

# Analysis of 16S rRNA gene mutations in a subset of *Aeromonas* strains and their impact in species delineation

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Received 18 May 2008 · Accepted 15 August 2008

**Summary.** Characterization of 999 *Aeromonas* strains using a published 16S rDNA RFLP identification method showed that 8.1% of the strains produced unexpected (hereafter called “atypical”) restriction patterns, making their identification uncertain. Atypical patterns were due to the presence of nucleotide polymorphisms among the *rrn* operons of the 16S rRNA gene (so-called microheterogeneities). Double sequencing signals at certain positions revealed the nucleotide composition was responsible for the microheterogeneities. Although the number of microheterogeneities was relatively low (0.06–0.66%), trees inferred from the 16S rRNA gene led either to a misidentification or to an inconclusive result for the majority of these strains. Strains with atypical patterns were, however, correctly identified using the *rpoD* gene sequences, as belonging to *Aeromonas caviae*, *A. veronii*, and *A. media*. All of them, but particularly the two former species, are associated with human disease. Microheterogeneities in 16S rRNA gene sequence were significantly ( $P < 0.01$ ) more prevalent in clinical than in environmental strains. This work also analyzed the effects of these microheterogeneities on the taxonomic position of the investigated strains. The results suggest the need for recording microheterogeneities in the 16S rRNA gene. [Int Microbiol 2008; 11(3):185-194]

**Key words:** *Aeromonas* · 16S rRNA gene microheterogeneities · *rpoD* gene

## Introduction

Direct sequencing of the 16S rRNA gene is generally accepted as a stable and specific marker for bacterial identification [32,59]. The 16S rRNA gene is often organized as a multi-gene family, with the copy number ranging from 1 to 15 rRNA (*rrn*) operons in the bacterial genomes [14]. Despite the general belief that the sequences of all the 16S *rrn* copies of an organism are identical or almost identical, scattered

nucleotide differences between the copies (so-called microheterogeneities or polymorphisms) have been described in many bacterial genera [2,6,11,13,14,32,33,42,56,57]. Most of those microheterogeneities are located in the variable regions V1, V2, V6, and, to a lesser extent, V3 and V4 of the 16S rRNA gene [14]. Nucleotide sequence divergence among the 16S rRNA gene copies of the majority of bacteria has been shown to be less than 1%, although in extreme cases, e.g., in some thermophilic bacteria, it may be as high as 11.6% [2]. Some authors have suggested that low-level microheterogeneities do not alter the inferred 16S rRNA gene phylogeny [2,14] while others have cautioned that it may lead to misidentification [6,11,32,41,57] particularly for species that are tightly defined [43].

*Aeromonas* is one such tightly defined genus because the 16S rDNA similarities among species range from 96.7 to 100% [34,35]. In this genus, the V2, V3 and V6 regions of the 16S rRNA gene contain the signature nucleotides that

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enable the identity of particular species to be established [34]. The taxonomy of the genus *Aeromonas* has undergone continuous changes due to the addition of new species and the introduction of new diagnostic criteria, such as those based on housekeeping genes [35–37,39,40,49]. Phenotypic similarity among the different species as defined by a limited number of test or miniaturized methods has led to unreliable biochemical identification [18,44] in contrast to the results of more extensive schemes [1]. This discrepancy has emphasized the need for molecular methods that can reliably identify all aeromonad species, particularly the rarer ones from environmental sources [5,18,21,27]. The *rpoD* gene [35,54], among other housekeeping genes [15,31,49,60], has proven to be an excellent molecular tool for inferring the taxonomy of *Aeromonas*. This was demonstrated using the sequences of 70 strains belonging to all of the species recognized at that time (including the type strains); all the strains were unambiguously identified, and the results further corroborated with the *gyrB* gene [54].

*Aeromonas* are ubiquitous waterborne organisms that have gained importance as human pathogens due to their ability to cause a broad spectrum of human infections, including gastroenteritis, cellulitis, wound infections, hepatobiliary infections, and septicemia [18]. *Aeromonas hydrophila* is the most frequently recorded clinical species, but this is an erroneous by-product of inappropriate identification methods [18,29,44]. DNA-DNA reassociation analyses demonstrated that 31% of the phenotypically identified *A. hydrophila* strains instead belong to *A. veroni*, in a study by Kuijper et al. [29]. The prevailing species in human feces according to molecular methods are *A. caviae*, *A. veronii*, *A. hydrophila*, and *A. media* [18,29]. PCR-based sequencing of the 16S rRNA gene is increasingly described in the literature as a tool for the diagnosis of *Aeromonas* infections [4,17, 25,48]. It was recently indicated, however, that 16S rDNA sequences are not the best choice for identifying *Aeromonas* because intragenomic heterogeneity is ca. 1.5% for the five 16S *rrn* operons of *A. veronii* and 1.4% among the six *rrn* operons of *Aeromonas media* [41]. By contrast, only two (0.13%) base differences were found among the ten 16S *rrn* operons described in the complete genome of *Aeromonas hydrophila* type strain ATCC 7966 [53]. Intragenomic variations also have been described for *Aeromonas popoffii* [16], *Aeromonas media* [54], *Aeromonas molluscorum* [40], *Aeromonas culicicola* [22] (now considered as *A. veronii* [26]), *Aeromonas bestiarum*, *Aeromonas salmonicida* [38], and *Aeromonas allosaccharophila* [50]. The extent to which microheterogeneities occur in *Aeromonas* spp., and thereby possibly affect proper identification, is not known.

The identification of almost 1000 strains using the 16S rDNA RFLP method, described by Borrell et al. [5] and

Figueras et al. [21], should have produced a unique pattern for 14 species of *Aeromonas* described up to the year 2000; however, several strains produced unexpected atypical restriction patterns. Five new species described since that time have not been evaluated with this method, i.e., *A. simiae* [24], *A. molluscorum* [40], *A. bivalvium* [39], *A. aquariorum* [37], and *A. tecta* [15]. Therefore, the aims of this study were: to identify *Aeromonas* strains showing atypical restriction patterns using the sequence of the *rpoD* gene, to characterize microheterogeneities in the 16S rRNA gene of those strains, and to re-evaluate how they can affect signature regions of the 16S rDNA sequences of *Aeromonas* spp.

## Materials and methods

**Bacterial strains and genetic identification.** A previously described 16S rDNA RFLP protocol [5,21] was used to genetically identify 999 strains of *Aeromonas* spp., 447 of which were clinical isolates and 552 of environmental origin. Environmental isolates were confirmed as belonging to *Aeromonas* on the basis of Gram staining, cytochrome oxidase, catalase, and growth in nutrient broth with and without 6% NaCl. Clinical isolates of intestinal (n = 369) and extra-intestinal (n = 78) origin were received from different Spanish hospitals for their genetic identification. All the strains with an atypical RFLP pattern were further confirmed as *Aeromonas* using a genus probe described by Chacón et al. [9].

**Amplification, sequencing, and analysis of the 16S rRNA and *rpoD* genes.** DNA from a single colony of each strain was extracted using InstaGene Matrix (Bio-Rad, Hercules, CA) following the manufacturer's instructions. The 16S rRNA gene (1503 bp) of 27 randomly chosen strains with atypical RFLP patterns (Table 1-SI), representing 21–100% of the strains with each of the 12 pattern types (Fig. 1), was amplified and sequenced as described by Martínez-Murcia et al. [34]. The *rpoD* gene (820 bp) of those 27 strains, together with 19 additional strains with atypical patterns (n = 46), representing 42.8–100% of the strains with each RFLP pattern type (Table 1-SI), were amplified and sequenced as described by Soler et al. [54]. PCR products were confirmed by electrophoresis on 1% agarose gels in TAE (Tris-acetate-EDTA) buffer, with 100-bp DNA Ladder Plus (Fermentas, Hanover, MD) as the molecular size marker. Chromatograms of the 16S rRNA gene were carefully analyzed to recognize ambiguous bases (i.e., double sequencing signals or microheterogeneities). In such cases, they were annotated in the sequence of each strain in two ways: i) noting the predominant base and ii) noting the ambiguous base, following the nomenclature of the International Union of Pure and Applied Chemistry (IUPAC code, i.e., Y = C or T, R = G or A, W = T or A, K = T or G, S = G or C, M = C or A). Numbering on the 16S rRNA gene (Table 2-SI) was based on the *Escherichia coli* nomenclature [7]. Sequences obtained for the 16S rDNA and *rpoD* genes were aligned with those of the type and reference strains of all the members of the genus *Aeromonas*, available in the GenBank [http://www.ncbi.nlm.nih.gov/Genbank/], using the CLUSTAL W program v. 1.83 [55]. Genetic distances were determined using Kimura's two-parameter model [28]; evolutionary trees were constructed by the neighbor-joining method [52], using the MEGA 3.1 program [30]. The constructed *rpoD* tree included 132 sequences (46 from this study and 70 from a previously published study [54], with six of the latter belonging to strains having atypical RFLP patterns, and 16 additional strains [36,49]). To evaluate the impact of microheterogeneities identified in the 16S rRNA gene phylogenetic analysis, two trees were constructed: (i) using sequences showing the predominant

base in those positions (Fig. 2A), and (ii) using sequences in which microheterogeneities were recorded following the IUPAC code (Fig. 2B).

**Statistical analysis.** The Pearson Chi ( $\chi$ )-square was applied to determine the significance of differences between the number of clinical and environmental strains showing an atypical RFLP pattern. Statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS 14, Chicago, IL, USA);  $P < 0.05$  was considered statistically significant.

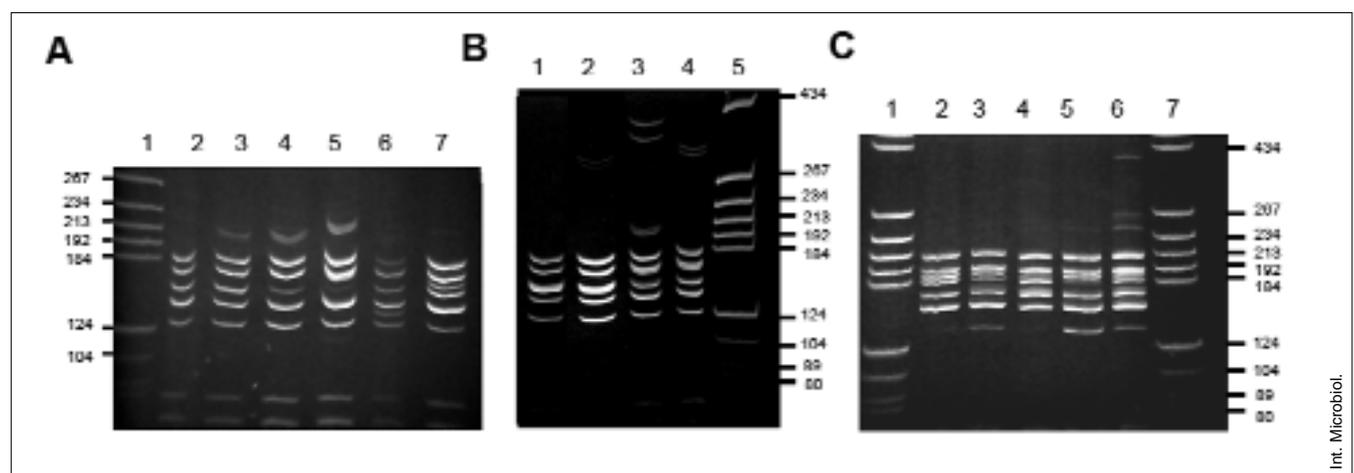
## Results

Of the 999 *Aeromonas* strains identified using the 16S rDNA RFLP method, 81 (57 of clinical origin, 24 environmental) had unexpected atypical restriction patterns. Atypical patterns were subdivided into 12 types and were predominantly associated with the clinical strains ( $P < 0.01$ ) (Fig. 1). The incidence of the different pattern types varied considerably. Pattern 1 was very common as it was observed in 37 of the 81 strains (45.7%), followed by pattern 2 (17.3%), pattern 3 (7.4%), patterns 4 and 5 (6.1% each), and pattern 11 (5%). The incidence of patterns 6, 7, 8, and 9 was low (2.5% each) while patterns 10 and 12 occurred in only one strain (1.2% each).

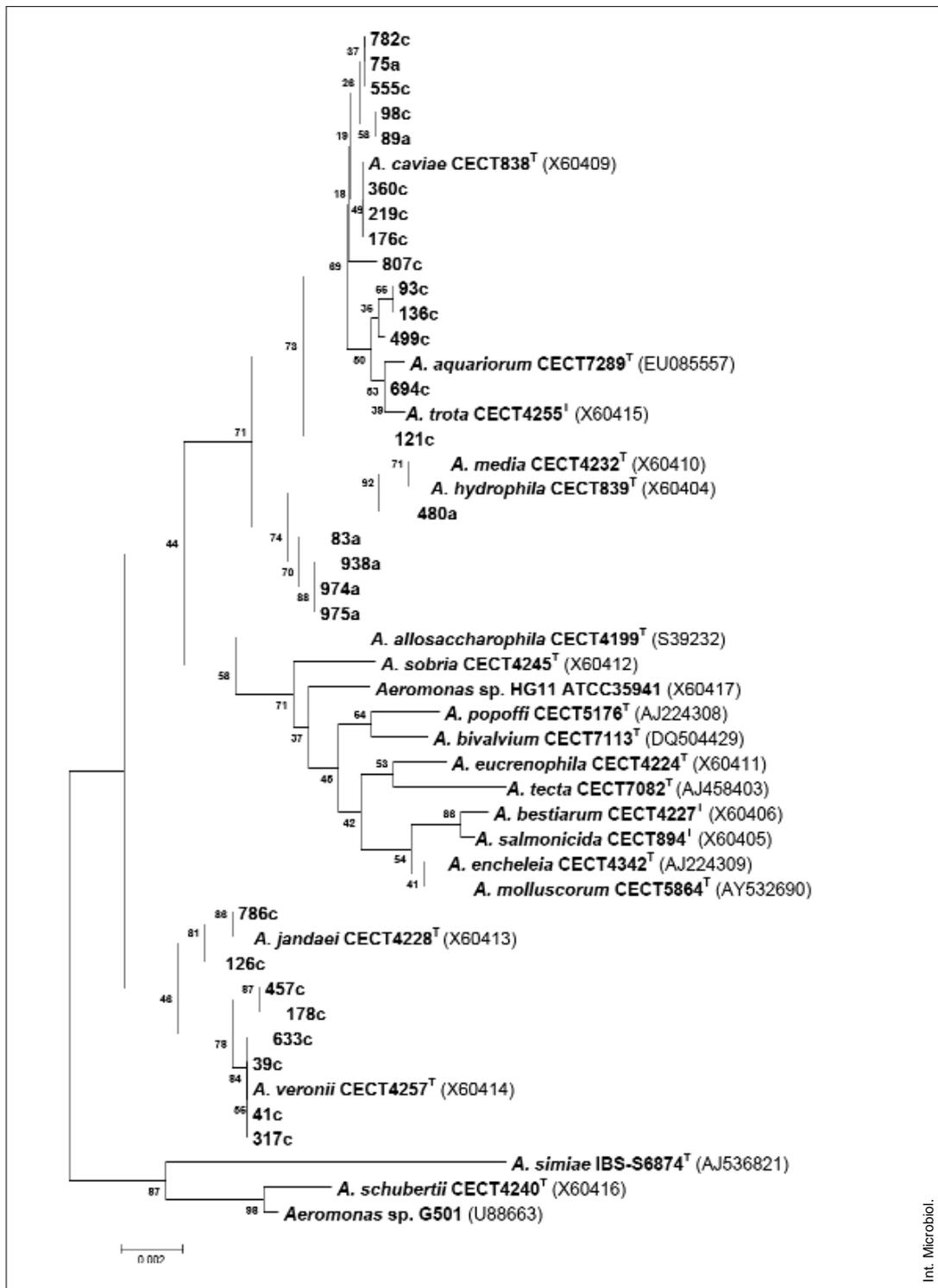
Chromatograms of the 16S rRNA gene sequences of the 27 strains belonging to each of the identified atypical pattern types showed a double sequencing signal (microheterogeneities) at determinate positions. Although secondary sig-

nals were slightly smaller, they were reproducible (in forward and reverse sequences in independent experiments) and are represented in Table 2-SI with small capital letters. The distribution of the different types of mutations, i.e., transitions [purine  $\leftrightarrow$  purine (A or G) and pyrimidine  $\leftrightarrow$  pyrimidine (C or T)] and transversions [purine  $\leftrightarrow$  pyrimidine], in the sequences of the 16S rRNA gene was analyzed. Among the 27 sequences of the 16S rRNA gene obtained in this study, 148 microheterogeneities were encountered, including 128 transitions (86.5%) and 20 transversions (13.5%), in 26 positions (1.8% of the 1503 positions sequenced) mostly located in the V3 region. The number of microheterogeneities per sequence ranged from 1 (0.06%) to 10 (0.66%) with a mean of  $5.48 \pm 2.88$  (SD).

Two 16S rRNA gene phylogenetic trees derived from sequences of the type strains of the 19 *Aeromonas* species described up to 2008 together with the 27 new sequences were constructed by assignment of the predominant base Fig. 2A or based on the microheterogeneities (IUPAC code) (Fig. 2B). In the first tree (Fig. 2A), 19 strains could not be properly assigned to a species. However, another six clustered with *A. veronii* (457c, 178c, 633c, 317c, 41c and 39c) and two (786c and 126c) with *A. jandaei*, each showing bootstrap values of around 80%. In the second tree (Fig. 2B), the taxonomic position of 20 strains could not be assessed. Six strains branched with *A. veronii* and one (786c) with *A. jandaei*, with bootstrap values of 89 and 77%, respectively. To deter-



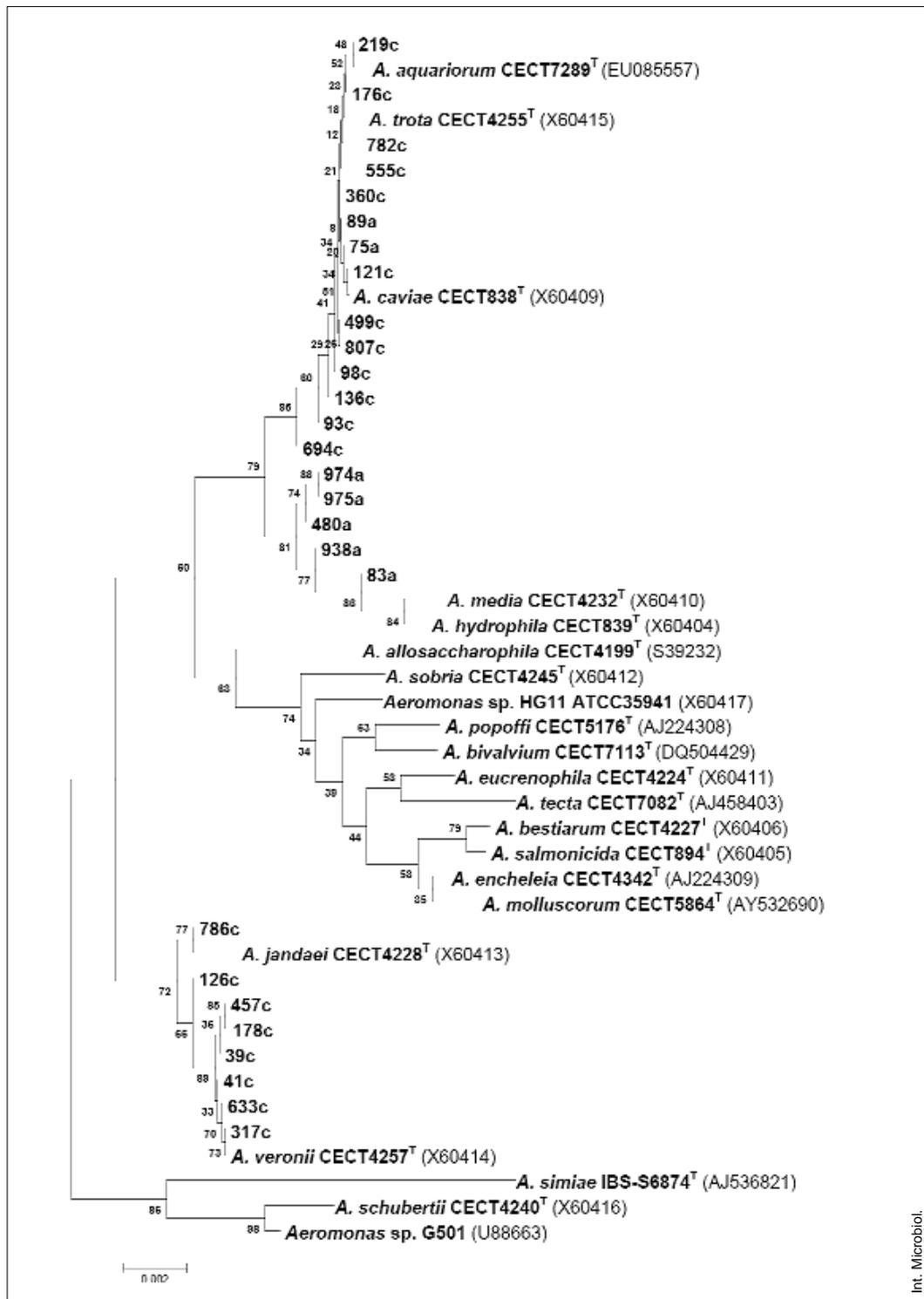
**Fig. 1.** Polyacrylamide (17%) gels showing the 12 types of atypical 16S rDNA RFLP patterns observed among 81 strains using endonucleases *AluI* and *MboI* and the typical patterns of *A. veronii*, *A. media*, and *A. caviae*. Strains with atypical patterns were identified using the *rpoD* gene. (A) *A. veronii*: lane 1, pBR322 DNA/*BsuRI* (*HaeIII*) marker 5; lane 2, CECT4257<sup>T</sup> showing the typical restriction pattern; lane 3, atypical pattern 5 of strain 39c; lane 4, atypical pattern 3 of strain 633c; lane 5, atypical pattern 11 of strain 126c; lane 6, atypical pattern 7 of strain 178c; lane 7, atypical pattern 10 of strain 457c. (B) *A. media*: lane 1 CECT 4232<sup>T</sup> showing the typical restriction pattern; lane 2, atypical pattern 9 of strain 239a; lane 3, atypical pattern 8 of strain 83a; lane 4, atypical pattern 4 of strain 975a; lane 5, pBR322 DNA/*BsuRI* (*HaeIII*) marker 5. (C) *A. caviae*: lane 1, pBR322 DNA/*BsuRI* (*HaeIII*) marker 5; lane 2, CECT 838<sup>T</sup> showing the typical restriction pattern; lane 3, atypical pattern 1 of strain 145c; lane 4, atypical pattern 6 of strain 121c; lane 5, atypical pattern 2 of strain 136c; lane 6, atypical pattern 12 of strain 807c; lane 7, pBR322 DNA/*BsuRI* (*HaeIII*) marker 5.



**Fig. 2A.** Phylogenetic tree constructed with 16S rDNA sequences of the type strains of all *Aeromonas* spp., the sequences (reflecting the predominant base at the microheterogeneity) of 27 strains with atypical 16S rDNA RFLP patterns. Numbers at nodes indicate bootstrap values (percentage of 1000 replicates).

mine the taxonomic position of strains showing atypical 16S rDNA RFLP patterns, the *rpoD* gene was sequenced from 46

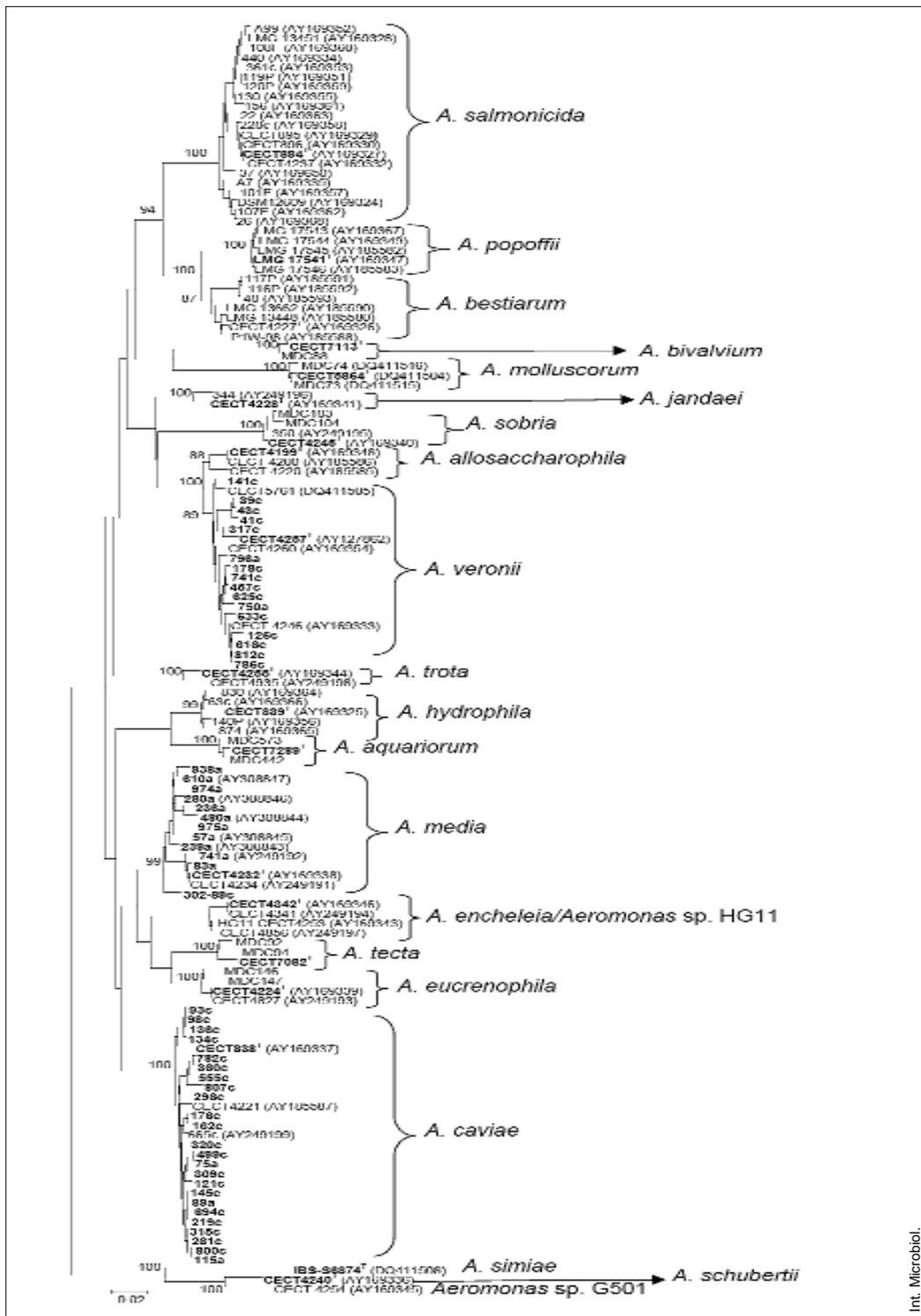
strains representing the 12 RFLP pattern types. Analysis of the *rpoD* gene also included six previously published



**Fig. 2B.** Phylogenetic tree constructed with 16S rDNA sequences of the type strains of all spp.; the sequences (reflecting the microheterogeneities using the IUPAC code) were those of 27 strains with atypical 16S rDNA RFLP patterns. Numbers at nodes indicate bootstrap values (percentage of 1000 replicates).

sequences from strains with atypical patterns (Table 1-SI). The derived neighbor-joining tree grouped all 52 strains with

a particular *Aeromonas* species (Fig. 3): 25 strains were grouped with *A. caviae*, 16 with *A. veronii*, and 11 with *A. media*, sup-



**Fig. 3.** Phylogenetic tree constructed with the *rpoD* sequences of 52 *Aeromonas* strains with atypical 16S rDNA RFLP patterns and available sequences of all type strains (bold text) and other references strains. Numbers at nodes indicate bootstrap values (percentage of 1000 replicates).

ported with bootstrap values of 100, 89, and 99%, respectively. None of the strains grouped with *A. jandaei*. *A. caviae* embraced

strains with atypical pattern types 1, 2, 6, and 12 (Table 1-SI). Pattern types 3, 5, 7, 10, and 11 belonged to *A. veronii*, and pat-

tern types 2, 4, 8 and 9 to *A. media*. Only the type 2 pattern was shared by two species (*A. media* and *A. caviae*). After *rpoD* analysis of 52 strains with atypical patterns, the four predominant *Aeromonas* species, accounting for 83.5% of the 999 strains, were *A. caviae* (372+25) (39.6%), *A. veronii* (260+16) (27.5%), *A. media* (82+11) (9.2%), and *A. hydrophila* (7.2%). The most predominant (45.7%) atypical pattern, type 1, belonged to *A. caviae*, the most frequently identified species in this study.

## Discussion

**Incidence of microheterogeneities in the 16S rRNA gene in *Aeromonas* spp.** According to the results of our study, 8.1% of 999 strains presented 16S rDNA RFLP patterns different from those previously defined for the 14 *Aeromonas* species described up to the year 2002 [5, 21,22]. Since then, five more *Aeromonas* species have been described: *A. simiae* [24], *A. molluscorum* [40], *A. bivalvium* [39], *A. aquariorum* [37], and *A. tecta* [15]. Although the RFLP identification method was not designed for those species, digestion of 1503 bp of their 16S rDNA with endonucleases *AluI* and *MboI* also produced specific patterns for *A. simiae* and *A. tecta*. However, the other three species could not be further differentiated using this method [5,21]. Sequencing showed that the atypical RFLP patterns were generated by the presence of reproducible microheterogeneities in the 16S rRNA gene, some of which were located at the targets of the endonucleases used (*AluI* and *MboI*) in the molecular identification, thus affecting the expected results. Thus, our study demonstrated that strains with atypical patterns had greater microheterogeneity, 26 positions in total, than the eight located in restriction sites revealed by the 16S rDNA RFLP method (Table 2-SI). In other words, the use of sequencing led to the identification of 3.4 times more variability than detected at the restrictions sites. The incidence of microheterogeneity found in our study, 8.1% (81 out of 999 strains), contrasted with the 21% reported by Morandi et al. [41], who analyzed 82 *Aeromonas* strains using a non-species-specific RFLP method [20,21,23]. Moreover, Morandi et al. [41] did not sequence a representative number of strains to confirm their estimates. Despite the heterogeneity observed, the 16S rDNA RFLP method [21] is considered to be a useful tool for the identification of *Aeromonas* species because more than 90% of the analyzed isolates could be identified.

The *rpoD* gene of 52 strains with atypical RFLP patterns enabled the strains to be identified as members of the species *A. caviae*, *A. veronii*, and *A. media*. This unambiguous identification also ruled out the possibility that the double

sequencing signals observed in the 16S rRNA chromatograms at certain positions arose from a mix of different strains. The characterized 16S rRNA gene microheterogeneities observed in those species ranged from 1 (0.06%) to 10 (0.66%), which is well within the range of microheterogeneities (from 1 to 11) described for other Gamma-proteobacteria [8]. This range was also in agreement with the values commonly described for some *Aeromonas* species; for instance, *Aeromonas popoffii* [16] has 2 positions (0.13%), *Aeromonas molluscorum* [40] 1–2 positions (0.06–0.13%), *Aeromonas culicicola* [22], now considered as *A. veronii* [26], 4–7 positions, (0.26–0.46%), *Aeromonas bestiarum* [38] 2 positions (0.13%), *Aeromonas salmonicida* [38] 2–8 positions (0.13%), *Aeromonas hydrophila* [53] 2 positions (0.13%), and *Aeromonas allosaccharophila* [50] 5 positions (0.33%). This corresponds to a range of 1 (0.06%) to 8 (0.52%) among those species. Considering the microheterogeneities observed in our study and those described in the literature, we can conclude that two positions (1011 and 1018) in the 16S rRNA gene, located in the V6 region, occur as microheterogeneities in all the above-mentioned species, with the exception of *A. hydrophila*.

### Impact of the 16S rRNA microheterogeneities on the taxonomic signature regions.

The allocation of most of the *Aeromonas* strains analyzed was ambiguous, i.e., 70% according to the predominant base in the sequences of the 16S rRNA gene phylogenetic tree (Fig 2A) and 74% according to the microheterogeneities (IUPAC code) in the other tree (Fig 2B). It should be noted that microheterogeneities using the IUPAC code are not considered at all in constructing alignments and, consequently, they are removed when phylogenetic trees are inferred [45]. These ambiguous allocations correspond to strains identified with the *rpoD* gene as *A. caviae* and *A. media*, which, on the basis of the 16S rRNA gene, are two tightly defined species. There are only three base-pair differences between *A. caviae* and *A. trota* type strains (position 649, 1011, and 1018) and between *A. hydrophila* and *A. media* type strains (positions 250, 471, and 476) (99.8% similarity in both cases) [34]. Thus, any heterogeneity at these signature positions, either alone or in combination with those corresponding to other species of the genus, will produce inconclusive results. Only six strains clustered in both phylogenetic trees with *A. veronii* (Fig. 2A,B), showing concordant results with *ropD*. In those strains, microheterogeneities were detected at two of the eight signature positions that, according to Martínez-Murcia et al. [34], differentiate *A. veronii* from its nearest neighbor, *A. jandaiei*. However, the presence of microheterogeneities in five or seven of these eight signature positions (strains 126c and 786c, respectively) affect identification. The heterogene-

ity found in these seven positions was previously described in *A. culicicola* [22], which probably accounts for its erroneous identification as a new species [46], but which later was synonymized with *A. veronii* [26].

Some authors have suggested that the existence of heterogeneity does not affect the taxonomy of the 16S rRNA gene [2,14], while others have disagreed [6,11,32,41,57]. It is clear from the results of this and previous studies [4,31,38,41] that microheterogeneity in the 16S rRNA gene of *Aeromonas* interferes with proper identification. To overcome this problem, the use of other genes, such as *rpoD* (this study), *gyrB*, *rpoB*, etc., previously shown to be useful for this purpose are necessary [4,31,49,54,60].

In our view, recognition of the heterogeneity detected in the sequences (using the IUPAC code) is important to avoid strain misidentification and faithful to the acknowledgement of diversity. In addition, recognizing microheterogeneities is essential to the design of rRNA-specific oligonucleotide probes as well as to the development of other identification methods, such as RFLPs [19]. Clarridge [12] suggested different ways to record sequence variations in relation to the type strain; these have been termed “sequevars.” This approach can also be adapted for recording microheterogeneities in sequences deposited in databases: (i) designating the position and base change [*A. caviae* 555c sequevar (1011T/C, 1018A/G, 1308T/C, 1329G/A)]; and (ii) combining this information with the percentage of positions showing microheterogeneities [*A. caviae* 555c, sequevar (1011T/C, 1018A/G, 1308T/C, 1329G/A) 0.26%].

It is clear that first we have to rule out that the presence of a double sequencing signal (microheterogeneity) in chromatograms of the 16S rRNA gene are not due to artifacts such as a “mixed culture” or a polymerase error. To that end, our study has shown that these double sequencing signals represent real base differences among different copies of the gene because: (i) molecular work was initiated from single colonies, (ii) no double signals in the single-copy gene *rpoD* sequences were observed, (iii) the signals were reproducible in different analyses achieved from different colonies of the same pure culture, and (iv) they explain the obtained atypical restriction patterns.

**Ecological and epidemiological significance of microheterogeneity.** The presence of sequence microheterogeneities between 16S *rrn* operons within single genomes has been observed in different bacteria (*Vibrio vulnificus* [58], genus *Bacillus* [47], *Streptococcus anginosus* [12], *Neisseria meningitidis* [51]) and has been linked to diverse potential roles, including an association with the virulence potential of individual isolates, as in the case of *V. vulnificus*

[58]. In the genus *Bacillus*, the existence of specific nucleotides in the 16S rRNA gene has been associated with the physiological properties of the isolates, related to their ability to grow at low or high temperature [47]. Microheterogeneities also have been related to the niche differences of clinical *S. anginosus* [12] and their analysis has proven to be a useful strategy for strain tracking in outbreaks of *N. meningitidis* [51].

*Aeromonas* spp. are autochthonous inhabitants of aquatic environments and are considered to be “emerging pathogens” [18]. The genus includes psychrophilic and mesophilic species, with most clinical isolates belonging to the species *A. veronii* (biovar *sobria*), *A. caviae*, and *A. hydrophila* [1,4,18,29,48]. Eleven other species and one biovar (*A. veronii* biovar *veronii*) have been associated sporadically with human infections [18,29]. The fact that the number of strains showing microheterogeneities in the 16S rRNA gene (atypical RFLP patterns) was significantly higher ( $P < 0.01$ ) in clinical isolates than in environmental ones is worthy of note.

Intragenomic microheterogeneity in the 16S rRNA gene was also found by other authors in an *A. caviae* strain associated with a case of cystitis [4]. Coincidentally, this was also the species that, in our study, had more strains with 16S rRNA gene heterogeneities, followed by *A. veronii*. Both species, together with *A. hydrophila*, have been frequently associated with human infections [18,29]. Although the reason for this is unknown, the prevalence of this species has been associated with the occurrence of particular virulence genes [3,10,18]. Our results emphasize the need to take into account the intragenomic diversity of the 16S rRNA gene, recording it in either of the proposed ways, and reinforce the usefulness of housekeeping genes, such as *rpoD*, but also *rpoB* and *gyrB*, in the identification of *Aeromonas* strains.

**Acknowledgements.** This research was supported by the Spanish Ministry of Health (FIS03/1183). We thank Dr. Jessica Boyd for providing the sequences of the rRNA operons of *A. salmonicida* A449.

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**Table 1-SI.** *Aeromonas* strains included in the phylogenetic analyses of 16S rRNA and *rpoD* genes and identification based on the latter gene

Strains	Origin	16S rDNA RFLP pattern type	GenBank accession no.		Identification based on <i>rpoD</i>
			16S rDNA	<i>rpoD</i>	
39c	Intestinal	5	EU488692	EU488635	<i>A. veronii</i>
41c	Intestinal	5	EU488697	EU488636	<i>A. veronii</i>
43c	Intestinal	5		EU488676	<i>A. veronii</i>
93c	Intestinal	2	EU488686	EU488637	<i>A. caviae</i>
98c	Intestinal	2	EU488687	EU488638	<i>A. caviae</i>
121c	Intestinal	6	EU488685	EU488639	<i>A. caviae</i>
126c	Intestinal	11	EU488699	EU488650	<i>A. veronii</i>
134c	Intestinal	1		EU488668	<i>A. caviae</i>
136c	Intestinal	2	EU488688	EU488640	<i>A. caviae</i>
141c	Intestinal	3		EU488674	<i>A. veronii</i>
145c	Intestinal	1		EU488675	<i>A. caviae</i>
162c	Intestinal	1		EU488672	<i>A. caviae</i>
176c	Intestinal	1	FJ168775	EU488670	<i>A. caviae</i>
178c	Intestinal	7	EU488694	EU488634	<i>A. veronii</i>
219c	Extra Intestinal	1	FJ168776	EU488671	<i>A. caviae</i>
261c	Intestinal	1		EU488667	<i>A. caviae</i>
296c	Intestinal	1		EU488669	<i>A. caviae</i>
302-89c	Extra Intestinal	8		EU488658	<i>A. media</i>
309c	Intestinal	2		EU488656	<i>A. caviae</i>
315c	Intestinal	1		EU488666	<i>A. caviae</i>
317c	Intestinal	3	EU488695	EU488641	<i>A. veronii</i>
320c	Intestinal	1		EU488673	<i>A. caviae</i>
360c	Intestinal	1	FJ168774	EU488665	<i>A. caviae</i>
457c	Intestinal	10	EU488696	EU488642	<i>A. veronii</i>
499c	Intestinal	1	EU488689	EU488643	<i>A. caviae</i>
555c	Intestinal	1	EU488690	EU488644	<i>A. caviae</i>
616c	Extra Intestinal	11		EU488657	<i>A. veronii</i>
625c	Intestinal	3		EU488655	<i>A. veronii</i>
633c	Intestinal	3	EU488693	EU488645	<i>A. veronii</i>
665c	Intestinal	1		AY249199 <sup>[54]</sup>	<i>A. caviae</i>
694c	Intestinal	1	FJ168772	EU488659	<i>A. caviae</i>
741c	Intestinal	7		EU488653	<i>A. veronii</i>
782c	Intestinal	1	FJ168777	EU488662	<i>A. caviae</i>
786c	Intestinal	11	FJ168771	EU488679	<i>A. veronii</i>
800c	Intestinal	1		EU488660	<i>A. caviae</i>
807c	Intestinal	12	EU488691	EU488649	<i>A. caviae</i>
812c	Intestinal	11		EU488664	<i>A. veronii</i>
57a	Freshwater	4		AY308845 <sup>[54]</sup>	<i>A. media</i>
75a	Sea water	1	FJ168770	EU488677	<i>A. caviae</i>
83a	Sea water	8	EU488681	EU488648	<i>A. media</i>
89a	Sea water	1	FJ168769	EU488678	<i>A. caviae</i>
115a	Sea water	1		EU488661	<i>A. caviae</i>
236a	Sea water	4		EU488680	<i>A. media</i>
239a	Freshwater	9		AY308843 <sup>[54]</sup>	<i>A. media</i>
280a	Freshwater	2		AY308846 <sup>[54]</sup>	<i>A. media</i>
480a	Sea water	9	FJ168773	AY308844 <sup>[54]</sup>	<i>A. media</i>
610a	Freshwater	2		AY308847 <sup>[54]</sup>	<i>A. media</i>
750a	Sea water	5		EU488663	<i>A. veronii</i>
796a	Freshwater	5		EU488654	<i>A. veronii</i>
938a	Sea water	4	EU488682	EU488646	<i>A. media</i>
974a	Freshwater	4	EU488683	EU488647	<i>A. media</i>
975a	Freshwater	4	EU488684	EU488652	<i>A. media</i>

**Table 2-SI.** Positions showing differences in the 16S rDNA sequences of strains with intragenomic heterogeneity among 16S *rrn* operons compared with sequences of the related type strains

Strain	No. of base differences	RFLP type	Localization <sup>a</sup> of microheterogeneities																				
			457	458	459	461 <sup>c</sup>	462 <sup>c</sup>	464 <sup>c</sup>	469	470	471	472	473	474	475 <sup>c</sup>	476 <sup>c</sup>	649 <sup>c</sup>	1004	1011	1018	1308 <sup>d</sup>	1329	1421
<i>A. trota</i> CECT4255 <sup>T</sup> (ATCC49657)		Typical <sup>b</sup>	C	A	G	A	G	T	T	C	T	G	C	T	G	G	G	A	C	G	T	A	G
<i>A. caviae</i> CECT838 <sup>T</sup> (ATCC15468)			C	A	G	A	G	T	T	C	T	G	C	T	G	G	A	A	T	A	T	A	G
75a	5 <sup>e</sup> (0.33%)	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T/C	A/G	T/C	G/A	-	
89a	5 <sup>e</sup> (0.33%)	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T/c	A/G	C/T	G/A	-	
176c	5 (0.33%)	1	-	-	-	-	-	-	-	-	-	-	-	-	G/A	-	-	T/C	A/G	T/C	A/G	-	
219c	6 <sup>e</sup> (0.4%)	1	-	-	-	-	-	-	-	-	-	-	-	-	G/A	-	-	T/c	A/G	T/c	A/G	-	
360c	4 (0.26%)	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T/C	A/G	T/C	A/G	-	
499c	6 (0.4%)	1	C/T	A/G	-	-	-	-	-	-	-	-	-	G/A	G/A	-	-	C/T	G/A	-	G	-	
555c, 782c	4 (0.26%)	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T/C	A/G	T/C	G/A	-	
694c	10 (0.66)	1	C/T	A/G	-	-	-	T/A	-	-	G/A	-	T/C	G/A	-	-	-	C/T	G/A	T/c	A/G	-	
93c, 136c	9 (0.6%)	2	C/T	A/G	-	-	-	T/A	-	-	-	-	T/C	G/A	-	-	-	C/T	G/A	C/T	G/A	-	
98c	8 (0.53%)	2	C/T	A/G	-	-	-	-	-	-	-	-	T/C	G/A	-	-	-	T/c	A/G	C/T	G/A	-	
121c	10 (0.66%)	6	T/c	G/A	T/G	G/A	-	-	C/T	-	C/T	G/c	C/A	T/c	G/A	-	-	-	-	-	-	-	
807c	5 <sup>e</sup> (0.33%)	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T/c	A/G	C/T	A/G	-	
<i>A. hydrophila</i> CECT839 <sup>T</sup> (ATCC7966)		Typical <sup>b</sup>	T	G	A	G	C	T	C	G	T	A	T	C	A	A	A	A	C	G	C	G	G
<i>A. media</i> CECT4232 <sup>T</sup> (ATCC33907)			T	G	A	G	C	T	C	G	C	A	T	C	A	G	A	A	C	G	C	G	G
83a	9 (0.6%)	8	-	-	G/A	A/G	G/c	-	A/c	C/G	T/c	G/A	C/T	-	-	A/G	-	-	-	-	-	-	
938a	10 <sup>e</sup> (0.66%)	4	-	-	G/A	A	G/c	-	A/c	C/G	T	G/A	C/T	-	-	A/G	-	-	T/c	A/G	-	-	
975a, 974a	2 (0.13%)	4	-	-	G	A	G	-	A	C	T	G	C	-	-	A	-	-	T/c	A/G	-	-	
480a	7 (0.46%)	9	-	-	A/G	A	C/G	-	C/A	G/c	T	A/G	T/c	-	-	A/G	-	-	T	A	-	-	
<i>A. jandaei</i> CECT4228 <sup>T</sup> (ATCC49568)		Typical <sup>b</sup>	C	A	G	A	G	T	T	C	T	G	C	T	G	G	A	A	C	G	C	G	G
<i>A. veronii</i> CECT4257 <sup>T</sup> (ATCC35624)			T	G	G	A	G	T	A	C	T	G	C	C	A	G	A	A	T	A	C	G	G
633c	3 (0.19%)	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T/C	A/G	-	-	G/A	
317c	1 (0.06%)	3	-	-	-	-	-	-	-	-	-	-	-	-	-	A/G	-	-	-	-	-	-	
39c, 41c	4 (0.26%)	5	-	-	-	-	T/G	-	-	-	-	-	-	-	-	A/G	-	T/C	A/G	-	-	-	
178c	1 (0.06%)	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G/A	C	G	-	-	-	
457c	1 (0.06%)	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	G	C/T	-	-	
126c	6 (0.4%)	11	C/T	A/G	-	-	-	T/A	-	-	-	-	T/C	G/A	-	A/G	-	-	-	-	-	-	
786c	8 (0.53%)	11	C/T	A/G	-	-	-	T/A	-	-	-	-	T/C	G/A	-	A/G	-	C/T	G/A	-	-	-	

The symbol “-” indicates the same base as the type strain of its species.

<sup>a</sup>Referred to *E. coli*. [7].

<sup>b</sup>Typical pattern indicates expected pattern for this species [5].

<sup>c</sup>This position affects restriction site *AluI*.

<sup>d</sup>This position affects restriction site *MboI*.

<sup>e</sup>Microheterogeneities detected in single strains in only one position are not illustrated: 75a (102G/C), 89a (181A/G), 219c (781A/G), 807c (824G/A), 938a (647T/C).