International Journal of Systematic and Evolutionary Microbiology (2003), **53**, 875–883 **DOI** 10.1099/ijs.0.02443-0

Phylogenetic analysis of members of the genus *Aeromonas* based on *gyrB* gene sequences

M. A. Yáñez,[1,2] V. Catalán,[2] D. Apráiz,[2] M. J. Figueras[3] and A. J. Martínez-Murcia[1]

Correspondence
A. J. Martínez-Murcia
ammurcia@umh.es

[1]Unidad de Diagnóstico Molecular, EPSO, Universidad Miguel Hernández, Ctra Beniel Km 3, E-03312 Orihuela (Alicante), Spain

[2]Labaqua SA, Alona 33, E-03007 Alicante, Spain

[3]Unidad de Microbiología, Departamento de Ciencias Médicas Básicas, Facultad de Medicina y Ciencias de la Salud, Universidad Rovira y Virgili, Reus, E-43201 Tarragona, Spain

The phylogenetic relationships of all known species of the genus *Aeromonas* were investigated by using the sequence of *gyrB*, a gene that encodes the B-subunit of DNA gyrase. Nucleotide sequences of *gyrB* were determined from 53 *Aeromonas* strains, including some new isolates, which were also characterized by analysis of the 16S rDNA variable regions. The results support the recognition of the family *Aeromonadaceae*, as distinct from *Plesiomonas shigelloides* and other enteric bacteria. This phylogenetic marker revealed strain groupings that are consistent with the taxonomic organization of all *Aeromonas* species described to date. In particular, *gyrB* results agreed with 16S rDNA analysis; moreover, the former showed a higher capacity to differentiate between species. The present analysis was useful for the elucidation of reported discrepancies between different DNA–DNA hybridization sets. Additionally, due to the sequence diversity found at the intraspecies level, *gyrB* is proposed as a useful target for simultaneous identification of species and strains. In conclusion, the *gyrB* gene has proved to be an excellent molecular chronometer for phylogenetic studies of the genus *Aeromonas*.

INTRODUCTION

The genus *Aeromonas* comprises a collection of oxidase- and catalase-positive, glucose-fermenting, facultatively anaerobic, Gram-negative, rod-shaped bacteria that are resistant to the vibriostatic agent O/129 and are generally motile by means of polar flagella (Popoff, 1984). Aeromonads are autochthonous to aquatic environments worldwide and have been implicated in the aetiology of a variety of fish and human diseases, frequently including diarrhoea and occasionally systemic infections (Janda, 1991). Over the past few years, interest in the genus *Aeromonas* as an emergent human pathogen has increased significantly (Abbott *et al.*, 1998; Janda & Abbott, 1998; Joseph & Carnahan, 2000).

Published online ahead of print on 29 November 2002 as DOI 10.1099/ijs.0.02443-0.

Abbreviation: HG, homology group.

The GenBank/EMBL/DDBJ accession numbers for the *gyrB* sequences of *Aeromonas* species determined in this study are AY101772–AY101824.

Tables showing percentage similarities and percentage nucleotide substitutions in *gyrB* in the genus *Aeromonas* are available as supplementary data in IJSEM Online.

The classification of the genus *Aeromonas* has been dogged by confusion and controversy. In *Bergey's Manual of Systematic Bacteriology* (Popoff, 1984), the genus was divided into three mesophilic and motile species (*Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas sobria*) and the psychrophilic, non-motile species *Aeromonas salmonicida*. Extensive DNA–DNA hybridization studies (Popoff *et al.*, 1981; Hickman-Brenner *et al.*, 1987, 1988; Kuijper *et al.*, 1989; Carnahan *et al.*, 1991) have resulted in the recognition of 14 so-called DNA homology groups (HGs): *A. hydrophila* (HG1), *Aeromonas* sp. (unnamed; HG2), *A. salmonicida* (HG3), *A. caviae* (HG4), *Aeromonas media* (HG5), *Aeromonas eucrenophila* (HG6), *A. sobria* (HG7), *Aeromonas veronii* biogroup sobria (HG8), *Aeromonas jandaei* (HG9), *A. veronii* biogroup veronii (HG10), *Aeromonas* sp. (unnamed; HG11), *A. schubertii* (HG12), *Aeromonas* group 501 (HG13; formerly Enteric group 501) and *Aeromonas trota* (HG14). The name *Aeromonas bestiarum* has been proposed for strains included in HG2 (Ali *et al.*, 1996). During the past decade, three novel species have been described: *Aeromonas allosaccharophila* (Martínez-Murcia *et al.*, 1992b), *Aeromonas encheleia* (Esteve *et al.*, 1995b) and *Aeromonas popoffii* (Huys *et al.*, 1997b). The species names *Aeromonas enteropelogenes* and *Aeromonas ichthiosmia* (Schubert *et al.*, 1990a, b) are now

Table 1. *Aeromonas* strains used in this study

Corresponding DNA HG is indicated. ATCC, American Type Culture Collection, Manassas, VA, USA; CCUG, Culture Collection of the University of Göteborg, Göteborg, Sweden; CDC, Centers for Disease Control, United States Public Health Service, Atlanta, GA, USA; CECT, Colección Española de Cultivos Tipo, Universidad de Valencia, Valencia, Spain; CIP, Collection Bactérienne de l'Institut Pasteur, Paris, France; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; LMG, Culture Collection of the Laboratorium voor Microbiologie Gent, Universiteit Gent, Gent, Belgium; NCIMB, National Culture Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, UK.

Species	16S rDNA	*gyrB* GenBank no.
***A. hydrophila* (HG1)**		
CECT 839^T (=ATCC 7966^T=DSM 30187^T=Popoff 543^T)	*A. hydrophila*	AY101778
Isolate 63c (=CECT 5216)	*A. hydrophila*	AY101789
***A. bestiarum* (HG2)**		
CECT 4227^T (=ATCC 51108^T=CDC 9533-76^T=LMG 3751^T=Popoff 218^T)	*A. salmonicida*/*A. bestiarum*	AY101774
LMG 13662 (=A295)	*A. salmonicida*/*A. bestiarum*	AY101786
LMG 13448 (=A169)	*A. salmonicida*/*A. bestiarum*	AY101822
***A. salmonicida* (HG3)**		
CECT 894^T (=ATCC 33658^T=CIP 103209^T=LMG 3780^T)	*A. salmonicida*/*A. bestiarum*	AY101773
CECT 5173 (=Popoff 316)	*A. salmonicida*/*A. bestiarum*	AY101810
Isolate 220c (=LMG 18998)	*A. salmonicida*/*A. bestiarum*	AY101816
subsp. *pectinolytica* DSM 12609 (=34MEL)	*A. salmonicida*/*A. bestiarum*	AY101790
subsp. *mausocida* CECT 896 (=ATCC 27013=CIP 103210=LMG 3782)	*A. salmonicida*/*A. bestiarum*	AY101784
subsp. *achromogenes* CECT 895 (=ATCC 33659=LMG 3781=NCIMB 1110)	*A. salmonicida*/*A. bestiarum*	AY101785
***A. caviae* (HG4)**		
CECT 838^T (=ATCC 15468^T=LMG 3775^T=Popoff 545^T)	*A. caviae*	AY101783
CECT 4221 (=ATCC 15467=CIP 76.15=LMG 3755=Popoff 544)	*A. caviae*	AY101794
***A. media* (HG5)**		
CECT 4232^T (=ATCC 33907^T=LMG 9073^T=NCIMB 2237^T)	*A. media*	AY101782
CECT 4234 (=ATCC 35950)	*A. media*	AY101824
***A. eucrenophila* (HG6)**		
CECT 4224^T (=ATCC 23309^T=LMG 3774^T=NCIMB 74^T=Popoff 546^T)	*A. eucrenophila*	AY101776
CECT 4854 (=A1651=CCUG 30343=LMG 13687)	*A. eucrenophila*	AY101813
CECT 4827 (=LMG 13057=CDC 9179=A914=CCUG 30341)	*A. eucrenophila*	AY101820
CECT 4825 (=A1655=CCUG 30347=LMG 13691)	*A. encheleia*	AY101793
***A. sobria* (HG7)**		
CECT 4245^T (=ATCC 43979^T=CIP 74.33^T=LMG 3783^T=Popoff 208^T)	*A. sobria*	AY101781
***A. veronii* bg. sobria (HG8)**		
CECT 4246 (=ATCC 9071=NCIMB 37=LMG 3785)	*A. veronii*	AY101775
CECT 4906 (=A64=CCUG 30357=LMG 13068)	*A. veronii*	AY101791
CECT 4911 (=A155=CCUG 30362=LMG 13073)	*A. allosaccharophila*	AY101792
***A. jandaei* (HG9)**		
CECT 4228^T (=A1642^T=ATCC 49568^T=LMG 12221^T)	*A. jandaei*	AY101780
***A. veronii* bg. veronii (HG10)**		
CECT 4257^T (=A901^T=ATCC 35624^T=LMG 9075^T)	*A. veronii*	AY101795
CECT 4260 (=ATCC 35625=LMG 16334)	*A. veronii*	AY101787
***Aeromonas* sp. (HG11)**		
CECT 4253 (=A902=ATCC 35941=NCIMB 13014)	*Aeromonas* sp. (HG11)	AY101779
CECT 4856 (=A926=LMG 13076=CCUG 30365)	*Aeromonas* sp. (HG11)	AY101808
CECT 4824 (=A1654=LMG 13062)	*Aeromonas* sp. (HG11)	AY101809
***A. schubertii* (HG12)**		
CECT 4240^T (=ATCC 43700^T=CDC 2446-81^T=LMG 9074^T)	*A. schubertii*	AY101772
***Aeromonas* sp. (HG13)**		
CECT 4254 (=ATCC 43946=CDC 2478-85)	*Aeromonas* sp.	AY101806
***A. trota* (HG14)**		
CECT 4255^T (=A1646^T=ATCC 49657^T=LMG 12223^T)	*A. trota*	AY101800
CECT 4935 (=LMG 13080=ATCC 49659=AS370)	*A. trota*	AY101805

Table 1. cont.

Species	16S rDNA	*gyrB* GenBank no.
***A. allosaccharophila* (HG15)**		
CECT 4199^T (=ATCC 51208^T=CCUG 31218^T=S289^T=LMG 14059^T)	*A. allosaccharophila*	AY101777
CECT 4220 (=ATCC 35942=CDC 715-84=LMG 14021)	*A. allosaccharophila*	AY101812
CECT 4200 (=S290=LMG 14058)	*A. allosaccharophila*	AY101823
***A. encheleia* (HG16)**		
CECT 4342^T (=ATCC 51929^T=S181^T=NCIMB 13442^T)	*A. encheleia*	AY101799
CECT 4341 (=ATCC 51930=S176=LMG 16329)	*A. encheleia*	AY101804
***A. popoffii* (HG17)**		
CECT 5176^T (=LMG 17541^T=IK-O-a-10-3^T)	*A. popoffii*	AY101801
LMG 17543	*A. popoffii*	AY101803
LMG 17544	*A. popoffii*	AY101821
***Aeromonas* sp.**		
Isolate 101F (=CECT 5219)	*A. salmonicida*/*A. bestiarum*	AY101788
Isolate A7 (=LMG 13452)	*A. salmonicida*/*A. bestiarum*	AY101815
Isolate 37	*A. salmonicida*/*A. bestiarum*	AY101819
Isolate 83	*A. caviae*	AY101802
Isolate 531c	Newly described (not shown)	AY101817
Isolate 610	Newly described (not shown)	AY101814
Isolate 520	Newly described (not shown)	AY101811
Isolate CFAE 35	Newly described (not shown)	AY101798
Isolate 350c	*A. sobria*	AY101807
Isolate 344	*A. jandaei*	AY101796
Isolate 16s-93	*A. veronii*	AY101797
CECT 5026 (=LMG 16405=KV 78=A1785)	Newly described (not shown)	AY101818

considered to be synonyms of *A. trota* and *A. veronii*, respectively (Carnahan, 1993; Collins *et al.*, 1993). Phylogenetic analyses based on 16S rRNA genes indicated that aeromonads are a very tight group of species (Martinez-Murcia *et al.*, 1992a). In almost all species of the genus, rDNA-derived relationships correlated well with DNA–DNA hybridization. DNA probes and RFLP profiles designed from 16S rDNA diagnostic regions have served to identify *Aeromonas* at the species level (Ash *et al.*, 1993a, b; Dorsch *et al.*, 1994; Borrell *et al.*, 1997; Khan & Cerniglia, 1997; Figueras *et al.*, 2000). However, there are reported discrepancies between different sets of DNA–DNA hybridization data (Hickman-Brenner *et al.*, 1987; Schubert & Hegazi, 1988; Esteve *et al.*, 1995a, b; Huys *et al.*, 1996a, b, 2001), and the fact that 16S rRNA is highly conserved (Martínez-Murcia, 1999) brings the latest descriptions of some species into question (Nair & Holmes, 1999).

It has been reported that *gyrB* (which encodes the B-subunit of DNA gyrase, a type-II DNA topoisomerase) could be a suitable phylogenetic marker for bacterial systematics (Yamamoto & Harayama, 1995, 1996, 1998; Huang, 1996; Venkateswaran *et al.*, 1998; Yamamoto *et al.*, 1999, 2000; Kasai *et al.*, 2000; Watanabe *et al.*, 2001; Stackebrandt *et al.*, 2002). This protein plays a crucial role in the DNA replication process, and its gene sequence has a mean synonymous substitution rate that is almost four times that of 16S rDNA (Yamamoto & Harayama, 1996).

In the present study, *gyrB* nucleotide sequences were determined from 53 *Aeromonas* strains, including type strains of all described species (or HGs), reference strains and some new isolates, which were also characterized by analysis of the 16S rDNA variable regions. This phylogenetic study was designed to clarify the interspecies phylogenetic relationships within *Aeromonas*, and to assess the congruence of *gyrB* groupings with those of both 16S rDNA sequencing and DNA–DNA hybridization.

METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 1. New isolates and reference strains from culture collections were identified genetically by 16S rDNA RFLP typing and further sequencing. Most *Aeromonas* strains were grown aerobically on trypticase soy agar (Oxoid) overnight at 28 °C. The only exception was the psychrophilic species *A. salmonicida*, which was incubated at 22 °C.

DNA extraction and purification. A single colony from a fresh culture was resuspended in 50 µl TE buffer, vortexed at high speed for 1 min and incubated at 96 °C for 10 min. The tube was vortexed again and centrifuged for 2 min at 12 000 ***g***. The supernatant was transferred to a fresh tube and stored at −20 °C.

Oligonucleotide primers. Primers gyrB3F and gyrB14R were designed from the most conserved regions in a preliminary alignment of published *A. hydrophila gyrB* sequences (GenBank accession numbers AF074917 and AF208250–AF208260). These primers were used to amplify a *gyrB* fragment of approximately 1100 bp. Primers

Table 2. Characteristics of primers used for PCR amplification and sequencing of *gyrB*

Primer	Position*	Sequence (5′–3′)
gyrB3F	334–354	TCCGGCGGTCTGCACGGCGT
gyrB7F	792–812	GGGGTCTACTGCTTCACCAA
gyrB9R	979–959	ACCTTGACGGAGATAACGGC
gyrB9Rs	980–960	CCTTGACCGAAATGACCGCC
gyrB14R	1464–1444	TTGTCCGGGTTGTACTCGTC

*According to *E. coli* numbering.

gyrB7F, gyrB9R and gyrB9Rs were then designed from alignments that included the first few *gyrB* sequences obtained in the present study. The five Cy5-labelled primers (Table 2) were purchased from Amersham Biosciences and were used for *gyrB* sequencing reactions as required. Primers for amplification and sequencing of 16S rDNA were described previously (Martínez-Murcia *et al.*, 1999).

PCR amplification and sequencing. PCR amplification was performed using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems). Each reaction was performed in a final volume of 50 µl, containing 50 mM KCl, 10 mM Tris/HCl (pH 9·0), 1·5 mM $MgCl_2$, 0·1 % Triton X-100, 0·2 mM each dNTP (Roche Diagnostics), 1 U *Taq* DNA polymerase (Amersham Biosciences), 20 pmol each primer and 1 µl DNA sample. The reaction mixture was subjected to 35 cycles of amplification as follows: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. Amplified products were analysed by electrophoresis on 1·2 % agarose–TBE gels, stained with ethidium bromide (0·5 µg ml^{-1}) and visualized on a UV transilluminator. PCR products were purified using the Concert Rapid PCR Purification system (Invitrogen Life Technologies), following the manufacturer's instructions. Nucleotide sequences were determined by using the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP in an ALFexpressII automatic DNA sequencer (both from Amersham Biosciences), according to the manufacturer's instructions, using Cy5-labelled primers.

Phylogenetic data analysis. The *gyrB* nucleotide sequences were aligned by using the CLUSTAL X program, version 1.8 (Thompson *et al.*, 1997). Genetic distances were obtained using 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).

RESULTS AND DISCUSSION

Nucleotide sequences of *gyrB* amplicons from 53 *Aeromonas* strains were determined (Table 1). Experiments were repeated at least twice from single colonies of original cultures, to confirm readings and solve ambiguities. The derived *gyrB* sequences were 960–1100 nt in length, covering more than 70 % of the ATPase domain (amino acid residues 2–392 in *Escherichia coli*; Huang, 1996) and 191 nt of the 3′ flanking region. Species identification by 16S rDNA sequence is shown in Table 1.

Phylogenetic location of the genus *Aeromonas*

A multiple alignment of the GyrB subunit amino acid sequences, deduced from the gene sequences, was obtained. Amino acid (289 aa) and nucleotide (869 bp) sequences from type strains of all known *Aeromonas* species (or HGs) were compared with the corresponding sequences of the species *E. coli*, *Vibrio cholerae*, *P. shigelloides*, *Neisseria gonorrhoeae* and *Myxococcus xanthus*. Percentage similarities for both proteins and genes are given in Table A, which is available as supplementary data in IJSEM Online. On average, protein sequences showed a higher level of conservation than nucleotide sequences. Ranges of nucleotide and protein sequence similarity values between *Aeromonas* and species of other genera were, respectively: 68·5–70·9 % and 75·1–77·2 % to *V. cholerae*; 72·0–74·0 % and 74·7–76·8 % to *P. shigelloides*; and 73·9–76·1 % and 75·7–76·5 % to *E. coli*. A phylogenetic tree constructed from the nucleotide alignment is shown in Fig. 1.

It is evident from the phylogenetic tree shown in Fig. 1 that the genus *Aeromonas* formed a distinct line, separate from *V. cholerae* and *E. coli*, as it did from *P. shigelloides*. Species of these four genera belong to the γ-subclass of the *Proteobacteria*, clearly separated from representatives of

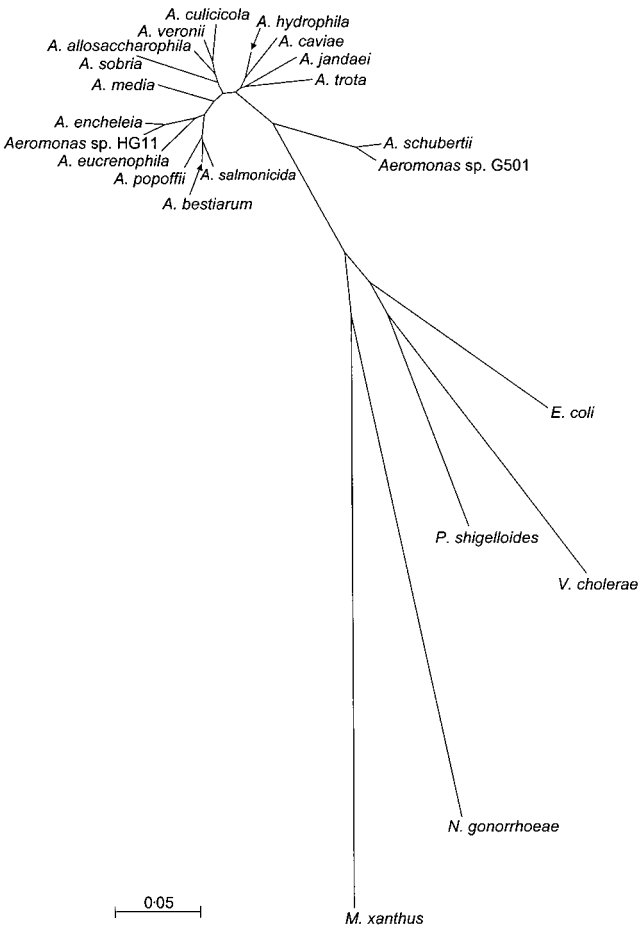

Fig. 1. Neighbour-joining tree based on *gyrB* sequences showing the relationships of type strains of all known *Aeromonas* species to some representative members of the class *Proteobacteria*.

the β- (*N. gonorrhoeae*) and δ- (*M. xanthus*) subclasses. These findings agree with a previous phylogenetic study based on 16S rRNA gene sequences (Martinez-Murcia *et al.*, 1992a) and support recognition of the family *Aeromonadaceae* (Colwell *et al.*, 1986). In addition, the aeromonads showed a lower interspecies *gyrB* substitution rate than that of other genera (Yamamoto *et al.*, 1999, 2000), in agreement with the former hypothesis that *Aeromonas* are a group of bacteria that have evolved relatively recently (Martínez-Murcia, 1999; Martínez-Murcia *et al.*, 1999).

Phylogenetic relationships of *Aeromonas* species

The *gyrB* sequences from all *Aeromonas* strains in this study were aligned, and percentage nucleotide substitutions (Table B, available as supplementary data in IJSEM Online) were calculated for a continuous stretch of 957 nt (positions 404–1364 according to the *E. coli* numbering). Sequence similarity between *Aeromonas* strains ranged from 86·7 to 100 %, corresponding to 0–127 nucleotide differences. At the intraspecies level, the rate of nucleotide substitution ranged from 0 to 2·6 %, and was <2 % for most *Aeromonas* species. However, interspecies nucleotide substitutions were usually >3 %, except for the following two pairs of species: *A. salmonicida* and *A. bestiarum* (2·2–3·3 %), and *A. encheleia* and *Aeromonas* sp. HG11 (2·1–2·7 %). Fig. 2 shows an unrooted tree that was constructed from the derived genetic distance matrix by the neighbour-joining method. The same topology (phylogenetic distances and branching points) was obtained when only 772 bp, corresponding to approximately 70 % of the ATPase domain, was used (not shown).

The results of this phylogenetic analysis demonstrate that, in the genus *Aeromonas*, *gyrB* sequences show a mean substitution rate that is approximately six times higher than that of 16S rRNA (Martinez-Murcia *et al.*, 1992a). This faster rate is an expected consequence of the chronometric nature of the *gyrB* gene as, although relatively conserved because it encodes a housekeeping enzyme, it is subject to a degenerative code which allows most silent mutations to occur (i.e. different codons may encode the same amino acid). Such evolutionary differences between the *gyrB* gene and protein chronometers are shown in Table A (available as supplementary data in IJSEM Online) where, apart from

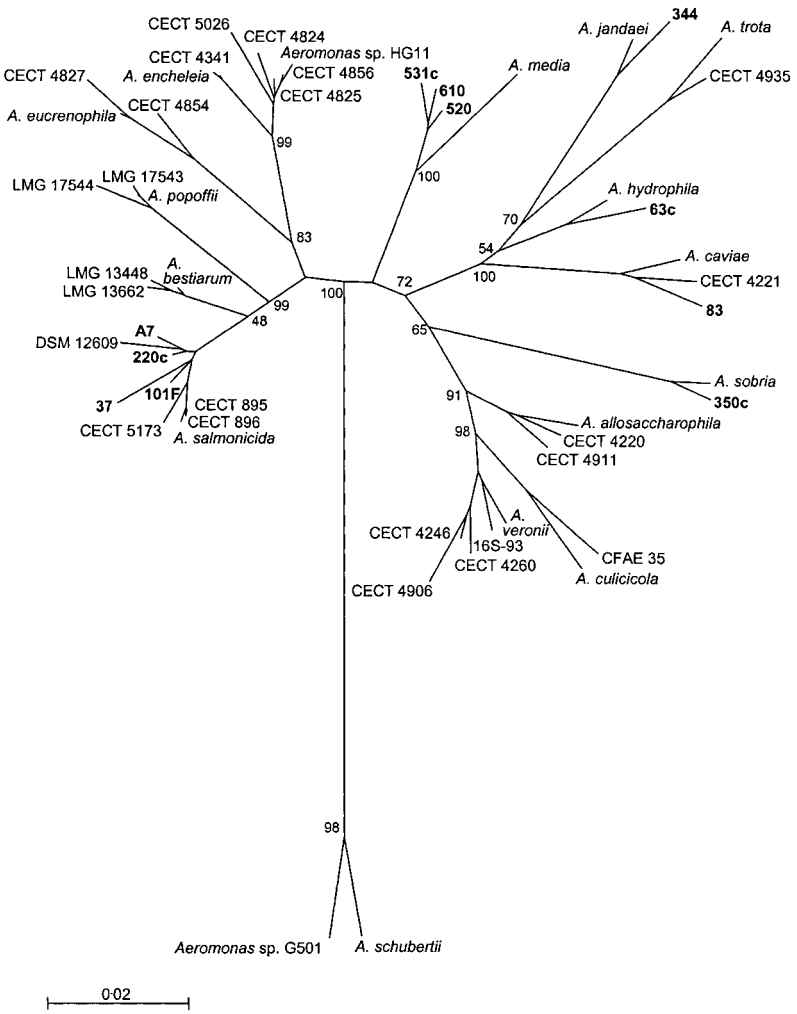

Fig. 2. Neighbour-joining tree based on *gyrB* sequences showing the inter- and intraspecies relationships of the genus *Aeromonas*. Species names correspond to type strains. Numbers in bold correspond to new *Aeromonas* isolates. Numbers shown next to each node indicate bootstrap values (percentage of 1000 replicates).

a higher level of protein conservation, different nucleotide sequences often encode almost-identical GyrB proteins. As a consequence, phylogenetic trees obtained from the protein sequences (not shown) show poor branching resolution and many strains of different species appear intermixed. Instead, *gyrB* gene sequences were found to be extremely useful in the current phylogenetic study of the genus *Aeromonas*.

Sequence analysis of *gyrB* shows considerable divergence between all *Aeromonas* species and DNA HGs. Moreover, apart from the strains *A. media* CECT 4234 and *A. allosaccharophila* CECT 4200, which showed sequences identical to those of the corresponding type strains of these species, a significant number of differences were found between strains of the same species. The two exceptions may suggest that these strains are duplicates of the type strains of their respective species. All other strains had a unique *gyrB* sequence; it therefore appears that *gyrB* sequences may be used not only for species identification, but also for simultaneous research of the phylogenetic depth of species. Despite the observed sequence diversity at the strain level, the groupings shown in Fig. 2 indicate a phylogenetic taxonomy consistent with that described in most previous genetic and phylogenetic studies of the genus *Aeromonas*. Almost all species formed separate lines on the basis of their *gyrB* sequences and clearly represent distinct genospecies. Due to the fact that *gyrB* sequence divergence is greater than that of 16S rRNA, we found the former gene more advantageous for *Aeromonas* species delineation. For example, *A. trota* and *A. caviae*, distinguishable by only a single nucleotide of their 16S rDNA sequences, are separated at a relatively large phylogenetic distance (Fig. 2) on the basis of partial *gyrB* sequence (6·0–7·2 % nucleotide substitutions, corresponding to 57–69 bp; see Table B in the supplementary data available in IJSEM Online). Similarly, *A. hydrophila* and *A. media*, which show only three nucleotide differences in 16S rDNA, are clearly separated by *gyrB* sequences (Fig. 2). *A. schubertii* clustered at the deepest branch of the genus (near the ancestral root), which is consistent with the 16S rRNA tree (Martínez-Murcia, 1999). The present analysis confirms that *A. veronii* biogroup *sobria* (HG8) and biogroup *veronii* (HG10) represent two heterogeneous phenotypes of a single species. Strain CECT 4911 (LMG 13073), largely classified as *A. veronii* (Huys *et al.*, 1996b), possessed a *gyrB* sequence that was clearly related to that of *A. allosaccharophila* (Fig. 2). 16S rDNA sequencing results (Table 1) confirmed that CECT 4911 actually belongs to *A. allosaccharophila*.

Our study included the *gyrB* sequence from the type strain of the recently described species *Aeromonas culicicola* (Pidiyar *et al.*, 2002), available in GenBank (accession no. AF175891). This taxon, together with isolate CFAE 35 (previously identified as *A. eucrenophila*; Singh & Sanyal, 1999), formed a separate phylogenetic line as shown in the tree in Fig. 2. The closest relatives of this species were *A. veronii* and *A. allosaccharophila* (3·4–4·0 and 3·9–4·4 % sequence divergence, respectively; Table B); therefore, these three species are equidistant from each other. The 16S rRNA gene of *A. culicicola* showed only a single nucleotide difference from that of *A. jandaei* CECT 4228^T. On the other hand, 56 differences (in approx. 1000 nt) were found in the *gyrB* gene, once again demonstrating the advantage of this gene in separating closely related species.

The species *A. allosaccharophila*

Previous amplified fragment length polymorphism (AFLP) and DNA–DNA hybridization studies by Huys *et al.* (1996b, 2001) have questioned the original proposal of the species *A. allosaccharophila*, which was based on phylogenetic and phenotypic analyses (Martínez-Murcia *et al.*, 1992b) and subsequent DNA–DNA reassociation results obtained by Esteve *et al.* (1995a). The latter study found that strains of *A. allosaccharophila* exhibited 0–40 % DNA relatedness with *A. veronii*, although later DNA–DNA hybridization studies by Huys *et al.* (2001) indicated a much higher range of 78–84 %, using the same reference strains. Apart from the striking lack of correlation between these hybridization studies, another inconsistency was found for the strain *A. allosaccharophila* CECT 4220 (ATCC 35942) when compared with DNA–DNA hybridization data obtained by Hickman-Brenner *et al.* (1987). It is worth nothing that Huys *et al.* often reported relatively high DNA relatedness values between very unrelated *Aeromonas* species (Martínez-Murcia, 1999; Huys *et al.*, 2001). In the original description of *A. allosaccharophila* (Martínez-Murcia *et al.*, 1992b), it was concluded that the species shows a clear phylogenetic divergence, demonstrated by qualitative evolutionary events (as well as the quantitative differences indicated) that support its phylogenetic distinctiveness from other species and particularly from *A. veronii*. The 16S rDNA of *A. allosaccharophila* shows six unique bases, i.e. different composition at positions where all other known *Aeromonas* species have the same conserved nucleotides (a fact that obviously gives more support to the distinctiveness of this species); also, *A. allosaccharophila* can be readily distinguished from *A. veronii* in region V2 (by two paired signature triplets at positions 154–156 and 167–165), the most relevant evolutionary event that splits the genus *Aeromonas* between *A. hydrophila* and *A. schubertii*. In the present study, *A. allosaccharophila*, although one of the closest phylogenetic relatives of *A. veronii*, undoubtedly formed a separate line on the basis of the *gyrB* sequence analysis, a result consistent with the phylogenetic analysis based on 16S rDNA (Martínez-Murcia *et al.*, 1992b). The rate of nucleotide differences in their *gyrB* sequences varies from 2·9 to 4·1 %, which is notable considering the mean intraspecies value in *Aeromonas* (approx. 2 %). In comparison, similar phylogenetic distances were found between *A. veronii* and *A. culicicola*, or between *A. popoffii* and *A. bestiarum*. In view of the present and previous studies, we consider that *A. allosaccharophila* shows sufficient phylogenetic divergence to be considered as a separate species.

Controversial taxonomic issues

An insertion of 3 bp at positions 484–485 (*E. coli* numbering) was only detected in all strains of the species *A. salmonicida* and *A. bestiarum* (triplet AGC), *A. popoffii* and *Aeromonas* sp. HG11 (triplet AGT) and *A. encheleia* and *A. eucrenophila* (triplet TCC). All of these species clustered together in the same sub-branch of the tree (Fig. 2). Although this codon encodes serine in all strains of these species, its composition was found to be variable, even in the first codon position (i.e. AGC, AGT and TCC). Because the insertion was found neither in other *Aeromonas* species nor in representatives of the other genera studied, we consider this to be an exclusive character of this group of species, and as support for the hypothesis that these species have evolved from each other relatively recently. Curiously, this group contains species that show the lowest range of *gyrB* nucleotide differences; this has been considered to be one of the most controversial taxonomic issues of the genus *Aeromonas*.

A. salmonicida and *A. bestiarum* have 2·2–3·3 % nucleotide substitutions in *gyrB*, which is on the border between the intra- and interspecies ranges observed in the present study. Previous 16S rDNA results have indicated that these two species are only distinguishable by two paired nucleotides at positions 1011 and 1018 (Martínez-Murcia, 1999). However, some strains of *A. salmonicida* have shown 16S rDNA sequences identical to that of the type strain of *A. bestiarum*, which is consistent with the difficulties found in the separation of strains of HG2 and HG3 by DNA–DNA hybridization (Hänninen, 1994; Ali *et al.*, 1996). Composition of the inserted codon in *gyrB* is the same for these two species (and different from all others), adding support for their close phylogenetic relationship. Strains of *A. popoffii* appear to be closely related to *A. bestiarum* on the basis of *gyrB* sequence (2·9–3·6 % divergence), in agreement with DNA–DNA hybridization data from the description of *A. popoffii* (Huys *et al.*, 1997b), which showed the highest value (51–63 %) with *A. bestiarum*. Although the species are very closely related, *A. popoffii* warrants separate species status, as supported by 16S rDNA phylogeny (Martínez-Murcia, 1999) and RFLP analysis of the 16S–23S intergenic spacer region (A. J. Martínez-Murcia and others, unpublished data).

A second example of controversy within the genus *Aeromonas* is represented by *A. encheleia* and the unnamed *Aeromonas* sp. HG11. The *gyrB* sequences determined in the present study indicate that these species are closely related, as the interspecies divergence was in the range 2·1–2·7 %. The composition of the inserted codon is different for these two species. Classification of strains into one of these species has been very difficult (Martínez-Murcia, 1999). For example, strain CECT 4824 (LMG 13062), formerly classified as *A. eucrenophila* (Huys *et al.*, 1996a), showed 94 % DNA–DNA hybridization with *A. encheleia* CECT 4342^T (Huys *et al.*, 1997a), but a value of 82 % hybridization with *A. eucrenophila* was reported in a previous study (Schubert & Hegazi, 1988). On the basis of *gyrB* sequence, this strain clearly clustered with *Aeromonas* sp. HG11, a result supported by 16S rDNA sequence analysis (Martínez-Murcia, 1999). Similarly, *A. eucrenophila* strain CECT 4825 (LMG 13691) has shown 50 % (Huys *et al.*, 1997a) and 80 % (Schubert & Hegazi, 1988) DNA–DNA hybridization with *A. eucrenophila* LMG 3774^T. The current *gyrB* sequence analysis indicates that this strain belongs to the *Aeromonas* sp. HG11 (Fig. 2), but its partial 16S rDNA sequence shows highest similarity to *A. encheleia* (Table 1). The 16S rDNA divergence between *A. encheleia* and *Aeromonas* sp. HG11 is based on eight nucleotide differences (Martínez-Murcia, 1999), which at first seems sufficient for a genus as tight as *Aeromonas*. All eight nucleotide differences were located at hypervariable positions (457–476) of a stem–loop with secondary structure base-pairing, rather than being dispersed around the molecule. This causes some concerns, as molecular clock 'distortions' (i.e. plesiomorphy) detected on variable stem–loops have previously been reported for closely related individuals (Sneath, 1993; Martínez-Murcia *et al.*, 1999). Consequently, further investigation using a considerable number of newly isolated *A. encheleia*/HG11 strains is recommended.

A similar case has been detected in the present study. Three isolates, 610, 520c and 531c, formed a separate cluster on the basis of *gyrB* sequences (Fig. 2), but the cluster was closely related to *A. media* (2·7–3·1 % substitutions). The distinctiveness of these isolates from *A. media* was also supported by 16S rDNA sequencing (data not shown); however, nucleotide differences were again located in the stem–loop at position 460 in the V3 region.

In conclusion, the *gyrB* gene sequence has proved to be an excellent molecular chronometer for phylogenetic inference in the genus *Aeromonas*. This phylogenetic marker revealed strain groupings consistent with the taxonomy proposed in most previous genetic and phylogenetic studies, particularly in agreement with 16S rDNA sequence analysis. The present analysis may serve to elucidate reported discrepancies between different DNA–DNA hybridization datasets. In addition, the observed sequence diversity at the intraspecies level allows us to propose that *gyrB* may be useful for strain differentiation and, at the same time, for identification of species following a phylogenetic frame.

ACKNOWLEDGEMENTS

M. A. Y. was the recipient of an MIT grant from Ministerio de Ciencia y Tecnología. This work has been supported by grants FIS 99/0944 from Ministerio de Salud y Consumo, and GV98-21-05 from Generalitat Valenciana. We would like to thank the Colección Española de Cultivos Tipo (CECT) and Dr Durg V. Singh, Rajiv Gandhi Centre for Biotechnology, Jagathy, Kerala, India, for providing strains.

REFERENCES

Abbott, S. L., Seli, L. S., Catino, M., Jr, Hartley, M. A. & Janda, J. M. (1998). Misidentification of unusual *Aeromonas* species as members

of the genus *Vibrio*: a continuing problem. *J Clin Microbiol* **36**, 1103–1104.

Ali, A., Carnahan, A. M., Altwegg, M., Lüthy-Hottenstein, J. & Joseph, S. W. (1996). *Aeromonas bestiarum* sp. nov. (formerly genomospecies DNA group 2 *A. hydrophila*), a new species isolated from non-human sources. *Med Microbiol Lett* **5**, 156–165.

Ash, C., Martínez-Murcia, A. J. & Collins, M. D. (1993a). Identification of *Aeromonas schubertii* and *Aeromonas jandaei* by using a polymerase chain reaction-probe test. *FEMS Microbiol Lett* **108**, 151–156.

Ash, C., Martínez-Murcia, A. J. & Collins, M. D. (1993b). Molecular identification of *Aeromonas sobria* by using a polymerase chain reaction-probe test. *Med Microbiol Lett* **2**, 80–86.

Borrell, N., Acinas, S. G., Figueras, M. J. & Martínez-Murcia, A. J. (1997). Identification of *Aeromonas* clinical isolates by restriction fragment length polymorphism of PCR-amplified 16S rRNA genes. *J Clin Microbiol* **35**, 1671–1674.

Carnahan, A. M. (1993). *Aeromonas* taxonomy: a sea of change. 4th International Workshop on *Aeromonas/Plesiomonas*, Atlanta, GA. *Med Microbiol Lett* **2**, 206–211.

Carnahan, A. M., Chakraborty, T., Fanning, G. R., Verma, D., Ali, A., Janda, J. M. & Joseph, S. W. (1991). *Aeromonas trota* sp. nov., an ampicilin-susceptible species isolated from clinical specimens. *J Clin Microbiol* **29**, 1206–1210.

Collins, M. D., Martinez-Murcia, A. J. & Cai, J. (1993). *Aeromonas enteropelogenes* and *Aeromonas ichthiosmia* are identical to *Aeromonas trota* and *Aeromonas veronii*, respectively, as revealed by small-subunit rRNA sequence analysis. *Int J Syst Bacteriol* **43**, 855–856.

Colwell, R. R., MacDonell, M. T., O'Brien, M. & De Ley, J. (1986). Proposal to recognize the family *Aeromonadaceae* fam. nov. *Int J Syst Bacteriol* **36**, 473–477.

Dorsch, M., Ashbolt, N. J., Cox, P. T. & Goodman, A. E. (1994). Rapid identification of *Aeromonas* species using 16S rDNA targeted oligonucleotide primers: a molecular approach based on screening of environmental isolates. *J Appl Bacteriol* **77**, 722–726.

Esteve, C., Gutiérrez, M. C. & Ventosa, A. (1995a). DNA relatedness among *Aeromonas allosaccharophila* strains and DNA hybridization groups of the genus *Aeromonas*. *Int J Syst Bacteriol* **45**, 390–391.

Esteve, C., Gutiérrez, M. C. & Ventosa, A. (1995b). *Aeromonas encheleia* sp. nov., isolated from European eels. *Int J Syst Bacteriol* **45**, 462–466.

Figueras, M. J., Soler, L., Chacón, M. R., Guarro, J. & Martínez-Murcia, A. J. (2000). Extended method for discrimination of *Aeromonas* spp. by 16S rDNA RFLP analysis. *Int J Syst Evol Microbiol* **50**, 2069–2073.

Hänninen, M.-L. (1994). Phenotypic characteristics of the three hybridization groups of *Aeromonas hydrophila* complex isolated from different sources. *J Appl Bacteriol* **76**, 455–462.

Hickman-Brenner, F. W., MacDonald, K. L., Steigerwalt, A. G., Fanning, G. R., Brenner, D. J. & Farmer, J. J., III (1987). *Aeromonas veronii*, a new ornithine decarboxlase-positive species that may cause diarrhea. *J Clin Microbiol* **25**, 900–906.

Hickman-Brenner, F. W., Fanning, G. R., Arduino, M. J., Brenner, D. J. & Farmer, J. J., III (1988). *Aeromonas schubertii*, a new mannitol-negative species found in human clinical specimens. *J Clin Microbiol* **26**, 1561–1564.

Huang, W. M. (1996). Bacterial diversity based on type II DNA topoisomerase genes. *Annu Rev Genet* **30**, 79–107.

Huys, G., Altwegg, M. Hänninen, M.-L. & 7 other authors (1996a). Genotypic and chemotaxonomic description of two subgroups in the species *Aeromonas eucrenophila* and their affiliation to *A. encheleia* and *Aeromonas* DNA hybridization group 11. *Syst Appl Microbiol* **19**, 616–623.

Huys, G., Coopman, R., Janssen, P. & Kersters, K. (1996b). High-resolution genotypic analysis of the genus *Aeromonas* by AFLP fingerprinting. *Int J Syst Bacteriol* **46**, 572–580.

Huys, G., Kämpfer, P., Altwegg, M., Coopman, R., Janssen, P., Gillis, M. & Kersters, K. (1997a). Inclusion of *Aeromonas* DNA hybridization group 11 in *Aeromonas encheleia* and extended descriptions of the species *Aeromonas eucrenophila* and *A. encheleia*. *Int J Syst Bacteriol* **47**, 1157–1164.

Huys, G., Kämpfer, P., Altwegg, M. & 7 other authors (1997b). *Aeromonas popoffii* sp. nov., a mesophilic bacterium isolated from drinking water production plants and reservoirs. *Int J Syst Bacteriol* **47**, 1165–1171.

Huys, G., Kämpfer, P. & Swings, J. (2001). New DNA-DNA hybridization and phenotypic data on the species *Aeromonas ichthiosmia* and *Aeromonas allosaccharophila*: *A. ichthiosmia* Schubert *et al.* 1990 is a later synonym of *A. veronii* Hickman-Brenner *et al.* 1987. *Syst Appl Microbiol* **24**, 177–182.

Janda, J. M. (1991). Recent advances in the study of the taxonomy, pathogenicity, and infectious syndromes associated with the genus *Aeromonas*. *Clin Microbiol Rev* **4**, 397–410.

Janda, J. M. & Abbott, S. L. (1998). Evolving concepts regarding the genus *Aeromonas*: an expanding panorama of species, disease presentations, and unanswered questions. *Clin Infect Dis* **27**, 332–344.

Joseph, S. W. & Carnahan, A. M. (2000). Update on the genus *Aeromonas*. *ASM News* **66**, 218–223.

Kasai, H., Ezaki, T. & Harayama, S. (2000). Differentiation of phylogenetically related slowly growing mycobacteria by their *gyrB* sequences. *J Clin Microbiol* **38**, 301–308.

Khan, A. A. & Cerniglia, C. E. (1997). Rapid and sensitive method for the detection of *Aeromonas caviae* and *Aeromonas trota* by polymerase chain reaction. *Lett Appl Microbiol* **24**, 233–239.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Kuijper, E. J., Steigerwalt, A. G., Schoenmakers, B. S. C. I. M., Peeters, M. F., Zanen, H. C. & Brenner, D. J. (1989). Phenotypic characterization and DNA relatedness in human fecal isolates of *Aeromonas* spp. *J Clin Microbiol* **27**, 132–138.

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.

Martínez-Murcia, A. J. (1999). Phylogenetic positions of *Aeromonas encheleia*, *Aeromonas popoffii*, *Aeromonas* DNA hybridization Group 11 and *Aeromonas* Group 501. *Int J Syst Bacteriol* **49**, 1403–1408.

Martinez-Murcia, A. J., Benlloch, S. & Collins, M. D. (1992a). Phylogenetic interrelationships of members of the genera *Aeromonas* and *Plesiomonas* as determined by 16S ribosomal DNA sequencing: lack of congruence with results of DNA-DNA hybridizations. *Int J Syst Bacteriol* **42**, 412–421.

Martínez-Murcia, A. J., Esteve, C., Garay, E. & Collins, M. D. (1992b). *Aeromonas allosaccharophila* sp. nov., a new mesophilic member of the genus *Aeromonas*. *FEMS Microbiol Lett* **91**, 199–205.

Martínez-Murcia, A. J., Antón, A. I. & Rodríguez-Valera, F. (1999). Patterns of sequence variation in two regions of the 16S rRNA multigene family of *Escherichia coli*. *Int J Syst Bacteriol* **49**, 601–610.

Nair, G. B. & Holmes, B. (1999). International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Vibrionaceae*. Minutes of the closed meeting, 19 May 1998, Atlanta, GA, USA. *Int J Syst Bacteriol* **49**, 1945–1947.

Pidiyar, V., Kaznowski, A., Badri Narayan, N., Patole, M. & Shouche, Y. S. (2002). *Aeromonas culicicola* sp. nov., from the midgut of *Culex quinquefasciatus*. *Int J Syst Evol Microbiol* **52**, 1723–1728.

Popoff, M. (1984). Genus III. *Aeromonas* Kluyver and Van Niel 1936, 398^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 545–548. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Popoff, M. Y., Coynault, C., Kiredjian, M. & Lemelin, M. (1981). Polynucleotide sequence relatedness among motile *Aeromonas* species. *Curr Microbiol* **5**, 109–114.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Schubert, R. H. W. & Hegazi, M. (1988). *Aeromonas eucrenophila* species nova, *Aeromonas caviae* a later and illegitimate synonym of *Aeromonas punctata*. *Zentbl Bakteriol Mikrobiol Hyg 1 Abt Orig A* **268**, 34–39.

Schubert, R. H. W., Hegazi, M. & Wahlig, W. (1990a). *Aeromonas enteropelogenes* species nova. *Hyg Med* **15**, 471–472.

Schubert, R. H. W., Hegazi, M. & Wahlig, W. (1990b). *Aeromonas ichthiosmia* species nova. *Hyg Med* **15**, 477–479.

Singh, D. V. & Sanyal, S. C. (1999). Virulence patterns of *Aeromonas eucrenophila* isolated from water and infected fish. *J Diarrhoeal Dis Res* **17**, 37–42.

Sneath, P. H. A. (1993). Evidence from *Aeromonas* for genetic crossing-over in ribosomal sequences. *Int J Syst Bacteriol* **43**, 626–629.

Stackebrandt, E., Frederiksen, W., Garrity, G. M. & 10 other authors (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Venkateswaran, K., Dohmoto, N. & Harayama, S. (1998). Cloning and nucleotide sequence of the *gyrB* gene of *Vibrio parahaemolyticus* and its application in detection of this pathogen in shrimp. *Appl Environ Microbiol* **64**, 681–687.

Watanabe, K., Nelson, J., Harayama, S. & Kasai, H. (2001). ICB database: the *gyrB* database for identification and classification of bacteria. *Nucleic Acid Res* **29**, 344–345.

Yamamoto, S. & Harayama, S. (1995). PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Appl Environ Microbiol* **61**, 1104–1109.

Yamamoto, S. & Harayama, S. (1996). Phylogenetic analysis of *Acinetobacter* strains based on the nucleotide sequences of *gyrB* genes and on the amino acid sequences of their products. *Int J Syst Bacteriol* **46**, 506–511.

Yamamoto, S. & Harayama, S. (1998). Phylogenetic relationships of *Pseudomonas putida* strains deduced from the nucleotide sequences of *gyrB*, *rpoD* and 16S rRNA genes. *Int J Syst Bacteriol* **48**, 813–819.

Yamamoto, S., Bouvet, P. J. M. & Harayama, S. (1999). Phylogenetic structures of the genus *Acinetobacter* based on *gyrB* sequences: comparison with the grouping by DNA–DNA hybridization. *Int J Syst Bacteriol* **49**, 87–95.

Yamamoto, S., Kasai, H., Arnold, D. L., Jackson, R. W., Vivian, A. & Harayama, S. (2000). Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. *Microbiology* **146**, 2385–2394.