R, Alperi A, Buján N, Romalde JL, Figueras MJ (2010) Comparison of phenotypical and genetic identification of *Aeromonas* strains isolated from diseased fish. Syst Appl Microbiol 33:149–153

Beaz-Hidalgo R, Martínez-Murcia A, Figueras, MJ (2013) Reclassification of *Aeromonas hydrophila* subsp. *dhakensis* Huys et al. 2002 and *Aeromonas aquariorum* Martínez-Murcia et al. 2008 as *Aeromonas dhakensis* sp. nov. comb nov. and emendation of the species *Aeromonas hydrophila*. Syst Appl Microbiol 36: 171–176

Borrell N, Acinas SG, Figueras MJ, Martínez-Murcia AJ (1997) Identification of *Aeromonas* clinical isolates by restriction fragment length polymorphism of PCR amplified 16SrRNA genes. J Clin Microbiol 35:1671–1674

Chacón MR, Castro-Escarpulli G, Soler L, Guarro J, Figueras MJ (2002) A DNA probe specific for *Aeromonas* colonies. Diagn Microbiol Infect Dis 44:221–225

Chacón MR, Figueras MJ, Castro-Escarpulli G, Soler L, Guarro J (2003) Distribution of virulence genes in clinical and environmental isolates of *Aeromonas* spp. Antonie Leeuwenhoek 84:269–278

Cízek A, Dolejska M, Sochorova R, Strachotova K, Piackova V, Vesely T (2010) Antimicrobial resistance and its genetic determinants in aeromonads isolated in ornamental (koi) carp (*Cyprinus carpio* koi) and common carp (*Cyprinus carpio*). Vet Microbiol 142:435–439

CLSI (Clinical and Laboratory Standards Institute) (2008) Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, approved standard 3rd edn. CLSI Document M31-A3, CLSI, Wayne, PA

Davin-Regli A, Bollet C, Chamorey E, Colonna D'Istria V, Cremieux A (1998) A cluster of cases of infections due to *Aeromonas hydrophila* revealed by combined RAPD and ERIC-PCR. J Med Microbiol 47:499–504

Dias C, Mota V, Martínez-Murcia A, Saavedra MJ (2012) Antimicrobial resistance patterns of *Aeromonas* spp. isolated from ornamental fish. J Aquac Res Dev 3:131

Figueras MJ, Soler L, Chacon MR, Guarro J, Martínez-Murcia AJ (2000) Extended method for discrimination of *Aeromonas* spp. by 16SrDNA-RFLP analysis. Int J Syst Evol Microbiol 50:2069–2073

Figueras MJ, Alperi A, Saavedra MJ, Ko WC, Gonzalo N, Navarro M, Martínez-Murcia A (2009) Clinical relevance of the recently described *Aeromonas aquariorum.* J Clin Microbiol 47:3742–3746

Fontes MC, Saavedra MJ, Martins C, Martínez-Murcia A (2011) Phylogenetic identification of *Aeromonas* from pigs slaughtered for consumption in slaughterhouses at the North of Portugal. Int J Food Microbiol 146:118–122

Garcia-Vallvé S, Palau J, Romeu A (1999) Horizontal gene transfer in glycosyl hydrolases inferred from codon usage in *Escherichia coli* and *Bacillus subtilis.* Mol Biol Evol 16:1125–1134

Garcia-Vallvé S, Puigloo P (2002) DendroUPGMA: a dendrogram construction utility, modified version Dec 2009. http://genomes.urv.cat/UPGMA/ (accessed 5 Dec 2013)

Hettiarachchi DC, Cheong CH (1994) Some characteristics of *Aeromonas hydrophila* and *Vibrio* species isolated from bacterial disease outbreaks in ornamental fish culture in Sri Lanka. J Natl Sci Counc Sri Lanka 22:361–369

Hu M, Wang N, Pan ZH, Lu CP, Liu YJ (2012) Identity and virulence properties of *Aeromonas* isolates from diseased fish, healthy controls and water environment in China. Lett Appl Microbiol 55:224–233

Jacobs L, Chenia HY (2007) Characterization of integrons and tetracycline resistance determinants in *Aeromonas* spp. isolated from South African aquaculture systems. Int J Food Microbiol 114:295–306

Janda JM, Abbott SL (2010) The genus *Aeromonas*: taxonomy, pathogenicity, and infection. Clin Microbiol Rev 23: 35–73

John N, Hatha AAM (2012) Prevalence, distribution and drug resistance of motile aeromonads in freshwater ornamental fishes. Indian J Fish 59:161–164

Kozińska A (2007) Dominant pathogenic species of motile aeromonads isolated from diseased and healthy fish cultured in Poland. J Fish Dis 30:293–301

Lewbart GA (2001) Bacteria and ornamental fish. Semin Avian Exotic Pet Med 10:48–56

Martínez-Murcia AJ, Saavedra MJ, Mota VR, Maier T, Stackebrandt E, Cousin S (2008) *Aeromonas aquariorum* sp. nov., isolated from aquaria of ornamental fish. Int J Syst Evol Microbiol 58:1169–1175

Martínez-Murcia A, Morena A, Saavedra MJ, Ocina R, López-Álvarez M, Lara E, Figueras MJ (2011) Multilocus phylogenetic analysis of the genus *Aeromonas.* Syst Appl Microbiol 34:189–199

Mazel D, Dychinco B, Webb VA, Davies J (2000) Antibiotic resistance in the ECOR collection, integrons and identification of a novel *aad* gene. Antimicrob Agents Chemother 44:1568–1574

Merino S, Aguillar A, Rubires X, Tomàs JM (1998) Mesophilic Aeromonas strains from different serogroups: the influence of growth temperature and osmolarity on lipopolysaccharide and virulence. Res Microbiol 149: 407–416

Musa N, Wei SL, Shaharom F, Wee W (2008) Surveillance of bacteria species in diseased freshwater ornamental fish from aquarium shop. World Appl Sci J 3:903–905

Nawaz M, Khan SA, Khan AA, Sung K, Tran Q, Kerdahi K, Steele R (2010) Detection and characterization of virulence genes and integrons in *Aeromonas veronii* isolated from catfish. Food Microbiol 27:327–331

Nielsen ME, Hoi L, Schmidt AS, Qian D, Shimada T, Shen JY, Larsen JL (2001) Is *Aeromonas hydrophila* the dominant motile *Aeromonas* species that causes disease outbreaks in aquaculture production in the Zhejiang Province of China? Dis Aquat Org 46:23–29

Pavel AB, Vasile CI (2012) PyElph — a software tool for gel images analysis and phylogenetics. BMC Bioinformatics 13:9

Puthucheary SD, Puah SM, Chua KH (2012) Molecular characterization of clinical isolates of *Aeromonas* species from Malaysia. PLoS ONE 7:e30205

Soler L, Figueras MJ, Chacón MR, Vila J, Marco F, Martínez-Murcia AJ, Guarro J (2002) Potential virulence and antimicrobial susceptibility of *Aeromonas popoffii* recovered from freshwater and seawater. FEMS Immunol Med Microbiol 32:243–247

Soler L, Figueras MJ, Chacón MR, Guarro J, Martínez-Murcia AJ (2003) Comparison of three molecular methods for typing *Aeromonas popoffii* isolates. Antonie Leeuwenhoek 83:341–349

Soler L, Yañez MA, Chacón MR, Aguilera-Arreola MG, Catalán V, Figueras MJ, Martínez-Murcia AJ (2004) Phylogenetic analysis of the genus *Aeromonas* based on two housekeeping genes. Int J Syst Evol Microbiol 54: 1511–1519

Sreedharan K, Phillip R, Bright Singh IS (2011) Isolation and characterization of virulent *Aeromonas veronii* from ascitic fluid of oscar *Astronotus ocellatus* showing signs of infectious dropsy. Dis Aquat Org 94:29–39

Sreedharan K, Phillip R, Bright Singh IS (2012) Virulence potential and antibiotic susceptibility pattern of motile aeromonads associated with freshwater ornamental fish culture systems: a possible threat to public health. Braz J Microbiol 43:754–765

Sreedharan K, Philip R, Bright Singh IS (2013) Characterization and virulence potential of phenotypically diverse *Aeromonas veronii* isolates recovered from moribund freshwater ornamental fishes of Kerala, India. Antonie Leeuwenhoek 103:53–67

Sri Lanka EDB (Export Development Board) (2014) Business portal: Trade information. SLEDB, Colombo. Available at www.srilankabusiness.com/en/find-sri-lankan-suppliers/product-profiles/aquarium-fish-aqua-culture-and-seafood (accessed 8 Jan 2013)

Szczuka E, Kaznowski A (2004) Typing of clinical and environmental *Aeromonas* spp. strains by random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR, and enterobacterial repetitive intergenic consensus sequence PCR. J Clin Microbiol 42: 220–228

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731–2739

Tso MD, Dooley JSG (1995) Temperature-dependent protein and lipopolysaccharide expression in clinical Aeromonas isolates. J Med Microbiol 42:32–38

Verner-Jeffreys DW, Welch TJ, Schwarz T, Pond MJ and others (2009) High prevalence of multidrug-tolerant bacteria and associated antimicrobial resistance genes isolated from ornamental fish and their carriage water. PLoS ONE 4:e8388

Versalovic J, Koeuth T, Lupski JR (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 19:6823–6831

Yáñez MA, Catalán V, Apraiz D, Figueras MJ, Martínez-Murcia AJ (2003) Phylogenetic analysis of members of the genus *Aeromonas* based on *gyrB* gene sequences. Int J Syst Evol Microbiol 53:875–883

Yi SW, You MJ, Cho HS, Lee CS, Kwon JK, Shin GW (2013) Molecular characterization of *Aeromonas* species isolated from farmed eels (*Anguilla japonica*). Vet Microbiol 164:195–200

Editorial responsibility: Catherine Collins, Aberdeen, UK

Submitted: July 8, 2013; Accepted: February 16, 2014
Proofs received from author(s): April 12, 2014