Phylogenetic relationships within the family *Halomonadaceae* based on comparative 23S and 16S rRNA gene sequence analysis

Rafael R. de la Haba,¹ David R. Arahal,² M. Carmen Márquez¹ and Antonio Ventosa¹

Correspondence
Antonio Ventosa
ventosa@us.es

¹Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, 41012 Sevilla, Spain

²Spanish Type Culture Collection (CECT) and Department of Microbiology and Ecology, University of Valencia, 46100 Valencia, Spain

A phylogenetic study of the family *Halomonadaceae* was carried out based on complete 16S rRNA and 23S rRNA gene sequences. Several 16S rRNA genes of type strains were resequenced, and 28 new sequences of the 23S rRNA gene were obtained. Currently, the family includes nine genera (*Carnimonas*, *Chromohalobacter*, *Cobetia*, *Halomonas*, *Halotalea*, *Kushneria*, *Modicisalibacter*, *Salinicola* and *Zymobacter*). These genera are phylogenetically coherent except *Halomonas*, which is polyphyletic. This genus comprises two clearly distinguished clusters: group 1 includes *Halomonas elongata* (the type species) and the species *Halomonas eurihalina*, *H. caseinilytica*, *H. halmophila*, *H. sabkhae*, *H. almeriensis*, *H. halophila*, *H. salina*, *H. organivorans*, *H. koreensis*, *H. maura* and *H. nitroreducens*. Group 2 comprises the species *Halomonas aquamarina*, *H. meridiana*, *H. axialensis*, *H. magadiensis*, *H. hydrothermalis*, *H. alkaliphila*, *H. venusta*, *H. boliviensis*, *H. neptunia*, *H. variabilis*, *H. sulfidaeris*, *H. subterranea*, *H. janggokensis*, *H. gomseomensis*, *H. arcis* and *H. subglaciescola*. *Halomonas salaria* forms a cluster with *Chromohalobacter salarius* and the recently described genus *Salinicola*, and their taxonomic affiliation requires further study. More than 20 *Halomonas* species are phylogenetically not within the core constituted by the *Halomonas sensu stricto* cluster (group 1) or group 2 and, since their positions on the different phylogenetic trees are not stable, they cannot be recognized as additional groups either. In general, there is excellent agreement between the phylogenies based on the two rRNA gene sequences, but the 23S rRNA gene showed higher resolution in the differentiation of species of the family *Halomonadaceae*.

The family *Halomonadaceae* forms a separate phylogenetic lineage within the *Gammaproteobacteria* according to 16S rRNA gene sequence analysis and is made up mostly of halophilic bacteria. Since its creation in 1988 (Franzmann *et al.*, 1988), the taxonomy of the family has been under continuous revision. At the time of writing, the family *Halomonadaceae* comprises nine genera, the most prominent being *Halomonas*, which contains 55 species, and *Chromohalobacter*, with nine species. Six genera are currently represented by single species; these are *Zymobacter palmae* (Okamoto *et al.*, 1993), *Carnimonas nigricans* (Garriga *et al.*, 1998; Arahal *et al.*, 2002b), *Cobetia marina* (Arahal *et al.*, 2002a), *Halotalea alkalilenta* (Ntougias *et al.*, 2007),

Modicisalibacter tunisiensis (Ben Ali Gam *et al.*, 2007) and *Salinicola socius* (Anan'ina *et al.*, 2007, 2008). Very recently, Sánchez-Porro *et al.* (2009) described the new genus *Kushneria*, which currently consists of four species, the novel species *Kushneria aurantia* as well as three species that were previously assigned to the genus *Halomonas*, *Kushneria avicenniae*, *K. indalinina* and *K. marisflavi*.

Arahal *et al.* (2002b) carried out a study of the phylogeny of species of the family *Halomonadaceae* whose names were validly published at that time (a total of 25) using comparative sequence analysis of the 23S and 16S rRNA genes. Since then, 49 novel species have been described and, in some cases, their phylogenetic relationships are not clearly established. The purpose of this article was to determine in detail the phylogenetic relationships of the species of the genera of the family *Halomonadaceae* and to clarify the current classification of this heterogeneous bacterial family using a comparative analysis based on 23S and 16S rRNA

The GenBank/EMBL/DDBJ accession numbers for the newly reported 16S and 23S rRNA gene sequences are detailed in Table 1.

Maximum-parsimony and maximum-likelihood trees based on 16S and 23S rRNA gene sequences are available as supplementary material with the online version of this paper.

gene sequences. Moreover, following the recommended minimal standards for the description of new members of the family *Halomonadaceae*, seven already-sequenced 16S rRNA genes of type strains have been sequenced again to resolve undetermined positions and to reach the established quality standards (Arahal *et al.*, 2007). In addition, 28 new complete 23S rRNA gene sequences have been obtained on this study. Finally, some suggestions are included about the recommended sequences to be used for future comparative phylogenetic analysis.

All strains used in this study were type strains of species with validly published names within the family *Halomonadaceae*. Most (23 strains) originated from culture collections and the rest (seven strains) were obtained directly from the authors that proposed them as types of novel species (Table 1). Strains were cultivated following the media and growth conditions recommended by the culture collections or the donor authors.

Chromosomal DNA was isolated and purified according to the following protocol. Cells were collected from 3 ml of an exponentially growing broth culture by centrifugation at 12 000 r.p.m. for 2 min and the pellet was resuspended in 558 µl TE buffer (pH 8.0). After that, 30 µl 10% (w/v) SDS, 6 µl proteinase K (20 mg ml⁻¹), 6 µl RNase (10 mg ml⁻¹) and 37 µl lysozyme (20 mg ml⁻¹) were added and an incubation step was performed at 37 °C for 30 min. This was followed by a second incubation at 65 °C for 15 min after the addition of 100 µl 5 M NaCl and 80 µl 10% (w/v) CTAB. Next, an equal volume of chloroform/isoamyl alcohol (24:1) was added to extract the total DNA. The contents of the tube were mixed vigorously and centrifuged at 12 000 r.p.m. for 5 min, and the aqueous fraction was recovered. When necessary, the extraction step was repeated to increase the yield of DNA. Subsequently, 0.6 vols cold isopropyl alcohol was added to precipitate the DNA and the mixture was centrifuged at 13 200 r.p.m. for 15 min. Finally, the precipitated DNA was dissolved in 50 µl TE buffer and checked by agarose gel electrophoresis. The gel was prepared dissolving 1% agarose (SeaKem) in 1 × TAE buffer by heating. Ethidium bromide (3 ml of a 10 mg ml⁻¹ solution) was added in order to visualize the genetic material. Each DNA solution (5 µl) was mixed with 2 µl loading buffer and loaded onto the gel (Sambrook & Russell, 2001). A 1 kb ladder (Invitrogen) was used as a marker.

For PCR amplification, approximately 100 ng total DNA was used with a *Taq* DNA polymerase kit (Eppendorf) following the recommendations of the manufacturer. The 16S rRNA gene was amplified from positions 8 to 1511 of the 16S rRNA by using the universal primers 16F27 and 16R1488. To obtain the complete 23S rRNA gene, two fragments were amplified, one from positions 12 to 2669 of the 23S rRNA gene and another from position 1091 of the 23S rRNA to position 49 of the 5S rRNA (*Escherichia coli* numbering). The 16S and 23S rRNA gene nucleotide sequences were determined by NBT-Newbiotechnics (Seville, Spain) using an automated

DNA sequencer model 3130XL (Applied Biosystems) and the sequences were then assembled by using the ChromasPro software (Technelysium) and corrected manually to resolve ambiguous positions. The amplification and sequencing primers used in this study are given in Table 2. Obtained 23S and 16S rRNA gene sequences were compared with reference 23S and 16S rRNA gene sequences retrieved from the GenBank and EMBL databases by BLAST searching (<http://www.ncbi.nlm.nih.gov/blast/>). The subsequent sequence analysis was performed by using the ARB program package (Ludwig *et al.*, 2004).

To construct the final phylogenetic trees, only sequences from type strains of species of the family *Halomonadaceae* whose names were validly published were taken into account and, when more than one sequence of the same gene and strain was available, only the most complete or the one that contained fewer ambiguous positions was used. The GenBank/EMBL/DDBJ accession numbers for the 16S and 23S rRNA gene sequences that were used in this study, which also includes those determined in our laboratory, are listed in Table 1. Sequences were aligned by using FastAligner version 1.03 and the alignments were corrected by hand. A distance matrix was obtained and evolutionary distances were computed using the formula of Jukes & Cantor (1969). Only sequence positions with 50% conservation or more within the family *Halomonadaceae* were taken into account by applying a filter. Evolutionary history was inferred using several treeing methods [maximum-parsimony (Fitch, 1971), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981)] on the basis of the recommendations of Ludwig *et al.* (1998). A global optimization of the maximum-parsimony analysis was carried out. A bootstrap test was performed by calculating 1000 replicate trees in order to assess the robustness of the topology. The application of these three treeing methods and changes of outgroups produced nearly identical tree topologies among the members of the family *Halomonadaceae*. Figs 1 and 2 show representative trees constructed using the neighbour-joining method with the 16S and 23S rRNA gene sequences, respectively. Maximum-parsimony (Supplementary Fig. S1) and maximum-likelihood (Supplementary Fig. S2) trees resulting from 16S and 23S rRNA gene sequence analyses are available in IJSEM Online.

Phylogenetic analyses of 16S rRNA and 23S rRNA gene sequences were performed on datasets consisting of 1446 and 2862 nucleotide positions (*E. coli* positions 24–1501 and 1–2904), respectively. The mean similarity scored between the different 16S rRNA gene sequences analysed in this study was one unit higher than that of the 23S rRNA gene sequences, suggesting a slower evolutionary rate for the 16S fraction of the rRNA gene. It has to be noted that, even in the post-genomic era, the number of publicly available 23S rRNA gene sequences is not very large: currently, there are about 12 500 complete or almost-complete 23S rRNA gene sequences available, whereas this number exceeds 324 000 in the case of the 16S rRNA gene. A recommended solution is the use of a database of good-quality and aligned sequences of the small

Table 1. Species of the family Halomonadaceae with validly published names included in this study

Sequences obtained in this study are highlighted in bold. When more than one sequence is available for the same species, we recommend the use of the sequence marked with an asterisk.

Species	Reference	16S rRNA gene			23S rRNA gene		
		Strain	Accession no.	Length (bp)	Strain	Accession no.	Length (bp)
<i>Carnimonas</i>							
<i>C. nigrificans</i>	Garriga <i>et al.</i> (1998)	CTCBS1 ^T	Y13299	1519	CECT 4437^T	AM941757	2913
<i>Chromohalobacter</i>							
<i>C. beijerinckii</i>	Peçonek <i>et al.</i> (2006)	ATCC 19372 ^T	AB021386	1495	DSM 7218^T	FN257758	2911
<i>C. canadensis</i>	Arahal <i>et al.</i> (2001a)	ATCC 43984 ^T	AJ295143*	1532	DSM 6769 ^T	AJ306870	2910
		DSM 6769 ^T	AF211861	1495			
<i>C. israelensis</i>	Arahal <i>et al.</i> (2001a)	ATCC 43985 ^T	AJ295144*	1532	DSM 6768 ^T	AJ306871	2909
		DSM 6768 ^T	AF211862	1492			
<i>C. japonicus</i>	Sánchez-Porro <i>et al.</i> (2007)	43 ^T	AB105159	1501			
<i>C. marismortui</i>	Ventosa <i>et al.</i> (1989)	ATCC 17056 ^T	X87219	1423	ATCC 17056 ^T	AJ306872	2998
<i>C. nigrandesensis</i>	Prado <i>et al.</i> (2006)	LTS-4N ^T	AJ277205	1505	CECT 5315^T	FN257760	3006
<i>C. salarius</i>	Aguilera <i>et al.</i> (2007)	CG4.1 ^T	AJ427626	1535			
<i>C. salexigens</i>	Arahal <i>et al.</i> (2001b)	DSM 3043 ^T	AJ295146	1531	DSM 3043 ^T	AJ306873	2908
<i>C. sarencensis</i>	Quillaguamán <i>et al.</i> (2004b)	LV4 ^T	AY373448	1448	LV4^T	FN257759	2910
<i>Cobetia</i>							
<i>C. marina</i>	Arahal <i>et al.</i> (2002a)	DSM 4741 ^T	AJ306890*	1536	DSM 4741 ^T	AJ306880	2910
		ATCC 25374 ^T	M93354	1483			
<i>Halomonas</i>							
<i>H. alimentaria</i>	Yoon <i>et al.</i> (2002)	YKJ-16 ^T	AF211860	1493	DSM 15356^T	FN257749	2908
<i>H. alkaliphila</i>	Romano <i>et al.</i> (2006)	18bAG ^T	AJ640133	1500			
<i>H. almeriensis</i>	Martínez-Checa <i>et al.</i> (2005)	M8 ^T	AY858696	1459	M8^T	FN257744	2909
<i>H. anticariensis</i>	Martínez-Cánovas <i>et al.</i> (2004b)	FP35 ^T	AY489405	1464	FP35^T	FN263244	2911
<i>H. aquamarina</i>	Dobson & Franzmann (1996)	DSM 30161 ^T	AJ306888*	1528	DSM 30161 ^T	AJ306874	2914
		ATCC 14400 ^T	M93352	1466			
<i>H. arcis</i>	Xu <i>et al.</i> (2007)	AJ282 ^T	EF144147	1404			
<i>H. axialensis</i>	Kaye <i>et al.</i> (2004)	Althfl ^T	AF212206	1438	CECT 5812^T	FN257753	2914
<i>H. boliviensis</i>	Quillaguamán <i>et al.</i> (2004a)	LC1 ^T	AY245449	1441	DSM 15516^T	FN257754	2913
<i>H. campaniensis</i>	Romano <i>et al.</i> (2005)	5AG ^T	AJ515365	1480	DSM 15293^T	FN257750	2909
<i>H. campisalis</i>	Mormile <i>et al.</i> (1999)	ATCC 700597 ^T	AF054286	1400	ATCC 700597^T	AM941752	2910
<i>H. caseinilytica</i>	Wu <i>et al.</i> (2008a)	AJ261 ^T	EF527874	1446			
<i>H. cerina</i>	González-Domenech <i>et al.</i> (2008b)	P4 ^T	EF613112	1449			
<i>H. cupida</i>	Dobson & Franzmann (1996)	DSM 4740^T	FN257742*	1464	DSM 4740 ^T	AJ306875	2910
		DSM 4740 ^T	L42615	1471			
<i>H. daqingensis</i>	Wu <i>et al.</i> (2008b)	DQD2-30 ^T	EF121854	1420			
<i>H. denitrificans</i>	Kim <i>et al.</i> (2007)	M29 ^T	AM229317	1489			
<i>H. desiderata</i>	Berendes <i>et al.</i> (1996)	FB2 ^T	X92417	1495	DSM 9502 ^T	AJ306876	2910
<i>H. elongata</i>	Vreeland <i>et al.</i> (1980)	ATCC 33173^T	AM941743*	1457	ATCC 33173 ^T	AJ306877	2910
		ATCC 33173 ^T	X67023	1470			
		ATCC 33173 ^T	M93355	1479			
<i>H. eurihalina</i>	Mellado <i>et al.</i> (1995)	ATCC 49336 ^T	X87218*	1473	ATCC 49336 ^T	AJ306878	2911
		ATCC 49336 ^T	L42620	1490			
<i>H. gomseomensis</i>	Kim <i>et al.</i> (2007)	M12 ^T	AM229314	1484			
<i>H. gudaonensis</i>	Wang <i>et al.</i> (2007a)	SL014B-69 ^T	DQ421808	1462			
<i>H. halmophila</i>	Dobson <i>et al.</i> (1990)	ATCC 19717 ^T	AJ306889*	1530	ATCC 19717 ^T	AJ306879	2920
		ATCC 19717 ^T	M59153	1540			
<i>H. halocynthiae</i>	Romanenko <i>et al.</i> (2002)	KMM 1376 ^T	AJ417388	1514	DSM 14573^T	FN257752	2908
<i>H. halodenitrificans</i>	Dobson & Franzmann (1996)	ATCC 13511 ^T	L04942	1531	CECT 5012^T	FN257751	2908
<i>H. halodurans</i>	Hebert & Vreeland (1987)	DSM 5160^T	FN257743*	1446	DSM 5160^T	FN257761	2910
		DSM 5160 ^T	L42619	1474			

Table 1. cont.

Species	Reference	16S rRNA gene			23S rRNA gene		
		Strain	Accession no.	Length (bp)	Strain	Accession no.	Length (bp)
<i>H. halophila</i>	Dobson & Franzmann (1996)	DSM 4770 ^T	FN257740*	1475	DSM 4770 ^T	FN257745	2908
		CCM 3662 ^T	AM941744	1461	CCM 3662 ^T	AM941750	2908
		DSM 4770 ^T	M93353	1478			
<i>H. hydrothermalis</i>	Kaye <i>et al.</i> (2004)	Slthf2 ^T	AF212218	1409	CECT 5814 ^T	AM941749	2912
<i>H. janggokensis</i>	Kim <i>et al.</i> (2007)	M24 ^T	AM229315	1478			
<i>H. kenyensis</i>	Boltyanskaya <i>et al.</i> (2007)	AIR-2 ^T	AY962237	1371			
<i>H. koreensis</i>	Lim <i>et al.</i> (2004)	SS20 ^T	AY382579	1399	JCM 12237 ^T	FN257747	2908
<i>H. korlensis</i>	Li <i>et al.</i> (2008)	XK1 ^T	EU085033	1517			
<i>H. kribbensis</i>	Jeon <i>et al.</i> (2007)	BH843 ^T	DQ280368	1417			
<i>H. lutea</i>	Wang <i>et al.</i> (2008a)	YIM 91125 ^T	EF674852	1479			
<i>H. magadiensis</i>	Duckworth <i>et al.</i> (2000)	21 MI ^T	X92150	1473	21 MI ^T	AM941747	2915
<i>H. maura</i>	Bouchotroch <i>et al.</i> (2001)	S-31 ^T	FN257741*	1455	S-31 ^T	FN257746	2908
		S-31 ^T	AJ271864	1396			
<i>H. meridiana</i>	James <i>et al.</i> (1990)	DSM 5425 ^T	AJ306891*	1528	DSM 5425 ^T	AJ306881	2914
		DSM 5425 ^T	M93356	1475			
<i>H. mongoliensis</i>	Boltyanskaya <i>et al.</i> (2007)	Z-7009 ^T	AY962236	1372			
<i>H. muralis</i>	Heyrman <i>et al.</i> (2002)	LMG 20969 ^T	AJ320530	1493	DSM 14789 ^T	FN257757	2923
<i>H. neptunia</i>	Kaye <i>et al.</i> (2004)	Eplume1 ^T	AF212202	1454	CECT 5815 ^T	FN257755	2920
<i>H. nitroreducens</i>	González-Domenech <i>et al.</i> (2008a)	11S ^T	EF613113	1441			
<i>H. organivorans</i>	García <i>et al.</i> (2004)	G-16.1 ^T	AJ616910	1472	G-16.1 ^T	AM941751	2908
<i>H. pacifica</i>	Dobson & Franzmann (1996)	DSM 4742 ^T	L42616*	1480	DSM 4742 ^T	AJ306882	2910
		ATCC 27122 ^T	EU373088	1449			
<i>H. pantelleriensis</i>	Romano <i>et al.</i> (1996)	AAP ^T	X93493	1477	DSM 9661 ^T	AJ306883	2912
<i>H. sabkhae</i>	Kharroub <i>et al.</i> (2008)	5-3 ^T	EF442769	1540			
<i>H. saccharovitans</i>	Xu <i>et al.</i> (2007)	AJ275 ^T	EF144149	1407			
<i>H. salaria</i>	Kim <i>et al.</i> (2007)	M27 ^T	AM229316	1492			
<i>H. salifodinae</i>	Wang <i>et al.</i> (2008b)	BC7 ^T	EF527873	1428			
<i>H. salina</i>	Dobson & Franzmann (1996)	F8-11 ^T	AJ295145*	1532	DSM 5928 ^T	AJ306884	2908
		ATCC 49509 ^T	L42617	1443			
		ATCC 49509 ^T	X87217	1478			
		ATCC 49509 ^T	AJ243447	1493			
		DSM 5928 ^T	AJ243448	1493			
<i>H. shengliensis</i>	Wang <i>et al.</i> (2007b)	SL014B-85 ^T	EF121853	1460			
<i>H. subglaciescola</i>	Franzmann <i>et al.</i> (1987)	DSM 4683 ^T	AJ306892*	1531	DSM 4683 ^T	AJ306885	2910
		DSM 4683 ^T	M93358	1481			
<i>H. subterranea</i>	Xu <i>et al.</i> (2007)	ZG16 ^T	EF144148	1381			
<i>H. sulfidaeris</i>	Kaye <i>et al.</i> (2004)	Esulfide1 ^T	AF212204	1430	CECT 5817 ^T	AM941748	2911
<i>H. taeanensis</i>	Lee <i>et al.</i> (2005)	BH539 ^T	AY671975	1408	BH539 ^T	AY829729	2907
<i>H. variabilis</i>	Dobson & Franzmann (1996)	DSM 3051 ^T	AJ306893*	1528	DSM 3051 ^T	AJ306886	2912
		DSM 3051 ^T	M93357	1470			
<i>H. ventosae</i>	Martínez-Cánovas <i>et al.</i> (2004a)	Al12 ^T	AY268080	1395	Al12 ^T	FN257748	3009
<i>H. venusta</i>	Dobson & Franzmann (1996)	DSM 4743 ^T	AJ306894*	1530	DSM 4743 ^T	AJ306887	2914
		DSM 4743 ^T	L42618	1479			
Halotalea							
<i>H. alkalilenta</i>	Ntougias <i>et al.</i> (2007)	AW-7 ^T	DQ421388	1491	CECT 7134 ^T	FN257762	3018
Kushneria							
<i>K. aurantia</i>	Sánchez-Porro <i>et al.</i> (2009)	A10 ^T	AM941746	1428	A10 ^T	AM941756	2914
<i>K. avicenniae</i>	Sánchez-Porro <i>et al.</i> (2009)	MW2a ^T	DQ888315	1433	MW2a ^T	AM941755	2912
<i>K. indalinina</i>	Sánchez-Porro <i>et al.</i> (2009)	CG2.1 ^T	AM941745*	1478	CG2.1 ^T	AM941754	2913
		CG2.1 ^T	AJ427627	1527			
<i>K. marisflavi</i>	Sánchez-Porro <i>et al.</i> (2009)	SW32 ^T	AF251143	1495	DSM 15357 ^T	AM941753	2914
Modicisalibacter							
<i>M. tunisiensis</i>	Ben Ali Gam <i>et al.</i> (2007)	LIT2 ^T	DQ641495	1531	CIP 109206 ^T	FN257756	2909

Table 1. cont.

Species	Reference	16S rRNA gene			23S rRNA gene		
		Strain	Accession no.	Length (bp)	Strain	Accession no.	Length (bp)
<i>Salinicola</i>							
<i>S. socius</i>	Anan'ina <i>et al.</i> (2007)	SMB35 ^T	DQ979342	1433			
<i>Zymobacter</i>							
<i>Z. palmae</i>	Okamoto <i>et al.</i> (1993)	ATCC 51623 ^T	AF211871*	1509	DSM 10491 ^T	AM941758*	2920
		T109 ^T	D14555	1503	ATCC 51623 ^T	AF211871	2560

and large rRNA subunits that can be retrieved from the SILVA rRNA database project (Pruesse *et al.*, 2007).

The 16S and 23S rRNA gene sequence analyses (Figs 1 and 2) demonstrated the phylogenetic distinctness of the family Halomonadaceae. As reported previously (Arahal *et al.*, 2002b), the genus *Halomonas* is not monophyletic and comprises two clearly separated phylogenetic groups that now contain larger numbers of species. Group 1 is formed by *Halomonas elongata* (the type species of the genus), *H. eurihalina*, *H. caseinilytica*, *H. halmophila*, *H. sabkhae*, *H. almeriensis*, *H. halophila*, *H. salina*, *H. organivorans*, *H. koreensis*, *H. maura* and *H. nitroreducens* (note that the abbreviation *H.* is used throughout to represent *Halomonas*; the genus name *Halotalea* is given in full throughout). The mean 16S rRNA gene sequence similarity for this group was 97.8%. A lower value was obtained with the 23S rRNA gene sequences (97.0%). The 16S rRNA gene sequence of *H. halophila* DSM 4770^T was

first determined by Dobson *et al.* (1993), showing a total of 76 ambiguous positions. A large number of unresolved positions and/or errors may be wrongly taken as evidence of a different species, hence the importance of including only high-quality (almost) complete 16S rRNA gene sequences in new taxonomic proposals (Arahal *et al.*, 2007). For this reason, we determined again the almost-complete 16S rRNA gene sequences of *H. halophila* strains DSM 4770^T and CCM 3662^T, equivalent designations of the type strain of the species, and newly determined the complete 23S rRNA gene sequences of these strains. In this study, the 16S and 23S rRNA gene sequences of these two strains showed 100% similarity to the respective sequences of the type strain of *H. salina*. However, although their 16S and 23S rRNA gene sequences are identical, it has been clearly demonstrated that *H. halophila* and *H. salina* constitute two different *Halomonas* species on the basis of their phenotypic and genotypic features (Valderrama *et al.*, 1991). This fact reinforces the importance in modern bacterial systematics of

Table 2. Amplification and sequencing primers used in this study

Each forward amplification primer was used together with the reverse primer below it in the table. Sequences are listed according to the IUPAC code for nucleotide ambiguities. Primer positions are given according to the *E. coli* numbering.

Primer	Sequence (5'→3')	Position	Use
16S rRNA gene			
16F27	AGAGTTTGATCMTGGCTCAG	8–27	Amplification, forward sequencing, 16S rRNA gene
16R1488	CGGTTACCTTGTAGGACTTCACC	1488–1511	Amplification, reverse sequencing, 16S rRNA gene
16F530	GTGCCAGCAGCCGCGG	515–530	Sequencing, 16S rRNA gene
16R343	ACTGCTGCCTCCCGTA	358–343	Sequencing, 16S rRNA gene
23S rRNA gene			
23SppioFw	TTSGGGTTATAKGGTCAAG	–12–7	Amplification, forward
985R	CCGGTCCTCTCGTACT	2654–2669	Amplification, reverse
1023V	GCGTAAYAGCTCACT	1091–1105	Amplification, forward
504R	SWGTTTCGRVAWGGGA	35–49	Amplification, reverse
992IRy97	TTCCCTCACRGCTACT	457–471	Sequencing, 23S rRNA gene
1020IRmz98	TCTGGGYTGTTYCCCT	975–990	Sequencing, 23S rRNA gene
1018Fw	GGGGGTAGAGCACTGTTT	855–872	Sequencing, 23S rRNA gene
1037IRm97	CTTACCCGACAAGGAATTTTCG	1934–1954	Sequencing, 23S rRNA gene
1027VIR	AAACCGACACAGGTRG	1608–1623	Sequencing, 23S rRNA gene
328IRvm97	TCCTAAGGTAGCGAAATTCCTTG	1923–1945	Sequencing, 23S rRNA gene
1042GPHI	GTTTGGCACCTCGATGTCGRCTC	2490–2512	Sequencing, 23S rRNA gene

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequence comparison, showing phylogenetic relationships between members of the family Halomonadaceae. Accession numbers are shown in parentheses. Bootstrap values >50 % are shown. Bar, 0.02 substitutions per nucleotide position.

adopting polyphasic approaches that integrate phenotypic (including chemotaxonomic markers) with genotypic methods (Arahal *et al.*, 2007).

A second group of species of the genus *Halomonas*, designated group 2, includes the 16 species *Halomonas aquamarina*, *H. meridiana*, *H. axialensis*, *H. magadiensis*, *H. hydrothermalis*, *H. alkaliphila*, *H. venusta*, *H. boliviensis*,

H. neptunia, *H. variabilis*, *H. sulfidaeris*, *H. subterranea*, *H. janggokensis*, *H. gomseomensis*, *H. arcis* and *H. subglaciescola*. This group displays mean similarities of 97.4 and 97.5 % for the 16S and 23S rRNA gene sequences, respectively. *H. aquamarina* DSM 30161^T, *H. meridiana* DSM 5425^T and *H. axialensis* Althfl^T (=CECT 5812^T) form a fairly stable clade within this group with very similar 16S rRNA (99.9 %) and 23S rRNA (99.3–99.7 %)

Fig. 2. Neighbour-joining phylogenetic tree, based on the 23S rRNA gene sequence comparison, showing phylogenetic relationships between members of the family Halomonadaceae. Accession numbers are shown in parentheses. Bootstrap values >50 % are shown. Bar, 0.02 substitutions per nucleotide position.

sequences. DNA–DNA hybridization values between the type strain of *H. axialensis* and those of *H. aquamarina* and *H. meridiana* was 28 and 32 %, respectively. Furthermore, *H. axialensis* is phenotypically different from the other two species (Kaye *et al.*, 2004), supporting its taxonomic status as a distinct *Halomonas* species.

The values of sequence similarity between the strains included in group 1 and those belonging to group 2 were 96.1–93.2 % for the 16S rRNA gene and 95.3–93.5 % for the 23S rRNA gene. These values would suggest that groups 1 and 2 of the genus *Halomonas* could constitute two different genera, with group 1 representing *Halomonas sensu stricto* since it contains the type species and therefore has priority to retain the name *Halomonas*. However, following the recommendations of Stackebrandt *et al.* (2002), the differentiation of taxa should be based on a polyphasic approach and sustained by phenotypic (including chemotaxonomic) features. Many attempts have been made to determine differential features between species of these two groups, but neither chemotaxonomic nor more general phenotypic studies have permitted their separation. All species belonging to groups 1 and 2 have phosphatidylglycerol and phosphatidylethanolamine as major polar lipids, ubiquinone 9 (except *H. alkaliphila*; Romano *et al.*, 2006) as the respiratory quinone and C_{16:0} and C_{18:1 ω 7c} (the latter not in *H. alkaliphila*; Romano *et al.*, 2006) as fatty acids (Franzmann & Tindall, 1990). In addition, species of groups 1 and 2 are catalase-positive and are strictly aerobic or facultatively anaerobic, oxidase-positive or -negative and may or may not be capable of reducing nitrates (Mata *et al.*, 2002; Arahal & Ventosa, 2006). In general, species from group 1 have a higher DNA G+C content (57–70 mol%) than species from group 2 (51.4–62 mol%), and the former are more halophilic than the latter. Furthermore, all species belonging to group 2 are motile, while the species of group 1 are motile or non-motile (Mata *et al.*, 2002; Arahal & Ventosa, 2006).

The other 27 species that are currently assigned to the genus *Halomonas* did not appear to be included clearly in either of these phylogenetic groups. One of these species, *Halomonas salaria*, always formed a separate group with the species *Chromohalobacter salarius* and the recently described *Salinicola socius* (Anan'ina *et al.*, 2007). The 16S rRNA gene sequence similarity between *H. salaria* M27^T and *Chromohalobacter salarius* CG 4.1^T and *S. socius* SMB35^T was 98.4 and 98.7 %, respectively. The mean 16S rRNA gene sequence similarity of this group to any of the *Halomonas* and *Chromohalobacter* species ranged from 93.0 to 96.4 % and 93.6 to 96.5 %, respectively, values low enough as to justify their placement in a single genus. In addition, the mean similarity values obtained between the 16S rRNA gene sequences of these three species and those of the four species of *Kushneria*, *Cobetia marina*, *Modicisalibacter tunisiensis*, *Zymobacter palmarum*, *Carnimonas nigrificans* and *Halotalea alkalilenta* were also very low (91.2–93.9, 94.3–95.2, 93.9–94.5, 93.0–93.4, 92.3–93.0 and 93.5–94.1 %, respectively). The phylogenetic

coherence of these three species, *Chromohalobacter salarius*, *H. salaria* and *S. socius*, was also indicated by their recovery as a cluster in all trees generated from the bootstrapped dataset (Fig. 1 and Supplementary Figs S1 and S2). On the basis of these results, a reconsideration of their taxonomic status seems necessary.

On the other hand, *Halomonas halodurans* DSM 5160^T constituted a very robust and stable cluster with *Cobetia marina* DSM 4741^T, the only species described to date within the genus *Cobetia* (Arahal *et al.*, 2002a). The 16S and 23S rRNA gene sequence similarities between these two strains were 100 %. However, when the 16S and 23S rRNA gene sequences of *H. halodurans* DSM 5160^T were compared to those of the species of the genera *Halomonas*, *Chromohalobacter*, *Modicisalibacter*, *Halotalea*, *Zymobacter*, *Carnimonas*, *Kushneria* and *Salinicola*, the similarity values were always very low (91.8–95.8 and 89.3–93.8 %, respectively). *Cobetia marina* was first described as '*Arthrobacter marinus*' by Cobet *et al.* (1970) and later proposed as *Pseudomonas marina* in an extensive study dealing with marine micro-organisms (Baumann *et al.*, 1972). In 1983, it was reclassified within the genus *Deleya* (Baumann *et al.*, 1983) and it was later moved again, to the genus *Halomonas* (Dobson & Franzmann, 1996). Finally, on the basis of comparative 16S and 23S rRNA gene phylogenetic analyses, as well as on phenotypic features, Arahal *et al.* (2002a) proposed the transfer of this species to the new genus *Cobetia*. From our data, there is not sufficient evidence to determine whether *H. halodurans* DSM 5160^T and *Cobetia marina* DSM 4741^T are members of the same or different species and thus an extensive study to compare these two species is required in order to define their taxonomic delineation.

It is also important to mention that the species *Halomonas muralis*, *Halomonas pantelleriensis*, *Halomonas lutea* and *Halomonas kribbensis* clustered together in all three 16S rRNA gene-based trees (Fig. 1 and Supplementary Figs S1 and S2), but this is not a robust branch, since it was not conserved when 23S rRNA gene sequence analysis was performed (Fig. 1 and Supplementary Figs S1 and S2). On the other hand, *Halomonas anticariensis* could be included in group 2 of the genus *Halomonas* based on the 23S rRNA gene, but the 16S rRNA gene sequence study reveals that it is located far away from other *Halomonas* species. A possible explanation for these different topologies is that a recombination event might have occurred in the 23S or 16S rRNA gene.

As mentioned previously by Lee *et al.* (2005), phylogenetic analysis based on 16S rRNA gene sequences showed that the species *Halomonas taeanensis* clustered together with *Cobetia marina*. Nevertheless, phylogenetic analysis based on 23S rRNA gene sequences as well as 23S and 16S rRNA gene sequence similarity values (96.9 % with *Halomonas ventosae* A112^T and 97.1 % with *Halomonas salifodinae* BC7^T, respectively) showed that *H. taeanensis* was more closely related to the genus *Halomonas* than to the genus *Cobetia*.

With respect to the genus *Chromohalobacter*, all the species described to date clustered together (the mean 16S and 23S rRNA gene sequence similarity of this group was 98.0 and 97.8%, respectively) with the only exception being *Chromohalobacter salarius*, as discussed previously.

Based on 16S rRNA gene sequence analysis and phenotypic evidence, Ben Ali Gam *et al.* (2007) proposed the creation of the genus *Modicisalibacter* with the new species *Modicisalibacter tunisiensis* in the family *Halomonadaceae*. However, after analysing the 23S rRNA gene sequence of the type strain of this species, we found that it is located within group 1 of *Halomonas* (maximum-parsimony and maximum-likelihood trees; Supplementary Figs S1 and S2). Our hypothesis is that horizontal gene transfer might have occurred with another species of the genus *Halomonas*.

The phylogenetic distinctness of the remaining genera currently included in the family *Halomonadaceae* (*Modicisalibacter*, *Halotalea*, *Zymobacter*, *Carnimonas*, *Cobetia*, *Kushneria* and *Salinicola*) was confirmed in this study, being stable in the trees produced from all methods of analysis.

The 16S rRNA signature nucleotide characteristics of the family *Halomonadaceae* were defined by Ben Ali Gam *et al.* (2007). Since then, new genera and species within the family have been described, but the 16S rRNA signature nucleotides that define the family have not changed: positions 484 (A/G), 486 (C/U), 640 (A/G), 660 (A), 668 (A), 669 (A), 737 (U), 738 (U), 745 (U), 776 (U), 1124 (U/G), 1297 (U), 1298 (C), 1423 (A), 1424 (C/U), 1439 (U/C), 1462 (A/G) and 1464 (C/U). This feature has been used in some studies as a key for differentiation of genera within the *Halomonadaceae*. However, it must be taken into consideration that this is an arbitrary feature that must be revised when new isolates are characterized and often requires emended descriptions of the genera (Ntougias *et al.*, 2007; Ben Ali Gam *et al.*, 2007). If the 16S rRNA sequence is to be used as a delineating trait at the rank of family or any other, it makes much more sense to use (almost) complete sequences and not only a few nucleotides.

In conclusion, we found that, although the resolution of the 23S rRNA gene sequence within the family *Halomonadaceae* is generally greater than that of the 16S rRNA sequence, it still does not allow resolution of the phylogenetic relationships of very closely related species. Furthermore, the continuous and rapid increase in the number of genera and species within this family makes it even more complicated. To overcome these limitations, the inclusion of further housekeeping genes, resulting in a multilocus sequencing analysis (MLSA), may help to clarify the phylogenetic relationships between the members of this heterogeneous family. In an attempt to carry out an MLSA of the species *H. variabilis*, Okamoto *et al.* (2004) determined the sequences of *gyrB*, *ectB* and *ectC* genes of ten strains of *H. variabilis*, finding that phylogenetic trees based on *gyrB* and *ectB* genes were very similar to that based on the 16S rRNA gene, but the *ectC*-based tree was inconsistent with the other topologies.

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