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TAXONOMIC DESCRIPTION
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Halorubrum chaoviator Mancinelli *et al.* 2009 is a later, heterotypic synonym of *Halorubrum ezzemoulense* Kharroub *et al.* 2006. Emended description of *Halorubrum ezzemoulense* Kharroub *et al.* 2006

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Abstract

A polyphasic comparative taxonomic study of *Halorubrum ezzemoulense* Kharroub *et al.* 2006, *Halorubrum chaoviator* Mancinelli *et al.* 2009 and eight new *Halorubrum* strains related to these haloarchaeal species was carried out. Multilocus sequence analysis using the five concatenated housekeeping genes *atpB*, *EF-2*, *glnA*, *ppsA* and *rpoB′*, and phylogenetic analysis based on the 757 core protein sequences obtained from their genomes showed that *Hrr. ezzemoulense* DSM 17463^T, *Hrr. chaoviator* Halo-G^{*T} (=DSM 19316^T) and the eight *Halorubrum* strains formed a robust cluster, clearly separated from the remaining species of the genus *Halorubrum*. The orthoANI value and digital DNA–DNA hybridization value, calculated by the Genome-to-Genome Distance Calculator (GGDC), showed percentages among *Hrr. ezzemoulense* DSM 17463^T, *Hrr. chaoviator* DSM 19316^T and the eight *Halorubrum* strains ranging from 99.4 to 97.9 %, and from 95.0 to 74.2 %, respectively, while these values for those strains and the type strains of the most closely related species of *Halorubrum* were 88.7–77.4 % and 36.1–22.3 %, respectively. Although some differences were observed, the phenotypic and polar lipid profiles were quite similar for all the strains studied. Overall, these data show that *Hrr. ezzemoulense*, *Hrr. chaoviator* and the eight new *Halorubrum* isolates constitute a single species. Thus, *Hrr. chaoviator* should be considered as a later, heterotypic synonym of *Hrr. ezzemoulense*. We propose an emended description of *Hrr. ezzemoulense*, including the features of *Hrr. chaoviator* and those of the eight new isolates.

The genus *Halorubrum* is classified within the family *Halorubraceae*, order *Haloferacales*, class *Halobacteria* [1, 2]. Currently this genus includes 37 species with validly published names, isolated from diverse hypersaline habitats, such as saline and soda lakes, salterns or saline soils, as well as from rock salt and salted food [3, 4]. Divergence patterns leading to speciation of *Halorubrum* populations have been previously studied based on phylogenetic, genomic and fingerprinting analyses [5, 6]. Recently, we carried out a study of 25 isolates belonging to the genus *Halorubrum* that were obtained from different hypersaline environments, and they were compared with the type strains of species of *Halorubrum* by using several taxonomic approaches: comparative 16S rRNA gene sequence analysis, multilocus sequence analysis (MLSA) based on the comparison of *atpB*, *EF-2*, *glnA*, *ppsA* and *rpoB′* housekeeping genes, average nucleotide identity (ANI), conventional DNA–DNA hybridization (DDH) and polar lipid profiles [7]. This study showed that several *Halorubrum* isolates, designated as phylogroup 1, clustered together and showed common features with the two species *Halorubrum ezzemoulense* and *Halorubrum chaoviator* [7]. *Hrr. ezzemoulense* was described by Kharroub *et al.* in 2006 [8] on the basis of the features of a single strain (designated 5.1^T), isolated from a water sample of Ezzemoul sabkha in Algeria, while *Hrr. chaoviator* was described by Mancinelli *et al.* in 2009 [9], based on the features of strain Halo-G^{*T}, isolated from an evaporitic salt crystal from the coast of Baja California, Mexico, and two additional strains isolated from a salt pool in Western Australia and a salt lake on the island of Naxos in Greece,

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Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization; GGDC, Genome-to-Genome Distance Calculator; GTR, general time reversible; HPTLC, high-performance thin-layer chromatography; JTT, Jones, Taylor, Thornton model; MLSA, multilocus sequence analysis; OGRI, overall genome relatedness indexes; TIM, transitional model.
Two supplementary tables and one supplementary figure are available with the online version of this article.

respectively. Our recent comparative study on the new isolates and the species of *Halorubrum* indicated that *Hrr. ezzemoulense* DSM 17463^T and *Hrr. chaoviator* Halo-G*^{T} (=DSM 19316^T) constitute a single species together with eight of the new isolates. In this paper we have compared in detail the type strains of both species of *Halorubrum* as well as eight representative strains of our previous study that were closely related to these species, in order to carry out a comprehensive polyphasic taxonomic study, which supports that *Hrr. chaoviator* should be considered as a later heterotypic synonym of *Hrr. ezzemoulense*, and that the eight new isolates are members of the species *Hrr. ezzemoulense*, for which we propose an emended description.

In this study we used the following type strains obtained from culture collections: *Hrr. ezzemoulense* DSM 17463^T and *Hrr. chaoviator* DSM 19316^T, as well as *Hrr. chaoviator* Halo-G*^{T} and the *Halorubrum* sp. strains C191, Ec15, Fb21, G37, Ga2p, Ga36, SD612 and SD683. The first six of the *Halorubrum* sp. strains were isolated from the hypersaline lake Aran-Bidgol, Iran, and the last two were obtained from water samples of a saltern in the Namib desert as previously described [7]. They were routinely cultured in modified SW20 medium [10] with 20 % (w/v) total salts, prepared using a salt mixture designated as SW 30 % (w/v) stock solution [11], which consists of (per litre): 234 g NaCl, 39 g $MgCl_2.6H_2O$, 61 g $MgSO_4.7H_2O$, 1 g $CaCl_2$, 6 g KCl, 0.2 g $NaHCO_3$ and 0.7 g NaBr. This solution was supplemented with 0.5 % (w/v) yeast extract (Difco) and 0.5 % (w/v) casamino acids. The pH was adjusted to 7.2 with 1 M KOH and the cultures were incubated at 37 °C. For solid media, 2.0 % (w/v) agar was used when necessary. The strains were maintained on the same medium in slant tubes, and for long-term preservation they were prepared as cryotubes for freezing at −80 °C as suspensions with 15 % glycerol [7].

The 16S rRNA and MLSA phylogenetic analyses were carried out as previously described [7]. The 16S rRNA gene nucleotide sequence of the strains was assembled with ChromasPro software version 1.5 and aligned using the ARB 6.0.5 software package [12]. Sequence similarities were determined by comparing the 16S rRNA gene sequence of *Hrr. ezzemoulense* CECT 7099^T and *Hrr. chaoviator* Halo-G*^{T} as well as the eight *Halorubrum* sp. isolates with known sequences of the *Halorubrum* species shown in Table S1 (available in the online version of this article), using ARB 6.0.5 and the EzBioCloud tool (http://www.ezbiocloud.net/identify; [13]). Analysis based on the almost-complete 16S rRNA gene sequences revealed the levels of similarity (Table S2). The 16S rRNA gene sequences of *Hrr ezzemoulense* CECT 7099^T and *Hrr. chaoviator* Halo-G*^{T} showed 99.7 % similarity; in addition, these two strains and all the eight new isolates showed levels of similarity in the range 99.6–100 %. Similarities equal to or lower than 99.4 % were obtained between those strains with the type strains of other species of *Halorubrum* and other haloarchaeal genera. Phylogenetic study based on 16S rRNA gene sequence comparisons was performed by reconstructing trees using the neighbour-joining [14], maximum-parsimony [15] and maximum-likelihood [16] algorithms with the ARB program package version 6.0.5 [12]. Maximum-likelihood analysis was performed using the Transitional Model 2 of nucleotide substitution with invariable sites, rate variation among sites and unequal base frequencies (TIM2+I+G+F) [17]. Base-frequency filters were applied in the sequence comparison analysis and the effects on the results were evaluated. To evaluate the robustness of the tree, a bootstrap analysis (1000 replications) was performed [18]. The inferred tree based on the 16S rRNA gene reconstructed with the maximum-likelihood algorithm showed that the eight *Halorubrum* sp. strains clustered with *Hrr. ezzemoulense* CECT 7099^T, *Hrr. chaoviator* Halo-G*^{T}, as well as with *Halorubrum californiense* SF3-213^T (Fig. 1). Bootstrap values were low in all cases. The topologies of the trees reconstructed using the neighbour-joining and maximum-parsimony algorithms were highly similar to that of the maximum-likelihood tree. As previously indicated, a comparison of 16S rRNA gene sequences does not allow us to determine in-depth phylogenetic relationships within the genus *Halorubrum* and thus an MLSA approach based on a comparison of partial sequences of the *atpB* (ATP synthase subunit B), *EF-2* (elongation factor 2), *glnA* (glutamine synthetase), *ppsA* (phosphoenolpyruvate synthase) and *rpoB′* (RNA polymerase subunit B′) housekeeping genes (Table S1) has been recently recommended for this genus [7]. PCR cycling conditions and amplification and sequencing primers for these genes are described elsewhere [6, 7]. Lengths of the resulting multiple alignments were 496, 507, 526, 514 and 522 bp for the *atpB*, *EF-2*, *glnA*, *ppsA* and *rpoB′* genes, respectively, with the concatenation of the five genes yielding a final alignment of 2565 bp. Fig. 2 shows the phylogenetic tree obtained by concatenation of these five housekeeping genes, reconstructed via the maximum-likelihood algorithm using the GTR+I+G substitution model, as implemented in PhyML version 3.1 [19]. This tree shows a better phylogenetic separation of the species of *Halorubrum*, and the eight *Halorubrum* sp. isolates are shown to constitute a cluster with the type strains of *Hrr. ezzemoulense* and *Hrr. chaoviator*. The percentage similarity of the five concatenated gene sequences between *Hrr. ezzemoulense* and *Hrr. chaoviator* was 99.7 % and those of these two species and the other eight related strains varied from 98.8 to 99.8 % and from 98.9 to 99.8 %, respectively. Overall, the percentages of MLSA similarity of the two *Halorubrum* species and the eight isolated strains that constitute a single cluster ranged from 98.8 to 99.8 % (Table S2).

To increase the resolution, we carried out a phylogenetic analysis based on the 757 core protein sequences obtained from the available genomes of *Hrr. ezzemoulense* DSM 17463^T, *Hrr. chaoviator* DSM 19316^T, the eight *Halorubrum* strains and the type strains of other related *Halorubrum* species (Table S1). All predicted protein sequences NCBI-annotated from each available genome were compared using an all-versus-all BLAST search by using the enveomic tool [20]. This analysis identified reciprocal best matches

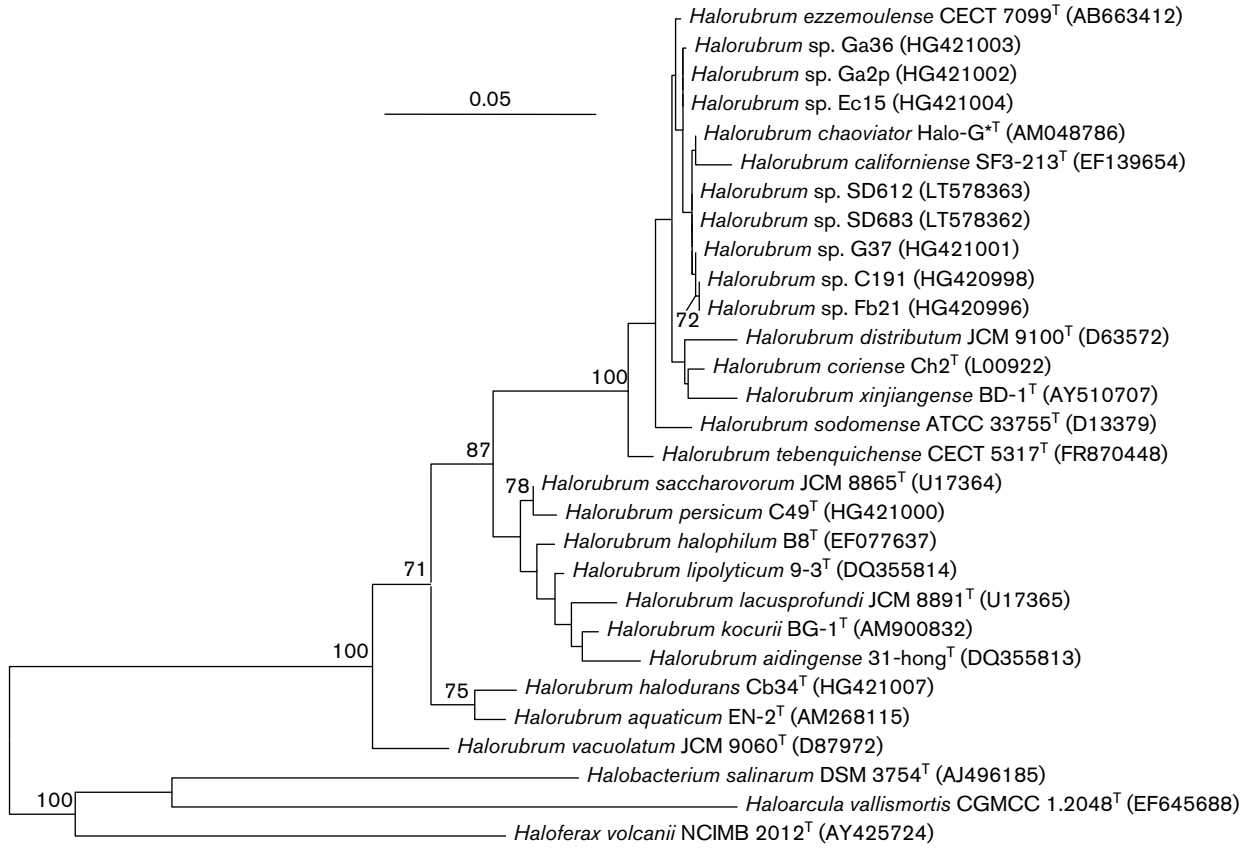

Fig. 1. Maximum-likelihood phylogenetic tree based on a comparison of 16S rRNA gene sequences showing the relationship between *Hrr. ezzemoulense* CECT 7099^T, *Hrr. chaoviator* Halo-G*^{T}, the new eight *Halorubrum* strains and other related species of the genus *Halorubrum* and other haloarchaea. The accession numbers of the sequences used are shown in parentheses after the strain designation. Bootstrap values (%) based on 1000 replicates are shown for branches with more than 70 % bootstrap support. The species *Haloarcula vallismortis*, *Haloferax volcanii* and *Halobacterium salinarum* were used as outgroups. Bar, 0.05 substitutions per nucleotide position.

(defined as >40 % amino acid identity) in all pairwise genome comparisons of the ten *Halorubrum* strains and the type strains of related *Halorubrum* species. From all those pairwise reciprocal best match proteins, the 757 shared proteins present in all the analysed genomes were selected to constitute the core orthologues. These core orthologous proteins were individually aligned using MUSCLE [21]. The resulting protein alignments were concatenated to create a core-protein alignment consisting of 250 398 amino acids, and the phylogenomic tree was reconstructed by the neighbour-joining method with the JTT model of amino acid substitution [22], as implemented in MEGA 5 [23]. As shown in Fig. 3, the overall topology of the phylogenetic tree was in agreement with the MLSA tree. The two *Halorubrum* species, *Hrr. ezzemoulense* DSM 17463^T and *Hrr. chaoviator* DSM 19316^T, and the eight *Halorubrum* strains formed a well-defined cluster, separate from the remaining species of the genus *Halorubrum*.

The use of Overall Genome Relatedness Indexes (OGRI), such as ANI and digital DDH, is currently recommended for delineation of prokaryotic species [24–29] and minimal standards have been recently reported [29]. The orthoANI percentages, determined according to Lee *et al.* [30] on the basis of a comparison of the genome sequences of *Hrr. ezzemoulense* DSM 17463^T, *Hrr. chaoviator* DSM 19316^T and the eight new *Halorubrum* isolates, indicate that the cluster formed by these strains has an ANI range of 99.4–97.9 %, while the range with respect to the type strains of related species of the genus *Halorubrum* was 88.7–77.4 % (Table 1). The threshold of 95–96 % defined for species delineation [24, 25, 29] clearly supports the placement of these strains within a single species.

On the other hand, we also calculated the digital DDH values, determined online (http://ggdc.dsmz.de/distcalc2.php) using the Genome-to-Genome Distance Calculator (GGDC) version 2.0 as described by Meier-Kolthoff *et al.* [27]. Estimated digital DDH values were calculated using formula 2 at the GGDC website, originally described by Auch *et al.* [26] and updated by Meier-Kolthoff *et al.* [27]. The GGDC values among *Hrr. ezzemoulense* DSM 17463^T,

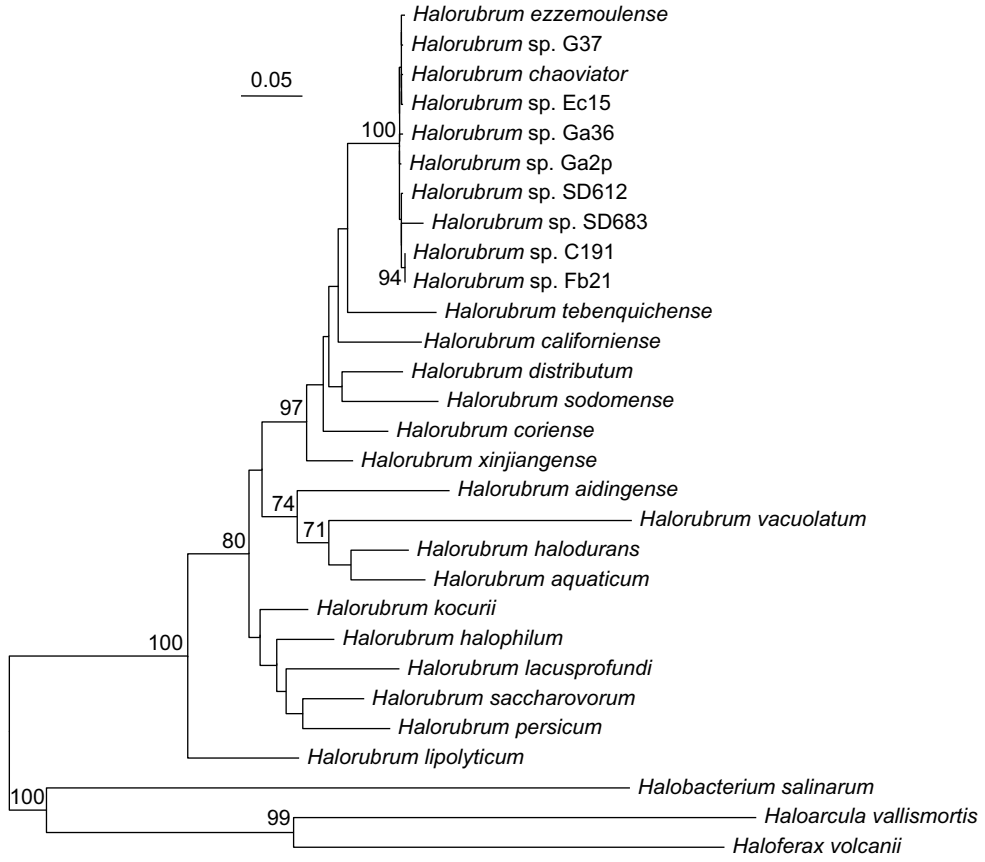

Fig. 2. Maximum-likelihood phylogenetic tree based on concatenated sequences of the five-housekeeping genes (*atpB*, *EF-2*, *glnA*, *ppsA* and *rpoB'*) showing the relationship between *Hrr. ezzemoulense*, *Hrr. chaoviator*, the new eight *Halorubrum* strains and other related species of the genus *Halorubrum* and other haloarchaea. The accession numbers of the sequences used are shown in Table S1. Bootstrap values >70 % are indicated. The species *Haloarcula vallismortis*, *Haloferax volcanii* and *Halobacterium salinarum* were used as outgroups. Bar, 0.05 substitutions per nucleotide position.

Hrr. chaoviator DSM 19316^T and the eight new *Halorubrum* strains ranged from 95.0 to 74.2 %, but the values among these strains and the type strains of related species of the genus *Halorubrum* were 36.1–22.3 % (Table 1). These percentages are lower than the 70 % cut-off established for species delineation [27, 29], and thus show unequivocally that the strains under study constitute a single species of *Halorubrum*, clearly separated from the remaining species of this genus. These data are in agreement with our recent study [7], showing an experimental DDH percentage of relatedness between *Hrr. ezzemoulense* DSM 17463^T and *Hrr. chaoviator* Halo-G*^{T} of 79 %, in contrast to the previously reported percentage of 39 % [9], in both cases using the same DDH competition procedure of the membrane filter method [7, 9].

Phenotypic characterization was carried out using standard taxonomic methods following the proposed minimal standards for *Halobacteria* recommended by Oren *et al.* [31]. Cell morphology and motility were examined in liquid medium after 7 days of growth by optical and phase-contrast microscopy (BX41; Olympus). Gram staining was performed using acetic-acid-fixed samples, as described by Dussault [32]. The growth and optimum requirements for NaCl, Mg^{2+}, pH and temperature were determined in routine modified SW20 medium, changing the recipe to test growth at different compound concentrations [33]. The range of NaCl (5–30 %, w/v) was tested at intervals of 5 %. The range of tolerance to Mg^{2+} was tested using $MgCl_2$ (0–10 %, w/v) at intervals of 1 % (w/v). Routine cultivation was performed at 37 °C and pH 7.5. The pH range for growth was assayed at pH 5.5–10.0, at intervals of 0.5 pH units, in liquid modified SW20 medium with various pH buffers: MES (pH 5.5–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5), CHES (pH 9.0–9.5) or CAPS (pH 10.0), each at a concentration of 50 mM. The range and optimum temperatures were determined by incubating at 4, 10, 20, 30, 37 and 45 °C in modified SW20 medium with optimal NaCl and Mg^{2+} concentrations and pH.

All phenotypic tests were carried out using the modified SW20 medium prepared with 20 % (w/v) total salts, at pH

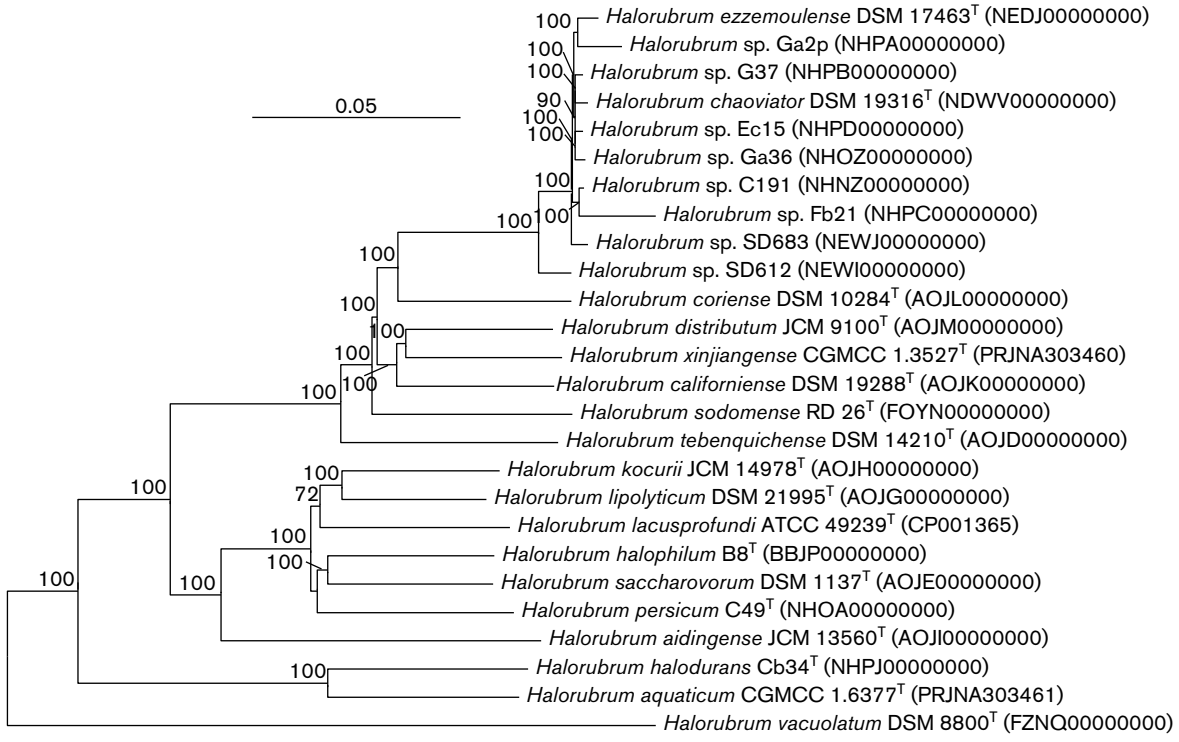

Fig. 3. Neighbour-joining core protein phylogenetic tree including the genomes of *Hrr. ezzemoulense*, *Hrr. chaoviator*, the eight new *Halorubrum* strains and other related species of the genus *Halorubrum*. This tree was based on the JTT distance calculated from the alignment of the translated 757 shared orthologous single-copy genes of these genomes. All genomes were retrieved from GenBank (Table S1). Bootstrap values over 70 % (based on 1000 pseudoreplicates) are shown above the branch. Bar, 0.05 substitutions per nucleotide position.

7.5 and at 37 °C. The type strain of the type species of *Halorubrum*, *Halorubrum saccharovorum* JCM 8865^T, was used as a reference for comparative purposes. Anaerobic growth was tested in the presence of nitrate and L-arginine by adding to the medium 3 % (w/v) KNO_3 or 4 % L-arginine, respectively, in filled stoppered tubes, as well plates of cultures incubated for 10 days at 37 °C in an anaerobic jar [31]. Catalase activity was determined by adding a 1 % (v/v) H_2O_2 solution to colonies on solid medium. The oxidase test was performed using a DrySlide assay (Difco). Hydrolysis of starch, gelatin, aesculin, casein, DNA and Tween 80 was determined as described by Barrow and Feltham [34]. Tests for indole production from tryptophan and urea hydrolysis were performed as described by Gerhardt *et al.* [35]. The methyl red, Voges–Proskauer and Simmons citrate tests were performed as described by Oren *et al.* [31]. H_2S formation was determined by monitoring the production of a black sulfide precipitate in modified SW20 medium containing 0.5 % (w/v) sodium thiosulfate, and the reduction of nitrate was detected by using sulfanilic acid and α-naphthylamine reagents [36]. To determine the utilization of different organic substrates such as carbohydrates, alcohols, amino acids and organic acids as the only source of carbon and energy, growth on medium containing 0.05 % (w/v) yeast extract and supplemented with 1 % (w/v) of the tested substrate (sterilized separately) was assessed as described by Ventosa *et al.* [37]. *Hrr. ezzemoulense* DSM 17463^T, *Hrr. chaoviator* DSM 19316^T and the eight new *Halorubrum* isolates were Gram-stain-negative motile rods, producing red-pigmented colonies. They were catalase- and oxidase-positive, not able to produce indole, nor able to hydrolyse gelatin, casein, DNA, aesculin or Tween 80. Voges–Proskauer, methyl red and urease tests were negative. The phenotypic features that showed variable results for the strains studied and their differential characteristics with respect to the type species of the genus *Halorubrum*, *Hrr. saccharovorum,* are shown in Table 2. Other phenotypic features are included in the emended description of the species.

For polar lipid analyses, cell biomass of the strains was obtained after 10 days of aerobic incubation in modified SW20 liquid medium under optimal conditions: 20 % (w/v) NaCl, 37 °C and pH 7.5. Polar lipids were extracted with chloroform/methanol following the method for extraction of membrane polar lipids of halophilic archaea previously described by Corcelli *et al.* [38]; the extracts were carefully dried using a SpeedVac Thermo Savan SPD111V device before weighing and then dissolved in chloroform to obtain

Table 1. OrthoANI (upper triangle in bold) and GGDC (lower triangle) values among the genomes of *Hrr. ezzemoulense* DSM 17463^T, *Hrr. chaoviator* DSM 19316^T and the eight new *Halorubrum* strains, as well as the type strains of related species of the genus *Halorubrum*

The main diagonal of the matrix is highlighted in grey. Genome accession numbers are presented in Table S1.

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1. *Hrr. ezzemoulense*	100	**98.8**	**98.8**	**99.0**	**98.7**	**99.0**	**98.5**	**98.8**	**98.2**	**98.8**	**81.5**	**81.0**	**88.1**	**88.4**	**88.0**	**80.8**	**82.9**	**82.7**	**81.7**	**82.9**	**82.6**	**82.6**	**87.2**	**86.0**	**77.8**	**87.3**
2. *Hrr. chaoviator*	90.2	100	**98.7**	**99.0**	**98.7**	**98.9**	**98.5**	**98.9**	**98.2**	**98.8**	**81.4**	**80.8**	**87.8**	**88.3**	**87.9**	**81.0**	**83.0**	**82.7**	**81.9**	**82.7**	**82.6**	**82.7**	**87.3**	**86.1**	**78.0**	**87.6**
3. *Halorubrum* sp. C191	89.3	88.5	100	**98.9**	**99.4**	**98.8**	**98.5**	**99.0**	**98.2**	**98.8**	**81.4**	**81.0**	**87.9**	**88.5**	**88.0**	**80.9**	**83.0**	**82.9**	**81.9**	**82.6**	**82.2**	**82.4**	**87.1**	**86.0**	**77.8**	**87.4**
4. *Halorubrum* sp. Ec15	91.1	91.1	89.9	100	**98.7**	**99.1**	**98.8**	**99.1**	**98.3**	**98.9**	**81.5**	**80.7**	**88.2**	**88.6**	**87.9**	**80.8**	**82.7**	**82.8**	**81.9**	**82.8**	**82.6**	**82.6**	**87.1**	**86.1**	**77.6**	**87.5**
5. *Halorubrum* sp. Fb21	89.1	89.0	95.0	89.4	100	**98.7**	**98.4**	**98.8**	**98.0**	**98.6**	**81.2**	**80.6**	**88.0**	**88.5**	**87.8**	**80.8**	**82.8**	**82.6**	**82.2**	**82.6**	**82.3**	**82.2**	**86.8**	**85.9**	**77.8**	**87.3**
6. *Halorubrum* sp. G37	91.1	90.9	90.4	92.0	89.6	100	**98.7**	**99.1**	**98.3**	**98.7**	**81.3**	**80.7**	**87.9**	**88.5**	**87.9**	**80.9**	**83.0**	**83.0**	**82.1**	**82.7**	**82.6**	**82.7**	**87.0**	**85.8**	**77.7**	**87.3**
7. *Halorubrum* sp. Ga2p	90.0	89.9	89.1	92.6	88.8	91.3	100	**98.8**	**97.9**	**98.5**	**81.1**	**80.5**	**87.7**	**88.2**	**87.4**	**80.5**	**82.7**	**82.7**	**81.9**	**82.5**	**82.1**	**82.3**	**87.0**	**85.6**	**77.4**	**86.9**
8. *Halorubrum* sp. Ga36	90.6	90.0	90.8	91.9	89.7	92.6	91.6	100	**98.3**	**98.8**	**81.6**	**80.8**	**88.0**	**88.5**	**87.8**	**80.7**	**83.0**	**82.8**	**82.0**	**82.9**	**82.5**	**82.4**	**87.3**	**86.2**	**77.7**	**87.4**
9. *Halorubrum* sp. SD612	75.2	74.9	75.1	75.8	74.2	75.6	75.8	75.5	100	**98.5**	**81.5**	**80.4**	**88.2**	**88.7**	**88.0**	**80.9**	**83.0**	**82.5**	**82.0**	**82.8**	**82.6**	**82.7**	**87.2**	**86.3**	**77.7**	**87.9**
10. *Halorubrum* sp. SD683	89.4	89.9	89.3	90.6	88.6	90.6	89.5	90.0	76.2	100	**81.2**	**80.4**	**87.9**	**88.7**	**87.8**	**80.7**	**82.7**	**82.3**	**81.8**	**82.7**	**82.3**	**82.4**	**87.1**	**86.1**	**77.4**	**87.4**
11. *Hrr. aidingense*	25.1	25.0	25.1	25.0	25.0	25.2	25.3	25.0	25.6	25.1	100	**80.5**	**81.7**	**81.4**	**81.7**	**80.6**	**83.4**	**83.3**	**83.2**	**84.0**	**83.3**	**83.2**	**81.6**	**81.7**	**77.7**	**81.7**
12. *Hrr. aquaticum*	25.1	24.8	25.1	24.8	24.3	25.2	25.1	24.9	24.6	24.3	24.9	100	**80.6**	**80.7**	**80.9**	**88.2**	**81.4**	**81.3**	**80.8**	**81.2**	**81.2**	**81.2**	**80.4**	**80.9**	**78.4**	**80.6**
13. *Hrr. californiense*	34.9	34.8	34.9	34.9	35.0	35.1	35.1	34.9	35.2	34.8	25.2	24.5	100	**88.1**	**88.8**	**80.7**	**83.0**	**82.8**	**81.9**	**82.8**	**83.0**	**82.4**	**87.5**	**86.5**	**78.0**	**88.4**
14. *Hrr. coriense*	35.8	35.9	36.0	36.1	35.5	36.1	36.1	35.9	36.0	36.1	25.3	24.6	35.5	100	**87.3**	**81.0**	**82.8**	**82.8**	**82.2**	**83.0**	**82.7**	**82.6**	**86.9**	**86.3**	**77.8**	**87.3**
15. *Hrr. distributum*	34.6	34.5	35.0	34.4	34.3	34.7	34.6	34.6	35.5	34.3	25.4	25.0	36.6	33.6	100	**81.4**	**83.6**	**83.2**	**82.7**	**83.6**	**82.8**	**83.2**	**88.5**	**86.7**	**78.0**	**89.3**
16. *Hrr. halodurans*	24.9	24.8	25.0	24.6	24.6	24.8	24.9	24.6	24.8	24.6	24.9	34.9	24.6	24.6	25.1	100	**81.4**	**81.1**	**80.6**	**81.5**	**81.2**	**81.5**	**80.8**	**81.0**	**78.4**	**80.9**
17. *Hrr. halophilum*	27.0	26.9	27.3	26.6	26.6	27.2	26.9	26.7	26.9	26.4	27.5	25.4	26.7	26.3	27.5	25.5	100	**87.9**	**87.6**	**88.6**	**88.0**	**88.8**	**82.7**	**83.1**	**77.8**	**83.1**
18. *Hrr. kocurii*	26.2	26.3	26.9	26.4	26.3	26.7	26.6	26.6	26.4	26.0	27.7	25.3	26.6	26.7	26.7	24.9	34.7	100	**87.3**	**89.4**	**87.4**	**87.6**	**82.6**	**82.6**	**77.9**	**83.2**
19. *Hrr. lacusprofundi*	25.3	25.4	25.5	25.3	25.5	25.4	25.6	25.5	25.6	25.3	27.2	24.4	25.6	25.3	26.0	24.3	34.0	33.4	100	**88.3**	**86.9**	**87.6**	**82.2**	**82.0**	**77.8**	**82.3**
20. *Hrr. lipolyticum*	26.3	26.2	26.5	26.4	26.2	26.5	26.7	26.4	26.8	26.2	28.2	25.3	26.6	26.5	27.2	25.1	36.5	38.5	34.9	100	**87.8**	**88.6**	**83.1**	**82.9**	**78.1**	**83.0**
21. *Hrr. persicum*	26.3	26.1	26.3	26.1	26.1	26.2	26.5	26.1	26.6	26.3	27.3	25.1	26.6	26.1	26.6	25.1	34.7	33.8	33.1	34.7	100	**87.8**	**82.6**	**82.7**	**78.1**	**82.8**
22. *Hrr. saccharovorum*	26.2	26.3	26.2	26.3	26.1	26.2	26.5	26.1	26.7	26.3	27.4	25.0	26.2	26.1	26.8	25.7	36.9	34.7	34.2	36.2	34.5	100	**82.7**	**82.9**	**77.8**	**82.8**
23. *Hrr. sodomense*	32.7	32.8	32.7	32.6	32.4	32.8	32.7	32.8	33.5	32.6	25.1	24.4	33.8	32.5	35.6	24.7	26.3	26.3	25.4	26.6	26.1	26.2	100	**86.5**	**77.5**	**87.8**
24. *Hrr. tebenquichense*	30.9	30.8	30.8	30.7	30.5	30.8	30.8	30.6	31.6	30.9	25.3	24.9	31.3	31.3	32.0	24.8	26.6	26.2	25.5	26.4	26.1	26.2	31.4	100	**77.5**	**86.5**
25. *Hrr. vacuolatum*	22.7	22.8	22.7	22.5	22.5	22.6	22.9	22.6	22.6	22.3	22.6	23.3	22.8	22.6	22.8	23.2	22.8	23.1	23.3	23.0	23.1	23.0	22.3	22.7	100	**77.8**
26. *Hrr. xinjiangensis*	33.4	33.4	33.6	33.5	33.3	33.8	33.7	33.7	34.6	33.5	25.5	24.6	35.7	33.4	37.2	24.9	26.7	26.7	25.5	26.8	26.4	26.4	34.2	31.5	22.9	100

Table 2. Differential features among *Hrr. ezzemoulense* DSM 17463^T, *Hrr. chaoviator* DSM 19316^T and the eight new strains, as well as the type species of the genus *Halorubrum*, *Hrr. saccharovorum* JCM 8865^T

Taxa: 1; *Hrr. ezzemoulense* DSM 17463^T; 2, *Hrr. chaoviator* DSM 19316^T; 3, strain C191; 4, strain Ec15; 5, strain Fb21; 6, strain G37; 7, strain Ga2p; 8, strain Ga36; 9, strain SD612; 10, strain SD683; 11, *Hrr. saccharovorum* JCM 8865^T. All data are from this study. +, Positive; −, negative; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
NaCl (%, w/v) range	15–25	20–30	15–30	15–30	20–30	15–30	20–30	15–30	15–30	15–30	10–30
Optimum NaCl (%, w/v)	20	20	25	25	25	25	25	25	20	20	25
Range of pH	6.5–9.0	7.0–8.0	7.0–8.0	7.0–8.0	7.0–8.0	7.0–8.0	7.0–8.0	7.0–8.0	6.5–8.0	6.5–8.0	6.5–8.0
Optimum pH	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	8.0
Range of temperature (°C)	25–45	25–40	20–40	20–40	20–40	20–40	20–40	20–40	20–40	20–40	30–45
Optimum temperature (°C)	40	37	37	37	37	37	37	37	37	37	40
Mg^{2+} requirement	+	+	−	−	−	−	−	−	+	+	+
Nitrate reduction	+	−	+	+	+	+	+	+	+	+	+
Starch hydrolysis	−	+	−	−	−	−	−	−	−	−	−
Indole production	−	−	−	−	−	−	−	−	−	−	+
Utilization as sole carbon and energy source of:											
D-Arabinose	+	+	−	−	−	−	−	−	+	+	−
D-Fructose	−	+	−	−	−	−	−	−	−	−	−
D-Galactose	−	+	−	−	−	−	−	−	−	−	+
D-Mannose	−	−	−	−	−	−	−	−	+	+	+
Maltose	+	+	−	−	−	−	−	−	+	−	+
Melezitose	+	+	−	−	−	−	−	−	+	+	ND
Lactose	−	+	−	−	−	−	−	−	+	+	+
Salicin	−	−	−	−	−	−	−	−	+	+	−
Sucrose	+	−	+	+	+	+	+	+	+	+	+
Glycerol	+	+	−	−	−	−	−	−	+	+	+
myo-Inositol	−	−	−	−	−	−	−	−	+	+	ND
D-Mannitol	+	−	+	+	+	+	+	+	+	+	−
Methanol	+	+	−	−	−	−	−	−	−	−	−
Acetate	+	−	−	−	−	−	−	−	−	−	+
Citrate	+	−	−	−	−	−	−	−	−	−	−
Fumarate	−	+	+	+	+	+	+	+	−	−	−
Succinate	−	−	−	−	−	−	−	−	−	−	+
DNA G+C content (mol%, genome)	66.6	66.5	66.0	67.7	69.3	67.0	67.8	67.7	70.1	69.0	69.9*

*Value obtained from the genome of *Hrr. saccharovorum* DSM 1137^T.

a concentration of 10 mg ml^{-1} of lipid dissolved in $CHCl_3$. The total lipid extracts were analysed by one-dimensional high-performance TLC (HPTLC) on Merck silica gel plates (Merck 10×20 cm; Art. 5626), and the plates were eluted in the solvent system chloroform/90 % methanol/acetic acid (65 : 4 : 35, v/v) [39, 40]. To detect all polar lipids, the plate was sprayed with 5 % (v/v) sulfuric acid in water and charred by heating at 160 °C [41]. Glycolipids appear as purple spots while the remaining polar lipids appear as brown spots after prolonged heating; alternatively, the polar lipids were developed by spraying the plate with a solution of primuline and the lipids detected upon excitation by UV light (336 nm) [42]. The following stains were used in order to identify the chemical nature of the lipids present in the HPTLC bands: (a) molybdenum-blue Sigma spray reagent for phospholipids [41]; (b) azure-A/sulfuric acid for sulfatides and sulfoglycolipids [43]; and (c) ninhydrin in acetone/lutidine (9 : 1) for free amino groups. To analyse the whole profiles of the strains studied, universal staining was performed with 20 % (w/v) phosphomolybdic acid (PMA) solution in ethanol and charred by heating at 160 °C. The high sensitivity of this staining allows all lipids to be detected even in small amounts.

HPTLC revealed that *Hrr. ezzemoulense* DSM 17463^T, *Hrr. chaoviator* Halo-G*^{T} and the eight *Halorubrum* strains possessed a similar polar lipid profile (Fig. S1), with the following major lipids: phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS) and one glycolipid chromatographically identical to sulfated mannosyl glycosyl diether (S-DGD-3). Biphosphatidylglycerol (BPG) was also

found as a minor component and minor phospholipids were also detected. The polar lipid profiles of all these strains had all major lipids described for neutrophilic species of the genus *Halorubrum* [44, 45], although some minor differences were observed in the minor polar lipids for the strains investigated which could be related to their different isolation habitats.

Overall, evidence from this polyphasic taxonomic study shows that *Hrr. ezzemoulense* and *Hrr. chaoviator* constitute a single species, the name *Hrr. ezzemoulense* having priority according to the Code of Nomenclature of Prokaryotes [46], and thus *Hrr. chaoviator* should be considered a later heterotypic synonym of *Hrr. ezzemoulense*. The eight new isolated strains are members of this species and thus we propose an emended description of the species *Hrr. ezzemoulense*, including the features of *Hrr. chaoviator* and those of the forementioned eight isolates.

EMENDED DESCRIPTION OF *HALORUBRUM EZZEMOULENSE* KHARROUB *ET AL.* 2006

Halorubrum ezzemoulense (ez.ze.mou.len′se. N.L. neut. adj. *ezzemoulense* pertaining to Ezzemoul sabkha, where the type strain was isolated).

The description is that of Kharroub *et al.* [8] with the following modifications: aerobic growth occurs at 15–30 % (w/v) NaCl, pH 6.5–9.0 and 20–45 °C. Optimum NaCl concentration, pH and temperature for growth are 20–25 % (w/v), pH 7.5 and 37–40 °C. Nitrate is generally reduced to nitrite, but nitrite is not reduced. Starch is generally not hydrolysed. Voges–Proskauer and methyl red tests are negative. Casein and DNA are not hydrolysed. D-Arabinose, D-fructose, D-galactose, D-mannose, maltose, melezitose, lactose, salicin, glycerol, *myo*-inositol, methanol, acetate, citrate and succinate are not generally utilized as sole carbon and energy source. Sucrose, D-mannitol and fumarate are generally utilized as sole carbon and energy source. Xylose, butanol, ethanol, methanol, propanol, sorbitol, benzoate, hippurate, propionate, succinate, valerate and tartrate are not utilized as sole carbon and energy source. The polar lipid profile includes: phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS) and one glycolipid chromatographically identical to sulfated mannosyl glycosyl diether (S-DGD-3), the main glycolipid of the genus *Halorubrum*. Biphosphatidylglycerol (BPG) is also found as a minor component, and minor phospholipids are also detected. The G+C content of the genomic DNA is 66.0–70.1 mol% (genome).

The type strain, 5.1^T (=CECT 7099^T=DSM 17463^T), was isolated from Ezzemoul sabkha in Algeria. The DNA G+C content of this strain is 66.6 mol% (genome).

The GenBank/EMBL/DDBJ accession numbers of the 16S rRNA gene sequence and complete genome sequence of the type strain CECT 7099^T/DSM 17463^T are AB663412 and NEDJ00000000, respectively.

Halorubrum chaoviator strain Halo-G*^{T} (=DSM 19316^T =NCIMB 14426^T=ATCC BAA-1602^T) is an additional strain of *Halorubrum ezzemoulense*, and *Halorubrum chaoviator* is a later heterotypic synonym of *Halorubrum ezzemoulense*. Strains C191, Ec15, Fb21, G37, Ga2p, Ga36 (isolated from the hypersaline lake Aran-Bidgol in Iran), SD612 and SD683 (isolated from a saltern in Namibia) are additional strains of this species.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
No experimental work with animals or humans was carried out in this study.

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