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NOTE

Occurrence of *Halococcus* spp. in the nostrils salt glands of the seabird *Calonectris diomedea*

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Abstract The nostrils of the seabird *Calonectris diomedea* are endowed with a salt-excreting gland that could produce a suitable environment for the colonization of extreme halophilic prokaryotes. We have studied in this organ the presence of extreme halophiles by means of culturing techniques. We could easily cultivate members of haloarchaea, and all cultures studied were identified as members of one of the two species *Halococcus morrhuae* and *Hcc. dombrowskii*. In order to reveal the diversity of these colonizers, we undertook a taxonomic study. Altogether, the results indicated that members of the genus *Halococcus* may constitute a part of the natural epizootic microbiota of *C. diomedea,* and that they exhibit such an important degree of taxonomic variability that appeals for a pragmatic species definition. This seabird nests in the west Mediterranean coasts, but its migratory habits, reaching locations as distant from the Mediterranean as the South Atlantic, may help in the dispersal mechanisms of haloarchaea through the Earth's surface.

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Introduction

Extremely halophilic organisms thrive in natural (e.g. salt lakes, evaporite basins, deep-sea basins,…) and artificial (solar salterns) hypersaline environments widely dispersed through the Earth geography (Javor 1989). Although the geographical distribution of these environments appears distantly scattered, in many cases the microbial composition revealed by molecular techniques seems to be highly similar (Pedrós-Alió 2005). Moreover, cultured members of distantly located brines as *Salinibacter ruber*, only showed very subtle geographical divergences, mainly due to the different expression of common metabolites as an indication of incipient allopatric speciation (Rosselló-Móra et al. 2008). The extreme similarities among distantly isolated strains led us to investigate possible mechanisms of dispersal of extremely halophilic microorganisms.

One of the possible mechanisms for extremely halophilic organisms' dispersal throughout the Earth's geography could be through the seawater streams that circulate around the world's oceans (Rodríguez-Valera et al. 1979). However, most of the extreme halophiles exhibit a relatively high-salt requirement for their growth. In general, as it has been demonstrated for cultures in the laboratory (Oren 1994), and for natural populations by molecular techniques (Antón et al. 2000), growth is absent below 10–15% salt. In this regard, the dispersal mechanism based on seawater currents may require that the extreme halophiles are transported in a dormant stage (Rodríguez-Valera et al. 1979). However, other haloarchaea may survive in low-salinity environments close to seawater salt

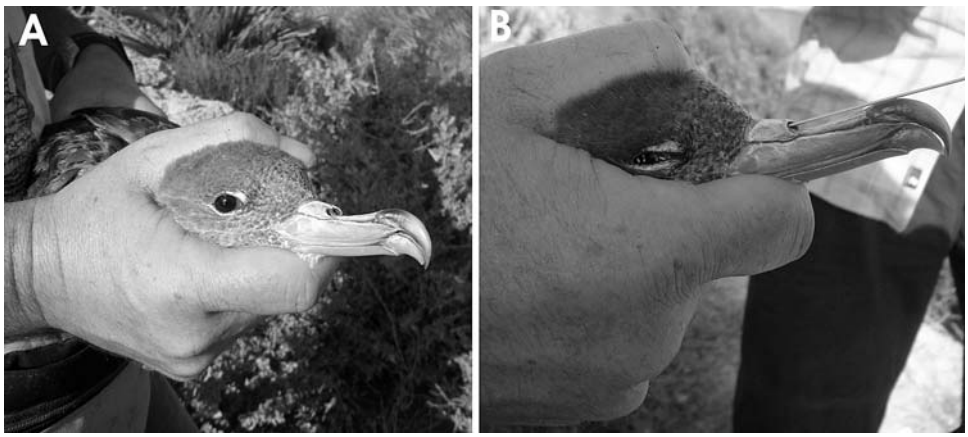

Fig. 1 *Calonectris diomedea* nostrils (**a**) and sampling procedure (**b**)

concentrations (Purdy et al. 2004), and thus transported as viable cells. Although this may be true for coastal high-salt environments, ocean transport may not explain the occurrence of extreme halophiles in salterns at high elevations. For example, widely spread extreme halophiles had been detected in high altitude salterns (Maturrano et al. 2006) at more than 3000 m above the sea level in the Andean cordillera. These organisms could have survived trapped in ancient evaporitic formations (Stan-Lotter et al. 2002). However, there are other mechanisms as aerial transport (Kellogg and Griffin 2006) that could act as plausible dispersal system.

In general, natural and artificial hypersaline environments are frequented by migratory birds that feed from the *Artemia* spp. thriving in their brines. Microorganisms can be trapped in their feathers or extremities and be dispersed through similar environments, as it has been hypothesized for cladocerans and bryozoan (Figuerola et al. 2005). The Balearic archipelago is scattered by small islands in where the seabird *Calonectris diomedea* nests. This species is a migratory bird that travels through long distances over the oceans, reaching locations as far as the South Atlantic (González-Solís et al. 2007). An important characteristic of this seabird for the main purpose of this work is the existence of a nostril salt-excreting gland that serves the bird to remove the excess of salt from its daily seawater ingests. The gland extrudes salt crystals that may serve as a potential habitat for extremely halophilic prokaryotes. For these reasons, we planed to screen nostril salt-excreting glands as a potential habitat of extreme halophilic organisms that may help in their dispersal. Our study revealed the occurrence of members of different species of the archaeal genus *Halococcus* that coexisted in this habitat. To our knowledge, this is the first report of seabirds carrying extremely halophilic microorganisms as possible microbiota. In addition, the study addressed issues about some basic tenets of the current view of the prokaryotic taxonomy.

Methods

Isolation of prokaryotes from nostril of *Calonectris diomedea* individuals, and reference strains used

A wild colony of the seabird *Calonectris diomedea* that nests on the Pantaleu islet, at the south-west coast of the Majorca Island was selected for the present study. Samples were taken from adult birds trying to produce as less stress as possible and no physical damage to the selected individuals (Fig. 1). Birds were sampled without anesthesia as the sample was taken superficially. Samples were obtained by introducing a sterile stab into the nostril of four different members of the colony. The stab biomass was spread onto 25% SW plates, containing per liter: 195 g NaCl, 34.6 g $MgCl_2{\cdot}6H_2O$, 49.5 g $MgSO_4{\cdot}7H_2O$, 0.72 g $CaCl_2$, 5 g KCl, 0.17 g $NaHCO_3$, 0.65 g NaBr, 0.2% yeast extract (pH 7.2), and 1.5% agar. The plates were incubated at 37°C in the dark during two to three weeks. Individual colonies were isolated in pure culture and further screened by bacterial and archaeal 16S rDNA PCR amplification as explained below. The reference strain *Hcc. dombrowskii* DSM 14522^T was obtained from the German culture collection (DSMZ), and the strains *Hcc. morrhuae* JCM 8876^T, *Hcc. qingdaonensis* JCM 13587^T and *Hcc. thailandensis* JCM 13552^T from the Japanese culture collection JCM.

Physiological and biochemical tests

Morphology of the organisms was observed under an optic microscope Zeiss Axio Imager A1. Salt requirement for growth was determined by growing the isolates in liquid SW medium with different concentrations of NaCl (5, 10, 15, 20, 25 and 30%). Similarly, the requirement for Mg^{+2} was tested in medium containing $MgCl_2$ at the following concentrations: 0, 5, 10, 50, 170 and 400 mM. The experiments were performed in duplicate in 96-well

microtiter plates. Two hundred microliters of each medium were inoculated with 5 µl of a well grown suspension of each strain ($OD_{600\ nm} = 1.0$). Growth was monitored spectrophotometrically at 600 nm after the incubation period (7–14 days at 37°C without shaking).The pH range for growth was determined in liquid 25% SW medium adjusted to the desired pH values (5.0, 5.2, 5.8, 6.2, 7.2, 7.4, 8.2 and 8.5). The microtiter plates were inoculated and incubated for 4 weeks as described above. Growth was also monitored spectrophotometrically at 600 nm.

Standard tests (cytochrome oxidase production, catalase production, nitrate reduction and gelatin liquefaction) were performed as previously described (Smibert and Krieg 1994; Stan-Lotter et al. 2002). The analytical systems API ZYM and API 20NE (BioMerieux) were used for the analysis of additional enzyme activities. The assimilation tests were performed as previously described (Stan-Lotter et al. 2002). Strips were inoculated with a cell culture grown in 4 M NaCl and brought to a suspension density recommended by the manufacturers, and incubated for up to 24 h (API ZYM) or 3 weeks (API 20NE). Tests were performed by triplicate for each strain.

DNA extraction, base composition and DNA-DNA hybridizations (DDH)

Cells from a well grown liquid culture of 200 ml for each strain were harvested by centrifugation (13,000 rpm for 3 min) after seven days since the inoculation. The cell wall disruption was made by grinding the pellets embedded in liquid nitrogen in a porcelain mortar and resuspended in potassium ethyl xanthogenate (XS) buffer (Leuko et al. 2007). Genomic DNA was prepared as previously indicated (Urdiain et al. 2008). G+C content was analyzed by hydrolysis of DNA to its nucleosides and quantified by HPLC, and DDH experiments were carried out following a microtiter plate non-radioactive method. Both protocols were followed as previously described (Urdiain et al. 2008).

Sequencing of the 16S rRNA genes and phylogenetic analyses

16S rRNA genes were amplified using primers 21F and 1492R for *Archaea*, and GM3 and GM4 for *Bacteria* (Table 1). In the case of stab scratched biomass, the cotton edge was immersed in 100 µl of distilled water to resuspend and lysate the biological material and boiled for 5 min. From this suspension, 1 µl was used for amplifications. PCR reactions were carried out in a Mastercycler personal thermocycler (Eppendorf) under the following standard conditions: 35 cycles (denaturation at 94°C for 15 s, annealing at 55°C for 30 s, extension at 72°C for 1 min) preceded by 2 min denaturation at 94°C and followed by 8 min extension at 72°C. PCR products obtained were purified with Qiaquick PCR purification kit (Qiagen) according to the manufacturer's protocol. For sequencing of the complete archaeal 16S rDNAs five primers were used: 21F, 344F, 915F, 915R and 1492R (Table 1). The nucleotide sequences were determined using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) according to manufacturer's recommendations and an ABIPRISM 310 DNA sequencer (Applied Biosystems).

Table 1 rRNA gene primers used in this study

Name	Specificity	Sense* and position+	Target	Sequence (5′–3′)	Reference
21F	*Archaea*	F: 7–26	16S rDNA	TTCCGGTTGATCCTGCCGGA	Benlloch et al. 2001
344F	*Archaea*	F: 344–363	16S rDNA	ACGGGGYGCAGCAGGCGCGA	Raskin et al. 1994
915F	*Archaea*	F: 915–934	16S rDNA	GTGCTCCCCCGCCAATTCC	Stahl and Amann 1991
915R	*Archaea*	R: 915–935	16S rDNA	GTGCTCCCCCGCCAATTCCT	Stahl and Amann 1991
GM3	*Bacteria*	F: 8–23	16S rDNA	AGAGTTTGATCMTGGC	Muyzer et al. 1995
GM4	*Bacteria*	R: 1492–1507	16S rDNA	TACCTTGTTACGACTT	Muyzer et al. 1995
Euryclus	*Archaea*	R: 24–26	23S rDNA	TCGCAGCTTRSCACGYCCTTC	Benlloch et al. 2001
1492R	*Bacteria*	R: 1492–1509	16S rDNA	TACGGYTACCTTGTTACG	Muyzer et al. 1995
atpB_409F	*Archaea*	F: 409–436	*atpB*	GACATCGTCGGTGAGSCVATSAACCC	Papke et al. 2004
atpB_906R	*Archaea*	R : 906–927	*atpB*	GCCAGGTCVGTRTACATGTA	Papke et al. 2004
gyrB_43F	*Archaea*	F: 43–59	*gyrB*	ATCGACGAGGCGCTT	This work
gyrB_1299R	*Archaea*	R:1299–1318	*gyrB*	CGGGTGTTTCTCGACGTT	This work
gyrB_339cF	*Archaea*	F: 339–358	*gyrB*	TCGTCGAGCGGTTTCAGG	This work
gyrB_998cR	*Archaea*	R: 998–1017	*gyrB*	GCCCTCGAAGTGATCATG	This work

F foward, *R* reverse

+Referred to *Escherichia coli* (for bacterial primers) or *Halobacterium salinarum* (for archaeal primers) 16S or 23S rRNA nucleotide position

Ribosomal internal transcribed spacer (ITS) analyses

The internal region between the 16S rRNA and 23S rRNA gene sequences plus the 16S rDNA were amplified by using primers 21F and Euryclus (Table 1) with the following conditions: 35 cycles (denaturation at 94°C for 15 s, annealing at 55°C for 30 s, extension at 72°C for 3 min) preceded by 2 min denaturation at 94°C and followed by 5 min extension at 72°C. Partial 16S rDNA and the complete ITS sequences were obtained for every strain tested.

Amplification and sequencing of protein-coding genes

Two housekeeping genes, subunit B of the ATP syntase (*atpB*) and the beta subunit of the gyrase B (*gyrB*) genes were selected on the basis of the availability of universal primers in the literature, and the amplifications were performed as recommended (Papke et al. 2004).

Sequence analyses

Sequences were revised and corrected with Sequencher v 4.7 (Gene Codes Corp.). 16S rRNA gene alignments were produced with the use of the ARB software package (Ludwig et al. 2004) (www.arb-home.de), introducing the new almost complete sequences into a preexisting alignment available of about 208,000 single sequences (Pruesse et al. 2007) (www.arb-silva.de). Housekeeping gene sequences for multilocus sequence analysis (MLSA) were aligned with the use of the program ClustalX 1.83, and the alignments were improved by removing hypervariable positions with the use of the online available program Gblocks (http://molevol.ibmb.csic.es/Gblocks_server.html) using the conditions previously published (Sória-Carrasco et al. 2007). Phylogenetic reconstructions based on sequence data were performed either online (http://atgc.lirmm.fr/phyml/) by the use of the PHYML program package (Guindon and Gascuel 2003), or using the neighbor joining, maximum likelihood, and maximum parsimony algorithms as implemented in the ARB software package (Ludwig et al. 2004). Tree topologies were compared and validated by the use of the different algorithms (and by using different datasets when analyzing 16S rRNA gene sequences). Bootstrap values were performed with the program PHYML and with a total of 100 replicates.

Results

Screening for prokaryotes from *Calonectris diomedea* salt glands, cultures and phylogenetic affiliation

Samples of nostrils from four different birds were smeared onto 25% SW plates and incubated during two to three weeks. After the incubation period, a high proportion of red-pink colonies grew on the plates. For each bird, about 20 pink-red colonies were isolated in pure culture. A first screening for *S. ruber* occurrence, checked through amplification with bacterial primers, did not yield any positive amplicon. All colonies yielded amplification products with the archaeal primers, and about 14 partial sequenced amplicons were related to members of the genus *Halococcus.* In addition to the 14 sequenced colonies, 10 randomly selected additional colonies were observed under the microscope. All 24 colonies showed a conspicuous coccoid morphology. From these, we selected seven light red colonies for further studies. Strains 2.0, 2.2, originated from the same bird sample, and the same was true for the pair of strains 3.0 and 3.1, and 4.0 and 4.1. The strain 5.0 was isolated from a fourth additional different bird. The 16S rRNA gene sequence reconstruction of the seven isolates affiliated them to the genus *Halococcus* (Fig. 2) with the closest relative species *Hcc. morrhuae* JCM 8876^T (D11106; 99.2-99.5 similarity), *Hcc. dombrowskii* DSM 14522^T (AJ420376; 99.5-99.9 similarity), and *Hcc. qingdaonensis* JCM 13587^T (AY243109; 99.1-99.4 similarity). In order to determine whether the new isolates were members of one of the three species, we followed a taxonomic study.

G+C content and DNA–DNA hybridization

The C+C mol% content of strains 2.0, 2.2, 4.0, 5.0 and 4.1 was close to 61 mol% and that of the strains 3.0 and 3.1 were 64.1 and 63.7 mol%, respectively. These values are in the range of the GC mol% of the *Halococcus* genus (Table 2). DDH experiments revealed that the seven isolates grouped with two of the three closely related *Halococcus* species: strains 2.0, 2.2, 3.0, 3.1, 4.0, and 5.0 were more related to the type strain *Hcc. morrhuae* JCM 8876^T, whereas strain 4.1 was related to *Hcc. dombrowskii* DSM 14522^T. Intraspecific genomic similarities varied between 90.5 and 71.3%, whereas interspecies similarities varied between 63.6 and 29.4 mol%.

MLSA

Three additional sequences (ITS, *gyrB* and *atpB*) were obtained for all the studied strains and used for phylogenetic reconstructions (Table 3). For this purpose, the sequences of *Hcc. thailandensis* strain JCM 13552^T, and *Hcc. quingdaonensis* JCM 13587^T were used as outgroups. Each gene alignment was individually reconstructed, as well as a concatenated of the three genes (data not shown). Finally, a concatenate of the three genes plus the 16S rRNA gene sequence, was reconstructed (Fig. 3). Altogether, the results show that the group of the new strains

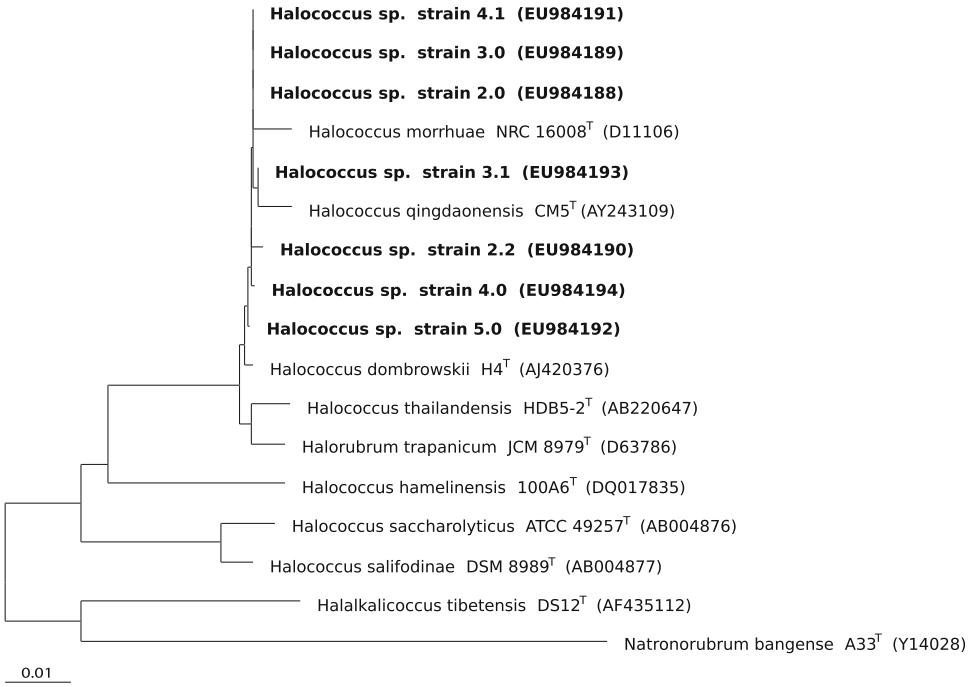

Fig. 2 Maximum likelihood reconstruction of the 16S rRNA gene alignment of the *Halococcus* species studied. The *bar* indicates 1% of sequence divergence

Table 2 DNA G+C content and DDH similarities among the strains used in this study

Strain[a]	G+C content (mol%)	DDH (%) with labeled strain		
		Hcc. morrhuae (JCM 8876^T)	*Hcc. dombrowskii* (DSM 14522^T)	*Hcc. qingdaonensis* (JCM 13587^T)
Hcc. morrhuae (JCM 8876^T)	(61–66)[b]	**100**	35.7 (34.1)[c]	49.0
2.0	61.1	**90.5**	41.0	55.4
2.2	61.6	**86.4**	38.1	54.9
3.0	64.1	**76.7**	34.2	59.2
3.1	63.7	**83.6**	34.2	57.1
4.0	61.5	**78.2**	41.3	56.6
5.0	61.6	**71.3**	29.4	51.2
Hcc. qingdaonensis (JCM 13587^T)	(61.2)[d]	63.6	37.5	100
4.1	61.5	55.7	**88.6**	36.0
Hcc. dombrowskii (DSM 14522^T)	61.3	43.1	**100**	56.5 (57.1)[d]

[a] Bird number followed by strain number, i.e. strains 2.0 and 2.0 were isolated from the same bird

[b] Data from Larsen, 1989 are in brackets

[c] Data from Namwong et al., 2007 are in brackets

[d] Data from Wang et al., 2007 are in brackets

isolated from the nostrils of the seabirds, and the two type strains of the closest relative species form a phylogenetic group that cannot be segregated in different clades. In general, the outgroups selected worked adequately (i.e. appeared clearly distant from the group of study, Fig. 3). The inclusion of the 16S rRNA gene sequence in the concatenate increased the stability of an identical topology of both trees, by raising the bootstrap values of some of the branches.

Physiological and biochemical characteristics

The new isolates grew aerobically at 37°C in liquid 25% SW medium with shaking. Optimum growth of strains occurred in the presence of NaCl concentrations ranging from 15 to 30%; no growth was observed in medium containing <15% NaCl. The strains required at least 1.7 mM Mg^{+2} in the medium containing 3.3 M NaCl. The optimal growth for all the strains occurred when Mg^{+2} was present in the medium

at concentrations ranging from 40 to 400 mM. However, individual strains differed in their Mg requirements for optimal growth (Table 4). Optimum pH values were 6.2 for strain 5.0, and 7.2–7.4 for the rest of the strains. Further phenotypic characteristics of strains are shown in Table 4, and compared with those of *Hcc. dombrowskii* DSM 14522^T, *Hcc. morrhuae* JCM 8876^T, *Hcc. qingdaonensis* JCM 13587^T and *Hcc. thailandensis* JCM 13552^T. All were catalase and oxidase positive, and urease negative. Hydrolysis of gelatin was positive, and the esterase activity was similar to *Hcc. morrhuae* JCM 8876^T and *Hcc. dombrowskii* DSM 14522^T. In addition, the new strains used glucose, acetate, lactate and D-fructose as the sole energy source.

Discussion

We have studied the suitability of the salt glands of the seabird *C. diomedea* as a potential habitat for cultivable extremely halophilic aerobic prokaryotes. All the microorganisms isolated using the methods described above were members of the *Archaea* domain, and all the efforts to culture *Bacteria* from the nostril samples were unsuccessful. The sequencing of the 16S rRNA gene in fourteen selected colonies affiliated the new isolates with *Halococcus* spp. Members of this genus have been found in environments as distinct as solar saltern brines (Javor 1984), stromatolites (Goh et al. 2006), fish sauce (Namwong et al. 2007), alpine salt deposits from the Permian age (Stan-Lotter et al. 2002), and salted codfish (Farlow 1880). All such findings indicate that *Halococcus* spp. may be very versatile as revealed by the enormous range of environments in where they thrive, or highly resistant to hostile environments as low-salinity systems (Rodríguez-Valera et al. 1979).

Seven of the fourteen strains that affiliated with the genus *Halococcus* were further studied. The phylogenetic reconstruction based on the nearly complete sequence of the 16S rRNA gene of the isolates affiliated them with the three species *Hcc. morrhuae* (Kocur and Hodgkiss 1973), *Hcc. dombrowskii* (Stan-Lotter et al. 2002), and *Hcc. quingdaonensis* (Wang et al. 2007). In particular for this group of species, the sequence divergence is so low (22 changes in homologous positions in the whole alignment) that no clear branching order could be revealed. Actually, all of them form a clade without resolution at the species level. The lack of resolution power at the species category level of the small subunit of the ribosome is well known, and thus DDH is appealed to solve the discrimination (Stackebrandt and Ebers 2006). In our case, the identification of the new isolates to the existing species was clear by using DDH. The results indicated that six of them could

Table 3 Genes used in this study for MLSA, their number of homologous positions in each alignment, number of informative positions, and accession numbers

Gene	Alignment positions	Informative positions	GenBank accession numbers
atpB	483	37	EU984195-06
gyr B	584	41	EU984207-18
ITS	257	32	EU984219-30
subtotal	1324	110	
SSU	1277	27	AJ420376; AB220647; AY243109; D11106
			EU984188-94
total	2601	137	

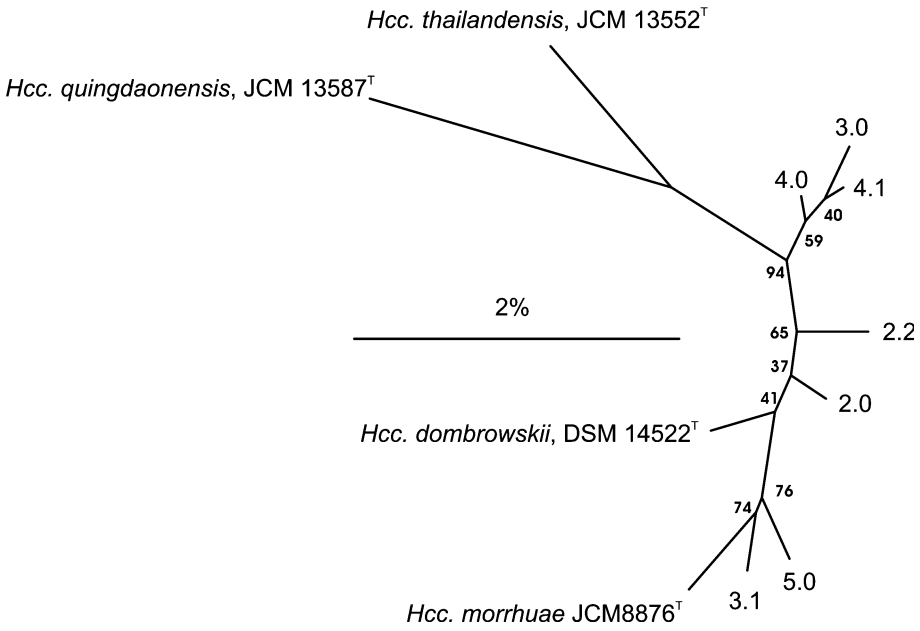

Fig. 3 PHYML-based phylogenetic reconstruction of a concatenate of the four genes studied. The bootstrap values are obtained after 100 calculations. The *bar* indicates 2% sequence divergence

Table 4 Comparison of phenotypic characteristics of \viously characterized *Halococcus* species and isolates

Characteristic	JCM 8876^T	2.0	2.2	3.0	3.1	4.0	5.0	DSM 14522^T	4.1	JCM 13552^T	JCM 13587^T
Optimum NaCl concentration (%, w/v)	20–30[a]	30	15–30	30	30	15–25	30	20–25	25–30	20–30[a]	18[a]
$MgCl_2$ requirement (mM)	80–500[a]	50–400	400	170	170–400	170	50	40–60	170–400	80[a]	ND
Optimal pH	ND	7.2	7.2	7.4	7.4	7.2	6.2	7.4	7.2	6.0–8.0[a]	ND
Oxidase	+	+	+	+	+	+	+	+	+	+/(−)[a]	−
Catalase	+	+	+	+	+	+	+	+	+	+/(−)[a]	+
Urease	−	−	−	−	−	−	−	−	−	−/(+)[a]	+
Nitrate reduction	+	−	−	+	+	+	+	+	+	+	−
Indole production	−	−	−	−	−	−	−	−	−	−	−
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	−	−
Cystine arylamidase	w (−)[b]	+	+	+	+	+	+	w (+)[b]	+	+	+
Acid phosphatase	w (−)[b]	−	−	−	−	−	−	w (+)[b]	−	w	w
Lipase esterase	+	−	−	+	+	−	−	V	−	+	+
Esterase	+	+	+	+	+	+	−	+	+	−	−
Utilization of											
D(+)Glucose	+	+	+	+	+	+	+	w (−)[b]	+	+	+
Acetate	ND	+	+	+	+	+	+	+	+	ND	ND
Fumarate	ND	+	+	+	+	+	−	−	−	ND	ND
Lactate	ND	+	+	+	+	+	+	−	+	ND	ND
D(−)Fructose	−	+	+	+	+	+	+	+	+	+	

For the type strains, the results were compared with the literature given in *brackets*: *Hcc. morrhuae* JCM 8876^T; *Hcc. qingdaonensis* JCM 13587^T, *Hcc. thailandensis* JCM 13552^T, *Hcc. dombrowskii* DSM 14522^T

Tests were performed at salinities of at least 15% NaCl. Positive reaction or growth (+); no reaction or growth (−); *w* weak reaction and *ND* not determined

[a] Namwong et al. 2007

[b] Stan-Lotter et al. 2002

be identified as members of *Hcc. morrhuae*, and one to *Hcc. dombrowskii*. However, the phenotypic traits studied showed that the strain collection was endorsed with a certain degree of diversity. Actually, the phenotypic set of traits that were initially clear to discriminate both species (Stan-Lotter et al. 2002) turned fuzzy once additional strains had been included. For example, traits as cystine arylamidase and acid phosphatase, or the use of D(−)fructose or D(+)glucose resulted not any more discriminative for the species as the new organisms showed discrepant metabolic behavior.

The DDH values shown in this work between the two sets of strains (*Hcc. morrhuae* JCM 8876^T, and strains 2.0, 2.2, 3.0, 3.1, 4.0, and 5.0; and *Hcc. dombrowskii* DSM 14522^T and strain 4.1) appear clearly below the recommended threshold of 70% to circumscribe species (Wayne et al. 1987), and thus both may be considered as different genospecies from the hybridization point of view. The problem is that due to the diverse metabolism shown by the strains in study, no clear phenotypic property is found to assure a distinct species status (Stackebrandt et al. 2002). The fact that genospecies cannot phenotypically be discriminated is not unusual for species that account with a large set of isolates, and this is especially evident in widely spread species with versatile metabolism (Rosselló-Móra and Amann 2001). In such cases when difficulties to discriminate genomic groups by means of phenotypic traits arise, the plausible classification will be as genomovars (Rosselló-Móra and Amann 2001). The difficulties in discriminating both species are even reinforced by our studies on MLSA. As derived from the different reconstructions, we could not separate them in two different clades as the strains appeared clearly mixed. MLSA is a method of promise to circumscribe species (Stackebrandt et al. 2002) in order to substitute DDH. However, as in our case, the low informative content of the studied genes cannot render clear results, and support the fact that both species might be better considered as genomovars of the same species.

The taxonomic problem raised here has no trivial solution. As *Hcc. dombrowskii*, numerous species are yearly classified basing their studies on a single strain, a fact that hampers the recognition of the taxon diversity, and endorses the species description with a high grade of rigidity (Christensen et al. 2001). The problems arise when

new closely related strains are isolated and their genetic and phenotypic profiles do not match that of the type strain leading to the species description emendation, or even to reclassification. Some microbiologists consider the current definition too coarse to circumscribe what may be a unit assumed to be species (Konstantinidis and Tiejde 2007). However, others appeal to the use of a pragmatic species circumscription for prokaryotes (Rosselló-Móra and Amann 2001) in where a certain degree of intraspecific variation is allowed. In our case, applying a relaxed definition would simplify the discussion on the species colonization of bird nostrils, as it would recognize just one colonizing species.

In any case, and independently of the fact that we may recognize one or two species colonizing nostrils, it is clear that this environment constitutes an ideal habitat for the colonization of haloarchaea due to the presence of the salt crystals produced by the salt gland. The isolation of closely related members from all nostrils sampled indicates that *Halococcus* are part of their natural culturable epizootic microbial community. Even if we consider that two species of the same genus coexist on the nostril mucosa, the observation is not surprising. As much as nine different species of *Deinococcus* had been demonstrated to coexist in the same environment (Rainey et al. 2005). In any case, all data analyzed here showed both species to be very closely related, phenotypically indistinguishable, and most probably sharing ecological niche.

Finally, the demonstration that the salt-excreting glands of seabirds constitute an appropriate habitat for extremely halophilic prokaryotes may have as well implications in their dispersion mechanism. The fact that the migratory habits of *C. diodomea* allows them to reach very distant environments in a constant yearly basis (González-Solís et al. 2007) may help in the dispersion of *Halococcus* spp. through the Earth's geography. This may constitute one of the most rapid and effective mechanisms to blurry allopatric speciation for the members of this archaeal species.

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References

Antón J, Rosselló-Móra R, Rodríguez-Valera F, Amann R (2000) Extremely halophilic bacteria in crystallizer ponds from solar salterns. Appl Environ Microbiol 66:3052–3057

Benlloch S, Acinas SG, Antón J, López-López A, Luz SP, Rodríguez-Valera F (2001) Archaeal biodiversity in crystallizer ponds from a solar saltern: culture versus PCR. Microb Ecol 41:12–19

Christensen H, Bisgaard M, Frederiksen W, Mutters R, Kuhnert P, Olsen E (2001) Is characterization of a single isolate sufficient for valid publication of a new genus or species? Proposal to modify recommendation 30b of the Bacteriological Code (1990 revision). Int J Syst Evol Microbiol 51:2221–2225

Farlow WG (1880) On the nature of the peculiar reddening of salted codfish during the summer season. US Fish Comm Rep for 1878, pp 969–973

Figuerola J, Green AJ, Michot TC (2005) Invertebrate eggs can fly: evidence of waterfowl-mediated gene flow in aquatic invertebrates. Am Nat 165:274–278

Goh F, Leuko S, Allen MA, Bowman JP, Kamekura M, Neilan BA, Burns BP (2006) *Halococcus hamelinensis* sp. nov., a novel halophilic archaeon isolated from stromatolites in Shark Bay, Australia. Int J Syst Evol Microbiol 56:1323–1329

González-Solís J, Croxall JP, Oro D, Ruiz X (2007) Transequatorial migration and mixing in the wintering area in a pelagic seabird. Front Ecol Environ 5:297–301

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696–704

Javor BJ (1984) Growth potential of halophilic bacteria isolated from solar salt environments: carbon sources and salt requirements. Appl Environ Microbiol 48:352–360

Javor BJ (1989) Hypersaline environments. Microbiology and Biogeochemistry. Brock/Springer series in contemporary bioscience. Springer, Berlin

Kellogg CA, Griffin DW (2006) Aerobiology and the global transport of desert dust. Trends Ecol Evol 21:638–644

Kocur M, Hodgkiss W (1973) Taxonomic status of the genus *Halococcus* Schoop. Int J Syst Bacteriol 23:151–156

Konstantinidis K, Tiejde JM (2007) Prokaryotic taxonomy and phylogeny in the genomic era: advancements and challenges ahead. Curr Opin Microbiol 10:504–509

Larsen H (1989) Genus IV *Halococcus*. In: Staley JT, Bryant MP, Pfennig N, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 3. Williams & Wilkins, Baltimore, pp 2228–2230

Leuko S, Goh F, Ibáñez-Peral R, Burns BP, Walter MR, Neilan BA (2007) Lysis efficiency of standard DNA extraction methods for *Halococcus* spp. in an organic rich environment. Extremophiles 12:301–308

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar Buchner A, Lai T, Steppi S, Jobb G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, König A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32:1363–1371

Maturrano L, Santos F, Rosselló-Móra R, Antón J (2006) Microbial diversity in Maras salterns, a hypersaline environment in the Peruvian Andes. Appl Environ Microbiol 72:3887–3895

Muyzer G, Teske A, Wirsen CO, Jannasch HW (1995) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch Microbiol 164:165–172

Namwong S, Tanasupawat S, Visessanguan W, Kudo T, Itoh T (2007) *Halococcus thailandensis* sp. nov., from fish sauce in Thailand. Int J Syst Evol Microbiol 57:2199–2203

Oren A (1994) The ecology of the extremely halophilic *Archaea*. FEMS Microbiol Rev 13:415–440

Papke RT, Koenig JE, Rodríguez-Valera F, Doolittle WF (2004) Frequent recombination in a saltern population of *Halorubrum*. Science 306:1928–1929

Pedrós-Alió C (2005) Diversity of microbial communities: the case of solar salterns. In: Gunde-Cimerman, Oren A, Plemenitas A (eds) Adaptation to life at high salt concentrations in *Archaea*, *Bacteria* and *Eucarya*. Springer, Dordrecht (The Netherlands), pp 71–90

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glöckner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35:7188–7196

Purdy KJ, Cresswell-Maynard TD, Nedwell DB, McGenity TJ, Grant WD, Timmis KN, Embley TM (2004) Isolation of haloarchaea that grow at low salinities. Environ Microbiol 6:591–595

Rainey FA, Ray K, Ferreira M, Gatz BZ, Nobre MF, Bagaley D, Rash BA, Park MJ, Earl AM, Shank NC, Small AM, Henk MC, Battista JR, Kämpfer P, Da Costa MS (2005) Extensive diversity of ionizing-radiation-resistant bacteria recovered from Sonoran desert soil, and description of nine new species of the genus *Deinococcus* obtained from a single soil sample. Appl Environ Microbiol 71:5225–5235

Raskin L, Stromley JM, Rittmann BE, Stahl DA (1994) Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. Appl Environ Microbiol 60:1232–1240

Rodríguez-Valera F, Ruiz-Berraquero F, Ramos-Cormenzana A (1979) Isolation of extreme halophiles from seawater. Appl Environ Microbiol 38:164–165

Rosselló-Móra R, Amann R (2001) The species concept for prokaryotes. FEMS Microbiol Rev 25:39–67

Rosselló-Móra R, Lucio M, Peña A, Brito-Echeverría J, López-López A, Valens-Vadell M, Frommberger M, Antón J, Schmitt-Kopplin P (2008) Metabolic evidence for biogeographic isolation of the extremophilic bacterium *Salinibacter ruber*. ISME J 2:242–253

Smibert RM, Krieg RN (1994) Phenotypic characterization. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds) Manual of methods for general microbiology. American Society for Microbiology, Washington DC, pp 607–654

Sória-Carrasco V, Valens-Vadell M, Peña A, Antón J, Amann R, Castresana R, Rosselló-Móra R (2007) Phylogenetic position of *Salinibacter ruber* based on concatenated protein alignments. Syst Appl Microbiol 30:171–179

Stackebrandt E, Ebers J (2006) Taxonomic parameters revisited: tarnished gold standards. Microbiol Today 33:152–155

Stackebrandt E, Frederiksen W, Garrity G, Grimont PAD, Kämpfer P, Maiden MCJ, Nesme X, Rosselló-Móra R, Swings J, Trüper HG, Vauterin L, Ward AC, Whitman WB (2002) Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int J Syst Evol Microbiol 52:1043–1047

Stahl DA, Amann RI (1991) Development and application of nucleic acid probes in bacterial systematics. In: Stackebrandt E, Goodfellow M (eds) Sequencing and hybridization techniques in bacterial systematics. Wiley, Chichester, pp 205–248

Stan-Lotter H, Pfaffenhuemer M, Legat A, Busse HJ, Radax C, Gruber C (2002) *Halococcus dombrowskii* sp. nov., an archaeal isolate from a Permian alpine salt deposit. Int J Syst Evol Microbiol 52:1807–1814

Urdiain M, López-López A, Gonzalo C, Busse HJ, Langer S, Kämpfer P, Rosselló-Móra R (2008) Reclassification of *Rhodobium marinum* and *Rhodobium pfennigii* as *Afifella marina* gen. nov. comb. nov. and *Afifella pfennigii* comb. nov., a new genus of photoheterotrophic *Alphaproteobacteria* and emended descriptions of *Rhodobium*, *Rhodobium orientis* and *Rhodobium gokarnense*. Syst Appl Microbiol 31:339–351

Wang Q, Li W, Yang H, Liu Y, Cao H, Dornmayr-Pfaffenhuemer M, Stan-Lotter H, Guo G (2007) *Halococcus qingdaonensis* sp. nov., a halophilic archaeon isolated from a crude sea-salt sample. Int J Syst Evol Microbiol 57:600–604

Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, Moore WEC, Murray RGE, Stackebrandt E, Starr MP, Trüper HG (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int J Syst Bacteriol 37:463–464