1 **Denitrification as an important taxonomic marker within the genus *Halomonas*.**

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7 **Running title:** Denitrification in *Halomonas* species.

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23 Two tables and two figures are available as supplementary material.

24

25 The nucleotide sequence data are available in the GenBank database under accession

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1 **Keywords:** *Halomonas;* denitrification; taxonomy; phylogeny.

2 **Summary**

3 We have made a comprehensive study of the denitrifying species of the genus *Halomonas*,
4 evaluating both the phylogenetic and phenotypic relationships amongst them and other
5 species of *Halomonas*. The phylogenetic analysis was based on the 16S rRNA gene sequence
6 as well as those of the three genes essential to a complete denitrification process: *narH, nirS*
7 and *nosZ*. The main aim of the phenotypic study was to improve our knowledge of some of the
8 species in question. To this end we investigated the type strain of each species, although in
9 the case of *Halomonas cerina* we also studied strains R53 and 15CR. In addition to this we
10 investigated some other strains phylogenetically related to *Halomonas ventosae, H.*
11 *denitrificans* and *H. koreensis* that were isolated during this study. We also looked into the
12 conditions under which all these bacteria denitrify. Our results indicate that these denitrifying
13 species of *Halomonas* are all closely related. A numerical analysis of the phenotypic data
14 demonstrates a high phenotypic similarity (73%) between most of them. In addition, all the
15 denitrifying strains have a high G+C content of between 63 and 74.3 mol%. The results of the
16 phylogenetic study point to two evolutionary lineages for the process. Although phenotypic
17 similarity does not always reflect phylogenetic relatedness, we have found significant
18 congruence between both features in *Halomonas*, making it clear that denitrifying ability should
19 be considered as an important phenotypic and phylogenetic discriminatory marker within this
20 genus

21

22 **Introduction**

23 The *Halomonadaceae* family belongs to the class *Gammaproteobacteria* and includes both
24 halophilic and non-halophilic bacteria: *Halomonas* [14], *Chromohalobacter* [55],
25 *Modicisalibacter* [5], *Cobetia* [2] and *Kushneria* [46] are made up of halophilic and halotolerant
26 species, whilst *Halotalea* [37], *Zymobacter* [38] and *Carnimonas* [17] comprise non-halophilic
27 bacteria. The genus *Halomonas* currently contains more than sixty species [15]. Its members
28 are Gram-negative, rod-shaped, non-sporulated chemo-organotrophs that exhibit
29 predominantly respiratory metabolism, using oxygen or nitrate as electron acceptors. Some
30 species are denitrifiers and a few have fermentative metabolism. Most *Halomonas* species
31 tend to be moderate halophiles, although some members of the genus could be classified as
32 halotolerant. Their G+C content ranges widely between 53 mol% in *H. venusta* to 74.3% in *H.*
33 *ventosae*. Their principal fatty acids are 16:1 cis 9, 16:0, 17:0 cyclo, 18:1 and 19:0 cyclo 11-

1 12. The major isoprenoid quinone is ubiquinone 9. *Halomonas* species are widely distributed
2 throughout hypersaline and marine environments and represent a high percentage of the
3 strains isolated in these habitats [4].
4
5 Denitrification constitutes one of the main branches of the global nitrogen cycle sustained by
6 prokaryotes and even by certain fungi. It is a dissimilatory process in which oxidised nitrogen
7 compounds (NO_3^- and NO_2^-) are used as terminal acceptors for electron transport. Nitrogen
8 oxides are reduced stepwise to gaseous products (NO, N_2O and N_2), leading to a loss of
9 nitrogen, which is introduced into the biosphere by biological and chemical fixation. In recent
10 years considerable effort has gone into developing techniques and methods to allow the
11 genes encoding the denitrification enzymes to be used as functional markers for phylogenetic
12 and ecological studies. Primer pairs and PCR protocols have been developed for all the
13 functional genes participating in this pathway [8, 22, 42, 48].
14
15 The main aim of this work has been to improve the taxonomic information available about all
16 current denitrifiers belonging to *Halomonas* by studying the phenotypic and phylogenetic
17 relationships among these denitrifying species and also with other *Halomonas* species. The
18 phylogenetic analysis was complemented with a comparison of 4 individual phylogenies based
19 on their 16S rRNA, *narH, nirS* and *nosZ* genes together with their concatenated alignment.
20 This provided a large set of sequences from denitrifying *Halomonas* bacteria, which in the
21 short term has allowed us to make more reliable assessments of their taxonomy and in the
22 long term will help us to embark upon more precise ecological studies of this genus. In
23 addition, we have characterized and included in this work new denitrifying strains selected from
24 more than one hundred isolates from a wide diversity of habitats.
25

26 **Materials and Methods**

27 ***Halomonas* strains**

28 The strains used in this study are listed in Table 1 together with their sites of isolation and
29 references. All these bacteria were kept on MY solid medium [43] with 7.5% w/v of a mixture
30 of sea salts [44] at 32ºC and pH 7-7.2.
31
32 For comparison's sake we included in the phenotypic study other species of *Halomonas*
33 representing the different phylotypes and phenotypes within the genus [3, 34]. With regard to

the phylogenetic analysis, we also included sequences obtained from data bases of *nar*H, *nir*S and *nos*Z genes of five other denitrifying species belonging to the class *Gammaproteobacteria* (Table 1).

Phenotypic characterization and numerical analysis

For this study we carried out 104 phenotypic tests described by Mata *et al*. [34].

Denitrifying ability: We carried out respiratory tests according to the method of Callies and Mannheim [11] as modified by Stanier *et al.* [51]. The strains were cultured in Weimberg tubes (anaerobic) and in haemolysis tubes with an inverted tube inside (microaerobic); with nitrate or nitrite as sole electron acceptor; and with two different carbon sources: glucose (1% w/v) and glycerol (1% w/v). Each assay was duplicated. In the former assays the tubes were inoculated with a 24-hour culture (50 μl) in liquid MY medium supplemented with nitrate or nitrite without shaking, and in the latter the tubes were inoculated with a culture (50 μl) obtained after 9 serial transfers (made every 24 hours) under the same conditions. Growth and gas production showed the ability to respire on nitrate and/or nitrite. In microaerobiosis we used the reactives α-naphthylamine and sulphanilic acid to detect residual nitrates and nitrites in the medium. Phenol red was used as pH indicator to rule out the possibility of a fermentative metabolism. We made additional experiments with *H. campisalis*, *H. campaniensis* and *H desiderata* at pH 8.5 and with *H. cupida* cultured with 3% w/v salts.

The phenotypic data were analysed numerically using the simple-matching coefficient (SSM) [50] and clustering was achieved by the unweighted-pair-group method of association (UPGMA) [49]. Computer analysis was undertaken with the NTSYSpc program version 2.0.1.5. (Applied Biostatistics Inc.).

Genomic DNA extraction

Genomic DNA was obtained using an AquaPure Genomic DNA isolation kit (Biorad®).

G+C content

The G+C content of the genomic DNA of the new isolates in this study and of *Halomonas denitrificans* DSM 18045^T were estimated from the midpoint value (T_m) [32] using the equation of Owen and Hill [40]. The G+C content of reference DNA from *Escherichia coli* NCTC 9001^T was taken to be 50.9 mol% [41].

DNA-DNA hybridization

DNA-DNA hybridization was done with strain AI13 by the spectroscopic DNA-DNA hybridization technique at DSMZ.

Transmission electron microscopy (TEM)

Cell size, morphology and flagella arrangement were studied for the strains 4CR and HGDK1, and for *H. campaniensis* DSM 15293^T, *H. campisalis* ATCC 700597^T and *H. saccharevitans* LMG 23976^T because their original descriptions did not include this information. Micrographs were obtained by TEM after staining with uranyl acetate.

PCR amplification, cloning and sequencing

We determined almost the entire 16S rRNA sequences for the selected strains (around 1,400 bp) as described in Bouchotroch *et al.* [7]. To amplify the *narH, nirS* and *nosZ* gene fragments we used the conditions and primers described in Table 2.

PCR amplifications were made using 50 µl reaction mixtures containing 20 to 100 ng of template DNA, 10 pmol each primer (Sigma®), 0.2 mM dNTP mix (Bioline®), 2mM $MgCl_2$, 5x PCR buffer (Bioline®) and 1.25 U of BioTaq™ DNA polymerase (Bioline®). Amplified PCR products from pure cultures were purified with the GFX-DNA and Gel Band Purification kit (GE Health Care®) and sequenced directly. When required, the PCR products were cloned in pGEMT-T cloning vector (Promega®) according to the manufacturer´s recommendations and transformed into *Escherichia coli* DH-5α. The clones were then submitted to sequence analysis.

The nucleotide sequences of the amplicons and inserts were determined by cycle sequencing with the Big Dye reagent (Applied Biosystems®, Foster City, CA, USA) and run in an Applied Biosystems ABI PRISM™ 3730 automated DNA sequencer and by capillary electrophoresis on a MegaBase 1000 using the DYENAMIC dye terminator cycle sequencing kit (GE Health Care®). The primers used were the same as those for PCR as well as new ones designed by us *ad hoc* (Table 2).

Sequence chromatograms were analysed using Chromas Lite version 2.01 (Technelysium Pty Ltd., Tewantin, QLD, Australia) and compared to reference sequences available in the

GenBank database using the BLAST search. All the sequences obtained were deposited in GenBank (Table 1).

Phylogenetic analysis

We created five alignments: four corresponding to the individual alignment of the sequences of the 16S rRNA, *nar*H, *nir*S and *nos*Z genes and one corresponding to the concatenation of all the gene sequences. All the alignments were made using the CLUSTALX program [54] and then refined both manually and automatically using the Gblocks software [12], which eliminated the poorly aligned positions and divergent regions of the alignments.

Phylogenetic analyses were undertaken in three ways: the neighbour-joining (NJ) and maximum-parsimony (MP) methods were undertaken using MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 software [53], whilst for the maximum-likelihood (ML) method we used the Dnaml program from the Phylogeny Inference PHYLIP package, version 3.68 [16]. Genetic distances were calculated by the Kimura two-parameter model [27]. We determined the reliability of an inferred tree by using bootstrap values based upon 1,000 replications, except with ML, where only 100 replicates were generated. Finally, we created a consensus tree from the NJ, MP and ML phylogenetic trees by using the Consense program, also contained in the above-mentioned PHYLIP package. We computed this phylogenetic tree by the majority-rule consensus tree method. Identity values between sequences were calculated using the software MegAlign in the DNASTAR package [10].

Results

Phenotypic characterization and numerical analysis

Numerical analysis was based on 104 phenotypic tests. The following features were not included in the numerical analysis due to their giving the same response for all the strains: Gram staining, presence of catalase, growth between 7.5 and 10% w/v sea salts in a range of temperatures between 20 and 37ºC at pH 8. The phenotypic features distinguishing between denitrifying species of *Halomonas* are included in Table S1.

The dendrogram obtained after numerical analysis is shown in Figure 1. The strains group into two phena (phenon A and phenon B) at 63% similarity level. Phenon A contains all the denitrifying species plus 12 other non-denitrifying species. The majority of the denitrifiers (18 strains) cluster into a subgroup within phenon A at 73% similarity level.

1

2 *Halomonas* species able to produce acids from sugars, as described by Mata *et al.* [34], are
3 grouped in phenon B. Three new isolates (4CR, F15 and HGD1) and *Halomonas koreensis,*
4 the only denitrifying bacteria which produced acids from sugars, fall into phenon A. All the
5 *Halomonas* denitrifying strains proved positive for oxidase except strain F15. They were
6 negative for hydrolysis of starch, aesculin and casein, respiration on fumarate, and production
7 of haemolysis. All strains were negative for lecithinase production with the exception of the
8 type strain of *H. fontilapidosi* and strain AI13. Metabolism was respiratory in all of them. None
9 of the strains produced acids from adonitol, L-arabinose, D-fructose, *myo*-inositol, lactose,
10 maltose, D-mannitol, D-mannose, D-melezitose, L-rhamnose, D-salicin, D-sorbitol, sorbose or
11 D-trehalose. They did not grow with methionine as sole carbon, nitrogen and energy source.
12 They were all susceptible to amoxycillin (25 µg), ampicillin (10 µg), chloramphenicol (30 µg),
13 nitrofurantoin (300 µg) and sulphamide (250 µg).
14
15 All the strains reduced nitrate and nitrite aerobically under all the conditions tested. In addition,
16 they all proved capable of denitrifying anaerobically, although the denitrification process in
17 *Halomonas campaniensis, H. campisalis and H. desiderata* only took place when we used an
18 inoculum transferred nine times. These three species did grow and denitrify to a greater extent
19 when we conducted all the experiments at pH 8.5, which is within their optimum pH range for
20 growth.
21
22 **G+C content**
23 The G+C content of *Halomonas denitrificans* DSM 18045^T was 68.9 mol%, as an average
24 value of three measurements. The G+C contents of the new isolates are listed in Table S2.
25
26 **Transmission electron microscopy (TEM)**
27 *Halomonas campaniensis*, *H. campisalis* and *H. saccharevitans*, and the strains 4CR and
28 HGDK1 have a single polar flagellum (Fig. S1). Transmission electron micrographs also show
29 cell morphology and size.
30
31 **DNA-DNA hybridization and taxonomic assignation of strain A13.**
32 The results of DNA–DNA hybridization between strain AI13 and *H. denitrificans* DSM 18045^T,
33 its closest phylogenetic relative, was 52%. This value, together with phenotypic, phylogenetic
34 and genotypic data, confirmed that strain AI13 belongs to the species *H. denitrificans*.

Phylogenetic analysis

The consensus phylogenetic tree based on 16S rRNA gene sequences and constructed from neighbour-joining, maximum-parsimony and maximum-likelihood methods is depicted in Figure 2. Our phylogenetic study included 48 *Halomonas* species. As can be seen, 47 of these species grouped into 3 phylotypes (A, B, and C). All the denitrifying species were phylogenetically related and appeared in the same phylotype (phylogroup A). *H. campaniensis, H. campisalis, H. desiderata* and *H. gudaonensis* form a distinct clade within this phylogroup.

We amplified the *narH*, *nirS* and *nosZ* genes belonging to denitrifying species of the genus *Halomonas* published to date. We also amplified the same genes from strains R53 and 15CR of *Halomonas cerina,* from the new isolates (strains HGD1, 4CR, C8, N64, F15, A13, and HDGK1) and from the type strain of *Marinobacter hydrocarbonoclasticus*.

The primers narH50F and narH1040R were successfully used to recover the *narH* gene sequences of all the strains assayed. With the *nirS* gene, the primer pair nirS1F and nirS6R yielded PCR fragments that agreed well with the expected length, except for *Halomonas campisalis* and *H. gudaonensis*. For these bacteria we associated a newly designed forward primer, nirS149F, with the reverse primer nirS6R. As far as the *nosZ* gene is concerned, primers nosZ661F and nosZ1773R were successful in retrieving PCR products except for *Halomonas koreensis*, for which a new pair of primers, nosZ126F and nosZ1527R had to be designed *ad hoc* (Table 2). We cloned the *nosZ* gene fragments from *H. fontilapidosi* and *H. ventosae* and then sequenced the insert with the universal primers SP6 and T7.

The distribution of identity values of the 16S rRNA nucleotide sequences between type strains of the denitrifying species of *Halomonas* ranged from 91.3% (*H. desiderata* with *H. halodenitrificans*) to 98.1% (*H. alimentaria* with *H. nitroreducens*). The identity percentages of the outgroups used were lower than 88.9%. The *narH* nucleotide sequence identities ranged from 86.7% to 95.3%. The identity value matrix for the *nirS* gene fragment showed two different groups of values. The highest identity percentage between any of the *H. campaniensis, H. campisalis*, *H. desiderata* and *H. gudaonensis* and the remaining species was 57.7%. The remaining denitrifying species of the genus (type strains) showed identity values close to those of the *narH* gene percentages. With the *nosZ* gene fragment the same trend was found as that with the *nirS* gene *H. campaniensis, H. campisalis*, *H. desiderata* and

1 *H. gudaonensis* showed very low identity values with the rest of the denitrifying species of the
2 genus.
3
4 Figure S2a depicts the consensus phylogenetic tree constructed by the three methods based
5 upon the 16S rRNA gene. As can be seen, the genus *Halomonas* constitutes a clearly
6 separate lineage, quite distant from the outgroups used. The *nar*H phylogeny (Fig. S2b) is
7 somewhat different from that obtained with with 16S rRNA if we analyse the subgroups that
8 appear. As far as the *nirS* gene (Fig. S2c) and *nos*Z gene are concerned (Fig. S2d), we found
9 a phylotype (phylotype alk) that includes *H. campaniensis*, *H. campisalis, H. desiderata* and *H.*
10 *gudaonensis*. This clade is supported by all three methods used and by quite high bootstrap
11 values. The remaining denitrifying strains of *Halomonas* group together in a similar way as that
12 found with the 16S rRNA gene (Fig. S2a).
13
14 Furthermore, after applying Gblocks with stringent conditions we combined all the genes into
15 one single analysis with 4,125 homologous positions (Fig. 3). The denitrifying species of
16 *Halomonas* are clearly separate from the outgroups, although they do not constitute a single
17 evolutionary lineage. *H. campaniensis, H. campisalis, H. desiderata* and *H. gudaonensis*
18 cluster together (phylotype alk). This clustering is supported by bootstrap values of nearly
19 100% in the NJ, MP and ML trees.
20
21 **Discussion**
22 The main aim of this work has been to establish the phenotypic and phylogenetic relationships
23 among the denitrifying strains of *Halomonas*. We considered this study to be essential
24 because some of these species were not adequately characterized phenotypically and/or were
25 not described according to the minimal standards established for *Halomonas* species [4]. As
26 far as the phenotypic study of the strains is concerned, we found several differences with
27 regard to the original descriptions of some of them. For example, *Halomonas gudaonensis*
28 and *H. shengliensis* grew at pH values lower than 8 (within a pH range of 6-10), whilst in the
29 original descriptions they only grew at alkaline pH values [56, 57]. We suspect that these
30 conflicting results may be due to the fact that the authors did not use the optimum salt-
31 concentration when they were testing growth at different pH values. In the same way, we did
32 not find any acid production from fructose by *H. denitrificans* [26], nor did *H. campaniensis*
33 grow when cultivated in media without sea-salts, as opposed to the claims of the original
34 publication [45]. Some other discrepancies are included in Table S1.

1 We have also characterized and included new denitrifying strains of *Halomonas* deriving from
2 various saline habitats. During the course of this work we were able to identify strain HDGK1
3 as *Halomonas koreeensis*, and strain A13 as *Halomonas denitrificans*. Strains HGD1, 4CR,
4 C8, N64 y F15 are phylogenetically closely related to *Halomonas ventosae* but they show
5 some important phenotypic differences from this species that preclude their inclusion in *H.*
6 *ventosae* until further studies are undertaken.
7
8 We observed a high phenotypic similarity between most of the denitrifying microorganisms
9 belonging to *Halomonas*, as depicted in Figure 1.
10
11 The G+C content of *Halomonas denitrificans* DSM 18045^T given in its original description by
12 Kim *et al.* [26] and determined by HPLC was 53.8 mol%. Nevertheless, we found a G+C
13 content of 68.9 mol%, which is within the G+C range of values for the other denitrifying
14 bacteria [4], values considerably higher than those of other *Halomonas* species. This
15 percentage is quite similar to the 66.05 mol% of the denitrifying strain Al13, which
16 phenotypically, genetically and phylogenetically is related to the species *H. denitrificans*.
17
18 We also determined the conditions under which these microorganisms were able to denitrify.
19 All the strains from this study reduced nitrate and nitrite aerobically. Nevertheless, the
20 denitrification process in *Halomonas campaniensis, H. campisalis and H. desiderata* only got
21 underway after 9 serial transfers and was more efficient when the pH of the medium was 8.5.
22 This pH dependence of denitrification was also observed by Berendes *et al.* [6] with *H.*
23 *desiderata* under anaerobic conditions. Strain HGD1, phenotypically and phylogenetically
24 affiliated to *H. ventosae*, reduced nitrate and nitrite aerobically but without producing a bubble
25 inside a Durham tube. This bubble is more related to the formation of N_2 than to other gases
26 such as N_2O or CO_2, which will only generate such a bubble when they exceed their solubility
27 in the medium [9]. Thus, strain HGD1 might produce low quantities of nitrous and nitric oxides
28 during aerobic denitrification and maintain these gases at relatively constant levels until the
29 more-oxidized compounds have been depleted. Another possible explanation may be the
30 extreme sensitivity of its nitrous oxide reductase to oxygen, as has been demonstrated for
31 other bacteria such as *Paracoccus denitrificans* [1]. Whatever the final explanation, according
32 to our observations the ability to denitrify can be considered as being a stable phenotypic
33 feature for any species in *Halomonas.*
34

In 2002 Arahal *et al*. [3] evaluated the phylogenetic status of the family *Halomonadaceae* using their 16S rRNA and 23S rRNA gene sequences. In this way they were able to establish two main phylotypes within the genus *Halomonas*. Since then new species of *Halomonas* have been discovered and therefore we have included some of them (19) that represent the different phylotypes and phenotypes within the genus [3, 34]. Figure 2 shows the three phylotypes (A, B, and C) obtained in this study. All the denitrifying strains fall into phylotype A, although *H. campaniensis, H. campisalis, H. desiderata* and *H. gudaonensis* form a distinct clade within this phylogroup.

Trüper and co-workers presented an analysis noting a few regular patterns that linked the physiological features of the halophilic microorganisms with their phylogenetic position [39]. On the basis of the data discussed above we feel we can complement their observations with some insights concerning the capacity for denitrification shown by some species within the genus *Halomonas*. Denitrification ability should therefore be recognised as an important marker among the recommended minimal standards for describing new taxa of the family *Halomonadaceae* [4].

The phylogenetic information provided by the denitrifying community of *Halomonas* was complemented with the sequences of some key genes in the denitrification process. We chose three genes, *narH, nirS* and *nosZ*, which participate in a complete denitrification pathway and have sufficient informative length for the alignments. In addition, their nucleotide and/or amino-acid sequences have already been widely used as molecular markers to evaluate the phylogeny of other species in comparison to the corresponding 16S rRNA gene data [8, 42, 48].

As far as the membrane-bound nitrate-reductase gene-based phylogeny of *Halomonas* spp. is concerned, the range of identities is high and close to the 16S rRNA gene of the same strains. With regard to the *nirS* gene, of particular interest are the extremely low values of *Halomonas campaniensis, H. campisalis, H. desiderata* and *H. gudaonensis* compared to the rest of the denitrifying species of the genus. This observation may suggest that the evolutionary rates of the *nirS* genes in these four species differed from those of the other species and this might explain why we could get no amplicon from *H. campisalis* and *H. gudaonensis* using the available *nirS* primers. Finally, in relation to *nos*Z sequence data, the same species cluster separately, in an order similar to that seen in *nir*S genes. All this goes to support the idea that

1 the *nirS* and *nosZ* genes have followed different evolutionary paths from that of *narH*. Petri and
2 Imhoff [42] emphasised the early origin of the *narH* gene whilst Delorme *et al.* [13]
3 hypothesised that the acquisition by bacteria of the capacity to reduce other nitrogen oxides
4 was probably a more recent event than that of nitrate reduction. We also conducted a
5 phylogenetic study based upon an analysis of the sequences resulting from the concatenation
6 of the 16S rRNA gene and the three denitrifying genes. This combined analysis concurs in
7 general with ribosomal phylogeny.
8
9 As mentioned before, *Halomonas campaniensis, H. campisalis, H. desiderata* and *H.*
10 *gudaonensis* form a distinct clade in the phylogenies obtained with the RNAr 16S gene and the
11 *nirS* and *nosZ* genes. Another point of interest is that the four species of this cluster were
12 isolated from alkaline habitats and grow best at pH values of 8 or even higher [6, 36, 45, 57].
13 This raises questions about how environmental factors may influence the evolution of
14 denitrification genes, and in fact Heylen *et al.* [23] and Jones *et al.* [25] have already
15 speculated about changes in the phylogenetic signal throughout a number of generations when
16 a microorganism finds itself under certain selective pressures within its habitat.
17
18 In conclusion, our results indicate that denitrifying activity and phylogenetic position are closely
19 related within the genus *Halomonas* and make it clear that denitrifying ability should be
20 considered as an important phylogenetic and phenotypic feature.
21
22
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30
31 **References**
32 [1] P.R. Alefounder, S.J. Ferguson, Electron transport-linked nitrous oxide synthesis and
33 reduction by *Paracoccus denitrificans* monitored with an electrode. Biochem Biophys. Res.
34 Commun. 104 (1982) 1149-1155.

1 [2] D.R. Arahal, A.M. Castillo, W. Ludwig, K.H. Schleifer, A. Ventosa. Proposal of *Cobetia*
2 *marina* gen. nov., comb. nov., within the family *Halomonadaceae*, to include the species
3 *Halomonas marina*. Syst. Appl. Microbiol. 25 (2002) 207-211.
4 [3] D.R. Arahal, W. Ludwig, K.H. Schleifer, A. Ventosa, Phylogeny of the family
5 *Halomonadaceae* based on 23S and 16S rDNA sequence analyses. Int. J. Syst. Evol.
6 Microbiol. 52 (2002) 241-249.
7 [4] D.R. Arahal, R.H. Vreeland, C.D. Litchfield, M.R. Mormile, B.J. Tindall, A. Oren, V. Béjar, E.
8 Quesada, A. Ventosa, Recommended minimal standards for describing new taxa of the family
9 *Halomonadaceae*. Int. J. Syst. Evol. Microbiol. 57 (2007) 2436-2446.
10 [5] Z. Ben Ali Gam, S. Abdelkafi, L. Casalot, J.L.Tholozan, R. Oueslati, M. Labat,
11 *Modicisalibacter tunisiensis* gen. nov., sp. nov., an aerobic, moderately halophilic bacterium
12 isolated from an oilfield-water injection sample, and emended description of the family
13 *Halomonadaceae* Franzmann *et al.* 1989 emend Dobson and Franzmann 1996 emend
14 Ntougias *et al.* 2007. Int. J. Syst. Evol. Microbiol. 57 (2007) 2307-2313.
15 [6] F. Berendes, G. Gottschalk, E. Heine-dobbernack, E.R.B. Moore, B.J. Tindall, *Halomonas*
16 *desiderata* sp. nov., a new alkaliphilic, halotolerant and denitrifying bacterium isolated from a
17 municipal sewage works. Syst. Appl. Microbiol. 19 (1996) 158-167.
18 [7] S. Bouchotroch, E. Quesada, A. Del Moral, I. Llamas, V. Béjar, *Halomonas maura* sp. nov.,
19 a novel moderately halophilic, exopolysaccharide-producing bacterium. Int. J. Syst. Evol.
20 Microbiol. 51 (2001) 1625-1632.
21 [8] G. Braker, J. Zhou, L. Wu, A.H. Devol, J.M. Tiedje, Nitrite reductase genes (*nirK* and *nirS*)
22 as functional markers to investigate diversity of denitrifying bacteria in pacific northwest marine
23 sediment communities. Appl. Environ. Microbiol. 66 (2000) 2096-2104.
24 [9] I. Brettar, M.G. Höfle, Nitrous oxide producing heterotrophic bacteria from the water column
25 of the central Baltic: abundance and molecular identification. Mar. Ecol. Prog. Ser. 94 (1993)
26 253-265.
27 [10] T.G. Burland, DNASTAR's Lasergene sequence analysis software. Methods Mol. Biol. 132
28 (2000) 71-91.
29 [11] E. Callies, E. Mannheim, Classification of the *Flavobacterium*-*Cytophaga* complex on the
30 basis of respiratory quinones and fumarate respiration. Int. J. Syst. Bacteriol. 28 (1978) 14-19.
31 [12] J. Castresana, Selection of conserved blocks from multiple alignments for their use in
32 phylogenetic analysis. Mol. Biol. Evol. 17 (2000) 540-552.

[13] S. Delorme, L. Philippot, V. Edel-Hermann, C. Deulvot, C. Mougel, P. Lemanceau, Comparative genetic diversity of the *narG*, *nosZ*, and 16S rRNA genes in fluorescent pseudomonads. Appl. Environ. Microbiol. 69 (2003) 1004-1012.

[14] S.J. Dobson, P.D. Franzmann, Unification of the genera *Deleya, Halomonas* and *Halovibrio* and the species *Paracoccus halodenitrificans* into a single genus, *Halomonas*, and placement of the genus *Zymobacter* in the family *Halomonadaceae*. Int. J. Syst. Bacteriol. 46 (1996) 550-558.

[15] J.P. Euzeby, List of Prokaryotic Names with Standing in Nomenclature. (2009) http://www.bacterio.cict.fr/

[16] J. Felsenstein, PHYLIP (Phylogenetic inference package), version 3,8. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA. 2008.

[17] M. Garriga, M.A. Ehrmann, J. Arnau , M. Hugas, R.F. Vogel, *Carnimonas nigrificans* gen. nov., sp. nov., a bacterial causative agent for black spot formation on cured meat products. Int. J. Syst. Bacteriol. 48 (1998) 677-686.

[18] M.J. Gauthier, B. Lafay, R. Christen, L. Fernandez, M. Acquaviva, P. Bonin, J.C. Bertrand, *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbondegrading marine bacterium. Int. J. Syst. Bacteriol. 42 (1992) 568–576.

[19] C.M. González-Domenech, V. Béjar, F. Martínez-Checa, E. Quesada, *Halomonas nitroreducens* sp. nov., a novel nitrate- and nitrite-reducing species. Int. J. Syst. Evol. Microbiol. 58 (2008) 872-876.

[20] C.M. González-Domenech, F. Martínez-Checa, E. Quesada, V. Béjar, *Halomonas cerina* sp. nov., a moderately halophilic, denitrifying, exopolysaccharide-producing bacterium. Int. J. Syst. Evol. Microbiol. 58 (2008) 803-809.

[21] C.M. González-Domenech, F. Martínez-Checa, E. Quesada, V. Béjar, *Halomonas fontilapidosi* sp. nov., a moderately halophilic, denitrifying bacterium. Int. J. Syst. Evol. Microbiol. 59 (2009) 1290-1296.

[22] C.M. Goregues, V.D. Michotey, P.C. Bonin, Molecular, biochemical, and physiological approaches for understanding the ecology of denitrification, Microb. Ecol. 49 (2005) 198-208.

[23] K. Heylen, D. Gevers, B. Vanparys, L. Wittebolle, J. Geets, N. Boon, P. De Vos, The incidence of *nirS* and *nirK* and their genetic heterogeneity in cultivated denitrifiers. Environ. Microbiol. 8 (2006) 2012-2021.

[24] H. Jeong, J.H. Yim, C. Lee, S.H. Choi, Y.K. Park, S.H. Yoon, C.G. Hur, H.Y. Kang, D. Kim, H.H. Lee, K.H. Park, S.H. Park, H.S. Park, H.K. Lee, T.K. Oh, J.F. Kim, Genomic blueprint of

1 *Hahella chejuensis*, a marine microbe producing an algicidal agent. Nucleic Acids Res. 33
2 (2005) 7066-7073.
3 [25] C.M. Jones, B. Stres, M. Rosenquist, S. Hallin, Phylogenetic analysis of nitrite, nitric oxide,
4 and nitrous oxide respiratory enzymes reveal a complex evolutionary history for denitrification.
5 Mol. Biol. Evol. 25 (2008) 1955-1966.
6 [26] K.K. Kim, L. Jin, H.C. Yang, S.T. Lee, *Halomonas gomseomensis* sp. nov., *Halomonas*
7 *janggokensis* sp. nov., *Halomonas salaria* sp. nov. and *Halomonas denitrificans* sp. nov.,
8 moderately halophilic bacteria isolated from saline water. *Int. J.* Syst. Evol. Microbiol. 57
9 (2007) 675-681.
10 [27] M. Kimura, A simple method for estimating evolutionary rate of base substitutions through
11 comparative studies of nucleotide sequences. J. Mol. Evol. 16 (1980) 111-120.
12 [28] H.K. Lee, J. Chun, E.Y. Moon, S.H. Ko, D.S. Lee, H.S. Lee, K.S. Bae, *Hahella chejuensis*
13 gen. nov., sp. nov., an extracellular-polysaccharide-producing marine bacterium. Int. J. Syst.
14 Evol. Microbiol. 51 (2001) 661-666.
15 [29] K.B. Lehmann, R. Neumann, Atlas und Grundriss der Bakteriologie und Lehrbuch der
16 speziellen bakteriologischen Diagnostik, 1st edn., J.F. Lehmann (eds). München, 1896.
17 [30] P. Lemanceau, R. Samson, C. Alabouvette, Recherches sur la re´sistance des sols aux
18 maladies. XV. Comparaison des populations de *Pseudomonas fluorescents* dans un sol
19 résistant et un sol sensible aux fusarioses vasculaires. Agronomie 8 (1988) 243–249.
20 [31] J.M. Lim, J.H. Yoon, J.C. Lee, C.O. Jeon, D.J. Park, C. Sung, C.J. Kim, *Halomonas*
21 *koreensis* sp. nov., a novel moderately halophilic bacterium isolated from a solar saltern in
22 Korea. Int. J. Syst. Evol. Microbiol. 54 (2004) 2037-2042.
23 [32] J. Marmur, P. Doty, Determination of the base composition of deoxyribonucleic acid from its
24 thermal denaturation temperature. J. Mol. Biol. 5 (1962) 109-118.
25 [33] M.J. Martínez-Cánovas, E. Quesada, I. Llamas, V. Béjar, *Halomonas ventosae* sp. nov., a
26 moderately halophilic, denitrifying, exopolysaccharide-producing bacterium. Int. J. Syst. Evol.
27 Microbiol. 54 (2004) 733-737.
28 [34] J.A. Mata, M.J. Martínez-Cánovas, E. Quesada, V. Béjar, A detailed phenotypic
29 characterisation of the type strains of *Halomonas* species. Syst. Appl. Microbiol. 25 (2002)
30 360-375.
31 [35] W. Migula, System der Bakterien, Vol. 2, Gustav Fischer, Jena, 1900.
32 [36] M.R. Mormile, M.F. Romine, M.T. Garcia, A. Ventosa, T.J. Bailey, B.M. Peyton,
33 *Halomonas campisalis* sp. nov., a denitrifying, moderately haloalkaliphilic bacterium. Syst.
34 Appl. Microbiol. 22 (1999) 551-558.

1 [37] S. Ntougias, G.I. Zervakis, C. Fasseas, *Halotalea alkalilenta* gen. nov., sp. nov., a novel
2 osmotolerant and alkalitolerant bacterium from alkaline olive mill wastes, and emended
3 description of the family *Halomonadaceae* Franzmann *et al.* 1989, emend. Dobson and
4 Franzmann 1996. Int. J. Syst. Evol. Microbiol. 57 (2007) 1975-1983.
5 [38] T. Okamoto, H. Taguchi, K. Nakamura, H. Ikenaga, H. Kuraishi, K. Yamasato,
6 *Zymobacter palmae* gen. nov., sp. nov., a new ethanol-fermenting peritrichous bacterium
7 isolated from palm sap. Arch. Microbiol. 160 (1993) 333-337.
8 [39] A. Oren, Microbial life at high salt concentrations: phylogenetic and metabolic diversity.
9 *Saline Systems*. 4 (2008) 2.
10 [40] R.J. Owen, L.R. Hill, The estimation of base compositions, base pairing and genome size of
11 bacterial deoxyribonucleic acids. In: F.A. Skinner, D.W. Lovelock (Eds.), Identification Methods
12 for Microbiologists, 2nd ed., Academic Press, London, 1979, pp. 277-296.
13 [41] R.J. Owen, D. Pitcher, Current methods for estimating DNA base composition and levels
14 of DNA-DNA hybridization. In: M. Good-Fellow, E. Minnikin (Eds.), Chemical methods in
15 bacterial systematic, Academic Press, New York, 1985, pp. 67-93.
16 [42] R. Petri, J.F. Imhoff, The relationship of nitrate reducing bacteria on the basis of *narH*
17 gene sequences and comparison of narH and 16S rDNA based phylogeny. Syst. Appl.
18 Microbiol. 23 (2000) 47-57.
19 [43] E. Quesada, V. Béjar, C. Calvo, Exopolysaccharide production by *Volcaniella eurihalina*.
20 Experientia 49 (1993) 1037-1041.
21 [44] F. Rodríguez-Valera, F. Ruiz-Berraquero, A. Ramos-Cormenzana, Characteristics of the
22 heterotropic bacterial populations in hypersaline environments of different salt concentration.
23 Microbiol. Ecol. 7 (1981) 235-243.
24 [45] I. Romano, A. Giordano, L. Lama, B. Nicolaus, A. Gambacorta, *Halomonas campaniensis*
25 sp. nov., a haloalkaliphilic bacterium isolated from a mineral pool of Campania Region, Italy.
26 Syst. Appl. Microbiol. 28 (2005) 610-618.
27 [46] C. Sánchez-Porro, R.R. de la Haba, N. Soto-Ramírez, M.C. Márquez, R. Montalvo-
28 Rodríguez, A. Ventosa, Description of *Kushneria aurantia* gen. nov., sp. nov., a novel member
29 of the family *Halomonadaceae*, and a proposal for reclassification of *Halomonas marisflavi* as
30 *Kushneria marisflavi* comb. nov., of *Halomonas indalinina* as *Kushneria indalinina* comb. nov.
31 and of *Halomonas avicenniae* as *Kushneria avicenniae* comb. nov. Int. J. Syst. Evol. Bacteriol.
32 59 (2009) 397-405.

1 [47] D.J. Scala, L.J. Kerkhof, Nitrous oxide reductase (*nosZ*) gene-specific PCR primers for
2 detection of denitrifiers and three *nosZ* genes from marine sediments. FEMS Microbiol. Lett.
3 162 (1998) 61-68.
4 [48] D.J. Scala, L.J. Kerkhof, Diversity of nitrous oxide reductase (*nosZ*) genes in continental
5 shelf sediments. Appl. Environ. Microbiol. 65 (1999) 1681–1687.
6 [49] P.H.A. Sneath, R.R. Sokal, Numerical taxonomy: The principles and practice of numerical
7 classification. W.H. Freeman and Co. Ltd., San Francisco, 1973.
8 [50] R.R. Sokal, C.D. Michener, A statistical method for evaluating systematic relationships.
9 Univ Kansas Sci Bull. 38 (1958) 1409-1438.
10 [51] R.Y. Stanier, N.J. Palleroni, M. Duodoroff, The aerobic pseudomonads: A taxonomic
11 study. *J Gen Micro Biol.* 43 (1966) 159-272.
12 [52] C.K. Stover, X.Q.T. Pham, A.L. Erwin, S.D. Mizoguchi, P. Warrener, M.J. Hickey, F.S.
13 Brinkman, W.O. Hufnagle, D.J. Kowalik, M. Lagrou, R.L. Garber, L. Goltry, E. Tolentino, S.
14 Westbrock-Wadman, Y. Yuan, LL. Brody, S.N. Coulter, K.R. Folger, A. Kas, K. Larbig, R. Lim,
15 K. Smith, D. Spencer, G.K. Wong, Z. Wu, I.T. Paulsen, J. Reizer, M.H. Saier, R.E. Hancock, S.
16 Lory, M.V. Olson, Complete genome sequence of *Pseudomonas aeruginosa* PA01, an
17 opportunistic pathogen. Nature 406 (2000) 959-964.
18 [53] K. Tamura, J. Dudley, M. Nei, S. Kumar, MEGA4: Molecular Evolutionary Genetics
19 Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24 (2007) 1596-1599.
20 [54] J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, The CLUSTAL_X
21 windows interface: flexible strategies for multiple sequence alignment aided by quality analysis
22 tools. Nucleic Acids Res. 25 (1997) 4876-4882.
23 [55] A. Ventosa, M.C. Gutiérrez, M.T. García, F. Ruiz-Berraquero, Classification of
24 "*Chromobacterium marismortui*" in a new genus, *Chromohalobacter* gen. nov., as
25 *Chromohalobacter marismortui* comb. nov., nom. rev. Int. J. Syst. Bacteriol. 39 (1989) 382-
26 386.
27 [56] Y.N. Wang, H. Cai, C.Q. Chi, A.H. Lu, X.G. Lin, Z.F. Jiang, X.L. Wu, *Halomonas*
28 *shengliensis* sp. nov., a moderately halophilic, denitrifying, crude-oil-utilizing bacterium. Int. J.
29 Syst. Evol. Microbiol. 57 (2007) 1222-1226.
30 [57] Y.N. Wang, H. Cai, S.L. Yu, Z.Y. Wang, J. Liu, X.L. Wu, *Halomonas gudaonensis* sp.
31 nov., isolated from a saline soil contaminated by crude oil. Int. J. Syst. Evol. Microbiol. 57
32 (2007) 911-915.

1 [58] J.H. Yoon, K.C. Lee, Y.H. Kho, K.H. Kang, C.J. Kim, Y.H. Park, *Halomonas alimentaria*
2 sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. *Int.* J. Syst. Evol.
3 Microbiol. 52 (2002) 123-130.
4 [59] X.W. Xu, Y.H. Wu, Z. Zhou, C.S. Wang, Y.G. Zhou, H.B. Zhang, Y. Wang, M. Wu,
5 *Halomonas saccharevitans* sp. nov., *Halomonas arcis* sp. nov. and *Halomonas subterranea*
6 sp. nov., halophilic bacteria isolated from hypersaline environments of China. Int. J. Syst. Evol.
7 Microbiol. 57 (2007) 1619-1624.

1 **Legends to Figures**

2

3 **Fig. 1.** Phenotypic dendrogram showing the clustering of the chosen species and strains of *Halomonas*
4 (denitrifying bacteria in bold upon grey shading).

5

6 **Fig. 2.** Phylogenetic consensus tree based on 16S rRNA gene sequences, showing the position of the
7 denitrifying bacteria (shaded grey) compared to other members of the genus *Halomonas.* The tree was obtained
8 from the neighbour-joining, maximum-parsimony and maximum-likelihood trees. GenBank/EMBL/DDBJ
9 accession numbers are given in parenthesis. The values at nodes indicate the number of methods where the
10 corresponding branch is supported.

11

12 **Fig. 3.** Consensus phylogenetic trees based upon the concatenation of the four selected genes. The analysis
13 was based upon an alignment of 4,125 homologous positions. The consensus tree was obtained from the
14 neighbour-joining, maximum-parsimony and maximum-likelihood trees. The values at nodes indicate the number
15 of methods where the corresponding branch is supported. Bootstrap values greater than 80% in the three
16 methods are shown by black circles in the consensus tree.

17

18 **Fig. S1.** Transmission electron micrograph showing the arrangement of the flagella, and cell morphology and
19 size of a) *Halomonas campaniensis* DSM 15293^{T}; b) *H. campisalis* ATCC 700597^{T}; c) *H. saccharevitans* LMG
20 23976^{T}; d) strain 4CR; e) strain HGDK1. Staining was done with uranyl acetate. Bar 1 μm.

21

22 **Fig. S2.** Consensus phylogenetic trees based on **a)**16S rRNA, **b)** *narH*, **c)** *nirS* and **d)** *nosZ* genes. The values at
23 nodes indicate the number of methods where the corresponding branch is supported. Bootstrap values greater
24 than 80% in the three methods are shown by black circles in the consensus tree.

25

Table1. Strains investigated in this study. Sequences FJ686133 to FJ686174 and GQ384036 to GQ384066 were obtained in this work.

Species	Strain designation	Original source	References	16S rRNA	*narH*	*nirS*	*nosZ*
H. alimentaria	DSM 15356^T	Jeotgal, a traditional Korean fermented seafood	[58]	AF211860**	FJ686135	FJ686149	FJ686163
H. campaniensis	DSM 15293^T	Mineral pool in the Campania region, Italy	[45]	AJ515365**	FJ686136	FJ686150	FJ686164
H. campisalis	ATCC 700597^T	Salt plain from Alkali Lake in Washington State, USA	[36]	AF054286	FJ686137	FJ686151	FJ686165
H. cerina	LMG 24145^T	Hypersaline soil from Santa Pola in Alicante, Spain	[20]	EF613112**	FJ686145	FJ686159	FJ686173
H. denitrificans	DSM 18045^T	Seawater at Anmyeondo, Korea	[26]	AM229317**	FJ686138	FJ686152	FJ686166
H. desiderata	DSM 9502^T	Water sample from sewage-treatment plant in Göttingen, Germany	[6]	X92417**	FJ686139	FJ686153	FJ686167
H. fontilapidosi	CECT 7341^T	Saline soil at Fuente de Piedra, Málaga, Spain	[21]	EU541349**	FJ686133	FJ686147	FJ686161
H. gudaonensis	LMG 23610^T	Saline soil contaminated with crude oil at Gudao, China	[57]	DQ421808**	FJ686141	FJ686154	FJ686168
H. halodenitrificans	CECT 5012^T	Meat-curing brines	[14]	L04942**	AB076402	FJ686155	FJ686169
H. koreensis	JCM 12237^T	Soil sample from a solar saltern in the Dangjin area, Korea	[31]	AY382579**	FJ686142	FJ686156	FJ686170
H. nitroreducens	CECT 7281^T	Soil sample from a solar saltern at Cahuil, Chile	[19]	EF613113**	FJ686134	FJ686148	FJ686162
H. saccharevitans	LMG 23976^T	Water sample from a salt lake on the Qinghai–Tibet plateau, China	[59]	EF144149**	ND	ND	ND
H. shengliensis	LMG 23897^T	Saline soil contaminated with crude oil from the Shengli oilfield at Shandong, China	[56]	EF121853**	FJ686144	FJ686158	FJ686172
H. ventosae	CECT 5797^T	Saline soil in Jaen, Spain	[33]	AY268080**	FJ686146	FJ686160	FJ686174
	R53[1] (LMG 24146)	Soil sample from Rambla Salada, Murcia, Spain	[20]	EF613110	GQ384044	GQ384052	GQ384060
	15CR[1] (LMG 24147)	Saline soil at Fuente de Piedra, Málaga, Spain	[20]	EF613111	GQ384037	GQ384046	GQ384055
	AI13[1] (CECT 7340)	Saline soil from Barranco de la Salina, Jaen, Spain	This study	EU541350	GQ384038	GQ384047	GQ384056
	C8	Soil sample from a solar saltern at Cahuil, Chile	This study	GQ384062	GQ384039	GQ384048	GQ384057
	F15	Saline soil at Fuente de Piedra, Málaga, Spain	This study	GQ384063	GQ384040	GQ384053	GQ384058
	HGD1	Hypersaline alkaline lake on the Altai Steppe, Siberia, Russia	This study	GQ384064	GQ384041	GQ384049	ND
	HGDK1[1]	Hypersaline alkaline lake on the Altai Steppe, Siberia, Russia	This study	GQ384066	GQ384042	GQ384050	ND

Table1. Continued.

Species	Strain designation	Original source	References	16S rRNA	*narH*	*nirS*	*nosZ*
	N64	Soil sample from a solar saltern at Sabinar, Almería, Spain	This study	GQ384065	GQ384043	GQ384051	GQ384059
	4CR	Saline soil at Fuente de Piedra, Málaga, Spain	This study	GQ384061	GQ384036	GQ384045	GQ384054
Hahella chejuensis	KCTC 2396^T	Marine sediment collected from Marado, in Cheju Island, Korea	[28]	AF195410	YP_435152	NC_007645*	YP_434563
Marinobacter hydrocarbonoclasticus	DSM 8798^T	Mediterranean seawater near a petroleum refinery, Gulf of Fos, France	[18]	X67022**	FJ686143	FJ686157	FJ686171
Pseudomonas aeruginosa	PA01	Various environments (ubiquitous microorganism)	[35]	NC_002516*	NP_252563	NP_249210	3797009-3798919
Pseudomonas fluorescens	C7R12	Rhizosphere of flax cultivated in the Châteaurenard, France	[30]	AM229082	AF197465	AF197466	AF056319
Pseudomonas stutzeri	DSM 5190^T	Clinical, spinal fluid; Copenhagen, Denmark	[29]	AJ308315**	AJ288126	X53676	M22628

* Sequences obtained from complete genome sequences of *Pseudomonas aeruginosa* strain PA01 [52] and from complete genome sequences of *Hahella chejuensis* KCTC 2396^T [24].

**The accession numbers correspond to the following type strains: *H. alimentaria*, YKJ-16; *H. campaniensis*, 5AG; *H. cerina*, SP4; *H. denitrificans,* M29; *H. desiderata*, FB2; *H. fontilapidosi*, 5CR; *H. gudaonensis,* SL014B-69; *H. halodenitrificans,* ATCC 13511^T; *H. koreensis*, SS20; *H. nitroreducens*, 11S; *H. saccharevitans,* AJ275; *H. shengliensis*, SL014B-85; *H. ventosae,* Al12; *Marinobacter hydrocarbonoclasticus,* ATCC 49840^T and *Pseudomonas stutzeri,* ATCC17588^T.

[1] Strain Al13 is assigned to *Halomonas denitrificans* and strain HGDK1 to *H. koreensis*, according to phenotypic, phylogenetic and genotypic analyses shown in this study. Strain R53 and 15CR belongs to *H. cerina* [20].

Table 2. Oligonucleotides used for amplification and sequencing the *narH*, *nirS* and *nosZ* genes.

Oligonucleotide[a]	Position[b]	Primer sequence (5′-3′)	Reference
PCR primers			
narH50F	43-59	AARTGYATCGGYTGCCA	[42]
narH1040R	1029-1043	GTNCGRTAYTCNGG	[42]
nirS1F	763–780	CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T	[8]
nirS6R [c]	1638–1653	CGTTGAACTT(A/G)CCGGT	[8]
nirS149F [c]	145	CTTCCTGGTCAACGTC	This study
nosZ661F [c]	661	CGGCTGGGGGCTGACCAA	[47]
nosZ1773R [c]	1773	ATRTCGATCARCTGBTCGTT	[47]
nosZ126F	126	CAACGACAAGDYCAA	This study
nosZ1527R	1527	CTGRCTGTCGADGAACAG	[47]
Sequencing primers			
narH444F	444	TTCATGATGTACCTGCCG	This study
nosZHal2F	661	CACGGCCTCAACACCTC	This study
nosZ555F	555	CAAGACTATTGGTGACTCC	This study
SP6	-	CGATTTAGGTGACACTATAG	Promega®
T7	-	TAATACGACTCACTATAGGG	Promega®

[a]The primers are indicated for the *nar*H, *nir*S and *nos*Z gene. Forward and reverse primers are indicated by the last letters F and R, respectively

[b]Positions in the *narH* gene of *Escherichia coli* (X16181), *nirS* and *nosZ* genes of *Pseudomonas stutzeri ZoBell* (X53676 and X65277, respectively), except for narH444F, nirS149F, nosZ126F, nosZHal2 and nosZ555F positions, which are related to the *narH* gene of *H. ventosae* (FJ686146), *nirS* gene of *H. desiderata* (FJ686153), *nosZ* genes of *H. ventosae* (FJ686174), *H. halodenitrificans* (FJ686169) and *Marinobacter hydrocarbonoclasticus* (FJ686171) respectively.

[c]The annealing temperature for the pair of primers nirS149F and nirS6R was 54ºC and for the *nosZ* gene in *H. alimentaria* and *H. desiderata*, it was more suitable to use 53.5ºC.

Figure 1

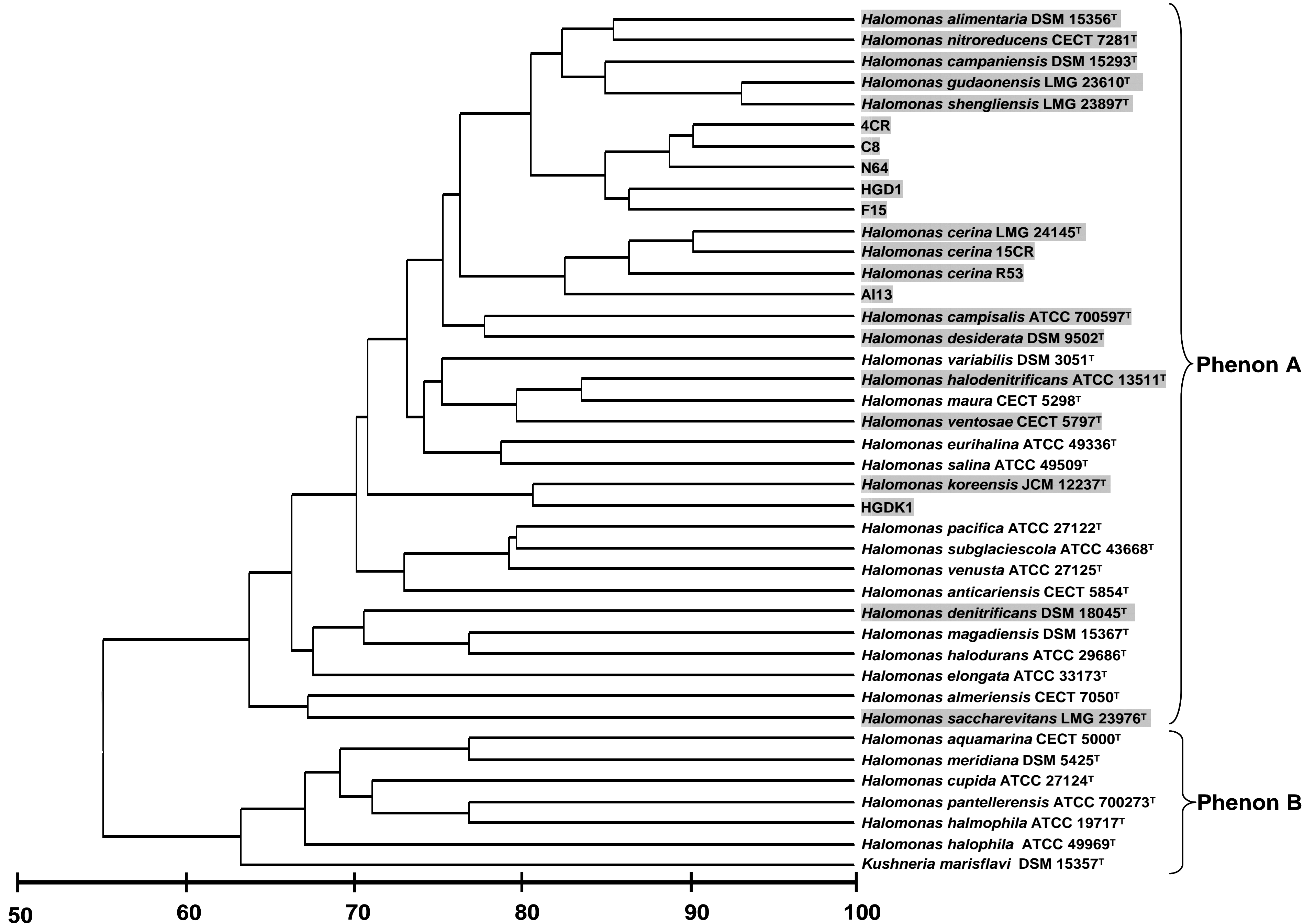
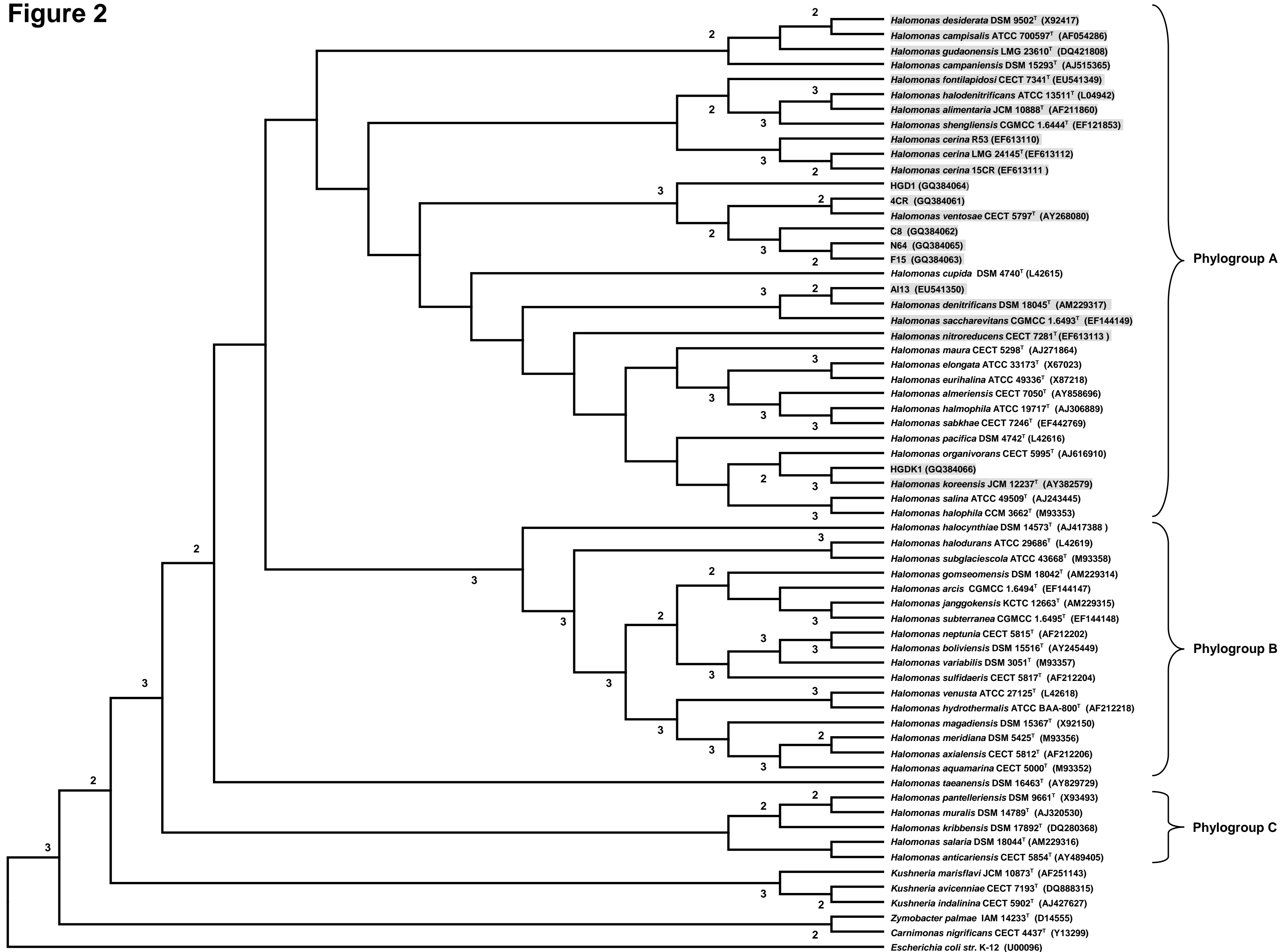


Figure 2



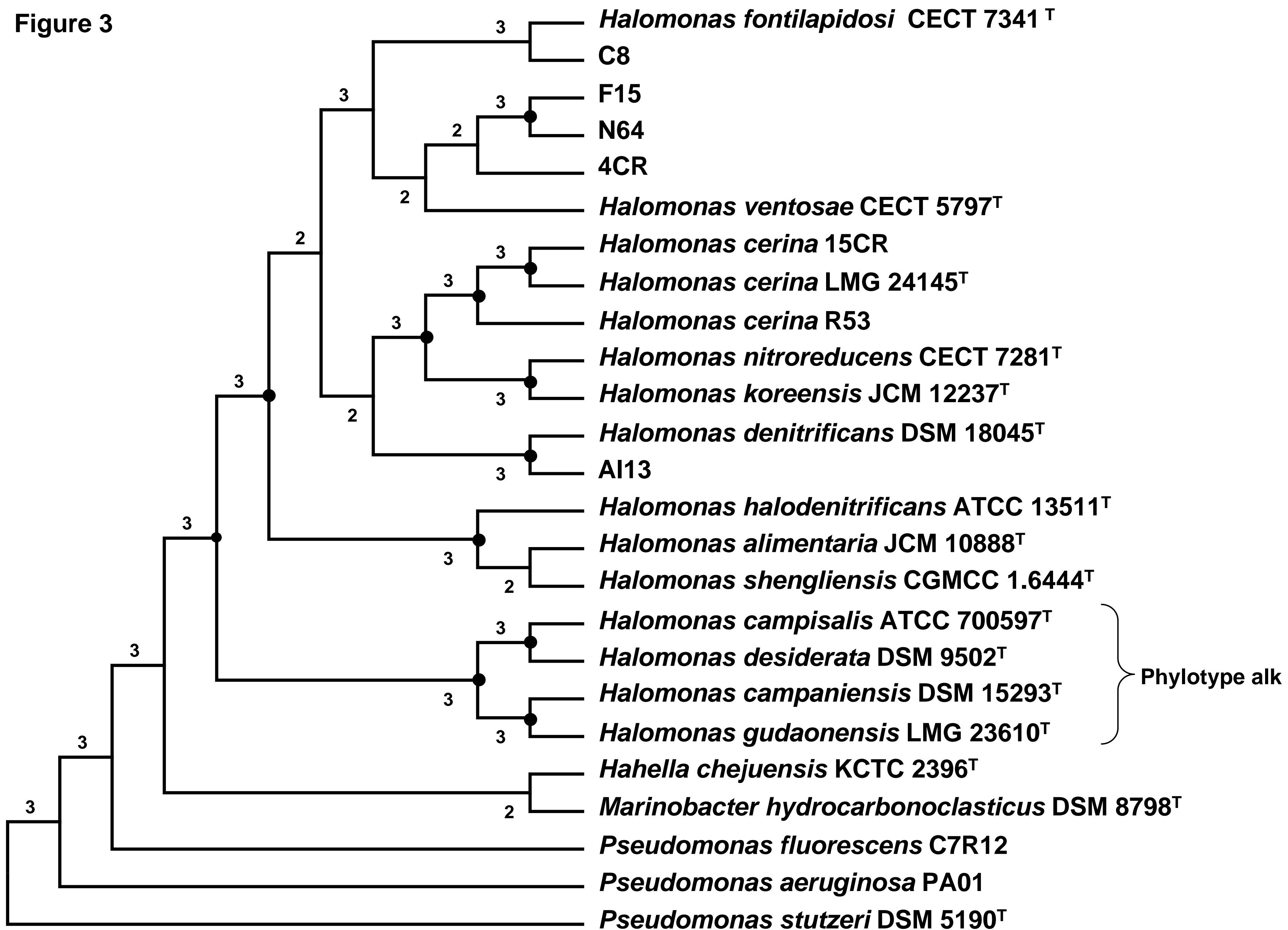


Figure 3
Halomonas fontilapidosi CECT 7341 T
C8
F15
N64
4CR
Halomonas ventosae CECT 5797T
Halomonas cerina 15CR
Halomonas cerina LMG 24145T
Halomonas cerina R53
Halomonas nitroreducens CECT 7281T
Halomonas koreensis JCM 12237T
Halomonas denitrificans DSM 18045T
Al13
Halomonas halodenitrificans ATCC 13511T
Halomonas alimentaria JCM 10888T
Halomonas shengliensis CGMCC 1.6444T
Halomonas campisalis ATCC 700597T
Halomonas desiderata DSM 9502T
Halomonas campaniensis DSM 15293T
Halomonas gudaonensis LMG 23610T
Phylotype alk
Hahella chejuensis KCTC 2396T
Marinobacter hydrocarbonoclasticus DSM 8798T
Pseudomonas fluorescens C7R12
Pseudomonas aeruginosa PA01
Pseudomonas stutzeri DSM 5190T

Table S1. Phenotypic characteristics distinguishing type strains of denitrifying species within the genus *Halomonas*

Species: **1**, *H. alimentaria;* **2**, *H.campaniensis;* **3**, *H. campisalis;* **4,** *H. cerina;* **5,** *H. denitrificans;* **6**, *H. desiderata*; **7**, *H. fontilapidosi*; **8,** *H. gudaonensis;* **9,** *H. halodenitrificans*; **10,** *H. koreensis;* **11,** *H. nitroreducens*; **12**, *H. saccharevitans*; **13,** *H. shengliensis*; **14,** *H. ventosae*. **+**, positive; -, negative; ND, not determined; I and D, intermediate and doubtful results respectively; EPS, extracellular polysaccharide production. Data from [6, 19, 20, 21, 26, 31, 33, 34, 45, 56, 57, 58, 59] and from this work. Data shaded in grey are not reported in the mentioned references; asterisks indicate results that are not in accordance with the previous descriptions.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Morphology	Coccus/ short rod	Straight rod	Rod	Short rod	Short rod	Short rod	Short rod	Short rod	Coccus/ short rod	Short rod	Short rod	Coccus/ short rod	Short rod	Short rod
Size (µm)	0.8-1.2 x 1.3-1.9	0.3-0.6 x 2.0-2.2	1.0 x 3.0-5.0	0.7-0.9 x 1.9-2.8	0.6-0.8 x 1.2-1.6	0.4-0.6 x 1.0-2.6	0.96 x 1.73	0.3-4.0 x 0.75-1.65	0.5-0.9 x 0.9-1.2	0.8-1.0 x 1.8-2.2	0.4-0.5 x 1.5-2.2	1–2 x 2–4	0.6-0.8 x 1.0-1.6	1.2-1.4 x 0.7-0.8
Pigmentation	Cream-yellow	Cream-pink	White	Wax-coloured	Brown-yellow	Cream	Cream-brown	Cream	Cream	Cream	Cream-white	Light yellow	Brown	Cream
EPS	-	-	-	+	-	-	-	-	-	-	+	+	-	+
Motility	-	+	+	-	+	+	+	+	-	+	-	+	+	+
Flagella	Absent	Polar	Polar	Absent	Peritrichous	Peritrichous	Polar	Lateral	Absent	Peritrichous	Absent	Polar	Lateral	Lateral
Sea-salt range (% w/v)	0-30*	0.5-25*	0.5-15	3-25	0-30*	0-20	3-25	0.5-30*	3-20	1-30*	0.5-30	0.5-15	0.5-30*	1-15
Sea-salt optimum (% w/v)	1-13	10	5	7.5-10	8-10	1-5	7.5-10	7.5-15	5-9	1-12	3-5	3-7.5	5-15	8
pH range	5-10	6-10*	8-11	5-10	5-10*	7-11	5-9	6-10*	5-10	5-10	5-10	5-10*	5-10*	6-10
pH optimum	6.5-7.5	7-9	9-10	7-8	6-9	9.5	6-8	8-10	7	5-8	7-9	7-8	7-8*	7-8
Temperature range (ºC)	4-45	4-45*	4-50	4-45	4-45	10-45	15-45	4-45*	5-37	15-45	4-45	4-48	4-45	15-50
Temperature optimum	30	37	30	20-32	25-35	37-42	32-45	30	32	35	20-32	30	30	32
Strictly halophilic	No	Yes*	Yes	Yes	No*	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Acids from:														
D-galactose	-	-	-	-	-	-	-	-	-	+*	-	-	-	-
D-glucose	-	-	-	-	-	-	-	-	-	+	-	-	-	-
sucrose	-	-	-	-	-	-	-	-	-	+*	-	-	-	-
Hydrolysis of:														
gelatine	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Tween 20	+	+	+	+	+	+	+	+	-	+	-	+	+	+
Tween 80	-	+	-	+	-	+	-	-	-	-	-	-	-	-
DNA	+	+	+	+	-	+	+	+	-	+	+	-	-	-
tyrosine	-	-*	-	+	+	+	+	-	+	+	+	-	-	+
tyrosine pigment	-	-	-	-	-	-	+	-	-	+	-	-	-	-
H_2S production	+	+	-	-	+*	+	+	+	+	+	+	-	+*	+
phosphatase	-	+	-	+	D	+	-	+	-	+	-	ND	+	-
lecithinase	-	-	-	-	-	-	+	-	-	-	-	-	-	-
urease	+	D	-	+	-	+	-	+	-	+	+	-	+	-
Gluconate oxidation	+	+	+	+	+	+	+	-	+	-	-	+	-	+
MacConkey agar growth	+	+	-	+	+	+	-	+	+	+	+	+	+	-
Cetrimide agar growth	-	-	-	-	+	+	-	+	-	+	+	+	+	-

Table S1. Continued.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Growth on [a]														
aesculin	-	-	-	-	-	-	-	-	+	-	-	ND	-	+
L-arabinose	+	-	-	-	-	+	+	-	-	ND	-	-	-	-
D-cellobiose	+	+	+	-	+	-	-	+*	-	+	-	-	+*	-
D-fructose	-	+	+	-	+	+	+	-	+	ND	+	-	-	+
D-galactose	+	-	-	-	+	-	+	+	+	-	+	-	+	+
D-glucose	+	+	+	+	-	+	+	+	+	+	+	-	+	+
lactose	+	-	-	-	+	+	+	+	-	-	+	-	+	+
maltose	+	+	+	+	-	+	+	+	-	ND	+	-	-	+
D-mannose	+	+	-	+	-	+	+	+	+	ND	+	-	+	-
D-salicin	-	-	-	+	+*	-	-	+	-	-	-	-	+	-
starch	+	+	+	-	+	+	+	+	-	+	+	ND	+	-
D-trehalose	+	+	+	-	+	+	-	+	+	+	+	-	+	+
D-xylose	+	-	-	ND	+	-	ND	-	+	-	D	-	+*	ND
acetate	+	+	+	+	+	+	-	+	+	+	+	-	+	+
citrate	+	+	+	+	+	-	+	+	+	+	+	-	-	+
formate	-	-	-	-	-	+	-	-	-	+	-	-	-	-
fumarate	+	+	+	+	+	+	-	+	-	+	+	+	+	+
gluconate	+	+	+	+	+*	+	+	+	+	+	+	-	+	+
malonate	+	-	-	-	+	+	-	+	+	-	-	-	+	+
propionate	+	+	+	-	+	-	-	+	+	+	+	-	+	+
succinate	+	+	+	+	+	+	+	+	+	+	+	-	+	+
adonitol	-	+	-	-	+	-	+	+	-	-	-	-	+	+
ethanol	+	-	-	+	+	-	-	+	+	+	+	-	+	-
glycerol	+	+	+	-	+	+	-	+	+	-	+	+	+	+
myo-inositol	-	+	+	-	+*	-	-	+	+	-	-	-	+*	+
D-mannitol	+	+	-	-	-	-	-	+	+	-	+	-	-	+
sorbitol	-	+	-	-	+*	-	+	+	+	-	+	-	+	+
L-alanine	+	+	+	-	+	-	-	+	+	+	+	+	+	-
L-histidine	+	+	-	-	-	-	-	+	-	+	+	-	-	-
DL-isoleucine	+	+	-	-	+	-	+	+	+	+	-	+	+	-
L-lysine	+	-	+	-	+	-	-	-	+	+	+	-	-	-
L-serine	+	+	+	+	+	+	+	+	+	+	+	+	+	-
L-valine	+	-	-	-	+	-	-	-	+	+	-	+	-	-
Susceptibility to														
erythromycin	+	+	+	+	I	+	ND	-	+	+	+	+	+	I
nalidixic acid	+	+	+	+	-	+	+	+	+	+	+	+	+	+
polymyxin B	+	+	+	+	-	+	-	+	+	+	+	+	+	+
rifampicine	+	+	+	+	+	+	+	+	+	+	+	-	+	+
streptomycin	-	-*	-	ND	-	+	ND	+	-	+	ND	-	+	+
tobramycin	-	+	+	-	-	+	+	-	+	+	-	-	-	-
trimetroprim-sulphametoxazol	+	+	+	+	-	+	+	+	+	+	+	+	+	+
DNA G+C content (mol%)	63.0	63.7	66.0	66.2	68.9*	66.0	65.7	64.0	64.0-66.0	70.0	65.37	65.9	66.6	72.6–74.6

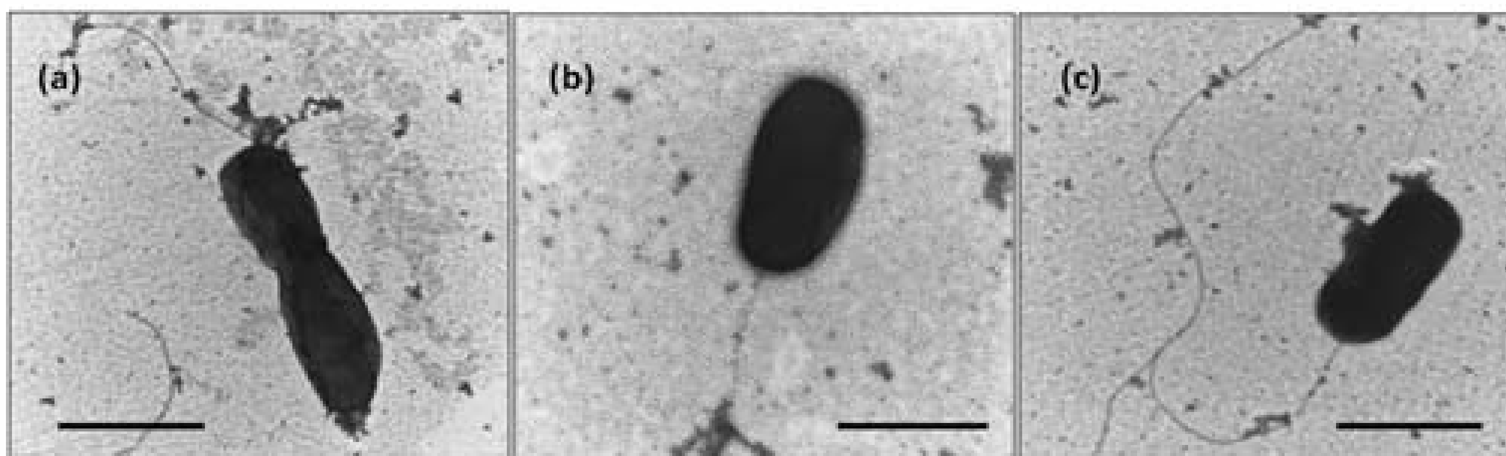
[a] When supplied as the sole source of carbon and energy, and carbon, nitrogen and energy.

Table S2. G+C content of the new denitrifying strains isolated in this study. The value shown is the average one of three measurements.

Strain	G+C content (mol %)
AI13	66,05±0,4
C8	67,41±0,2
F15	66,26±0,2
HGD1	66,00*
HGDK1	68,80*
N64	67,14±0,1
4CR	67,55±1,2

*Data provided by Dr. Sorokin. from TU Delft (Netherlands)

Figure S1



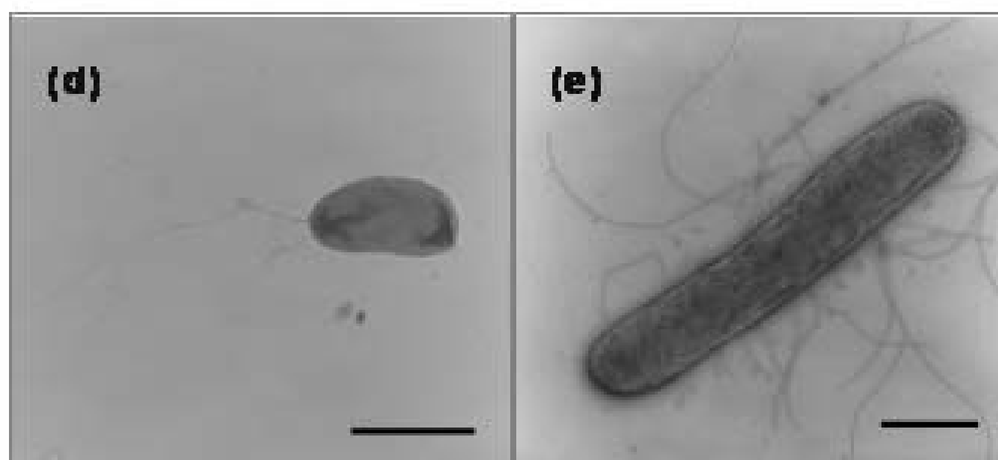


Figure S2

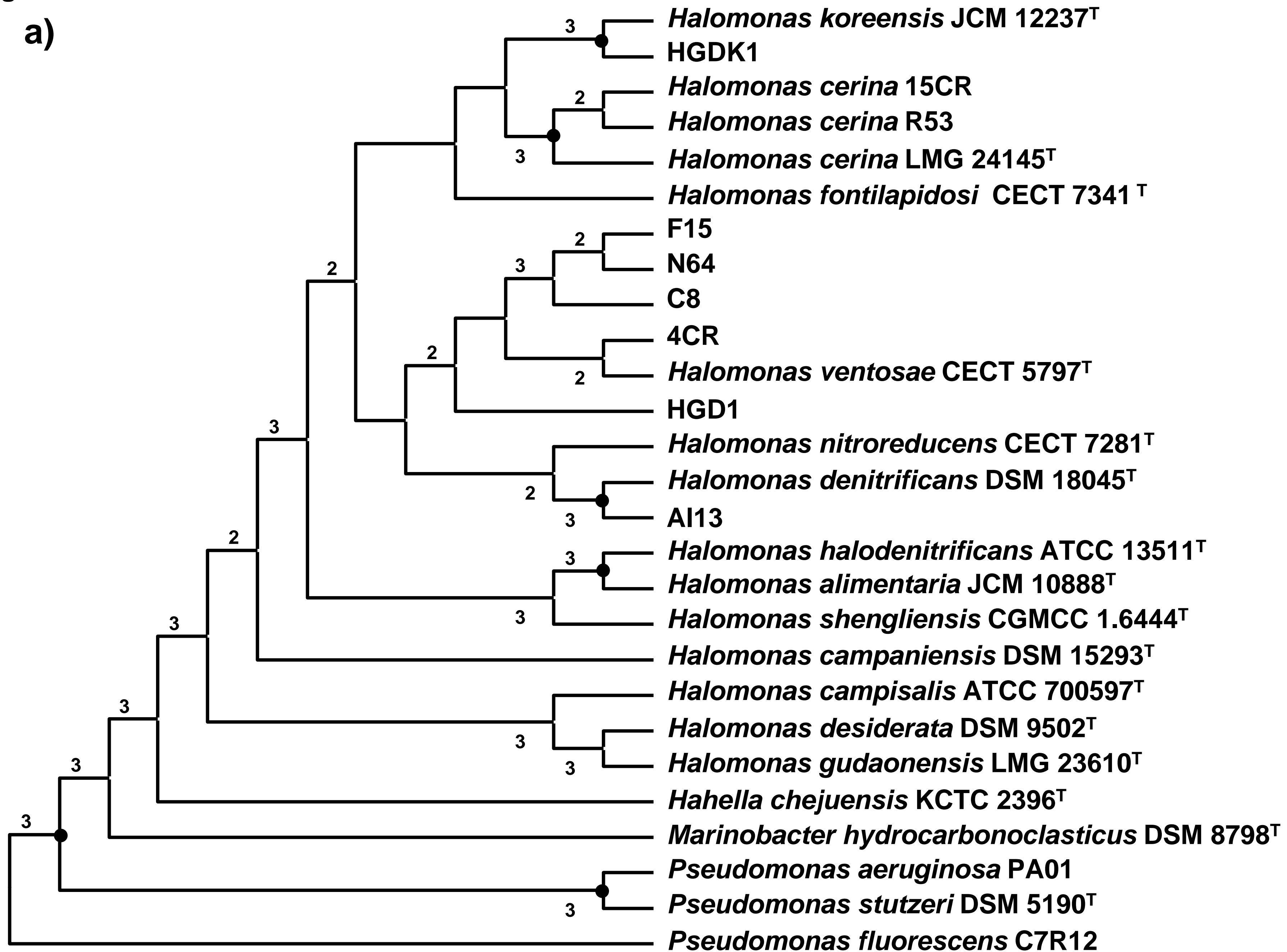


Figure S2

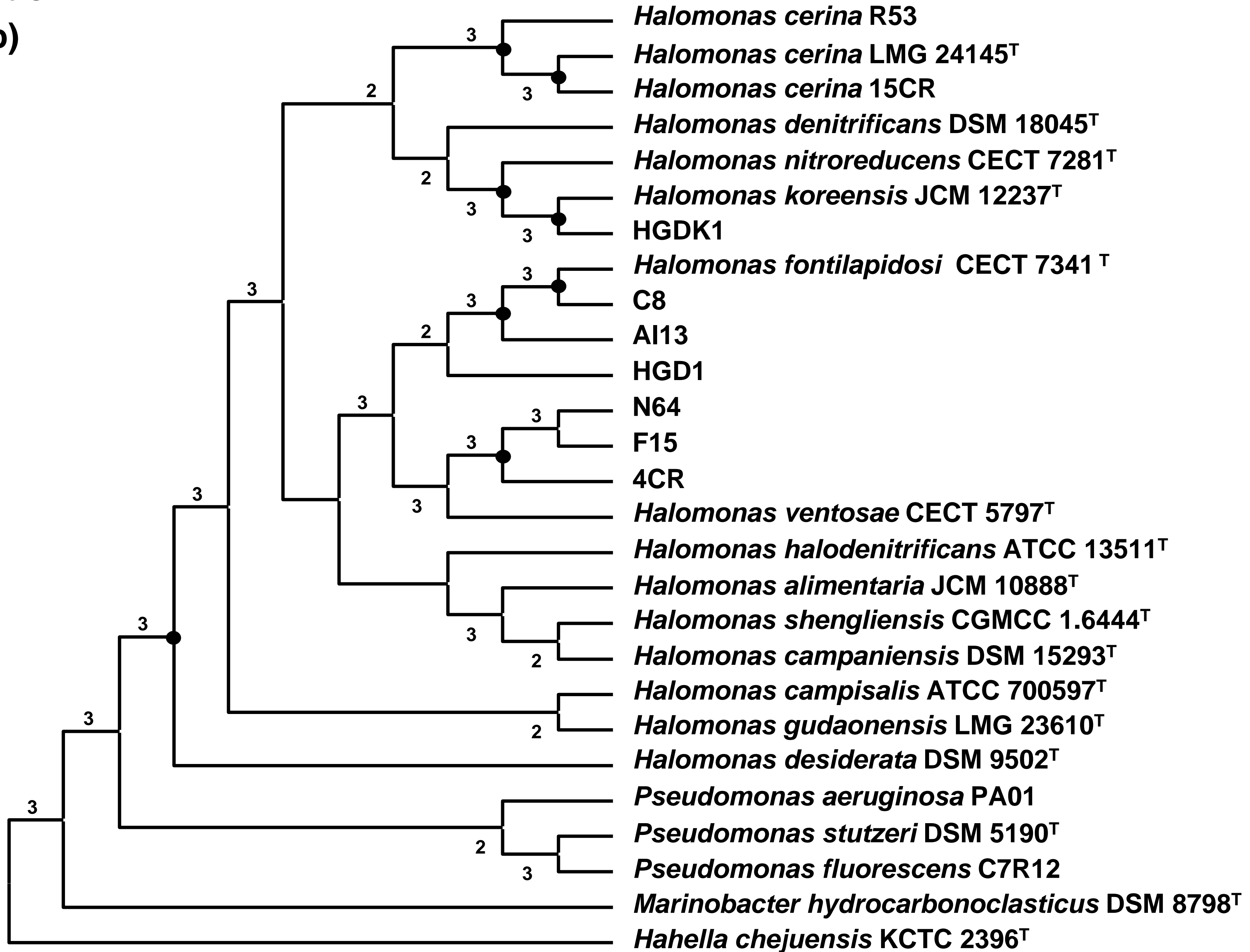
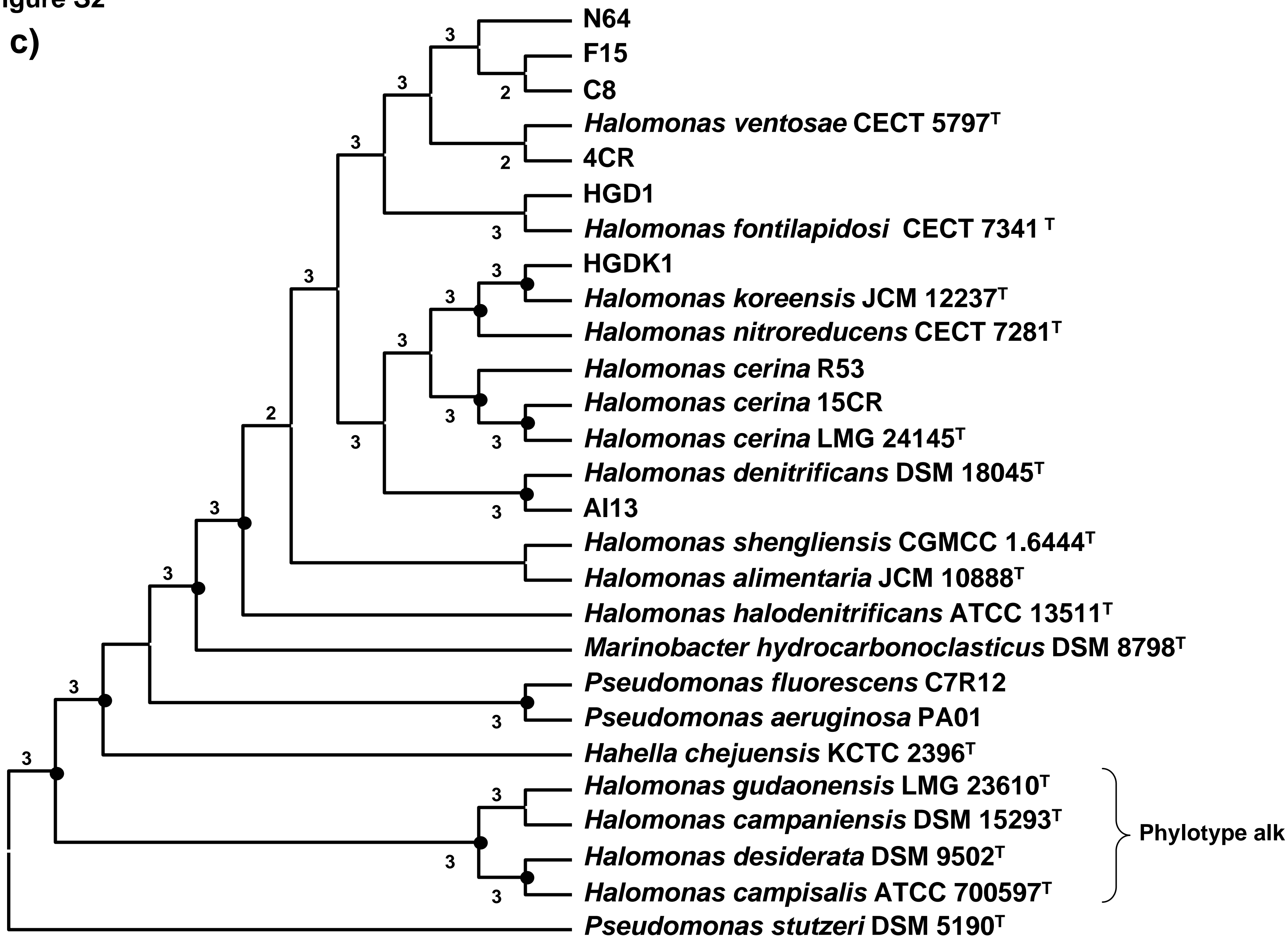


Figure S2



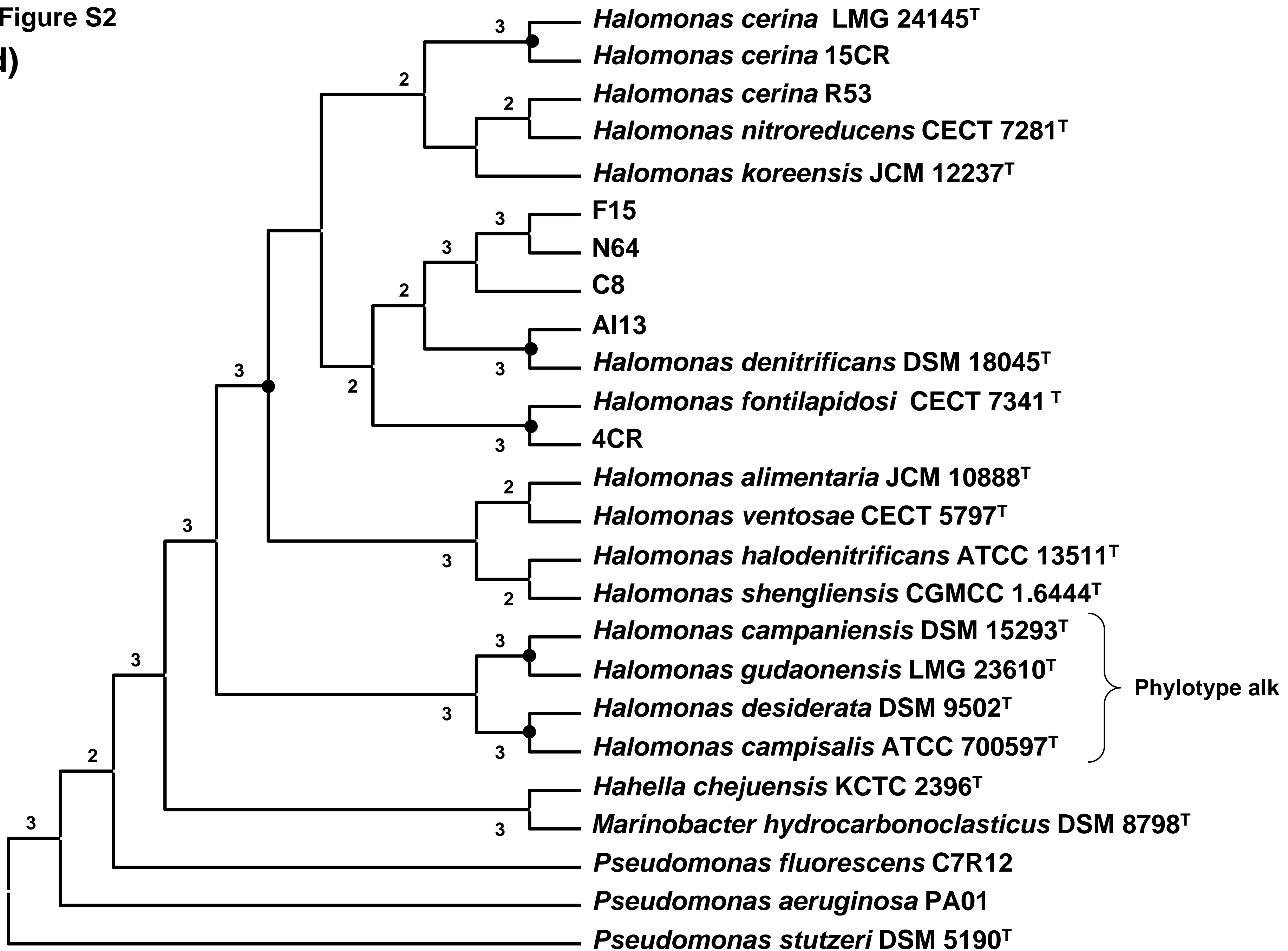


Figure S2
d)
Halomonas cerina LMG 24145T
Halomonas cerina 15CR
Halomonas cerina R53
Halomonas nitroreducens CECT 7281T
Halomonas koreensis JCM 12237T
F15
N64
C8
AI13
Halomonas denitrificans DSM 18045T
Halomonas fontilapidosi CECT 7341 T
4CR
Halomonas alimentaria JCM 10888T
Halomonas ventosae CECT 5797T
Halomonas halodenitrificans ATCC 13511T
Halomonas shengliensis CGMCC 1.6444T
Halomonas campaniensis DSM 15293T
Halomonas gudaonensis LMG 23610T
Halomonas desiderata DSM 9502T
Halomonas campisalis ATCC 700597T
Phylotype alk
Hahella chejuensis KCTC 2396T
Marinobacter hydrocarbonoclasticus DSM 8798T
Pseudomonas fluorescens C7R12
Pseudomonas aeruginosa PA01
Pseudomonas stutzeri DSM 5190T