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Phylogenetic analysis and identification of *Aeromonas* species based on sequencing of the *cpn60* universal target

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An analysis of the universal target (UT) sequence from the *cpn60* gene was performed in order to evaluate its usefulness in phylogenetic and taxonomic studies and as an identification marker for the genus *Aeromonas*. Sequences of 555 bp, corresponding to the UT region, were obtained from a collection of 35 strains representing all of the species and subspecies of *Aeromonas*. From the analysis of these sequences, a range of divergence of 0–23.3 % was obtained, with a mean of 11.2 ± 0.9 %. Comparative analyses between *cpn60* and *gyrB*, *rpoD* and 16S rRNA gene sequences were carried out from the same *Aeromonas* strain collection. Sequences of the *cpn60* UT region showed similar discriminatory power to *gyrB* and *rpoD* sequences. The phylogenetic relationships inferred from *cpn60* sequence distances indicated an excellent correlation with the present affiliation of *Aeromonas* species with the exception of *Aeromonas hydrophila* subsp. *dhakensis*, which appeared in a separate phylogenetic line, and *Aeromonas sharmana*, which exhibited a very loose phylogenetic relationship to the genus *Aeromonas*. Sequencing of *cpn60* from 33 additional *Aeromonas* strains also allowed us to establish intra- and interspecific threshold values. Intraspecific divergence rates were ⩽3.5 %, while interspecific divergence rates fell between 3.7 and 16.9 %, excluding *A. sharmana*. In this study, *cpn60* UT sequencing was shown to be a universal, useful, simple and rapid method for the identification and phylogenetic affiliation of *Aeromonas* strains.

INTRODUCTION

Classification of the genus *Aeromonas*, which belongs to the *Gammaproteobacteria*, remains complex from a taxonomic point of view because of the continuous description of novel species, the rearrangement of strains and species described so far and the discrepancies observed in different DNA–DNA hybridization studies (Huys *et al.*, 1997, 2001, 2005; Martínez-Murcia, 1999; Esteve *et al.*, 2003; Miñana-Galbis *et al.*, 2007). Sequence analysis of different housekeeping genes has been recommended for species delineation in addition to DNA–DNA hybridization in order to increase discriminatory power and the robustness of phylogenetic relationships with regard to 16S rRNA gene sequence analysis (Stackebrandt *et al.*, 2002). Recent studies based on the sequences of *dnaJ*, *gyrB*, *rpoB* and *rpoD* have shown that the use of several housekeeping genes is an effective approach for the classification of *Aeromonas* species (Küpfer *et al.*, 2006; Saavedra *et al.*, 2006; Nhung *et al.*, 2007).

The type I chaperonin Cpn60 (Hsp60 or GroEL) is a highly conserved protein found in bacteria, some archaea and organelles of endosymbiotic origin. Overexpressed under physiologically stressful conditions, it has been described as an intercellular signalling molecule and a potent immunogen, and has been implicated in inflammatory diseases (Hill *et al.*, 2004; Wick *et al.*, 2004; Horwich *et al.*, 2007). Analyses of *cpn60* sequences are useful for microbiological studies using different approaches, such as phylogeny, microbial detection and identification, as well as microbial ecology and evolution (Hill *et al.*, 2006; Thompson *et al.*, 2005; Fares & Travers, 2006; Goyal *et al.*, 2006; Gupta & Sneath, 2007). Hill *et al.* (2004) have analysed sequences of this gene from a wide variety of bacterial, archaeal and eukaryotic species. They concluded that a 549–567 bp region of the *cpn60* gene (the universal target or UT), amplified by universal PCR primers, is representative of the complete gene (approx. 1600 bp) in terms of phylogeneti-

Abbreviation: UT, universal target.

The GenBank/EMBL/DDBJ accession numbers for the *cpn60* UT sequences determined in this study are detailed in Table 1.

Scatter plots of JC69 distances and an extended consensus neighbour-joining tree of *cpn60* UT sequences are available as supplementary material with the online version of this paper.

cally informative sequence variation. Furthermore, these authors have designed and implemented a web-based chaperonin sequence database (cpnDB; http://cpndb.cbr.nrc.ca).

In the present study, we have sequenced and analysed a 555 nt sequence, corresponding to the UT region of the *cpn60* gene, in type and reference strains of all *Aeromonas* described to date. This should allow us to evaluate its applicability for species delineation and identification within *Aeromonas*. Sequencing of the *cpn60* gene not only increases the number of housekeeping genes sequenced from *Aeromonas* species, but may also facilitate simple and rapid *Aeromonas* species identification.

METHODS

Bacterial strains. The *Aeromonas* strains used in this study for sequencing of the *cpn60* UT region and their GenBank accession numbers are listed in Table 1.

DNA extraction, PCR amplification and sequencing. DNA extraction was performed as described previously (Miñana-Galbis *et al.* 2007). PCR amplification and sequencing of the *cpn60* UT region were conducted using a modification of previously described methods (Brousseau *et al.*, 2001; Hill *et al.*, 2002). Two primers were used for PCR amplification and sequencing, C175 (5′-GAAATYGAACTGG-AAGACAA-3′) and C938 (5′-GTYGCTTTTTCCAGCTCCA-3′). These primers were designed from the complete *cpn60* sequences of *Aeromonas salmonicida* NCIMB 835 and *Escherichia coli* (GenBank accession numbers AF030975 and X07850, respectively), and correspond to nucleotides 175–194 and 938–920, respectively, of the complete *cpn60* gene. PCR amplification was carried out in a total volume of 50 µl containing 50 mM KCl, 15 mM Tris/HCl, pH 8.0, 1.5 mM $MgCl_2$, 0.25 mM dNTPs (Amersham Biosciences), 2.5 U Ampli*Taq* Gold DNA polymerase (Applied Biosystems) and 50 pmol of each primer (Isogen Life Science). The reaction mixtures were subjected to the following thermal cycling program in a 2720 Thermal Cycler (Applied Biosystems): denaturation at 95 °C for 10 min, 35 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min and a final extension step at 72 °C for 10 min. The amplified products were purified using the MSB Spin PCRapace kit (Invitek) and sequencing was performed using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) with the following thermal cycling conditions: 96 °C for 1 min and 25 cycles of 96 °C for 10 s, 52 °C for 5 s and 60 °C for 4 min. Nucleotide sequences were determined in an ABI PRISM 3730 DNA analyser by the Genomics Unit of the Scientific and Technical Services of the University of Barcelona.

Data analyses. Multiple DNA and protein alignments were obtained by using CLUSTAL W software (Thompson *et al.*, 1994). DNA polymorphism data and G+C content determination were conducted with DnaSP software (Rozas & Rozas, 1999). Phylogeny calculations, including synonymous and non-synonymous substitutions, the *Z*-test of neutrality (dS=dN) and neighbour-joining and maximum-parsimony trees of DNA sequence alignments were performed using MEGA software, version 3.1 (Kumar *et al.*, 2004). *Escherichia coli* sequences were used as an outgroup. Statistical analysis, including correlations and regression analysis, were conducted using Excel XP (Microsoft) and R computing language (Ihaka & Gentleman, 1996). When applied, bootstrap analysis was computed with 1000 replicates.

RESULTS AND DISCUSSION

Comparative analysis of *cpn60* UT sequences

In this study, we sequenced the UT region of the *cpn60* gene, a 555 bp fragment corresponding to positions 274–828 of the complete gene, in 35 strains representing all species and subspecies of the genus *Aeromonas* (Table 1). The same two primers designed in this study were used for amplification and sequencing. Analyses of these 35 sequences (Table 2) revealed 32 unique sequences; no insertions or deletions were detected. These sequences exhibited 189 polymorphic sites (34.1 %), 136 of which were parsimony-informative sites, with a total number of 274 mutations. The pairwise differences ranged from 0 to 111 nucleotides (0–20 %), with the mean number of nucleotide differences of 57.2 ± 4.0. In accordance with Nei (1996), distances were calculated based on the Jukes–Cantor (JC69) model (Jukes & Cantor, 1969), since the number of nucleotides was high ($n>500$), the number of nucleotide substitutions per site (d) was below 0.25 (0.233) and the transition to transversion ratio (R) was less than five ($R=2.0$).

Using the same collection of *Aeromonas* strains, analyses of *cpn60* sequences were compared to those obtained from *gyrB*, *rpoD* and 16S rRNA genes, either sequenced previously in our laboratory or taken from GenBank. The range and mean pairwise JC69 distances for these genes are shown in Table 2. Differences between distances obtained when comparing all four genes were statistically significant ($P<0.001$; Wilcoxon's signed rank test) and, although the range of sequence divergences of *cpn60* was smaller than those of *gyrB* and *rpoD*, they proved significantly greater than that of the 16S rRNA gene. Correlations and regression curves between pairwise JC69 distances of *cpn60*, *gyrB*, *rpoD* and 16S rRNA gene sequences were generated by Pearson's product–moment correlation coefficient (Supplementary Fig. S1, available in IJSEM Online). A significant correlation was obtained between the different genetic loci ($r>0.6$, $P<0.001$). The test for synonymous and non-synonymous analysis (dS=dN for a null hypothesis and dS>dN for an alternative hypothesis) was highly significant ($P<0.001$), indicating the presence of purifying selection (Table 2).

After conducting a pairwise comparison of *cpn60*, *gyrB*, *rpoD* and 16S rRNA gene sequences (595 comparisons), we calculated the percentage divergence of the number of nucleotide differences per sequence (Fig. 1). Histograms represent the sequence divergence of the pairwise comparisons for the 35 strains included in this study. The distance matrix for *cpn60* showed six distances of 0 with respect to the 14 obtained for the 16S rRNA gene. In the case of *gyrB* and *rpoD*, all distances were above 0. Moreover, the divergence distribution of *cpn60* was smoother than those of the other genes compared.

As *cpn60* was initially proposed as a possible alternative to the 16S rRNA gene (Hill *et al.*, 2004), these results demonstrate that the *cpn60* UT sequences provide much

Table 1. *Aeromonas* strains used for *cpn60* UT sequencing

Strain	GenBank accession number
A. allosaccharophila	
CECT 4199^T	EU306795
CECT 4200	EU741624
A. bestiarum	
112A*	EU741625
628A*	EU306797
CECT 4227^T	EU306796
A. bivalvium	
665N	EU306798
868E^T	EU306799
A. caviae	
A10Cl*	EU741626
706OP*	EU741627
CECT 838^T	EU306800
A. culicicola CIP 107763^T	EU306840
A. encheleia	
CECT 4342^T	EU306801
CECT 4343	EU741628
CECT 4824	EU741629
A. enteropelogenes	
CECT 4487^T	EU306837
CECT 4935	EU741630
CECT 4936	EU741631
CECT 4937	EU741632
A. eucrenophila	
CECT 4224^T	EU306803
CECT 4853	EU741633
CECT 4854	EU741634
A. hydrophila CECT 5236	EU741635
A. hydrophila subsp. *dhakensis*	
CECT 5744^T	EU306806
LMG 19558	EU741636
A. hydrophila subsp. *hydrophila* CECT 839^T	EU306804
A. hydrophila subsp. *ranae* CIP 107985^T	EU306805
A. ichthiosmia CECT 4486^T	EU306841
A. jandaei	
CECT 4228^T	EU306807
CECT 4813	EU741637
CECT 4814	EU741638
CECT 4815	EU741639
CECT 4838	EU741640
A. media	
CECT 4232^T	EU306808
CECT 4234	EU741641
A. molluscorum	
093M	EU306809
431E	EU306810
848T^T	EU306811
849T	EU306812
869N	EU306813
A. popoffii	
LMG 17541^T	EU306814
LMG 17542	EU306815
LMG 17543	EU306816
A. salmonicida	
083C*	EU306817
087M*	EU306818
621A*	EU306819
635A*	EU306820
670N*	EU306821
818E*	EU306822
856T*	EU306823
CECT 5173	EU741642
A. salmonicida subsp. *achromogenes* LMG 14900^T	EU306824
A. salmonicida subsp. *masoucida* CECT 896^T	EU306825
A. salmonicida subsp. *pectinolytica* CECT 5752^T	EU306827
A. salmonicida subsp. *salmonicida* CECT 894^T	EU306828
A. salmonicida subsp. *smithia* CIP 104757^T	EU306829
A. schubertii	
CECT 4933	EU741643
CECT 4934	EU741644
CIP 103437^T	EU306830
A. sharmana DSM 17445^T	EU306831
A. simiae	
CIP 107797	EU306832
CIP 107798^T	EU306833
A. sobria	
CECT 4245^T	EU306834
CECT 4248	EU741645
A. trota CECT 4255^T	EU306836
A. veronii biovar sobria CECT 4246	EU306838
A. veronii biovar veronii CECT 4257^T	EU306839
Aeromonas sp. HG11 CECT 4253	EU306802
Aeromonas sp. HG13 CECT 4254	EU306835

*See Miñana-Galbis *et al.* (2002, 2004b) for further information on these strains.

better discrimination than the 16S rRNA gene between the species of the genus *Aeromonas*. Moreover, *cpn60* UT sequences showed similar discriminatory power to that obtained with *gyrB* and *rpoD*.

The mean DNA G+C content of the *cpn60* gene sequences (59.6±0.3 mol%) was within the range of G+C content reported for the genus *Aeromonas* (57–63 mol%; Martin-Carnahan & Joseph, 2005).

Peptide translations of the partial *cpn60* sequences were also obtained. Of 185 amino acids, 153 (82.7 %) were conserved in all sequences, while 32 (17.3 %) showed variability, 16 of which were singleton sites. With the exception of *A. sharmana*, which exhibited a histidine residue in position 93 (codon 277, 278, 279), the translated peptide sequences lacked histidine and tryptophan residues.

Phylogenetic relationships

Fig. 2 shows the JC69 neighbour-joining tree obtained with the UT sequences of the *cpn60* gene, clustering together all

Table 2. Analysis of 35 *Aeromonas* sequences from *cpn60*, *gyrB*, *rpoD* and 16S rRNA genes

Sequence information	*cpn60*	*gyrB*	*rpoD*	16S rRNA
Number of sites	555	942	799	1544
Number of polymorphic sites	189 (34.1 %)	334 (35.5 %)	337 (42.2 %)	103 (6.7 %)
Number of nucleotide differences				
Range	0–111	1–209	0–207	0–79
Mean ± SEM	57.2 ± 4.0	85.0 ± 5.0	96.9 ± 5.3	20.9 ± 2.4
Jukes–Cantor distance (*d*)				
Range	0–0.233	0.001–0.263	0–0.318	0–0.057
Overall mean ± SEM	0.112 ± 0.009	0.098 ± 0.006	0.134 ± 0.008	0.014 ± 0.002
Transition/transversion ratio (*R*)	2.0	1.7	1.8	2.1
dS*	0.476 ± 0.043	0.430 ± 0.030	0.701 ± 0.057	NA
dN*	0.021 ± 0.005	0.022 ± 0.004	0.034 ± 0.004	NA
dS>dN†	$P<0.001$	$P<0.001$	$P<0.001$	NA

NA, Not applicable.

*Synonymous substitutions per synonymous site (dS) and non-synonymous substitutions per non-synonymous site (dN) (means ± SEM) (Nei–Gojobori method using Jukes–Cantor distance).

†Acceptance probability of a null hypothesis of dS=dN with dS>dN as the alternative hypothesis, using a *Z*-test.

of the *Aeromonas* species and subspecies with a bootstrap value of 92 %, except in the case of *A. sharmana*. Dendrograms generated by Kimura two-parameter, Tamura–Nei and maximum-parsimony showed almost identical topologies to that obtained using JC69 (not shown).

Aeromonas bivalvium and *Aeromonas molluscorum* strains clustered in separate groups in the dendrogram. These results were expected, since these species can be easily separated from the remaining *Aeromonas* species based on phenotypic characteristics, FAFLP fingerprinting and sequence analysis of genes such as 16S rRNA, *gyrB* and *rpoD* (Miñana-Galbis *et al.*, 2004a, 2007; Saavedra *et al.*, 2006). Five nucleotide differences (0.9 % divergence) were observed between the two *A. bivalvium* strains, and 5–23 differences (0.9–4.3 % divergence) among the five *A. molluscorum* strains. Therefore, the *cpn60* UT sequence afforded a clear differentiation between *A. bivalvium* and *A. molluscorum* strains.

Aeromonas caviae, *A. media*, *A. eucrenophila* and *A. encheleia* displayed related but different phylogenetic lines in the dendrogram (Fig. 2), with 27–45 interspecies nucleotide differences (4.6–8.6 % divergence). In agreement with previous studies (Huys *et al.*, 1997; Soler *et al.*,

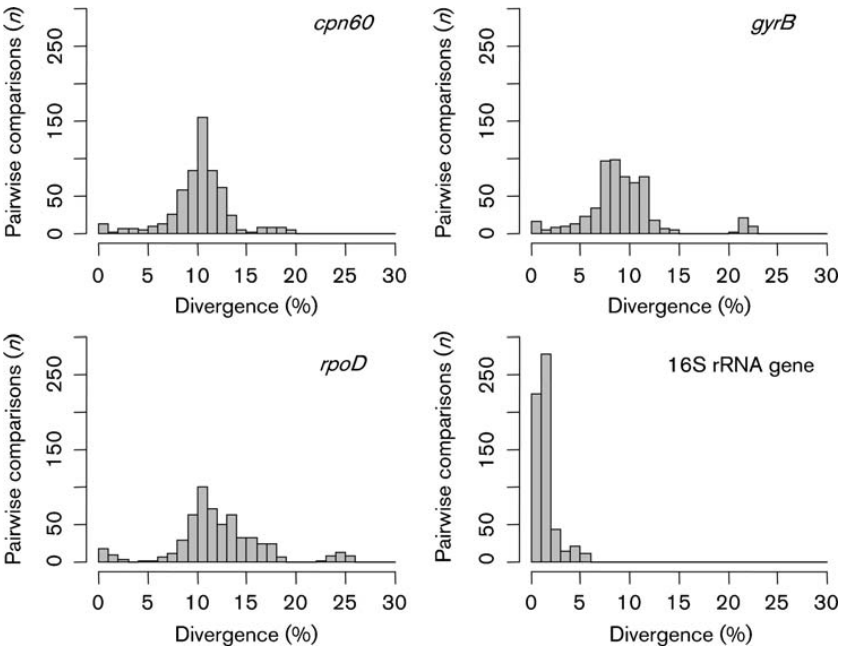

Fig. 1. Distribution of pairwise sequence divergence comparisons of *cpn60*, *gyrB*, *rpoD* and 16S rRNA genes. The horizontal axes represent classes of sequence divergence.

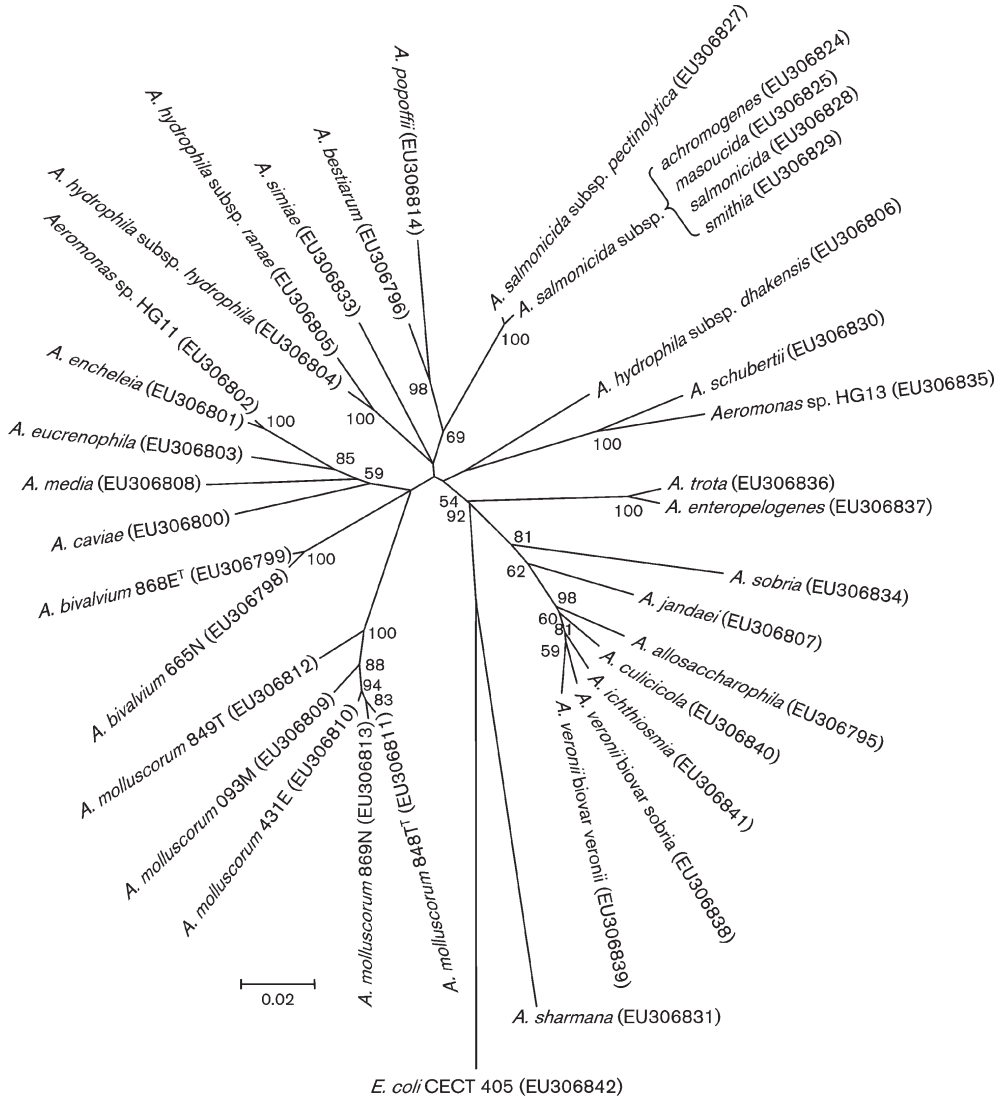

Fig. 2. Consensus neighbour-joining phylogenetic tree (JC69 distance) obtained from 35 *cpn60* UT sequences, encompassing all of the species and subspecies of the genus *Aeromonas*. GenBank accession numbers are indicated in parentheses. Bar, distance of 0.02, as calculated by MEGA. Bootstrap values (>50 %) after 1000 replicates are shown as percentages. Further strain details are given in Table 1.

2004; Küpfer *et al.*, 2006), *Aeromonas* HG11 (as represented by strain CECT 4253) can be regarded as belonging to *A. encheleia* based on *cpn60* UT sequence analysis, since its sequence exhibits only four nucleotide differences (0.7 % divergence) from that of the *A. encheleia* type strain.

UT sequences obtained from the type strains of the *A. salmonicida* subspecies were identical to the corresponding *cpn60* gene sequence from the complete genome of *A. salmonicida* subsp. *salmonicida* A449 with the exception of *A. salmonicida* subsp. *pectinolytica*, which exhibited three nucleotide differences (0.5 % divergence). This cluster was close to those of *Aeromonas bestiarum* and *Aeromonas popoffii*, with a bootstrap value of 69 %. UT sequences for these three species showed a divergence range of 5.4–9.6 %.

The sequence obtained from *Aeromonas hydrophila* subsp. *hydrophila* was identical to that from the complete genome of *A. hydrophila* subsp. *hydrophila* ATCC 7966^T. The *A. hydrophila* cluster grouped together the type strains of *A. hydrophila* subsp. *hydrophila* and *A. hydrophila* subsp. *ranae*, with 14 nucleotide differences (2.6 % divergence) between them, in a position close to the *A. salmonicida* group. However, the *cpn60* UT sequence from the type strain of *A. hydrophila* subsp. *dhakensis* revealed 37–40 nucleotide differences (7–7.6 % divergence) when it was compared with those of the other *A. hydrophila* subspecies, thereby clustering this subspecies in a separate phylogenetic line. These results, together with those obtained for the 16S rRNA, *gyrB* and *rpoD* genes (Miñana-Galbis *et al.*, 2004a; D. Miñana-Galbis, M. Farfán, M. C. Fusté and J. G. Lorén,

unpublished results), suggest that *A. hydrophila* subsp. *dhakensis* can be considered as a novel *Aeromonas* species.

The *cpn60* UT sequence therefore allowed the accurate differentiation of species within the '*A. hydrophila* complex' (Martínez-Murcia *et al.*, 2005), similar to that obtained by FAFLP fingerprinting and sequence analysis of *gyrB*, *dnaJ* and *rpoD* (Huys & Swings, 1999; Saavedra *et al.*, 2006; Nhung *et al.*, 2007). This result is remarkable since species of this complex, primarily *A. salmonicida* and *A. bestiarum*, are very difficult to distinguish by phenotypic identification, 16S rRNA gene sequencing or DNA–DNA hybridization (Miñana-Galbis *et al.*, 2002; Valera & Esteve, 2002; Abbott *et al.*, 2003; Martínez-Murcia *et al.*, 2005). The type strain of *Aeromonas simiae* was also included in the group containing *A. salmonicida*, *A. bestiarum*, *A. hydrophila* and *A. popoffii*, albeit with a bootstrap value of 14 %.

The strain *Aeromonas* sp. CECT 4254 (HG13) clustered close to the type strain of *Aeromonas schubertii* (100 % bootstrap) in a separate phylogenetic line, consistent with previous analyses (Hickman-Brenner *et al.*, 1988; Martínez-Murcia, 1999; Miñana-Galbis *et al.*, 2004a; Küpfer *et al.* 2006; Saavedra *et al.*, 2006). Sequences obtained from these strains differed in 30 nucleotides (5.6 % divergence).

The type strain of *Aeromonas trota* joined to *Aeromonas enteropelogenes* in the same cluster (100 % bootstrap). The two sequences differed by only 10 nucleotides (1.8 % divergence). *Aeromonas enteropelogenes* has been reported to be a later heterotypic synonym of *Aeromonas trota* (Collins *et al.*, 1993; Huys *et al.*, 2002),

All the type or reference strains of *Aeromonas veronii* bv. veronii, *A. veronii* bv. sobria, *A. culicicola* and *A. ichthiosmia* were clustered together, differing by 14–20 nucleotides (2.6–3.7 % divergence). These results were expected, considering that *Aeromonas ichthiosmia* and *Aeromonas culicicola* are regarded as later heterotypic synonymous of *Aeromonas veronii* (Collins *et al.*, 1993; Huys *et al.*, 2001, 2005; Miñana-Galbis *et al.*, 2004a). Moreover, *Aeromonas allosaccharophila* appeared in very close proximity to the *A. veronii* group (98 % bootstrap) in the phylogenetic tree, differing by 20–24 nucleotides (3.7–4.5 % divergence) with respect to *A. veronii* strains. This result suggests that *A. allosaccharophila* occupies a taxonomically uncertainty position with respect to *A. veronii*, which is consistent with AFLP genotyping and *dnaJ* sequencing studies (Huys *et al.*, 1996; Nhung *et al.*, 2007). Likewise, *Aeromonas jandaei* and *A. sobria* were located relatively close to the *A. veronii* group (81 % bootstrap), in independent phylogenetic lines.

Recently, the taxonomic status of *A. sharmana* as a member of the genus *Aeromonas* has been questioned on the basis of 16S rRNA gene, *rpoD* and *gyrB* sequences (Martínez-Murcia *et al.*, 2007; D. Miñana-Galbis, M. Farfán, M. C. Fusté and J. G. Lorén, unpublished results). Analysis of *cpn60* UT sequences in *Aeromonas* has provided new evidence that *A. sharmana* can no longer be regarded as a member of this genus. In this study, the *cpn60* UT sequence of *A. sharmana* DSM 17445^T exhibited 86–111 nucleotide differences (17.4–23.3 % divergence), with a mean of 98.4 ± 1.1 (20.2 ± 0.3 % divergence). These values are significantly higher than the mean (54.7 ± 0.6 nt or 10.6 ± 0.1 % divergence) obtained among the other *Aeromonas* species (*t*-test, $P<0.001$). The *A. sharmana* UT sequence exhibited 29 unique nucleotides that were absent from the other *Aeromonas* sequences. This clear separation of *A. sharmana* from the genus *Aeromonas* can also be inferred from Fig. 2 and Supplementary Fig. S1. Furthermore, when we determined the amino acid composition of the UT sequences, *A. sharmana* was the only one to contain a histidine residue.

Usefulness of *cpn60* UT for *Aeromonas* species identification

In order to validate the usefulness of *cpn60* UT sequencing for *Aeromonas* identification, we sequenced 33 additional *Aeromonas* strains (Table 1). These 33 sequences, as well as two sequences obtained from the cpnDB (GenBank accession numbers AF030975 and DQ074967), were compared with the 35 *Aeromonas* sequences analysed previously in this work. The addition of the 33 new sequences did not modify the topology of the phylogenetic tree (Fig. 2 and Supplementary Fig. S2).

Based on the results obtained in this study, threshold values were established to permit *Aeromonas* species discrimination based on *cpn60* UT sequence divergence. Intraspecific divergence rates were $\leqslant 3.5$ % ($\leqslant 19$ nt differences), while interspecific divergence rates ranged from 3.7 to 16.9 % (20–84 nt differences), excluding *A. sharmana* (Table 3). These results were similar to those obtained from *dnaJ*, *gyrB* and *rpoD* sequence analyses, with interspecific threshold values of 5.2 % for *dnaJ* and 3 % for *gyrB* and *rpoD* (Soler *et al.*, 2004; Nhung *et al.*, 2007). The intraspecific threshold value showed three exceptions, since the *A. culicicola* and *A. ichthiosmia cpn60* sequences exhibited a divergence of 3.7 % and, in the case of *A. molluscorum*, the sequence of strain 849T exhibited a divergence of 4.1 % with respect to strain 869N and 4.3 % with respect to strain 848T^T.

Pairwise comparison of *cpn60* UT sequences also allowed us to discriminate between the type and reference strains of the different *Aeromonas* species, except in the case of *A. salmonicida* (Supplementary Fig. S2). Therefore, it was possible to differentiate between *A. encheleia* and *Aeromonas* sp. HG11, *A. hydrophila* subsp. *hydrophila* and *A. hydrophila* subsp. *ranae*, *A. trota* and *A. enteropelogenes*, *A. veronii*, *A. culicicola*, *A. ichthiosmia*, *A. veronii* bv. sobria and *A. veronii* bv. veronii. In the case of the *A. salmonicida* subspecies, all of them except *A. salmonicida* subsp. *pectinolytica* exhibited identical *cpn60* sequences and therefore could not be differentiated.

In addition to other housekeeping genes such as *dnaJ*, *gyrB*, *rpoB* and *rpoD* (Küpfer *et al.*, 2006; Saavedra *et al.*, 2006;

Table 3. Intra- and interspecific ranges of nucleotide differences and Jukes–Cantor distances of all *cpn60* sequences analysed in this study

A. encheleia includes *Aeromonas* sp. HG11, *A. hydrophila* includes *A. hydrophila* subsp. *hydrophila* and *A. hydrophila* subsp. *ranae*, *A. salmonicida* includes *A. salmonicida* subsp. *achromogenes*, *A. salmonicida* subsp. *masoucida*, *A. salmonicida* subsp. *pectinolytica*, *A. salmonicida* subsp. *salmonicida* and *A. salmonicida* subsp. *smithia*, *A. trota* includes *A. enteropelogenes* and *A. veronii* includes *A. culicicola*, *A. ichthiosmia*, *A. veronii* biovar sobria and *A. veronii* biovar veronii. NA, Not applicable.

Species	*n*	Nucleotide differences (*n*)		JC distance (%)	
		Intraspecies	Interspecies	Intraspecies	Interspecies
A. allosaccharophila	2	0	20–75	0	3.7–14.9
A. bestiarum	3	8–14	27–65	1.5–2.6	5.0–12.7
A. bivalvium	2	5	36–78	0.9	6.8–15.6
A. caviae	3	6–8	34–75	1.1–1.5	6.4–14.9
A. encheleia	4	0–6	23–77	0–1.1	4.3–15.3
A. eucrenophila	3	0–10	23–74	0–1.8	4.3–14.7
A. hydrophila	3	7–14	33–66	1.3–2.6	6.2–12.9
A. hydrophila subsp. *dhakensis*	2	6	36–71	1.1	6.8–14.0
A. jandaei	5	0–13	27–72	0–2.4	5.0–14.2
A. media	2	0	36–79	0	6.8–15.8
A. molluscorum (without 849T)	4	5–15	48–80	0.9–2.8	9.2–16.0
A. molluscorum 849T	1	15–23	39–76	2.8–4.3	7.4–15.1
A. popoffii	3	0–9	27–79	0–1.6	5.0–15.8
A. salmonicida	15	0–8	29–70	0–1.6	5.4–13.8
A. schubertii	3	0–1	29–84	0–0.2	5.4–16.9
A. simiae	2	0	39–73	0	7.4–14.5
A. sobria	2	19	43–84	3.5	8.2–16.9
A. trota	5	0–11	46–73	0–2.0	8.8–14.5
A. veronii	4	14–20	20–72	2.6–3.7	3.7–15.8
Aeromonas sp. HG13	1	NA	29–77	NA	5.4–15.3
A. sharmana	1	NA	86–111	NA	17.4–23.3
E. coli	1	NA	94–117	NA	19.2–24.8

Nhung *et al.*, 2007), the present study has demonstrated that *cpn60* sequence analysis offers an effective method for discriminating species of *Aeromonas*, inferring their phylogenetic relationships and contributing to further taxonomic clarification of certain controversial taxa found in this genus. Furthermore, from an experimental standpoint, the determination of *cpn60* UT sequences is a simple and rapid technique that requires a unique sequencing reaction, due to its own relatively short sequence (555 bp).

Finally, we recommend UT *cpn60* gene sequencing be included in the description of any novel *Aeromonas* species, since it represents a suitable alternative for the identification and phylogenetic study of *Aeromonas* species. Moreover, *cpn60* is a universal gene that allows the establishment of a web-based taxonomic database within the cpnDB (http://cpndb.cbr.nrc.ca).

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REFERENCES

Abbott, S. L., Cheung, W. K. W. & Janda, J. M. (2003). The genus *Aeromonas*: biochemical characteristics, atypical reactions, and phenotypic identification schemes. *J Clin Microbiol* **41**, 2348–2357.

Brousseau, R., Hill, J. E., Préfontaine, G., Goh, S. H., Harel, J. & Hemmingsen, S. M. (2001). *Streptococcus suis* serotypes characterized by analysis of chaperonin 60 gene sequences. *Appl Environ Microbiol* **67**, 4828–4833.

Collins, M. D., Martínez-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.

Esteve, C., Valera, L., Gutiérrez, C. & Ventosa, A. (2003). Taxonomic study of sucrose-positive *Aeromonas jandaei*-like isolates from faeces, water and eels: emendation of *A. jandaei* Carnahan *et al.* 1992. *Int J Syst Evol Microbiol* **53**, 1411–1419.

Fares, M. A. & Travers, S. A. (2006). A novel method for detecting intramolecular coevolution: adding a further dimension to selective constraints analyses. *Genetics* **173**, 9–23.

Goyal, K., Qamra, R. & Mande, S. C. (2006). Multiple gene duplication and rapid evolution in the *groEL* gene: functional implications. *J Mol Evol* **63**, 781–787.

Gupta, R. S. & Sneath, P. H. (2007). Application of the character compatibility approach to generalized molecular sequence data: branching order of the proteobacterial subdivisions. *J Mol Evol* **64**, 90–100.

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.

Hill, J. E., Seipp, R. P., Betts, M., Hawkins, L., Van Kessel, A. G., Crosby, W. L. & Hemmingsen, S. M. (2002). Extensive profiling of a complex microbial community by high-throughput sequencing. *Appl Environ Microbiol* **68**, 3055–3066.

Hill, J. E., Penny, S. L., Crowell, K. G., Goh, S. H. & Hemmingsen, S. M. (2004). cpnDB: a chaperonin sequence database. *Genome Res* **14**, 1669–1675.

Hill, J. E., Paccagnella, A., Law, K., Melito, P. L., Woodward, D. L., Price, L., Leung, A. H., Ng, L. K., Hemmingsen, S. M. & Goh, S. H. (2006). Identification of *Campylobacter* spp. and discrimination from *Helicobacter* and *Arcobacter* spp. by direct sequencing of PCR-amplified *cpn60* sequences and comparison to cpnDB, a chaperonin reference sequence database. *J Med Microbiol* **55**, 393–399.

Horwich, A. L., Fenton, W. A., Chapman, E. & Farr, G. W. (2007). Two families of chaperonin: physiology and mechanism. *Annu Rev Cell Dev Biol* **23**, 115–145.

Huys, G. & Swings, J. (1999). Evaluation of a fluorescent amplified fragment length polymorphism (FAFLP) methodology for the genotypic discrimination of *Aeromonas* taxa. *FEMS Microbiol Lett* **177**, 83–92.

Huys, G., Coopman, R., Janssen, P. & Kersters, K. (1996). 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. (1997). 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. & 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.

Huys, G., Denys, R. & Swings, J. (2002). DNA–DNA reassociation and phenotypic data indicate synonymy between *Aeromonas enteropelogenes* Schubert *et al.* 1990 and *Aeromonas trota* Carnahan *et al.* 1991. *Int J Syst Evol Microbiol* **52**, 1969–1972.

Huys, G., Cnockaert, M. & Swings, J. (2005). *Aeromonas culicicola* Pidiyar *et al.* 2002 is a later subjective synonym of *Aeromonas veronii* Hickman-Brenner *et al.* 1987. *Syst Appl Microbiol* **28**, 604–609.

Ihaka, R. & Gentleman, R. (1996). R: a language for data analysis and graphics. *J Comput Graph Stat* **5**, 299–314.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.

Küpfer, M., Kuhnert, P., Korczak, B. M., Peduzzi, R. & Demarta, A. (2006). Genetic relationships of *Aeromonas* strains inferred from 16S rRNA, *gyrB* and *rpoB* gene sequences. *Int J Syst Evol Microbiol* **56**, 2743–2751.

Martin-Carnahan, A. & Joseph, S. W. (2005). Genus I. *Aeromonas* Stanier 1943, 213^{AL}. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2, part B, pp. 557–578. Edited by G. M. Garrity, D. J. Brenner, N. R. Krieg & J. T. Staley. New York: Springer.

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.

Martínez-Murcia, A. J., Soler, L., Saavedra, M. J., Chacón, M. R., Guarro, J., Stackebrandt, E. & Figueras, M. J. (2005). Phenotypic, genotypic, and phylogenetic discrepancies to differentiate *Aeromonas salmonicida* from *Aeromonas bestiarum*. *Int Microbiol* **8**, 259–269.

Martínez-Murcia, A. J., Figueras, M. J., Saavedra, M. J. & Stackebrandt, E. (2007). The recently proposed species *Aeromonas sharmana* sp. nov., isolate GPTSA-6^{T}, is not a member of the genus *Aeromonas*. *Int Microbiol* **10**, 61–64.

Miñana-Galbis, D., Farfán, M., Lorén, J. G. & Fusté, M. C. (2002). Biochemical identification and numerical taxonomy of *Aeromonas* spp. isolated from environmental and clinical samples in Spain. *J Appl Microbiol* **93**, 420–430.

Miñana-Galbis, D., Farfán, M., Fusté, M. C. & Lorén, J. G. (2004a). *Aeromonas molluscorum* sp. nov., isolated from bivalve molluscs. *Int J Syst Evol Microbiol* **54**, 2073–2078.

Miñana-Galbis, D., Farfán, M., Fusté, M. C. & Lorén, J. G. (2004b). Genetic diversity and population structure of *Aeromonas hydrophila*, *Aer. bestiarum*, *Aer. salmonicida* and *Aer. popoffii* by multilocus enzyme electrophoresis (MLEE). *Environ Microbiol* **6**, 198–208.

Miñana-Galbis, D., Farfán, M., Fusté, M. C. & Lorén, J. G. (2007). *Aeromonas bivalvium* sp. nov., isolated from bivalve molluscs. *Int J Syst Evol Microbiol* **57**, 582–587.

Nei, M. (1996). Phylogenetic analysis in molecular evolutionary genetics. *Annu Rev Genet* **30**, 371–403.

Nhung, P. H., Hata, H., Ohkusu, K., Noda, M., Shah, M. M., Goto, K. & Ezaki, T. (2007). Use of the novel phylogenetic marker *dnaJ* and DNA–DNA hybridization to clarify interrelationships within the genus *Aeromonas*. *Int J Syst Evol Microbiol* **57**, 1232–1237.

Rozas, J. & Rozas, R. (1999). DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics* **15**, 174–175.

Saavedra, M. J., Figueras, M. J. & Martínez-Murcia, A. J. (2006). Updated phylogeny of the genus *Aeromonas*. *Int J Syst Evol Microbiol* **56**, 2481–2487.

Soler, L., Yáñez, M. A., Chacon, M. R., Aguilera-Arreola, M. G., Catalán, V., Figueras, M. J. & Martínez-Murcia, A. J. (2004). Phylogenetic analysis of the genus *Aeromonas* based on two housekeeping genes. *Int J Syst Evol Microbiol* **54**, 1511–1519.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A. D., Kämpfer, P., Maiden, M. C. J., Nesme, X., Rosselló-Mora, R., Swings, J. & 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., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Thompson, J. R., Pacocha, S., Pharino, C., Klepac-Ceraj, V., Hunt, D. E., Benoit, J., Sarma-Rupavtarm, R., Distel, D. L. & Polz, M. F. (2005). Genotypic diversity within a natural coastal bacterioplankton population. *Science* **307**, 1311–1313.

Valera, L. & Esteve, C. (2002). Phenotypic study by numerical taxonomy of strains belonging to the genus *Aeromonas*. *J Appl Microbiol* **93**, 77–95.

Wick, G., Knoflach, M. & Xu, Q. (2004). Autoimmune and inflammatory mechanisms in atherosclerosis. *Annu Rev Immunol* **22**, 361–403.