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

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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)
Carnimonas							
C. nigrificans	Garriga *et al.* (1998)	CTCBS1^T	Y13299	1519	**CECT 4437^T**	**AM941757**	**2913**
Chromohalobacter							
C. beijerinckii	Peçonek *et al.* (2006)	ATCC 19372^T	AB021386	1495	**DSM 7218^T**	**FN257758**	**2911**
C. canadensis	Arahal *et al.* (2001a)	ATCC 43984^T	AJ295143*	1532	DSM 6769^T	AJ306870	2910
		DSM 6769^T	AF211861	1495			
C. israelensis	Arahal *et al.* (2001a)	ATCC 43985^T	AJ295144*	1532	DSM 6768^T	AJ306871	2909
		DSM 6768^T	AF211862	1492			
C. japonicus	Sánchez-Porro *et al.* (2007)	43^T	AB105159	1501			
C. marismortui	Ventosa *et al.* (1989)	ATCC 17056^T	X87219	1423	ATCC 17056^T	AJ306872	2998
C. nigrandesensis	Prado *et al.* (2006)	LTS-4N^T	AJ277205	1505	**CECT 5315^T**	**FN257760**	**3006**
C. salarius	Aguilera *et al.* (2007)	CG4.1^T	AJ427626	1535			
C. salexigens	Arahal *et al.* (2001b)	DSM 3043^T	AJ295146	1531	DSM 3043^T	AJ306873	2908
C. sarecensis	Quillaguamán *et al.* (2004b)	LV4^T	AY373448	1448	**LV4^T**	**FN257759**	**2910**
Cobetia							
C. marina	Arahal *et al.* (2002a)	DSM 4741^T	AJ306890*	1536	DSM 4741^T	AJ306880	2910
		ATCC 25374^T	M93354	1483			
Halomonas							
H. alimentaria	Yoon *et al.* (2002)	YKJ-16^T	AF211860	1493	**DSM 15356^T**	**FN257749**	**2908**
H. alkaliphila	Romano *et al.* (2006)	18bAG^T	AJ640133	1500			
H. almeriensis	Martínez-Checa *et al.* (2005)	M8^T	AY858696	1459	**M8^T**	**FN257744**	**2909**
H. anticariensis	Martínez-Cánovas *et al.* (2004b)	FP35^T	AY489405	1464	**FP35^T**	**FN263244**	**2911**
H. aquamarina	Dobson & Franzmann (1996)	DSM 30161^T	AJ306888*	1528	DSM 30161^T	AJ306874	2914
		ATCC 14400^T	M93352	1466			
H. arcis	Xu *et al.* (2007)	AJ282^T	EF144147	1404			
H. axialensis	Kaye *et al.* (2004)	Althf1^T	AF212206	1438	**CECT 5812^T**	**FN257753**	**2914**
H. boliviensis	Quillaguamán *et al.* (2004a)	LC1^T	AY245449	1441	**DSM 15516^T**	**FN257754**	**2913**
H. campaniensis	Romano *et al.* (2005)	5AG^T	AJ515365	1480	**DSM 15293^T**	**FN257750**	**2909**
H. campisalis	Mormile *et al.* (1999)	ATCC 700597^T	AF054286	1400	**ATCC 700597^T**	**AM941752**	**2910**
H. caseinilytica	Wu *et al.* (2008a)	AJ261^T	EF527874	1446			
H. cerina	González-Domenech *et al.* (2008b)	P4^T	EF613112	1449			
H. cupida	Dobson & Franzmann (1996)	**DSM 4740^T**	**FN257742***	**1464**	DSM 4740^T	AJ306875	2910
		DSM 4740^T	L42615	1471			
H. daqingensis	Wu *et al.* (2008b)	DQD2-30^T	EF121854	1420			
H. denitrificans	Kim *et al.* (2007)	M29^T	AM229317	1489			
H. desiderata	Berendes *et al.* (1996)	FB2^T	X92417	1495	DSM 9502^T	AJ306876	2910
H. elongata	Vreeland *et al.* (1980)	**ATCC 33173^T**	**AM941743***	**1457**	ATCC 33173^T	AJ306877	2910
		ATCC 33173^T	X67023	1470			
		ATCC 33173^T	M93355	1479			
H. eurihalina	Mellado *et al.* (1995)	ATCC 49336^T	X87218*	1473	ATCC 49336^T	AJ306878	2911
		ATCC 49336^T	L42620	1490			
H. gomseomensis	Kim *et al.* (2007)	M12^T	AM229314	1484			
H. gudaonensis	Wang *et al.* (2007a)	SL014B-69^T	DQ421808	1462			
H. halmophila	Dobson *et al.* (1990)	ATCC 19717^T	AJ306889*	1530	ATCC 19717^T	AJ306879	2920
		ATCC 19717^T	M59153	1540			
H. halocynthiae	Romanenko *et al.* (2002)	KMM 1376^T	AJ417388	1514	**DSM 14573^T**	**FN257752**	**2908**
H. halodenitrificans	Dobson & Franzmann (1996)	ATCC 13511^T	L04942	1531	**CECT 5012^T**	**FN257751**	**2908**
H. halodurans	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)
H. halophila	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			
H. hydrothermalis	Kaye *et al.* (2004)	Slthf2^T	AF212218	1409	**CECT 5814^T**	**AM941749**	**2912**
H. janggokensis	Kim *et al.* (2007)	M24^T	AM229315	1478			
H. kenyensis	Boltyanskaya *et al.* (2007)	AIR-2^T	AY962237	1371			
H. koreensis	Lim *et al.* (2004)	SS20^T	AY382579	1399	**JCM 12237^T**	**FN257747**	**2908**
H. korlensis	Li *et al.* (2008)	XK1^T	EU085033	1517			
H. kribbensis	Jeon *et al.* (2007)	BH843^T	DQ280368	1417			
H. lutea	Wang *et al.* (2008a)	YIM 91125^T	EF674852	1479			
H. magadiensis	Duckworth *et al.* (2000)	21 MI^T	X92150	1473	**21 MI^T**	**AM941747**	**2915**
H. maura	Bouchotroch *et al.* (2001)	**S-31^T**	**FN257741***	**1455**	**S-31^T**	**FN257746**	**2908**
		S-31^T	AJ271864	1396			
H. meridiana	James *et al.* (1990)	DSM 5425^T	AJ306891*	1528	DSM 5425^T	AJ306881	2914
		DSM 5425^T	M93356	1475			
H. mongoliensis	Boltyanskaya *et al.* (2007)	Z-7009^T	AY962236	1372			
H. muralis	Heyrman *et al.* (2002)	LMG 20969^T	AJ320530	1493	**DSM 14789^T**	**FN257757**	**2923**
H. neptunia	Kaye *et al.* (2004)	Eplume1^T	AF212202	1454	**CECT 5815^T**	**FN257755**	**2920**
H. nitroreducens	González-Domenech *et al.* (2008a)	11S^T	EF613113	1441			
H. organivorans	García *et al.* (2004)	G-16.1^T	AJ616910	1472	**G-16.1^T**	**AM941751**	**2908**
H. pacifica	Dobson & Franzmann (1996)	DSM 4742^T	L42616*	1480	DSM 4742^T	AJ306882	2910
		ATCC 27122^T	EU373088	1449			
H. pantelleriensis	Romano *et al.* (1996)	AAP^T	X93493	1477	DSM 9661^T	AJ306883	2912
H. sabkhae	Kharroub *et al.* (2008)	5-3^T	EF442769	1540			
H. saccharevitans	Xu *et al.* (2007)	AJ275^T	EF144149	1407			
H. salaria	Kim *et al.* (2007)	M27^T	AM229316	1492			
H. salifodinae	Wang *et al.* (2008b)	BC7^T	EF527873	1428			
H. salina	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			
H. shengliensis	Wang *et al.* (2007b)	SL014B-85^T	EF121853	1460			
H. subglaciescola	Franzmann *et al.* (1987)	DSM 4683^T	AJ306892*	1531	DSM 4683^T	AJ306885	2910
		DSM 4683^T	M93358	1481			
H. subterranea	Xu *et al.* (2007)	ZG16^T	EF144148	1381			
H. sulfidaeris	Kaye *et al.* (2004)	Esulfide1^T	AF212204	1430	**CECT 5817^T**	**AM941748**	**2911**
H. taeanensis	Lee *et al.* (2005)	BH539^T	AY671975	1408	BH539^T	AY829729	2907
H. variabilis	Dobson & Franzmann (1996)	DSM 3051^T	AJ306893*	1528	DSM 3051^T	AJ306886	2912
		DSM 3051^T	M93357	1470			
H. ventosae	Martínez-Cánovas *et al.* (2004a)	Al12^T	AY268080	1395	**Al12^T**	**FN257748**	**3009**
H. venusta	Dobson & Franzmann (1996)	DSM 4743^T	AJ306894*	1530	DSM 4743^T	AJ306887	2914
		DSM 4743^T	L42618	1479			
Halotalea							
H. alkalilenta	Ntougias *et al.* (2007)	AW-7^T	DQ421388	1491	**CECT 7134^T**	**FN257762**	**3018**
Kushneria							
K. aurantia	Sánchez-Porro *et al.* (2009)	A10^T	AM941746	1428	A10^T	AM941756	2914
K. avicenniae	Sánchez-Porro *et al.* (2009)	MW2a^T	DQ888315	1433	MW2a^T	AM941755	2912
K. indalinina	Sánchez-Porro *et al.* (2009)	**CG2.1^T**	**AM941745***	**1478**	CG2.1^T	AM941754	2913
		CG2.1^T	AJ427627	1527			
K. marisflavi	Sánchez-Porro *et al.* (2009)	SW32^T	AF251143	1495	DSM 15357^T	AM941753	2914
Modicisalibacter							
M. tunisiensis	Ben Ali Gam *et al.* (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)
Salinicola							
S. socius	Anan'ina *et al.* (2007)	SMB35^T	DQ979342	1433			
Zymobacter							
Z. palmae	Okamoto *et al.* (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	CGGTTACCTTGTTAGGACTTCACC	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	SWGTTCGRVAWGGGA	35–49	Amplification, reverse
992IRry97	TTCCCTCACRGTACT	457–471	Sequencing, 23S rRNA gene
1020IRrmz98	TCTGGGYTGTTYCCCT	975–990	Sequencing, 23S rRNA gene
1018Fw	GGGGGTAGAGCACTGTTT	855–872	Sequencing, 23S rRNA gene
1037IRrm97	CTTACCCGACAAGGAATTTCG	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

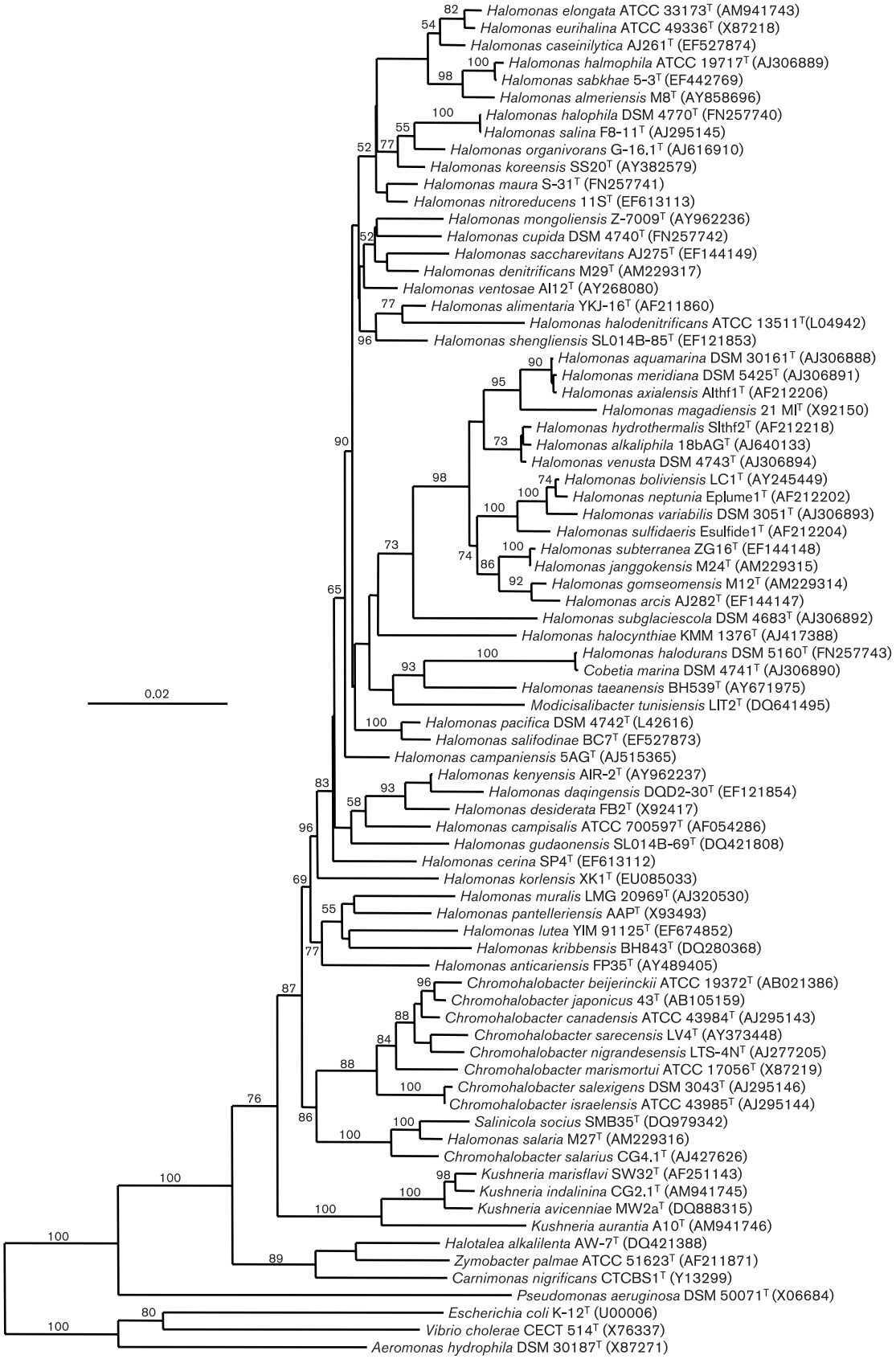
Halomonas elongata ATCC 33173T (AM941743)
Halomonas eurihalina ATCC 49336T (X87218)
Halomonas caseinilytica AJ261T (EF527874)
Halomonas halmophila ATCC 19717T (AJ306889)
Halomonas sabkhae 5-3T (EF442769)
Halomonas almeriensis M8T (AY858696)
Halomonas halophila DSM 4770T (FN257740)
Halomonas salina F8-11T (AJ295145)
Halomonas organivorans G-16.1T (AJ616910)
Halomonas koreensis SS20T (AY382579)
Halomonas maura S-31T (FN257741)
Halomonas nitroreducens 11ST (EF613113)
Halomonas mongoliensis Z-7009T (AY962236)
Halomonas cupida DSM 4740T (FN257742)
Halomonas saccharevitans AJ275T (EF144149)
Halomonas denitrificans M29T (AM229317)
Halomonas ventosae AI12T (AY268080)
Halomonas alimentaria YKJ-16T (AF211860)
Halomonas halodenitrificans ATCC 13511T(L04942)
Halomonas shengliensis SL014B-85T (EF121853)
Halomonas aquamarina DSM 30161T (AJ306888)
Halomonas meridiana DSM 5425T (AJ306891)
Halomonas axialensis Althf1T (AF212206)
Halomonas magadiensis 21 MIT (X92150)
Halomonas hydrothermalis Slthf2T (AF212218)
Halomonas alkaliphila 18bAGT (AJ640133)
Halomonas venusta DSM 4743T (AJ306894)
Halomonas boliviensis LC1T (AY245449)
Halomonas neptunia Eplume1T (AF212202)
Halomonas variabilis DSM 3051T (AJ306893)
Halomonas sulfidaeris Esulfide1T (AF212204)
Halomonas subterranea ZG16T (EF144148)
Halomonas janggokensis M24T (AM229315)
Halomonas gomseomensis M12T (AM229314)
Halomonas arcis AJ282T (EF144147)
Halomonas subglaciescola DSM 4683T (AJ306892)
Halomonas halocynthiae KMM 1376T (AJ417388)
Halomonas halodurans DSM 5160T (FN257743)
Cobetia marina DSM 4741T (AJ306890)
Halomonas taeanensis BH539T (AY671975)
Modicisalibacter tunisiensis LIT2T (DQ641495)
Halomonas pacifica DSM 4742T (L42616)
Halomonas salifodinae BC7T (EF527873)
Halomonas campaniensis 5AGT (AJ515365)
Halomonas kenyensis AIR-2T (AY962237)
Halomonas daqingensis DQD2-30T (EF121854)
Halomonas desiderata FB2T (X92417)
Halomonas campisalis ATCC 700597T (AF054286)
Halomonas gudaonensis SL014B-69T (DQ421808)
Halomonas cerina SP4T (EF613112)
Halomonas korlensis XK1T (EU085033)
Halomonas muralis LMG 20969T (AJ320530)
Halomonas pantelleriensis AAPT (X93493)
Halomonas lutea YIM 91125T (EF674852)
Halomonas kribbensis BH843T (DQ280368)
Halomonas anticariensis FP35T (AY489405)
Chromohalobacter beijerinckii ATCC 19372T (AB021386)
Chromohalobacter japonicus 43T (AB105159)
Chromohalobacter canadensis ATCC 43984T (AJ295143)
Chromohalobacter sarecensis LV4T (AY373448)
Chromohalobacter nigrandesensis LTS-4NT (AJ277205)
Chromohalobacter marismortui ATCC 17056T (X87219)
Chromohalobacter salexigens DSM 3043T (AJ295146)
Chromohalobacter israelensis ATCC 43985T (AJ295144)
Salinicola socius SMB35T (DQ979342)
Halomonas salaria M27T (AM229316)
Chromohalobacter salarius CG4.1T (AJ427626)
Kushneria marisflavi SW32T (AF251143)
Kushneria indalinina CG2.1T (AM941745)
Kushneria avicenniae MW2aT (DQ888315)
Kushneria aurantia A10T (AM941746)
Halotalea alkalilenta AW-7T (DQ421388)
Zymobacter palmae ATCC 51623T (AF211871)
Carnimonas nigrificans CTCBS1T (Y13299)
Pseudomonas aeruginosa DSM 50071T (X06684)
Escherichia coli K-12T (U00006)
Vibrio cholerae CECT 514T (X76337)
Aeromonas hydrophila DSM 30187T (X87271)
0.02

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* Althf1^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 %)

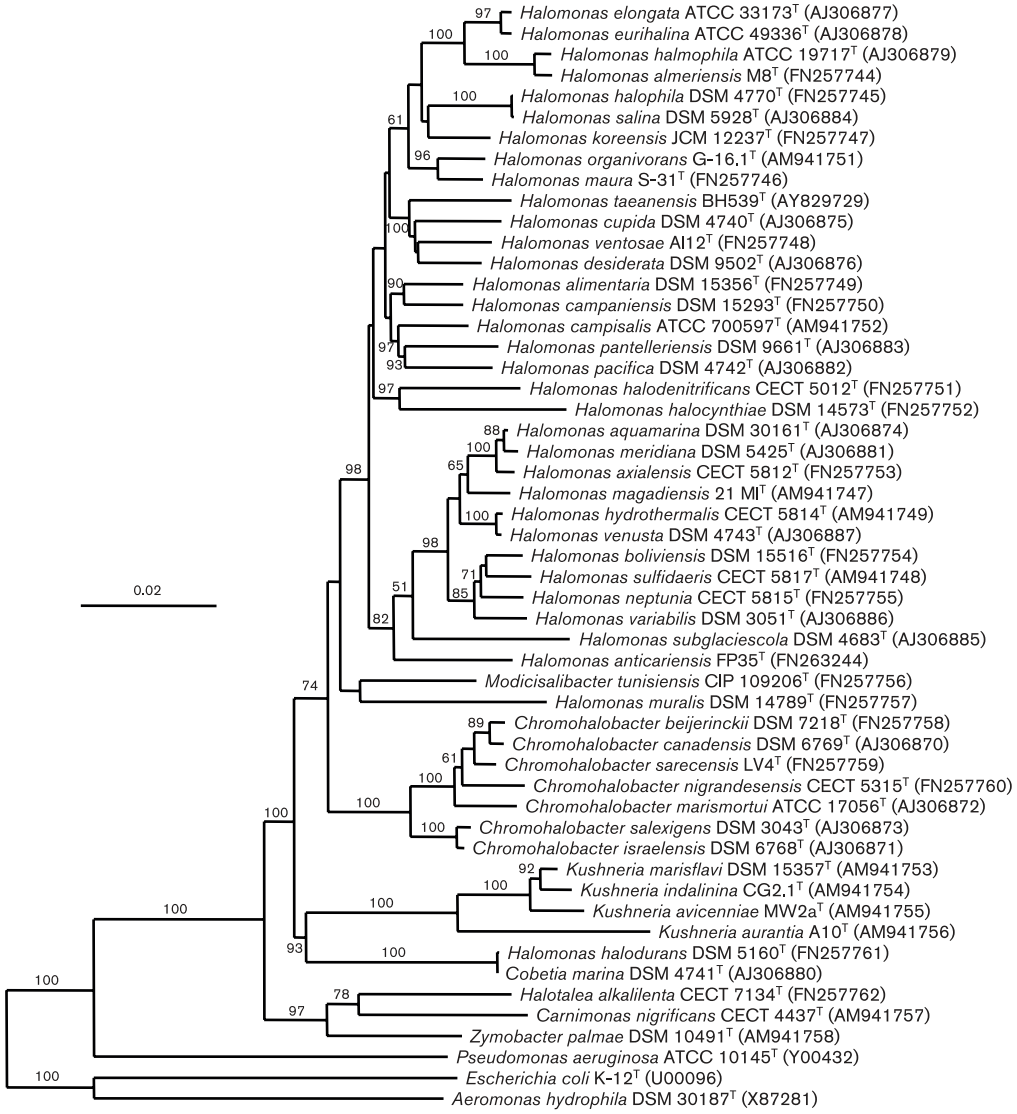

Fig. 2. Neighbour-joining phylogenetic tree, based on the 23S rRNA gene sequence comparison, showing phylogenetic relationships between members of the family *Halomonadaceae*. Accession numbers are shown in parentheses. Bootstrap values >50 % are shown. Bar, 0.02 substitutions per nucleotide position.

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 $C_{18:1}\omega7c$ (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 palmae*, *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* Al12^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.

Acknowledgements

R. R. d. l. H. was a recipient of a fellowship from the Spanish Ministerio de Educación y Ciencia. We thank Dr Paul de Vos for advice and Dr R. Thane Papke for critical reading of the manuscript. This study was supported by grants from the Spanish Ministerio de Educación y Ciencia (BIO2006-06927) and the Junta de Andalucía (P06-CVI-01829).

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