Updated phylogeny of the genus *Aeromonas*

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Recent phylogenetic studies of the genus *Aeromonas* based on *gyrB* and *rpoD* gene sequences have improved the phylogeny based on 16S rRNA gene sequences first published in 1992, particularly in the ability to split closely related species. These studies did not include the recently described species *Aeromonas simiae* and *Aeromonas molluscorum* and only a single strain of *Aeromonas culicicola* was available for analysis at that time. In the present work, these *Aeromonas* species and newly isolated strains of *A. culicicola* were examined. Sequence analysis indicates that *A. simiae* and *A. molluscorum* belong to non-described phylogenetic lines of descent within this genus, which supports the original description of both species. The most closely related species are *Aeromonas schubertii* and *Aeromonas encheleia*, respectively, which is consistent with 16S rRNA gene sequencing results. However, while the five strains of *A. molluscorum* showed nucleotide differences in their *gyrB* and *rpoD* gene sequences, the only two known *A. simiae* strains exhibited identical gene sequences, suggesting that they are isolates of the same strain. On the basis of the *rpoD* gene sequence phylogeny, *A. culicicola* strains from the original description and new isolates from drinking water and ornamental fish clustered within the species *Aeromonas veronii*, suggesting inconsistencies with previous results. Other strains with previously controversial taxonomy and new isolates from other studies were included in this study in order to clarify their phylogenetic affiliation at the species level.

Species of the genus *Aeromonas* are common inhabitants of aquatic environments and have been described in connection with fish and human diseases (Altwegg, 1999; Austin & Adams, 1996; Figueras, 2005; Saavedra *et al.*, 2004). This genus belongs to the family *Aeromonadaceae* (Colwell *et al.*, 1986; Martínez-Murcia *et al.*, 1992a; Yáñez *et al.*, 2003) and, over the last two decades, the number of recognized species has expanded very rapidly. According to the last edition of *Bergey's Manual of Systematic Bacteriology* (Martin-Carnahan & Joseph, 2005), the genus comprises the following species: *Aeromonas hydrophila*, *Aeromonas bestiarum*, *Aeromonas salmonicida*, *Aeromonas caviae*, *Aeromonas media*, *Aeromonas eucrenophila*, *Aeromonas sobria*, *Aeromonas veronii* (biovars *sobria* and *veronii*), *Aeromonas jandaei*, *Aeromonas schubertii*, *Aeromonas trota*, *Aeromonas allosaccharophila*, *Aeromonas encheleia*, *Aeromonas popoffii* and the two DNA homology groups, *Aeromonas* sp. HG11, *Aeromonas* sp. HG13 (formerly Enteric Group 501) which remain without a species name. *Aeromonas ichthiosmia* (Schubert *et al.*, 1990b) and

Aeromonas enteropelogenes (Schubert *et al.*, 1990a) are now considered to be synonyms of *A. veronii* and *A. trota*, respectively (Carnahan, 1993; Collins *et al.*, 1993; Huys *et al.*, 2001, 2002; Martin-Carnahan & Joseph, 2005). Three novel species, *Aeromonas culicicola* (Pidiyar *et al.*, 2002), *Aeromonas simiae* (Harf-Monteil *et al.*, 2004) and *Aeromonas molluscorum* (Miñana-Galbis *et al.*, 2004), have recently been described. It has been proposed that *A. culicicola* is a synonym of *A. veronii* (Huys *et al.*, 2005), but only the type strain was investigated. In addition, some judicial decisions on certain species names are pending because no Request for an Opinion has yet been submitted to the Judicial Commission of the International Committee on Systematic Bacteriology (<http://www.bacterio.cict.fr/a/aeromonas.html>).

The taxonomy of the genus *Aeromonas*, then, is complex and has been dogged by confusion and controversy. Several DNA–DNA hybridization studies have contributed to the elucidation of the inter-relationships between *Aeromonas* species, although discrepancies have been reported between different sets of data using the same strains (Esteve *et al.*, 1995a, b; Huys *et al.*, 1996a, b, 2001; Martínez-Murcia, 1999;

Abbreviations: AFLP, amplified fragment length polymorphism; HG, homology group.

Martínez-Murcia *et al.*, 1992a, 2005). Phylogenetic analyses based on 16S rRNA gene sequencing have indicated that this is a very tight group of species (Martínez-Murcia *et al.*, 1992a). Although most 16S rRNA gene sequence-derived relationships have correlated well with the DNA homology groups (HG), difficulties have been encountered in splitting closely related species (Martínez-Murcia, 1999; Martínez-Murcia *et al.*, 2005). DNA probes and restriction fragment length polymorphism (RFLP) profiles designed from 16S rRNA gene diagnostic regions have served to identify *Aeromonas* at the species level (Ash *et al.*, 1993a, b; Borrell *et al.*, 1997; Dorsch *et al.*, 1994; Figueras *et al.*, 2000; Khan & Cerniglia, 1997; Lee *et al.*, 2002; Soler *et al.*, 2002). Recent sequencing analyses based on the gene sequences of *gyrB* (encoding the B subunit of DNA gyrase, a type II DNA topoisomerase) and *rpoD* (encoding the σ^{70} factor, one of the sigma factors that confers promoter-specific transcription initiation on RNA polymerase) have demonstrated that they are excellent molecular markers for phylogenetic inference in the genus *Aeromonas* (Soler *et al.*, 2004; Yáñez *et al.*, 2003) and these chronometers seem to be well synchronized. The recently described species *A. simiae* (Harf-Monteil *et al.*, 2004) and *A. molluscorum* (Miñana-Galbis *et al.*, 2004) were not included in these previous studies and only the type strain of *A. culicicola* (Pidiyar *et al.*, 2002) was available at that time. Therefore, in the present phylogenetic study, an analysis was made of the *gyrB*, *rpoD* and 16S rRNA gene sequences of all of the currently recognized species of the genus *Aeromonas*, new isolates of *A. culicicola* (Figueras *et al.*, 2005) and other *Aeromonas* strains of uncertain classification (Valera & Esteve, 2002).

The strains used in this study are listed in Table 1. Strains of *A. encheleia* and snail isolates were kindly supplied by Drs Esteve and Kodjo; those of *A. molluscorum*, *A. culicicola* and *A. simiae* were from Drs Miñana-Galbis, Pidiyar and Harf-Monteil, respectively. Strains were cultivated on tryptic soy agar (Oxoid) at 30 °C for 24 h. Cultures were harvested and washed in deionized sterile water. Total DNA was extracted and purified. PCR amplification and sequencing of the *gyrB*, *rpoD* and 16S rRNA genes were performed following previously described methods (Martínez-Murcia *et al.*, 1999; Soler *et al.*, 2004; Yáñez *et al.*, 2003) although we used the BigDye Terminator V3.1 cycle sequencing kit in the ABI 3100-Avant Genetic Analyser (Applied Biosystems), according to the manufacturer's instructions, and the services supplied by the Molecular Diagnostics Center (MDC), Orihuela, Spain. Nucleotide sequences were aligned by the CLUSTAL_X program, version 1.8 (Thompson *et al.*, 1997). For alignments, previously published reference sequences (Martínez-Murcia, 1999; Martínez-Murcia *et al.*, 1992a; Soler *et al.*, 2004; Yáñez *et al.*, 2003) were used. Genetic distances were obtained by Kimura's two parameter model (Kimura, 1980) and evolutionary trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) with the MEGA program (Kumar *et al.*, 2001).

The *gyrB* and *rpoD* gene sequences showed similar substitution rates, confirming that they are well synchronized as previously demonstrated (Soler *et al.*, 2004; Yáñez *et al.*, 2003). Unrooted phylogenetic trees derived from *gyrB* (Fig. 1) and *rpoD* (Fig. 2) gene sequences showed a strain grouping in agreement with all species of the genus *Aeromonas* described to date, except for *Aeromonas culicicola*. The original description of *A. culicicola* (Pidiyar *et al.*, 2002) was based on strain MTCC 3249^T (=CECT 5761^T) from *Culex quinquefasciatus* and two strains, SH (MDC56) and SLH (MDC57), from *Aedes aegyptii*. However, inconsistencies found by Figueras *et al.* (2005) and now confirmed at the MDC laboratory, suggest that some of the 16S rRNA gene sequences of *A. culicicola* strains could be affiliated to *A. veronii*. Previous phylogenetic studies based on *gyrB* gene sequences (Soler *et al.*, 2004; Yáñez *et al.*, 2003) included the sequence of strain MTCC 3249^T (GenBank accession number AF175891 obtained by V. Pidiyar, M. Patole, A. Kaznowski, and Y. S. Shouche, 2003) which showed five nucleotide differences from that obtained at the MDC laboratory. A duplicate type strain was obtained from the Colección Española de Cultivos Tipo (CECT) and the same differences were confirmed. Similarly, the *gyrB* gene sequence of strains SH and SLH (GenBank accession numbers AY130993 and AF488548, respectively) showed a single nucleotide difference. However, the sequences obtained at MDC showed no differences at all and were identical to the sequence submitted under accession number AY130993. The present study included the three original strains (Pidiyar *et al.*, 2002), two isolates, MDC59 and MDC60, from drinking water (Figueras *et al.*, 2005) and three strains recently isolated from ornamental fish (MDC78, MDC285 and MDC364). Strains SH, SLH and the two strains isolated from drinking water clearly clustered within the species *A. veronii* in phylogenetic trees constructed using *gyrB* and *rpoD* gene sequences, in agreement with trees constructed from 16S rRNA gene sequences. Following the *gyrB* gene sequence phylogeny, the type strain of *A. culicicola* and the three strains isolated from ornamental fish formed a subcluster that was borderline with the group containing the *A. veronii* strains (Fig. 1). The *rpoD* gene sequence tree unequivocally grouped all strains within the confines of the *A. veronii* species (Fig. 2). The present *rpoD* gene sequence results using seven additional *A. culicicola* strains were in agreement with the recently published proposal that *Aeromonas culicicola* Pidiyar *et al.* 2002 should be considered to be a later subjective synonym of *Aeromonas veronii* Hickman-Brenner *et al.* 1988 (Huys *et al.*, 2005). The proposal, which used only the type strain, was based on evidence from biochemical characterization, fatty acid analysis, DNA–DNA hybridization and previously reported amplified fragment length polymorphism (AFLP) (Miñana-Galbis *et al.*, 2004) and *gyrB/rpoD* gene sequence (Soler *et al.*, 2004; Yáñez *et al.*, 2003). DNA–DNA hybridization between the type strains of these two species ranged from 79 to 88 %, data that contradict the findings of Pidiyar *et al.* (2002), who reported a much lower DNA–DNA hybridization value (44 ± 3 %). This represents a further example of

Table 1. Bacterial strains used for *gyrB/rpoD* gene sequencing in this study and equivalent designations

CECT, Colección Española de Cultivos Tipo, Universidad de Valencia, Valencia, Spain; LMG, BCCM/LMG Culture Collection, Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; CIP, Collection Bactérienne de l'Institut Pasteur, Paris, France; MDC, Molecular Diagnostics Center, Orihuela, Alicante, Spain; CCUG, Culture Collection, University of Göteborg, Sweden; MTCC, Microbial Type Culture Collection & Gene Bank, India.

Strain	Strain designation as received
MDC20 ^T	<i>A. molluscorum</i> 848T ^T (=CECT 5864 ^T =LMG 22214 ^T)
MDC43	<i>A. molluscorum</i> 93M (=CECT 5865=LMG 22215)
MDC72	<i>A. molluscorum</i> 431E (=CECT 5866=LMG 22216)
MDC73	<i>A. molluscorum</i> 849T (=CECT 5867=LMG 22217)
MDC74	<i>A. molluscorum</i> 869N (=CECT 5868=LMG 22218)
MDC28 ^T	<i>A. culicicola</i> CECT 5761 ^T (=MTCC 3249 ^T)
MDC56	<i>A. culicicola</i> SH
MDC57	<i>A. culicicola</i> SLH
MDC59	<i>A. culicicola</i> 02424
MDC60	<i>A. culicicola</i> 03037
MDC78	<i>Aeromonas</i> sp. O ₄
MDC285	<i>Aeromonas</i> sp. A ₃
MDC364	<i>Aeromonas</i> sp. B ₂₉
MDC54 ^T	<i>A. simiae</i> CIP 107798 ^T (=IBS S6874 ^T =CCUG 47378 ^T)
MDC55	<i>A. simiae</i> CIP 107797 (=IBS S6652)
MDC49	<i>A. hydrophila</i> E99 (=Kodjo L3)
MDC50	<i>A. hydrophila</i> E100 (=Kodjo L15)
MDC51	<i>A. caviae</i> E103 [=Kodjo Ob3(2)]
MDC52	<i>Aeromonas</i> sp. E104 [=Kodjo B3(41)]
MDC61	<i>A. encheleia</i> CECT 4825 (=LMG 13691=CCUG 30347)
MDC62	<i>A. encheleia</i> CECT 4824 (=LMG 13062=CCUG 30346)
MDC64	<i>Aeromonas</i> sp. CECT 5028 (=LMG 17060)
MDC65	<i>A. encheleia</i> CECT 5030 (=LMG 17065)
MDC66	<i>A. encheleia</i> CECT 4826 (=LMG 13061=CCUG 30345)
MDC67	<i>Aeromonas</i> sp. CECT 4987 (=LMG 17058)
MDC69	<i>Aeromonas</i> sp. CECT 5027 (=LMG 16406)
MDC70	<i>Aeromonas</i> sp. CECT 5025 (=LMG 16403)
MDC71	<i>Aeromonas</i> sp. CECT 4988 (=LMG 17063)
MDC24	<i>Aeromonas</i> sp. HG11 CECT 4856 (=A926=LMG 13076)
MDC98	<i>A. veronii</i> bv. sobria LMG 13074 (=CECT 4912)
MDC99	<i>A. veronii</i> bv. sobria LMG 13071 (=CECT 4909)
MDC100	<i>A. veronii</i> bv. sobria LMG 13073 (=CECT 4911)
MDC45	<i>Aeromonas</i> sp. S-1
MDC234	<i>Aeromonas</i> sp. C ₈ A ₁
MDC380	<i>Aeromonas</i> sp. AD ₁ A ₃
MDC561	<i>Aeromonas</i> sp. 10562

inconsistency between different sets of DNA–DNA hybridization data published by different authors using the same reference strains (Martínez-Murcia *et al.*, 2005).

The description of the species *A. simiae* (Harf-Monteil *et al.*, 2004) includes the 16S rRNA gene sequences of the only available strains, S6874 and S6652, which had a single nucleotide difference. The sequences of the 16S rRNA gene and those of the *gyrB* and *rpoD* genes obtained in this study were identical for this pair of strains, suggesting that the two strains may belong to the same clone. This has been further

confirmed by using several genotyping techniques (Figueras *et al.*, 2006). In the present study, *A. simiae* constituted a robust phylogenetic branch with considerable sequence divergence as shown by the two molecular chronometers under evaluation (Figs 1 and 2). *A. schubertii* was found to be the most closely related species, in agreement with the 16S rRNA gene sequence data in the original description (Harf-Monteil *et al.*, 2004).

The species *A. molluscorum* comprises strains 93M, 431E, 869N, 849T and the type strain, 848T^T (Miñana-Galbis *et al.*,

Fig. 1. Unrooted phylogenetic tree based on *gyrB* gene sequences, showing the relationships of all described *Aeromonas* species. Numbers shown next to each node indicate bootstrap values (percentage of 1000 replicates). GenBank accession numbers are in parentheses. Bar, 0.02 substitutions per site.

2004). In the species description, genetic typing by AFLP showed that these strains are different from each other and this has since been confirmed by using other genotyping methods (Figueras *et al.*, 2006). The *gyrB* gene sequence phylogeny indicated that the *A. molluscorum* strains are different from each other and that they form a tight group

Fig. 2. Unrooted phylogenetic tree based on *rpoD* gene sequences, showing the relationships of all described *Aeromonas* species. Numbers shown next to each node indicate bootstrap values (percentage of 1000 replicates). GenBank accession numbers are in parentheses. Bar, 0.02 substitutions per site.

with a relatively long phylogenetic line (Fig. 1), branched close to *A. encheleia*, in agreement with the tree based on 16S rRNA gene sequences (Miñana-Galbis *et al.*, 2004). The *rpoD* gene sequences of the *A. molluscorum* strains were also different from each other and confirmed the phylogenetic distinctiveness of this species (Fig. 2). This finding represents a new case of *gyrB* (and *rpoD*) gene sequences providing the means to split closely related *Aeromonas* species, even at the intra-species level, when the ability to do so on the basis of 16S rRNA gene sequence was limited.

In previous studies based on AFLP fingerprinting and DNA–DNA hybridization (Huys *et al.*, 1996b, 2001), the original description of the species *A. allosaccharophila* (Martínez-Murcia *et al.*, 1992b) was questioned because a group of strains, including LMG 13071 (=CECT 4909), LMG 13073 (=CECT 4911) and LMG 13074 (=CECT 4912), received as *A. veronii* bv. *sobria*, clustered with the two known strains of *A. allosaccharophila*. This incongruity with the results from previous DNA–DNA hybridization studies (Esteve *et al.*, 1995a) has been extensively discussed elsewhere (Yáñez *et al.*, 2003). In the present study, the three LMG culture collection strains mentioned above undoubtedly clustered with *A. allosaccharophila* on the basis of *gyrB* gene sequence analyses, a cluster clearly separated from that of *A. veronii*, which also contained the reference strain of *A. veronii* bv. *sobria*. Similar *gyrB* gene sequence inter-cluster distances have been shown for other *Aeromonas* species, for instance *A. salmonicida* and *A. bestiarum* (Fig. 1) with sufficient evidence to consider them as separate species (Martínez-Murcia *et al.*, 2005). Sequencing of the 16S rRNA gene regions that include diagnostic nucleotides for *A. allosaccharophila* (Martínez-Murcia *et al.*, 1992b) also supports this affiliation (data not shown). This is of particular concern as this material may be part of the AEROLIB laboratory database, a reference for AFLP analysis carried out by the identification service of the Belgian Coordinated Collection of Microorganisms. Recently, thanks to the resolution of *gyrB* gene sequencing, for the first time since the original species description, we have identified not only 17 *A. allosaccharophila* strains (e.g. MDC45, MDC234 and MDC380 in Fig. 1; MDC234 in Fig. 2) isolated in a survey from pig carcasses and the processing machinery of a slaughterhouse in the North of Portugal, but also a single strain (MDC561) recently isolated from a clinical case of gastroenteritis (Saavedra *et al.*, 2006). Although the phylogenetic tree based on *rpoD* gene sequences showed a borderline relationship to the *A. veronii*, the *gyrB* gene sequence results provided evidence that supports the original proposal of *A. allosaccharophila*. This species has rarely been isolated before, but is perhaps more widely distributed and only detectable if approaches such as sequencing of housekeeping genes are used.

Finally, four isolates from snails identified by Kodjo *et al.* (1997) (Table 1), four strains formerly classified as *A. encheleia* (Demarta *et al.*, 2004) and five aeromonads of uncertain classification on the basis of numerical taxonomy

(Valera & Esteve 2002; Phenon 19, *Aeromonas* sp.) were included in the *gyrB* gene sequence analysis (Fig. 1). The four strains isolated from snails, MDC49–MDC52, clustered with *A. caviae*, while the other nine strains formed a cluster with the reference strain of *Aeromonas* DNA homology group 11 (HG11), a group very closely related to the two original *A. encheleia* strains as previously reported (Huys *et al.*, 1997; Martínez-Murcia, 1999).

As demonstrated in the present phylogenetic analysis, the use of several housekeeping genes is an invaluable approach for the classification of *Aeromonas* species and for the proper identification of novel closely related isolates. The resolution of the 16S rRNA gene sequence to split species has been superseded by the more variable and synchronized sequences of the *gyrB* and *rpoD* genes. As taxonomic conclusions, the present data add support to the species descriptions of *A. allosaccharophila*, *A. simiae* and *A. molluscorum*. However, the relationship between *A. culicicola* and *A. veronii* remains unclear.

Note added in proof

While this paper was in press, *Aeromonas sharmiana* sp. nov. has been described [Saha & Chakrabarti, *Int J Syst Evol Microbiol* 56 (2006), 1905–1909]. However, in our opinion, the 16S rRNA gene phylogenetic tree presented in the paper indicates that this novel species does not belong to the genus *Aeromonas*.

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