A multilocus sequence analysis approach to the phylogeny and taxonomy of the *Halobacteriales*

R. Thane Papke,¹ Emma White,¹ Prajwal Reddy,¹ Griffin Weigel,¹ Masahiro Kamekura,² Hiroaki Minegishi,³ Ron Usami³ and Antonio Ventosa⁴

Correspondence
R. Thane Papke
thane@uconn.edu

¹Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269, USA

²Halophiles Research Institute, Noda, Chiba 278-0043, Japan

³Bio-Nano Electronics Research Center, Toyo University, Kujirai, Kawagoe, Saitama 350-8585, Japan

⁴Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla, 41012 Sevilla, Spain

Members of the order *Halobacteriales* are obligate extreme halophiles that belong to the domain *Archaea*. The classification of the *Halobacteriales* currently relies on a polyphasic approach, which integrates phenotypic, genotypic and chemotaxonomic characterization. However, the most utilized genetic marker for phylogeny, the 16S rRNA gene, has multiple drawbacks for use with the *Halobacteriales*: the species of many genera exhibit large intragenic differences between multiple ribosomal RNA operons, the gene is too conserved to discriminate reliably at the species level and it appears to be the most frequently recombined gene between closely related species. Moreover, the *Halobacteriales* is a rapidly expanding group due to recent successes at cultivating novel strains from a diverse set of hypersaline environments; a fast, reliable, inexpensive, portable molecular method for discriminating species is required for their investigation. Recently, multilocus sequence analysis (MLSA) has been shown to be an effective tool for strain identification and taxonomic designation, even for those taxa that experience frequent lateral gene transfer and homologous recombination. In this study, MLSA was utilized for evolutionary and taxonomic investigation of the *Halobacteriales*. Efficacy of the MLSA approach was tested across a hierarchical gradient using 52 halobacterial strains, representing 33 species (including names without standing in nomenclature) and 14 genera. A subset of 21 strains from the genus *Haloarcula* was analysed separately to test the sensitivity and relevance of the MLSA approach among closely related strains and species. The results demonstrated that MLSA differentiated individual strains, reliably grouped strains into species and species into genera and identified potential novel species and also family-like relationships. This study demonstrates that MLSA is a rapid and informative molecular method that will probably accommodate strain analysis at any taxonomic level within the *Halobacteriales*.

INTRODUCTION

Members of the order *Halobacteriales* are aerobic, heterotrophic and obligately halophilic archaea. Commonly known as haloarchaea, these halophiles are found in environments with a minimum salt concentration of 1.5 M, such as salt lakes, soda flats, hypersaline soils, underground salt deposits

Abbreviations: AU, approximately unbiased; LGT, lateral gene transfer; ML, maximum-likelihood; MLSA, multilocus sequence analysis; MP, maximum-parsimony.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this study are detailed in Table 1.

A supplementary table is available with the online version of this paper.

and evaporation ponds for the production of sea salt (Oren, 1994). Furthermore, they are the dominant members in those communities. *In situ* community characterization of hypersaline environments as typically revealed through 16S rRNA gene sequences (Casamayor *et al.*, 2002), protein markers (Papke *et al.*, 2003; Pasić *et al.*, 2007) and, more recently, metagenomics (Bodaker *et al.*, 2010; Cuadros-Orellana *et al.*, 2007; Legault *et al.*, 2006) has discovered a surprising amount of haloarchaeal diversity, which is reflected in the growing number of described species and genera (Ochsenreiter *et al.*, 2002; Oren, 2008). As of July 2010, 29 genera and 101 species names are recognized (<http://www.the-icsp.org/taxa/halobacterlist.htm>).

Use of the 16S rRNA gene sequence to assess phylogenetic relationships is universal in taxonomic descriptions of the *Halobacteriales*. However, due to the highly conserved nature of the 16S rRNA gene [e.g. only five polymorphisms among 1473 positions between *Haloferax volcanii* and *Haloferax alexandrinus* (Asker & Ohta, 2002)], sequence data cannot be used reliably to differentiate closely related species, and evidence suggests that it is the most frequently transferred gene among close relatives (Papke, 2009), which confounds species identification. Additionally, it was discovered that single strains from many genera of the *Halobacteriales*, including *Haloarcula*, *Halosimplex* and *Natrinema*, have highly divergent, paralogous rRNA operons that exhibit over 6% sequence divergence, several indels and extensive evidence for intragenic and interspecies recombination (Boucher *et al.*, 2004; López-López *et al.*, 2007), making phylogeny and taxonomy difficult when based on this marker alone. Because of these complications, the essential single-copy RNA polymerase *rpoB'* was proposed to complement or replace 16S rRNA gene analysis (Case *et al.*, 2007; Enache *et al.*, 2007; Minegishi *et al.*, 2010). However, single-gene phylogenies are limited to the evolution of the analysed gene, which, in the light of lateral gene transfer (LGT), probably does not correspond to the cumulative evolutionary history of the investigated organisms. For instance, comparison of 16S rRNA and *rpoB'* gene phylogenies among taxa of the *Halobacteriales* revealed that the overall phylogenetic topologies were incongruent (Walsh *et al.*, 2004).

A recently developed molecular method for taxonomy, called multilocus sequence analysis (MLSA), sidesteps many of the limitations of single-gene phylogenies by using four to 12 concatenated gene fragments and imparts an averaged phylogeny of those loci. By focusing on gene segments, rapid and rigorous analysis for taxonomic purposes is facilitated (Maiden *et al.*, 1998). Though MLSA was originally designed for epidemiological studies, it was demonstrated to be robust for circumscribing species and assessing their evolutionary history. MLSA was effective for identifying species clusters of highly recombinogenic *Neisseria* populations (Hanage *et al.*, 2005), while also demonstrating evolutionary trends of 'despeciation' in rapidly evolving populations of *Campylobacter jejuni* and *C. coli* (Sheppard *et al.*, 2008). Furthermore, it can be used to differentiate between closely related strains and species that exhibit little or no 16S rRNA gene sequence divergence (Papke *et al.*, 2004, 2007).

Applied to the genus *Halorubrum* of the *Halobacteriales*, an MLSA approach led to the discovery of three well-supported, highly recombinogenic, species-like phylogenetic clusters that were identical or nearly identical at the 16S rRNA gene locus (Papke *et al.*, 2007). In view of the potential strength of MLSA for identifying and classifying species of the *Halobacteriales*, it is important to test the protocol across a large diversity of species and genera. Therefore, the main purpose of this study was to develop an MLSA approach for the *Halobacteriales* that could be utilized for all current and future described species.

METHODS

Strains. Twenty-one closely related strains from seven species whose names have been validly published belonging to the genus *Haloarcula* and an additional group of 31 strains representing 13 other genera in the order *Halobacteriales* were analysed in this study (Table 1). Strains of species with validly published names were cultivated in media recommended by the online catalogue of the Japan Collection of Microorganisms (JCM; <http://www.jcm.riken.jp/>). Strains of species without validly published names or that have not been deposited in culture collections were grown in media listed in Supplementary Table S1, available in IJSEM Online.

Amplification and sequencing. Genomic DNA from cultures was isolated using standard methods (e.g. Qiagen kit), quantified and checked for purity using a Nanodrop spectrophotometer ND-1000 at 260/280 nm and diluted with 1 M Tris/HCl to 20 ng μl^{-1} for subsequent PCR analysis. From each strain, five gene fragments were amplified and sequenced: *atpB*, *EF-2*, *radA*, *rpoB'* and *secY*. These genes were chosen for analysis because they are housekeeping genes that were previously investigated in halobacteria (e.g. Kamaishi *et al.*, 1996; Minegishi *et al.*, 2010; Nakamura *et al.*, 1999; Renesto *et al.*, 2001; Sandler *et al.*, 1999). The PCR and sequencing primers used in this study were adapted from Papke *et al.* (2004, 2007) to accommodate a wider taxonomic range (Table 2). Names of primers include information on the region amplified for each gene.

Amplification was performed with Invitrogen *Taq* DNA Polymerase Native or Roche FastStart Universal SYBR Green Master (Rox). For the majority of loci, the following recipe was used: 5 μl 10 $\times$ buffer, 1.5 μl MgCl_2 (50 mM stock), 1 μl dNTPs, 2.0 μl each primer (10 μM), 1 μl *Taq* polymerase (5 U μl^{-1}), 36.5 μl distilled water and 1 μl template (20 ng μl^{-1}). The *secY* gene primers were added as 3.0 μl aliquots (10 μM stock). All reactions were performed in an Eppendorf Mastercycler EPgradient thermocycler. The annealing temperature for the thermal profile was optimized for each primer set (Table 2). The general thermal profile included an initial denaturation step (1 min, 94 °C) followed by 30 cycles of denaturation (1 min, 94 °C), annealing (1 min) and extension (1 min, 72 °C) and a final extension period (5 min, 72 °C).

PCR amplicons were purified using standard procedures and sequenced in both directions using BigDye chemistry and an ABI 3130xl DNA Analyzer or an ABI 3730xl DNA Analyzer through Genewiz (<http://www.Genewiz.com>). Sequencing primers are listed in Table 2. Each sequence was edited using the software package Geneious (<http://www.geneious.com/>). Amplicon lengths in nucleotide base pairs are: *atpB*, 493; *EF-2*, 507; *radA*, 498; *rpoB'*, 519; *secY*, 495.

Multisequence alignment. DNA sequences were translated into amino acid sequences, aligned using Muscle version 3.6 (Edgar, 2004a, b) and edited using Mesquite version 2.71 (Maddison & Maddison, 2009). Nucleotide sequences from *Haloarcula* strains were aligned separately using the amino acid alignment as a template. Regions of sequence alignments present in less than 50% of the taxa were removed for phylogenetic analysis. Alignments of each gene for both the comprehensive *Halobacteriales* dataset and the *Haloarcula* subset were concatenated in the following order: *atpB*, *EF-2*, *radA*, *rpoB'* and *secY*. Alignments are available upon request from the corresponding author.

Phylogenetic reconstruction and congruence. Phylogenetic reconstructions for the *Halobacteriales* dataset and the *Haloarcula* subset were performed on each gene and the concatenated loci. Optimal models of evolution were estimated from the nucleotide data of the *Haloarcula* subset using jModelTest version 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008), considering 11 substitution types, and from the amino acid data of the *Halobacteriales* dataset using ProtTest

Table 1. Analysed strains of the *Halobacteriales* and other archaea, with accession numbers

Genera are abbreviated following the recommendations of the ICSP Subcommittee on the taxonomy of *Halobacteriaceae* (Oren & Ventosa, 2000) as follows: *Har.*, *Haloarcula*; *Hbt.*, *Halobacterium*; *Hcc.*, *Halococcus*; *Hfx.*, *Haloferax*; *Hmc.*, *Halomicrobium*; *Hqr.*, *Haloquadratum*; *Hrd.*, *Halorhabdus*; *Hrr.*, *Halorubrum*; *Htg.*, *Haloterrigena*; *Nab.*, *Natrialba*; *Nnm.*, *Natrinema*; *Nbt.*, *Natronobacterium*; *Nmn.*, *Natronomonas*; *Nrr.*, *Natronorubrum*. ND, Not determined (unable to obtain a PCR product). Accession numbers from this study begin HQ.

Strain	<i>atpB</i>	<i>EF-2</i>	<i>radA</i>	<i>rpoB'</i>	<i>secY</i>
' <i>Har. aidinensis</i> ' A5	HQ149341	HQ149387	HQ149430	HQ149469	HQ149498
<i>Har. amylolytica</i> JCM 13557 ^T	HQ149372	HQ149388	HQ149431	AB477142	HQ149499
<i>Har. argentinensis</i> JCM 9737 ^T	HQ149342	HQ149389	HQ149432	HQ149481	HQ149500
' <i>Har. californiae</i> ' JCM 8912	HQ149343	HQ149390	HQ149433	AB477144	HQ149501
<i>Har. hispanica</i> ATCC 33960 ^T	HQ149344	HQ149391	HQ149434	HQ149480	HQ149502
<i>Har. japonica</i> JCM 7785 ^T	HQ149345	HQ149392	HQ149435	AB477146	HQ149503
<i>Har. marismortui</i> ATCC 43049 ^T	HQ149346	HQ149393	HQ149436	AY596297	NC_006396
<i>Har. quadrata</i> JCM 11048 ^T	HQ149348	HQ149395	HQ149438	HQ149482	HQ149504
' <i>Har. sinaiensis</i> ' ATCC 33800	HQ149350	HQ149397	HQ149440	HQ149483	HQ149505
<i>Haloarcula</i> sp. Aidin-2	HQ149336	HQ149382	HQ149425	HQ149471	HQ149494
<i>Haloarcula</i> sp. Aidin-5	HQ149337	HQ149383	HQ149426	HQ149472	HQ149495
<i>Haloarcula</i> sp. Aidin-9	HQ149338	HQ149384	HQ149427	ND	HQ149496
<i>Haloarcula</i> sp. Aidin-11	HQ149335	HQ149381	HQ149424	HQ149470	HQ149493
<i>Haloarcula</i> sp. NG5	HQ149339	HQ149385	HQ149428	HQ149473	ND
<i>Haloarcula</i> sp. port	HQ149347	HQ149394	HQ149437	HQ149474	ND
<i>Haloarcula</i> sp. Safaga-17	HQ149367	HQ149421	HQ149466	HQ149477	HQ149521
<i>Haloarcula</i> sp. shark2	HQ149349	HQ149396	HQ149439	HQ149475	ND
<i>Haloarcula</i> sp. Toen-14	HQ149368	HQ149422	HQ149467	HQ149478	HQ149522
<i>Haloarcula</i> sp. Toen-15	HQ149369	HQ149423	HQ149468	HQ149479	HQ149523
<i>Haloarcula</i> sp. XD1	HQ149340	HQ149386	HQ149429	ND	HQ149497
<i>Har. vallismortis</i> ATCC 29715 ^T	HQ149351	HQ149398	HQ149441	AB477148	HQ149506
<i>Hbt. salinarum</i> JCM 9120	HQ149380	X17148	AF090196	X57144	HQ149507
<i>Hbt. salinarum</i> NCIMB 2288	AM774415	HQ149399	HQ149442	CAP15004	HQ149508
<i>Halobacterium</i> sp. NRC-1	AE004437	AE004437	AE004437	AAG20693	AE004437
<i>Hcc. hamelinensis</i> JCM 12892 ^T	HQ149373	HQ149400	HQ149443	AB477156	HQ149509
<i>Hcc. morrhuae</i> JCM 8876 ^T	HQ149352	HQ149401	HQ149444	HQ149484	HQ149510
<i>Hcc. saccharolyticus</i> ATCC 49257 ^T	HQ149353	HQ149402	HQ149445	HQ149485	HQ149511
<i>Hfx. denitrificans</i> ATCC 35960 ^T	HQ149354	HQ149403	HQ149446	HQ149486	HQ149512
<i>Hfx. volcanii</i> NCIMB 2287 ^T	NC_013967	HQ149404	HQ149447	HQ149487	NC_013967
<i>Hmc. mukohataei</i> DSM 12286 ^T	HQ149355	HQ149405	HQ149448	CP001688	CP001688
<i>Hqr. walsbyi</i> DSM 16790	NC_008212	NC_008212	NC_008212	YP_659080	NC_008212
<i>Hrd. utahensis</i> DSM 12940 ^T	HQ149356	CP001687	HQ149449	HQ149488	CP001687
<i>Hrr. aidingense</i> JCM 13560 ^T	HQ149374	HQ149406	HQ149450	AB477176	HQ149514
<i>Hrr. coriense</i> JCM 9275 ^T	HQ149357	HQ149407	HQ149451	HQ149490	HQ149515
<i>Hrr. distributum</i> JCM 9100 ^T	HQ149358	HQ149408	HQ149452	HQ149491	ND
<i>Hrr. lacusprofundi</i> ATCC 49239 ^T	HQ149375	NC_012029	HQ149453	AB477181	NC_012029
<i>Hrr. saccharovororum</i> JCM 8865 ^T	HQ149376	HQ149409	HQ149454	AB477184	HQ149516
<i>Hrr. sodomense</i> JCM 8880 ^T	HQ149377	HQ149410	ND	AB477185	HQ149517
<i>Halorubrum</i> sp. TP009	AM777270	AM777546	AM777664	HQ149489	HQ149513
<i>Hrr. vacuolatum</i> JCM 9060 ^T	HQ149378	HQ149411	ND	AB477190	HQ149518
<i>Htg. turkmenica</i> GSL-11	HQ149359	HQ149412	HQ149455	HQ149492	ND
<i>Htg. turkmenica</i> ATCC 51198 ^T	HQ149360	HQ149413	HQ149456	AB477198	NC_013743
<i>Natrialba</i> sp. Aidin-3	HQ149361	HQ149414	HQ149457	HQ149476	HQ149520
<i>Nab. magadii</i> ATCC 43099 ^T	HQ149379	NC_013922	HQ149458	AB477206	NC_013922
<i>Nnm. pallidum</i> NCIMB 784	HQ149363	HQ149418	HQ149462	AB477210	ND
<i>Natrinema</i> sp. XA3-1	HQ149362	HQ149417	HQ149461	AB295622	HQ149524
<i>Nnm. versiforme</i> JCM 10478 ^T	HQ149364	ND	HQ149463	AB477212	HQ149519
<i>Nbt. gregoryi</i> NCIMB 2189 ^T	HQ149371	HQ149416	HQ149460	AB477213	ND
<i>Natronobacterium</i> sp. SSL6	HQ149370	HQ149415	HQ149459	AJ809525	ND
<i>Nmn. pharaonis</i> JCM 8858 ^T	NC_007426	NC_007426	NC_007426	AJ809526	NC_007426
<i>Natronorubrum</i> sp. JCM 10938	HQ149365	HQ149419	HQ149464	AJ809527	ND

Table 1. cont.

Strain	<i>atpB</i>	<i>EF-2</i>	<i>radA</i>	<i>rpoB'</i>	<i>secY</i>
<i>Natronorubrum</i> sp. JCM 10944	HQ149366	HQ149420	HQ149465	ND	ND
<i>Archaeoglobus fulgidus</i> DSM 4304 ^T	NC_000917	NC_000917	NC_000917	NC_000917	NC_000917
<i>Methanosarcina acetivorans</i> C2A ^T	NC_003552	NC_003552	NC_003552	NC_003552	NC_003552
<i>Methanosarcina barkeri</i> Fusaro	CP000099	CP000099	CP000099	CP000099	CP000099
<i>Methanosarcina mazei</i> Go1	AE008384	AE008384	AE008384	AE008384	AE008384
<i>Methanothermobacter thermautotrophicus</i> ΔH^T	CP002278	CP002278	CP002278	CP002278	CP002278
<i>Methanococcus vannielii</i> SB	CP000742	CP000742	CP000742	CP000742	CP000742

version 2.4 (Abascal *et al.*, 2005; Guindon & Gascuel, 2003) with the slow optimization strategy. Phylogenies reconstructed with maximum-likelihood (ML) mapping used RAxML version 7.0.4 (Stamatakis, 2006) with 100 bootstrap replicates. These phylogenies were based on the GTR nucleotide model of evolution and the WAG amino acid model of evolution. The inclusion of an alpha parameter and invariable sites was determined by the results of jModelTest version 0.1.1 and ProtTest version 2.4, respectively.

Additional support for ML phylogenies was determined through bootstrap analysis with 100 pseudoreplicates using distance and maximum-parsimony (MP) criteria, which were calculated using PAUP* version 4.0b10 (Swofford, 2003). The evolutionary models for

both distance and MP criteria were recommended by the results from jModelTest version 0.1.1 and ProtTest version 2.4. All bootstrap values equal to or greater than 70% that corresponded to clades represented in the ML phylogenies were mapped onto those trees.

Optimal substitution models were approximated from the nucleotide data from the *Haloarcula* strains using jModelTest and from the amino acid data from all of the strains in this study using ProtTest; the results of these tests are shown in Table 3. The models proposed for the nucleotide data included TN (Tamura & Nei, 1993), TIM2 and TIM3, and GTR (Tavaré, 1986). These models all consider unequal base frequencies and two different transition rates, but vary in the number of transversion rates deemed necessary to model evolution.

Table 2. Amplification and sequencing primers

Primer names are derived from the locus they amplify, followed by the position in the alignment that they prime, whether they were forward (F) or reverse (R) and whether they were used for sequencing (seq) or had polylinker (poly) or M13 sequences used for sequencing. T_A is the annealing temperature as determined by gradient PCR. M13 and polylinker sequences are indicated in bold.

Primer	T_A (°C)	Sequence (5'–3')
atpB_409Fa	54	GACATCGTCGGTGAGSCVATSAACCC
atpB_906Ra	54	GCCAGGTCVGTRTACATGTA
atpB_409Fb_poly	60	GCATGCTGAATTCATAGATGCCGGG GACATCGTCGGTGAGSCVATSAACCC
atpB_906Rb_poly	60	GACGTCGCATGCTGAATTCATAGATGCCGGG GCCAGGTCVGTRTACATGTA
atpB_409Fb_seq	–	GACATCGTCGGTGAGSCVATSAACCC
atpB_906Rb_seq	–	GCCAGGTCVGTRTACATGTA
EF-2_634Fa	56	TCCGCGCTBTAYAASTGG
EF-2_1147Ra	56	GTGGTCGATGGWYTCGAAHGG
EF-2_634Fb_poly	58	TGAATTCATAGATGCCGGGGT TCCGCGCTVTACAARTGG
EF-2_1147Rb_poly	58	TGAATTCATAGATGCCGGGAT GTGGTGATSGACTCGAASGG
EF-2_634Fb_seq	–	TCCGCGCTVTACAARTGG
EF-2_1147Rb_seq	–	GTGGTCGATSGACTCGAASGG
radA_prSJS252Fa	54	ACSGARKTSTWCGSGARTTCGGSKCSGGSAA
radA_prSJS253Ra	54	GTCSGGGTTSMSAMSACCTGGTTSCT
radA_M13Fb	59	TGTAAAACGACGGCCAGT ACCGARKTRTACGSGAGTTGSBKCSGGSAA
radA_M13Rb	59	CAGGAAACAGCTATGAC CRCTCVGGSGMSKVSACCTGRTTBGT
rpoB'_940Fa	56	TCGAYGARCCVGACGACATGG
rpoB'_1462Ra	56	AGCGGGCGTGS AKYTTGTT
rpoB'_962Fb_M13	58	TGTAAAACGACGGCCAGT TCTGAAGAGCCGGACGACATGG
rpoB'_1504Rb_M13	58	CAGGAAACAGCTATGAC CGGTGACACCTGBACCGGNCC
secY_356Fa	53	TCTATCAGGGVCKNCAGAAG
secY_856Ra	53	GCRTARATGAGCTTBACCGGG
secY_356Fb_poly	60	GACGTCGCATGCTGAATTCATAGATGCCGGT TCTATCAGGGVCKNCAGAAG
secY_856Rb_poly	60	ACGTCGCATGCTGAATTCATAGATGCCGGG CR TARATGAGCTTBACCGGG
secY_856Rc_M13	60	CAGGAAACAGCTATGAC CGTTACATGAGCTTBACCGGG
secY_356Fb_seq	–	TCTATCAGGGVCKNCAGAAG

Table 3. jModelTest- and ProtTest-determined evolutionary models used for constructing the phylogeny of each locus and dataset

Locus	<i>Haloarcula</i> (jModelTest)	<i>Halobacteriales</i> (ProtTest)
Concatenated	TN+ Γ +I	LG+ Γ +I
<i>atpB</i>	GTR+ Γ +I	LG+ Γ +I
<i>EF-2</i>	TN+ Γ +I	LG+ Γ
<i>radA</i>	TIM2+ Γ +I	LG+ Γ
<i>rpoB'</i>	TN+ Γ	LG+ Γ
<i>secY</i>	TIM3+ Γ	LG+ Γ +I

All of the models proposed included the gamma shape parameter, and all but *rpoB'* and *secY* considered invariable sites. The proposed models for the amino acid data of the entire dataset were all LG (Le & Gascuel, 2008). All of the models recommended the gamma shape parameter, but only half included invariable sites (the concatenated data, *AtpB* and *SecY*).

Congruence between phylogenetic signals represented in tree topologies and sequence alignments were evaluated using the approximately unbiased (AU) test (Shimodaira, 2002). Site-wise log-likelihood values of all ML topologies were calculated using RAXML version 7.0.4 and the AU test was calculated using Consel version 0.1 (Shimodaira & Hasegawa, 2001); the *Haloarcula* subset was evaluated separately. A *P*-value of 1.00 indicates congruence, whereas a *P*-value of <0.05 is considered statistically incongruent. Incongruence between one topology and the sequence data of a different gene indicates a discrepancy between the evolutionary histories of those genes, which could be interpreted as evidence for LGT, though other explanations are possible.

RESULTS AND DISCUSSION

A major goal of this study was to develop the molecular analysis protocol MLSA for phylogeny and taxonomy of the *Halobacteriales* to be robust to the known diversity and future discovery of strains, species and genera. To test MLSA effectively as a new molecular protocol for taxonomy of the *Halobacteriales*, it was necessary to test both narrow and broad taxonomic diversity. Such analysis would determine how precise the method is for differentiating small genetic differences between environmental strains from the same putative species and for identifying phylogenetic clusters as strains organized into species, and species structured into genera. It would also test the primers designed for their ability to amplify a wide diversity of haloarchaea. Therefore, 21 closely related strains from seven recognized species belonging to the genus *Haloarcula* and 31 additional strains from 23 species with validly published names spanning 13 genera throughout the order *Halobacteriales* were investigated (Table 1).

Effectiveness of PCR primers

Members of the order *Halobacteriales* exhibit extensive sequence evolution among known species and environmental strains. To develop effective PCR primers for

analyses, identification of targeted loci common to all taxa necessarily had to strike a balance between using genes conserved enough to develop effective primers and those that are modified enough to be phylogenetically informative. Five loci were targeted for sequence analysis: *atpB*, *EF-2*, *radA*, *rpoB'* and *secY*. All loci except *atpB* had at least one allele for which the sequence could not be obtained, no matter how many attempts were made. PCR and sequencing success was improved by the addition of polylinkers or M-13 sequencing primers to the 5' end, probably due to increased stability of the primers during amplification annealing steps, and exact primer matches (e.g. no degeneracy) during sequencing.

It is not known why some loci were amplified and sequenced more successfully. Primer design could account for some aspects, because attempts to amplify all loci across a diverse group like the *Halobacteriales* can be difficult. Attempts were made to limit the primer degeneracy to 16 $\times$; however, some primer sets that were more degenerate worked better. LGT could also play a role if a homologue was acquired recently from a distant, non-*Halobacteriales* relative. However, both of those explanations seem unsatisfactory, because they do not clarify why, within the genus *Haloarcula*, for example, there was differential amplification success for alleles that one would expect to be highly similar. Nonetheless, a 100% success rate is not necessary to analyse and classify new strains effectively using the MLSA approach, as demonstrated below.

Phylogenetic reconstruction of *Haloarcula* species

Three phylogenetic trees with 100 bootstrap pseudoreplicates were constructed for each gene and the concatenated alignment using different criteria: ML, distance and MP. Results containing nodes with bootstrap values $\geq 70\%$ are mapped onto the ML trees (Fig. 1). The ML trees for the *Haloarcula* subset were built with the GTR substitution model and the gamma shape parameter, with invariable sites included when recommended by the jModelTest results. The distance and MP trees were constructed using the models proposed by the jModelTest.

It has been suggested that concatenation of multiple loci in phylogenetic reconstruction overcomes many of the limitations of single-gene analyses and therefore defines relationships more robustly among taxa for classification purposes (Hanage *et al.*, 2005). Visual inspection of the *Haloarcula* concatenated alignment tree reveals that a majority of the strains and species fall into two well-supported clades, indicated in Fig. 1(a) by filled and shaded bars; subsequently, members of the 'black' and 'grey' clusters were identified and marked similarly in the single-gene tree phylogenies (Fig. 1b–f). Members of the 'black' and 'grey' clusters form monophyletic clusters with strong bootstrap support for a majority of the strains in the individual gene phylogenies; however, there were exceptions. Loci from strains that deviated from the concatenated phylogeny, and from each other, are interpreted to have experienced LGT. Within the

Fig. 1. Analysis of *Haloarcula* polarized by *Haloferax*. ML trees based on nucleotide sequence alignments of a concatenation of all five genes (a), *atpB* (b), *EF-2* (c), *radA* (d), *rpoB* (e) and *secY* (f). Bootstrap values $\geq 70\%$ were obtained from three separate analyses and are presented in the order ML/distance/MP; –, $<70\%$. See Table 1 for genus abbreviations, strain details and sequence accession numbers.

'black' and 'grey' clades, there are 'core' strains that remain relatively constant as a cluster, are closely related to each other, are related to *Haloarcula amylolytica* and *Haloarcula marismortui*, respectively, often have identical sequences at individual loci and nearly always form a monophyletic group (with exceptions).

Within the 'black' clade, *Haloarcula hispanica* and *Haloarcula* sp. XD1 consistently fall outside the 'core', indicating that the MLSA molecular data coincide with the uniqueness of *Har. hispanica* as being a species separate from *Har. amylolytica*.

Further, it indicates that *Haloarcula* sp. XD1 needs to be examined more fully for taxonomic description. Moreover, '*Haloarcula aidinensis*', a species whose name has not been validly published, along with each of the 'core' strains of the 'black' clade, clusters together tightly with *Har. amylolytica*.

The 'grey' clade, however, does not appear as clear-cut in identifying and defining species, probably because of frequent homologous recombination between strains. The major conflicting data are that *Har. marismortui* and *Har. quadrata* have nearly identical DNA sequences (see *atpB*,

Table 4. AU test results for congruence between loci across *Haloarcula* strains

Comparisons with a *P*-value <0.05 are rejected as incongruent and are in bold.

Dataset	Concatenated	<i>atpB</i>	<i>EF-2</i>	<i>radA</i>	<i>rpoB'</i>	<i>secY</i>
Concatenated	0.673	0.059	0.000	0.000	0.001	0.006
<i>atpB</i>	0.000	0.891	0.000	0.000	0.001	0.001
<i>EF-2</i>	0.000	0.000	0.958	0.000	0.000	0.000
<i>radA</i>	0.016	0.001	0.000	0.927	0.000	0.000
<i>rpoB'</i>	0.149	0.031	0.080	0.034	0.958	0.000
<i>secY</i>	0.557	0.277	0.086	0.135	0.049	0.998

rpoB') and so do *Har. marismortui* and *Har. vallismortis* (see *EF-2* and *radA*). Furthermore, '*Haloarcula californiae*', '*Har. sinaiensis*' and *Haloarcula* sp. Safaga-17 consistently cluster with either *Har. marismortui* or *Har. quadrata* or both; the decision as to which of the two taxonomic species to which they might belong is their correct home is filled with complexity and cannot be sorted out by this analysis. It is clear from this analysis that the different species in the 'grey' clade with validly published names are not genetically isolated; they continue to recombine frequently with other members of the community and form more of a continuum rather than the discrete clusters needed for taxonomic circumscription.

Of the strains that do not fall into the 'black' and 'grey' clusters, only one represents a species with a validly published name (*Haloarcula argentinensis*). One strain, Toen-14, is closely related to *Har. argentinensis* and is probably another isolate of that species. However, *Haloarcula* sp. NG5 and *Haloarcula* sp. port are sufficiently genetically divergent from all other *Haloarcula* strains and species that further analysis should be performed to assess their taxonomic standing.

These results demonstrate that MLSA is an effective method for rapid identification and it is sensitive for species circumscription. In this study, the concatenated phylogeny demonstrates clustering patterns that provide important insight into the organization of these strains within the genus *Haloarcula*, and can address taxonomic questions. Novel species can be identified by comparing the genetic distance of unnamed strains to the type strains of species. For example, *Haloarcula* sp. port, NG5 and XD1 are sufficiently distant from all known species to warrant their placement within separate species, should additional phenotypic and biochemical data support this claim. Additionally, rapid and inexpensive identification of uncharacterized environmental strains can be carried out within species with validly published names; for example, single-gene and concatenated phylogenies cluster *Haloarcula* sp. Toen-14 with *Har. argentinensis*, suggesting a renaming as *Har. argentinensis* Toen-14.

MLSA also addressed issues of taxonomic uncertainty and raised questions regarding named species. In this analysis, *Har. amylolytica* and '*Har. aidinensis*' were in the same cluster. This cluster encompasses very little sequence divergence, and the number of nucleotide substitutions between *Har. amylolytica* and '*Har. aidinensis*' is only 9 out

of 2511 bases analysed. According to Ventosa (2001), '*Har. aidinensis*' was proposed as a species, but its name was not validly published because the characterization attempted revealed insufficient differences. Each of the five genes analysed in this study strongly suggests that '*Har. aidinensis*' A5 in fact belongs to *Har. amylolytica*, as do all of the unnamed strains within the 'black' cluster 'core'. Interestingly, this observation suggests that enormous phenotypic or metabolic diversity exists within limited genotypic diversity.

Within the 'grey' cluster, MLSA easily identified two strains as members of separate species, *Har. vallismortis* and *Haloarcula* sp. shark2. *Har. vallismortis* is the type species of the genus; however, the strain shark2 is remarkably distinct and requires further analysis, as it probably represents a novel taxon. However, there is much more ambiguity within the grey cluster 'core'. Clearly, *Har. quadrata* and *Har. marismortui* are different species based on other evidence (Oren *et al.*, 1990, 1999); however, what is not so clear is whether the unnamed 'grey' clade 'core' strains should be divided between them or whether they require their own designations, or possibly that there are no discernible discrete differences because they form a gradient of diversity, with *Har. quadrata* and *Har. marismortui* representing the extremes. The major contributing factor to the taxonomic uncertainty is homologous recombination among the strains analysed. This is not a unique situation among the *Halobacteriales*, as *Halorubrum* strains also demonstrate this phenomenon (Papke *et al.*, 2004, 2007). A likely scenario is that members of the 'grey' clade core were once a single species in the not too distant past and, through mutation and frequent recombination with each other and with close relatives, they diversified. Rather than demonstrating an easy-to-understand bifurcating evolutionary history, they reveal a network of relationships that complicates their taxonomic placement. However, the observation that cells of '*Har. sinaiensis*' and '*Har. californiae*' display a box-shaped morphology suggests they probably belong to the species *Har. quadrata*, rather than *Har. marismortui*, which is pleomorphic (Oren *et al.*, 1999).

The single-gene phylogenies show variation between gene histories that can provide insight into species diversification and genus stability. Primarily, the gene phylogenies are incongruent, according to the AU tests (Table 4), probably

indicating different evolutionary histories. While the 'black' clade and the 'grey' clade are monophyletic in the concatenated phylogeny, they are paraphyletic in many of the single-gene phylogenies. For example, in the *EF-2* phylogeny, *Haloarcula* sp. XD1 is found within the 'black' clade, probably because of an LGT event. Furthermore, in the 'grey' clade, not only is *Har. marismortui* separate from the rest of the 'grey' clade, but it is also identical to *Har. vallismortis*, which is probably the result of a more recent LGT event. The *secY* gene, or portions of it, appears to be highly mobile among the members of the 'grey' clade, as it does not form a cohesive group. Therefore, LGT can be understood as a diversifying force. While there are similar examples of LGT across all of the analysed loci, the majority signal in the concatenated phylogeny strongly supports the cohesion of these clades. This could be due to recombination events occurring more frequently with close relatives, rather than distant ones. The observation that most recombination events within the genus *Haloarcula* do not come from other genera suggests that LGT, while making some species taxonomy difficult, also acts as a homogenizing force that reinforces genetic cohesiveness at the genus level.

Phylogenetic reconstruction of the *Halobacteriales*

Similar to the nucleotide data presented in the analysis of *Haloarcula*, phylogenetic trees were constructed from the amino acid data under three different criteria: ML, distance and MP. The ML trees were built using the WAG substitution model (Whelan & Goldman, 2001), the gamma shape parameter and invariable sites when recommended. The distance and MP trees were also constructed using the models predicted by ProtTest. Bootstrap analysis values $\geq 70\%$ from those methodologies were mapped onto the ML trees (Fig. 2). The concatenated phylogeny (Fig. 2a) shows that most species cluster according to their assigned genus. The exceptions to this pattern were strains within the genera *Haloterrigena*, *Natrialba* and *Natrinema*. Species clustered according to genera in all single-gene phylogenies (Fig. 2b–f) except those belonging to the three genera noted above. The only other exception was the genus *Halococcus* in the *EF-2* tree (Fig. 2c), in which *Halococcus saccharolyticus* appears to have received a gene from a member of *Halobacterium*. There are also two sets of genera that cluster together (designated by open and filled bars) with significant support in the concatenated phylogeny (Fig. 2a), and these groups coincide with clades I and II, respectively, proposed by Walsh *et al.* (2004). Members of those clades are similarly identified in the single-gene phylogenies (Fig. 2b–f). Both clades are reconstructed in the *RadA* and *RpoB'* phylogenies (Fig. 2d, e); however, only clade I has significant bootstrap support. The species that do not cluster according to genus (see above) are all members of clade I, implying that there is a considerable amount of gene flow among members of the group. The observation that clade I is still observed in the concatenated phylogeny, but is not supported, suggests that

its cohesion may be facilitated by biased LGT among its members [e.g. see above and Andam *et al.* (2010)].

The results of the *secY* analysis, for both the *Haloarcula* subset and the *Halobacteriales* complete set, exhibited significant variation in clustering patterns compared with the other four genes. Interestingly, the *secY* phylogeny for the *Halobacteriales* demonstrated that four members of the assumed outgroup (*Archaeoglobus fulgidus*, *Methanosarcina acetivorans*, *Methanosarcina barkeri* and *Methanosarcina mazei*) clustered within the order *Halobacteriales*, suggesting a gene transfer from the *Halobacteriales*. However, there is no bootstrap support for the backbone of the tree, and further investigation is therefore warranted before any firm conclusions should be made. Use of *secY* in an MLSA set of useful genes appears suboptimal; however, it is too early to make a firm conclusion, and it is nonetheless helpful in understanding gene flow. For instance, use of recombination, like a biological species concept in sexually reproducing organisms, may lead to better species taxonomy. Removal of *secY* from the concatenated datasets had no discernible effect on how strains clustered (not shown).

By investigating a wide range of diversity, the efficacy of MLSA for broad levels of taxonomic organization was explored. Probably the most important aspect of this testing is that the primers designed are capable of amplifying a large number of halobacterial taxa and thus are likely to amplify the entire breadth of known and unknown species. Additionally, generally speaking, each analysed locus, and the concatenated data, organized each of the strains and species into their respective genera and provided an advantage over the 16S rRNA gene for the reasons discussed above (e.g. contain highly divergent paralogous copies, too conserved to differentiate closely related strains and species, intragenically and interspecifically recombinogenic). There was very little statistical support for relationships between genera, except for two groups, which correspond to clades I and II from Walsh *et al.* (2004). These groups are pronounced in the concatenated phylogeny, but are only replicated in the *RadA* and *RpoB'* phylogenies. The cumulative data support organization above the genus level within the *Halobacteriales*, despite the fact that the individual gene histories provide conflicting results, as indicated by the AU test results (Table 5). Intergeneric recombination appears to be reorganizing the evolutionary past of these genes, but perhaps the residual ancestral signal is still strong enough to capture family-level organization. If additional data continue to support evidence for clades 1 and 2, perhaps a family designation should be applied.

Conclusions

MLSA as tested proves to be an effective method for reconstructing the phylogeny of the *Halobacteriales* across taxonomic hierarchical levels. The multilocus approach utilized here was sensitive enough to differentiate closely

Fig. 2. Analysis of *Halobacteriales* polarized by euryarchaea. ML trees based on amino acid sequence alignments of a concatenation of all five deduced amino acid sequences (a), AtpB (b), EF-2 (c), RadA (d), RpoB' (e) and SecY (f). Bootstrap values $\geq 70\%$ were obtained from three separate analyses and are presented in the order ML/distance/MP; –, $< 70\%$. See Table 1 for genus abbreviations, strain details and sequence accession numbers. Sequences from six strains were chosen as the outgroup to the *Halobacteriales* and are referred to as *Euryarchaeota* in the tree: *Methanosarcina acetivorans*, *Methanosarcina barkeri*, *Methanosarcina mazei*, *Archaeoglobus fulgidus*, *Methanothermobacter thermautotrophicus* and *Methanococcus vannielii*. Genera that formed clades I and II of Enache *et al.* (2007) and Walsh *et al.* (2004) are indicated by bars; open bars indicate representatives of clade I and filled bars indicate representatives of clade II. Additional genus abbreviations in (f): A., *Archaeoglobus*; M., *Methanosarcina*.

related strains from the same species and to identify strains of sufficient uniqueness that they probably belong to novel species. Furthermore, it could recognize family-like hierarchy within the order. These two attributes, fine-scale

sensitivity and broad-scale application, make MLSA a highly versatile method for taxonomy. Furthermore, by analysing multiple strains from the same species, a better understanding of population dynamics, evolutionary processes and the

Table 5. AU test results for congruence between loci across *Halobacteriales* strains

Comparisons with a *P*-value < 0.05 are rejected as incongruent and are in bold.

Dataset	Concatenated	AtpB	EF-2	RadA	RpoB'	SecY
Concatenated	0.978	0.001	0.000	0.000	0.001	0.000
AtpB	0.000	0.901	0.000	0.000	0.000	0.000
EF-2	0.000	0.082	0.984	0.004	0.069	0.037
RadA	0.006	0.160	0.03	0.929	0.258	0.391
RpoB'	0.002	0.193	0.031	0.029	0.901	0.170
SecY	0.041	0.240	0.011	0.145	0.072	0.758

biological relevance of operational taxonomic units and species becomes accessible. For instance, examining multiple strains that comprised the 'black' and 'grey' clades in the genus *Haloarcula* led to fruitful insight into their taxonomic standing. Additionally, through the examination of tens of *Halorubrum* strains, Papke *et al.* (2007) demonstrated the existence of phylogenetic clusters in which recombination was more prevalent within than between clusters, suggesting a quasi-biological species concept. Thus, application of MLSA to the detection of recombination in the *Halobacteriales* could facilitate an important, currently unrecognized character for species circumscription. However, irrespective of recombination, MLSA in general provides more insight into the dynamics of speciation and species evolution, which are critical for stable species nomenclature.

There has been growing recognition of the power of MLSA for epidemiological, ecological and taxonomic practices. This method is rapid and portable and generates storable data that are highly sensitive and phylogenetically relevant (Maiden *et al.*, 1998; Urwin & Maiden, 2003). It has been suggested that MLSA could be an effective tool to supplement or even supplant DNA–DNA hybridization (DDH), which does not generate storable data, for species circumscription in the recommended polyphasic approach to taxonomy (Young *et al.*, 2008). Of course, more comparative analyses between MLSA and DDH need to be done before such a recommendation should be made for its use in the *Halobacteriales*.

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