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1 Novel haloarchaeal viruses from Lake Retba infecting *Haloferax* and
2 *Halorubrum* species

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23 **Summary**

24 The diversity of archaeal viruses is severely undersampled compared to that of viruses infecting bacteria and
25 eukaryotes, limiting our understanding on their evolution and environmental impacts. Here we describe the
26 isolation and characterization of four new viruses infecting halophilic archaea from the saline Lake Retba,
27 located close to Dakar on the coast of Senegal. Three of the viruses, HRPV10, HRPV11 and HRPV12, have
28 enveloped pleomorphic virions and should belong to the family *Pleolipoviridae*, whereas the fourth virus,
29 HFTV1, has an icosahedral capsid and a long non-contractile tail, typical of bacterial and archaeal members
30 of the order *Caudovirales*. Comparative genomic and phylogenomic analyses place HRPV10, HRPV11 and
31 HRPV12 into the genus *Betapleolipovirus*, whereas HFTV1 appears to be most closely related to the
32 unclassified *Halorubrum* virus HRTV-4. Differently from HRTV-4, HFTV1 encodes host-derived
33 minichromosome maintenance helicase and PCNA homologs, which are likely to orchestrate its genome
34 replication. HFTV1, the first archaeal virus isolated on a *Haloferax* strain, could also infect *Halorubrum* sp.,
35 albeit with an eight-fold lower efficiency, whereas pleolipoviruses nearly exclusively infected autochthonous
36 *Halorubrum* strains. Mapping of the metagenomic sequences from this environment to the genomes of
37 isolated haloarchaeal viruses showed that these known viruses are underrepresented in the available
38 viromes.

39

40 Introduction

41 Hypersaline environments, where salt concentration is close to saturating, harbor a high number of virus-like
42 particles (VLP) but rather low microbial diversity (Oren, 2002; Pagaling *et al.*, 2007; Sime-Ngando *et al.*, 2011;
43 Ventosa *et al.*, 2015; Roux *et al.*, 2016). Although these environments are dominated by archaea and bacteria,
44 some eukaryotes are present, e.g. the salt-adapted unicellular green alga *Dunaliella salina*, some fungi and
45 yeast, protozoa, and the brine shrimp *Artemia* (Triantaphyllidis *et al.*, 1998; Gunde-Cimerman *et al.*, 2018).
46 Haloarchaeal virus predation is among the most important factors driving the genetic variation of different
47 haloarchaeal species. For instance, most of the differences between the closely related species are mapped
48 to the genes encoding for cell surface structures and their modification, which directly affect virus-host
49 interactions (Cuadros-Orellana *et al.*, 2007; Dyll-Smith *et al.*, 2011; Tschitschko *et al.*, 2018). As of today,
50 more than 100 viruses have been isolated from hypersaline environments of which the majority infect
51 extremely halophilic euryarchaea, all belonging to the class Halobacteria (Tang *et al.*, 2004; Pagaling *et al.*,
52 2007; Atanasova *et al.*, 2015b; Dyll-Smith *et al.*, 2019). Halophilic viruses are well adapted to high salinity
53 and some of them remain infectious even in saturated salt (Demina *et al.*, 2016a). Some viruses can even
54 survive in low salinities, a beneficial trait under changing environmental conditions (Pietilä *et al.*, 2013c).
55 Halophilic archaeal viruses fall into four different morphological groups: spindle-shaped (genus
56 *Salterprovirus*), pleomorphic (family *Pleolipoviridae*), tailless icosahedral (family *Spherolipoviridae*), and
57 tailed icosahedral (order *Caudovirales*) (Pietilä *et al.*, 2014; Pietilä *et al.*, 2016; Demina *et al.*, 2017). However,
58 filamentous and some exceptional morphotypes not reminiscent of any isolated virus, e.g. hairpin-shaped,
59 bacilliform, and chain-like VLPs, have also been visualized in hypersaline water bodies (Oren *et al.*, 1997;
60 Sime-Ngando *et al.*, 2011; Di Meglio *et al.*, 2016). Significant number of archaeal virus genes have no obvious
61 homologs in public sequence databases (Krupovic *et al.*, 2018). Thus, classification of viral sequences in
62 metagenomic datasets is still a challenge due to the high genetic diversity, despite of remarkable advances
63 in next-generation sequencing and bioinformatics. In addition, the relatively low number of described
64 halophilic archaeal virus isolates with determined complete genome sequences limits the utility of sequence
65 similarity-based analyses. For example, only few similarities to the NCBI non-redundant database at either

66 the nucleotide or amino acid level were reported following the analyses of the metaviromes from hypersaline
67 Lake Retba (Sime-Ngando *et al.*, 2011; Roux *et al.*, 2016) and halite endoliths in the Atacama Desert (Crits-
68 Christoph *et al.*, 2016).

69 Two groups of haloarchaeal viruses have been frequently isolated from geographically remote locations.
70 These include members of the family *Pleolipoviridae* and the order *Caudovirales*, respectively. Membrane-
71 containing virions of pleolipoviruses resemble extracellular membrane vesicles able to carry the virus
72 genome from host to host (Pietilä *et al.*, 2012). They represent a unique archaeal virus group with a recently
73 established taxonomic position as the first family containing viruses with either single-stranded (ss) or
74 double-stranded (ds) DNA genomes (linear or circular forms) (Pietilä *et al.*, 2016; Bamford *et al.*, 2017). The
75 non-lytic pleomorphic virus life-cycle starts with fusion between the viral and host membranes and they exit
76 the host cell most probably by budding, preserving the host membrane integrity (Svirskaitė *et al.*, 2016; El
77 Omari *et al.*, 2019). Their simplistic mechanism of nucleic acid transmission resembles the function of the
78 recently described “infectious” plasmid membrane vesicles isolated from Antarctic species of haloarchaea
79 (Erdmann *et al.*, 2017), supporting the tight evolutionary relationships between viruses and non-viral mobile
80 genetic elements (Iranzo *et al.*, 2016b). Pleolipoviruses share a conserved core of four to five genes, mainly
81 encoding major structural proteins of which one is the spike protein responsible for host attachment and
82 membrane fusion (Pietilä *et al.*, 2012; Sencilo *et al.*, 2012; El Omari *et al.*, 2019).

83 The tailed icosahedral dsDNA viruses represent the most numerous archaeal virus group described today
84 (Atanasova *et al.*, 2015a). Intriguingly, all these viruses infect halophiles or methanogens of the phylum
85 Euryarchaeota (Prangishvili *et al.*, 2017). However, the identified proviruses and metagenomic studies
86 suggest wider association of archaeal tailed viruses across different orders within the Euryarchaeota, but also
87 with members of the phylum Thaumarchaeota (Krupovic *et al.*, 2010a; Krupovic *et al.*, 2011b; Danovaro *et*
88 *al.*, 2016; Filosof *et al.*, 2017; Vik *et al.*, 2017; Abby *et al.*, 2018; Ahlgren *et al.*, 2019; Lopez-Perez *et al.*,
89 2019). This group of viruses shares the same architectural principles with the icosahedral tailed dsDNA
90 bacteriophages of the order *Caudovirales* (Pietilä *et al.*, 2013b). All three different tail structures initially

91 characterized for bacteriophages, have been found among the archaeal viruses: long contractile
92 (myoviruses), long non-contractile (siphoviruses), and short non-contractile tails (podoviruses) (Atanasova *et al.*,
93 *et al.*, 2012). Genomes of archaeal caudoviruses are mosaics of genes with different evolutionary histories and
94 their gene contents and genome lengths differ considerably, and consequently, also differ their capsid sizes,
95 making this group genetically very diverse (Krupovic *et al.*, 2010a; Pietilä *et al.*, 2013c; Sencilo *et al.*, 2013;
96 Dyall-Smith *et al.*, 2019). Although their capsid structures are very conserved, their receptor binding proteins
97 have a high genetic plasticity allowing them to adapt to new hosts. Particularly, archaeal myoviruses with
98 contractile tails have very broad host ranges crossing the genus boundary (Atanasova *et al.*, 2012; Atanasova
99 *et al.*, 2015c). Furthermore, several myoviruses were shown to encode an invertible tail fiber gene module,
100 which allows these viruses to alternate between different variants of the tail fiber proteins with distinct host
101 specificities (Rossler *et al.*, 2004; Dyall-Smith *et al.*, 2018; Dyall-Smith *et al.*, 2019).

102 Both culture-independent and culture-dependent approaches indicate that haloviruses represent a globally
103 distributed reservoir of orphan genes encoding novel functions (Aalto *et al.*, 2012; Atanasova *et al.*, 2012;
104 Roux *et al.*, 2016). In addition, many halophilic archaea carry proviruses in their chromosomes (Krupovic *et al.*,
105 *et al.*, 2010a; Dyall-Smith *et al.*, 2011; Makarova *et al.*, 2014; Liu *et al.*, 2015; Maier *et al.*, 2015; Demina *et al.*,
106 2016a; Atanasova *et al.*, 2018b). The co-evolution of viruses and host cells in the presence of high
107 recombination frequency in halophilic microbes have resulted in a globally distributed complex network of
108 viruses, proviruses, membrane vesicle, transposons, and plasmids sharing the common genetic pool and
109 displaying dynamic interplay across time and space (Zhang *et al.*, 2012; Forterre *et al.*, 2014; Atanasova *et al.*,
110 2015c; Liu *et al.*, 2015; Iranzo *et al.*, 2016b; Atanasova *et al.*, 2018b; Dyall-Smith and Pfeiffer, 2018; Wang
111 *et al.*, 2018a; Wang *et al.*, 2018b). Here we report on the isolation of four new haloarchaeal viruses from
112 saline Lake Retba. Morphological and genomic characterization of these viruses allowed their tentative
113 taxonomic assignments. The siphovirus HFTV1, to the best of our knowledge, is the first virus isolated on a
114 *Haloferax* strain, and it should belong in the order *Caudovirales* with other archaeal and bacterial tailed
115 dsDNA viruses. Bacterial and archaeal caudoviruses together with eukaryotic herpesviruses form the HK97–
116 like virus lineage (Abrescia *et al.*, 2012). The pleomorphic archaeal viruses might belong to a tentative new

117 virus lineage comprising membrane vesicle-like archaeal viruses of the family *Pleolipoviridae*. Collectively,
118 our results further expand the knowledge on the genomic diversity and host range of haloarchaeal viruses
119 and provide insights into their genome evolution.

120 Results and Discussion

121 Isolation of novel archaeal viruses of *Halorubrum* and *Haloferax*

122 The viruses designated HRPV10, HRPV11, HRPV12 and HFTV1 were isolated together with their host strains
123 from saline Lake Retba (14°50'14" N, 17°14'55" W), close to Dakar, the capital of Senegal, in May, 2011 (Table
124 1). Sample LR1 collected from the center of the Lake Retba contained grey water and a grey sediment mixed
125 with salt, whereas sample LR2 (purple water with white sediment) was collected close to the site where salt
126 is collected for trade. The salinity of the LR1 and LR2 samples was 290-300 g/L and 250 g/L, respectively,
127 whereas the temperature (27°C) and pH (7.8) were the same at both sampling sites.

128 The haloarchaeal isolates were obtained by directly plating of the samples on MGM plates (see Methods).
129 The obtained colonies were colony-purified on solid media. The pure cultures of the isolated halophilic
130 archaeal strains (19 in total) were identified as members of the class *Halobacteria* by partial 16S rRNA gene
131 sequence analysis (Fig. 1, Table 2). The isolates belong to three of the six families of the class Halobacteria:
132 Halorubraceae (11 isolates), Haloferacaceae (7 isolates) and Halobacteriaceae (1 isolate). All isolates from
133 Halorubraceae were identified as *Halorubrum* spp., eight of which form a clade with *Halorubrum*
134 *lacusprofundi*.

135 LR2-19 clusters with *Hrr. sodomense*. LR1-22 clusters with an uncharacterized species of *Halorubrum* and
136 LR2-20 did not cluster with any other strain. Among the isolates within Haloferacaceae, all seven were
137 classified as *Haloferax* spp., clustering with *Hfx. volcanii*. LR2-15, the only representative of Halobacteriaceae,
138 clustered with *Halomicroarcula limicola* and was classified as *Halomicroarcula* sp. LR2-15. The viruses were
139 isolated on the endogenous Lake Retba strains (Table 2) using the same Lake Retba samples (see Methods
140 for details). The host strains of the viruses HRPV10, HRPV11, and HRPV12 are *Halorubrum* spp. LR2-17, LR2-
141 12, and LR1-23, respectively (Table 1), whereas HFTV1 infects *Haloferax* sp. LR2-5, making HFTV1 the first
142 known virus isolated on *Haloferax* strain. The defective proviruses identified in *Hfx. mediterranei* (Li *et al.*,

143 2013) and a variant of Halorubrum virus HF1 capable of infecting *Hfx. volcanii* [(Nuttall and Dyll-Smith, 1993)
144 not available to our knowledge, personal communication], are the only reports on *Haloferax* viruses.

145

146 HRPV10, HRPV11, and HRPV12 produce hazy plaques that are 3-10 mm in diameter, whereas the HFTV1
147 plaques are clear (Table 1; Supplementary Fig. 1). The virus isolate plaque morphologies were different from
148 each other, and HRPV10 and HRPV11, in particular, produce very hazy plaques difficult to document as
149 figures, but they are visible in optimal lightning conditions (Supplementary Fig. 1A and B). The plaque
150 morphologies of the HRPV10, HRPV11, and HRPV12 resemble the plaques of the members in the family
151 *Pleolipoviridae* suggesting that also the plaques of the new pleomorphic virus isolates have non-lytic life cycle
152 and the plaque is a consequence of the host cell growth retardation due to the virus infection (Pietilä *et al.*,
153 2009; Svirskaitė *et al.*, 2016). The plaques were purified by three consecutive time to obtain pure virus
154 cultures (see Methods). The virus stocks gave typical titers of 10^{11} - 10^{12} pfu/ml (Table 1), suggesting that they
155 might be promising model systems for studies on haloarchaeal virus functions and virus-host interactions.
156 Infectivity of the viruses remained unchanged at 4 °C for a period of four weeks (data not shown).

157 For virus purification, virions were collected from the virus stocks by using two-step polyethylene glycol-NaCl
158 precipitation and purified to near homogeneity by rate zonal centrifugation in sucrose followed by
159 equilibrium centrifugation in CsCl. In the case of HRPV10, HRPV11 and HRPV12, this approach yielded highly
160 pure virion preparations based on the specific infectivities (2 - 5×10^{13} pfu/mg of protein; Table 3), negative
161 staining and transmission electron microscopy (TEM) analysis of the purified particles (Fig. 2A-C), and SDS-
162 PAGE gel analysis (Fig. 3A-C). Specific infectivities and protein patterns of the purified HRPV10, HRPV11, and
163 HRPV12 viruses were comparable with data reported for pleomorphic viruses purified by using the
164 comparable precipitation and preparative ultracentrifugation techniques (e.g. viruses HRPV-1, HRPV-2,
165 HRPV-3, HRPV-6, HHPV-1, His2, and HHPV4) yielding highly pure virus material (specific infectivities 2 - 5×10^{13}
166 pfu/mg of protein) (Pietilä *et al.*, 2012; Atanasova *et al.*, 2018b). HFTV1 virus particles were purified in high
167 numbers based on TEM (Fig. 3D) and protein quantities (Table 3) but the purified particles had specific

168 infectivity of $\sim 2 \times 10^9$ pfu/mg of protein (Table 3), which is 3-4 magnitudes lower than e.g. the specific
169 infectivities of the purified virus samples of haloarchaeal tailed virus HSTV-1 ($\sim 9 \times 10^{12}$ pfu/mg of protein) and
170 icosahedral membrane-containing virus HCIV-1 ($\sim 1 \times 10^{12}$ pfu/mg of protein), of which have been analyzed
171 structurally (Pietilä *et al.*, 2013b; Demina *et al.*, 2016b; Santos-Perez *et al.*, 2019). The negative staining and
172 TEM of the purified HFTV1 particles revealed that some of particles had lost their genome explaining partly
173 the loss of infectivity (Fig. 2D).

174

175 **Membrane vesicle-like virions of HRPV10, HRPV11 and HRPV12**

176 The purified HRPV10, HRPV11 and HRPV12 virions were tailless round particles with a diameter of ~ 55 nm
177 (Fig. 2A-C). The virion morphologies resembled one another and those of viruses in the family *Pleolipoviridae*
178 (Pietilä *et al.*, 2012). All three virion types equilibrated in CsCl density gradients (mean density of 1.30-1.35
179 g/ml) suggested that they contain lipids as one of their structural components. HRPV11 and HRPV12 were
180 sensitive to chloroform, a widely used organic solvent, whereas HRPV10 was resistant (Table 1). Infectivity
181 of all three viruses in the presence of non-ionic detergents Nonidet P-40 or Triton X-100 decreased by 7-11
182 orders of magnitude (Table 1).

183 The lipid compositions of the viruses and their host strains were verified by thin-layer chromatography and
184 ammonium molybdate staining. The major polar lipids of *Haloarcula hispanica* – phosphatidylglycerol (PG),
185 phosphatidylglycerophosphate methyl ester (PGP-Me), phosphatidylglycerosulfate (PGS), and triglycosyl
186 glycerodiether (TGD) – have been previously identified (Bamford *et al.*, 2005) and were used as a control (Fig.
187 3A-C). The patterns of lipid species of *Halorubrum* sp. LR2-17, LR2-12, and LR1-23 were identical to each
188 other, containing probably PG, PG-Me and PGS (Fig. 3A-C). In all three viruses, lipids were found to be a
189 structural component of the virions (Fig. 3A-C). The virus lipid profiles were identical with each other and
190 resembled the lipid profiles of their respective hosts suggesting that viruses use non-selective lipid uptake as
191 also shown previously for other pleolipoviruses (Pietilä *et al.*, 2010; Pietilä *et al.*, 2012). Virions of all three
192 viruses contained two major structural protein species, which were ~ 60 kDa and ~ 7 kDa in mass when

193 resolved in polyacrylamide gel (Fig. 3D-F). The patterns were different from each other and the major
194 structural proteins were identified based on the gene homology to those of other pleolipoviruses (see below).

195

196 **Three new pleomorphic viruses are members of the genus *Betapleolipovirus***

197 The nucleic acids extracted from the purified virions of HRPV10, HRPV11, and HRPV12 were sensitive to RQ1
198 DNase and Exonuclease III, resulting in complete degradation or extensive fragmentation, respectively. Mung
199 bean nuclease, which is specific to ssDNA (Fig. 4A), digested the HRPV10, HRPV11, and HRPV12 genomes into
200 discrete fragments (Fig. 4B-D), suggesting that the genomes are dsDNA molecules with nicks or single-
201 stranded regions. Sequencing of the genomes and assembly of the reads using a *de novo* assembly algorithm
202 (with default parameters) implemented in the CLC Genomics Workbench (QIAGEN Bioinformatics) resulted
203 in single contigs for each genome. Each contig contained direct terminal repeats of 18-20 bp. The sequencing
204 reads overlapping both termini were as abundant as those from other genomic positions, indicating that the
205 genomes are circular. Consistently, assembly of the sequencing reads with the SPAdes algorithm (Bankevich
206 *et al.*, 2012) resulted in contigs identical to those assembled with CLC Genomics Workbench, albeit with
207 alternative start positions and terminal repeats, confirming the circular nature of the genomes
208 (Supplementary Fig. 2).

209 To assess the relationship between the 3 new pleolipoviruses and the previously described members of the
210 family *Pleolipoviridae*, we calculated intergenomic distances between pairs of viruses by pairwise
211 comparisons of their nucleotide sequences and constructed the corresponding phylogenomic tree using
212 VICTOR(Fig. 5A), a Genome BLAST Distance Phylogeny method (Meier-Kolthoff and Goker, 2017). The
213 clustering of the pleolipoviruses in the resultant tree is consistent with previous classification based on the
214 presence of genes for the putative replication-initiation or DNA polymerase proteins and relatedness of the
215 VP3-like proteins (Pietilä *et al.*, 2016). HRPV10, HRPV11 and HRPV12 form a well-supported clade with
216 members of the genus *Betapleolipovirus*, namely, *Halorubrum* virus HRPV-3 and *Halogeometricum* virus

217 HGPV-1. The clade also includes *Haloarcula* virus HHPV3 and *Natrinema* virus SNJ2, two tentative members
218 of the *Betapleolipovirus* genus (Liu *et al.*, 2015; Bamford *et al.*, 2017).

219 The genomes of HRPV10, HRPV11, and HRPV12 were 9296, 9368, and 9944 bp in length, respectively, and
220 their GC% contents were 55.2-55.7%. Genomes were predicted to contain 13-16 ORFs oriented in both
221 transcriptional directions forming at least two putative operons (Fig. 5B; Supplementary Table 1). Genomes
222 of HRPV10, HRPV11 and HRPV12 are very similar to each other (92-95% nucleotide identity over the whole
223 length), but different from other characterized pleolipoviruses. The most closely related virus HGPV1 shares
224 68% identity over just 14% of the genome as determined by BLASTN. Consistently, comparison of the
225 HRPV10, HRPV11 and HRPV12 proteins against the proteomes of all other known pleolipoviruses has
226 revealed betapleolipoviruses as the closest relatives, with the largest number of sequence matches to
227 betapleolipoviruses HGPV-1 (gene 2, ORF5, ORF6, ORF7, ORF9, ORF13) and HRPV3 (ORF12 and ORF9),
228 whereas ORF4 was most similar to the homolog encoded by alphapleolipovirus HHPV-2 (Supplementary
229 Table 2). The presence of the signature gene encoding the putative replication protein (ORF11 in HRPV10)
230 unequivocally relates HRPV10, HRPV11 and HRPV12 to betapleolipoviruses. However, single-gene
231 phylogenies reconstructed for the core proteins, namely, spike protein (El Omari *et al.*, 2019) (Supplementary
232 Fig. 3A) and the putative NTPase (Supplementary Fig. 3B), were not entirely consistent with this assignment
233 (Fig. 5A), most likely reflecting occasional recombination between pleolipoviruses belonging to different
234 genera, consistent with previous observations (Wang *et al.*, 2018a). Comparison of HRPV ORFs to the non-
235 redundant protein sequence database reveal that the most similar sequences are found in the genomes of
236 *Halorubrum coriense*, *Halorubrum terrestre* and *Halorubrum* sp. T3, indicating the presence of related
237 proviruses within these organisms (Supplementary Table 1). Proviruses related to pleolipoviruses have been
238 described in haloarchaeal strains (Liu *et al.*, 2015; Demina *et al.*, 2016a; Atanasova *et al.*, 2018a; Wang *et al.*,
239 2018a).

240 The close genetic similarity between HRPV10, HRPV11 and HRPV12 allows tracing the evolutionary events
241 which took place in a relatively recent past. In particular, HRPV11 and HRPV12 share two small genes

242 (HRPV11-ORF9 and HRPV12-ORF9; HRPV11-ORF14 and HRPV12-ORF15), encoding putative DNA-binding
243 proteins carrying zinc-binding domains, which are absent in HRPV10, whereas ORF13 of HRPV12 is not found
244 in the two other viruses (Supplementary Table 1). Notably, the closest homolog of the latter gene is encoded
245 by an uncultivated tailed haloarchaeal virus eHP-27 (51% identity; $E=3e-57$) (Garcia-Heredia *et al.*, 2012),
246 followed by homologs from diverse haloarchaea. Given that HRPV12 ORF13, which encodes a putative
247 AdoMet-dependent methyltransferase (Supplementary Table 1), is not present in any other pleolipovirus
248 (Fig. 5B), in all likelihood, it has been introduced into the HRPV12 genome horizontally from an unrelated
249 haloarchaeal virus, following the divergence of HRPV12 from a common ancestor with HRPV10 and HRPV11.
250 By contrast, the homolog of HRPV11 ORF9 has been apparently lost from the HRPV10 genome due to an
251 inactivating point mutation, resulting in a long intergenic region between ORFs 8 and 9. Furthermore, analysis
252 of the nucleotide similarity pattern along the HRPV10, HRPV11 and HRPV12 genomes uncovered a
253 hypervariable region within ORF4, which encodes for a putative receptor-binding spike protein, one of the
254 two major virion proteins suggested to be involved in host recognition and virus entry (Pietilä *et al.*, 2010).
255 Notably, ORF4 homologs in HRPV11 and HRPV12 do not display appreciable similarity within the central
256 region (Fig. 5B), pinpointing a highly variable protein domain, which is most likely to be critical for host
257 recognition and binding; a similar conservation pattern is also observed in alphapleolipoviruses (e.g.,
258 compare HRPV-2 and HRPV-6 in Fig. 5B).

259 Comparative genomics analysis has shown that besides the five core genes conserved in all pleolipoviruses
260 (except for His2, which contains four core genes), HRPV10, HRPV11 and HRPV12 encode several putative
261 proteins specific to members of the genus *Betapleolipovirus*. These include homologs of HRPV10 ORF8 and
262 ORF11, which are conserved in all currently known betapleolipoviruses, as well as ORF10 and ORF13,
263 conserved in a subset of betapleolipoviruses, but not in viruses from the two other genera (Fig. 5B). Previous
264 sequence analyses did not provide insights into the putative functions of the four conserved proteins. Indeed,
265 HRPV10 ORF11-like proteins, which were suggested to represent replication initiation proteins of
266 betapleolipoviruses (Krupovic *et al.*, 2018), remain recalcitrant to functional annotation based on sequence
267 similarity searches. However, profile-profile comparisons initiated with the sequence of HRPV10 ORF8

268 revealed homology to various PD-(D/E)XK family nuclease, including type II restriction endonucleases
269 (Supplementary Table 1). Notably, the protein is not restricted to betapleolipoviruses, but is also conserved
270 in several other groups of unrelated haloarchaeal viruses, including members of the *Caudovirales* (HHTV-1)
271 and *Sphaerolipoviridae* (SH1, PH1, HCIV-1, HHIV-2). HRPV10 ORF10 and ORF13 encode putative DNA-binding
272 proteins with winged helix-turn-helix and ribbon-helix-helix domains, respectively (Supplementary Table 1),
273 and may be involved in transcriptional regulation of the viral and/or host genes.

274

275 **The first virus isolated on *Haloferax* has an icosahedral head, non-contractile tail and circularly**
276 **permuted dsDNA genome**

277 Micrographs of the purified HFTV1 virions revealed icosahedral particles with a long non-contractile tail
278 typical of the siphovirus morphotype (Fig. 2D). The diameter of the head was ~50 nm and the tail length was
279 ~60 nm. The major protein species of HFTV1 virions were approximately 50, 40, 22 and 16 kDa in mass (Fig.
280 3G). The infectivity of HFTV1 in the presence of chloroform, Nonidet P-40, or Triton X-100 remained
281 unchanged (Table 1), suggesting that the virion does not contain a membrane moiety and consists only of
282 proteins and nucleic acid.

283 The nucleic acid extracted from purified HFTV1 virions was sensitive to RQ1 DNase treatment, but resistant
284 to Exonuclease III and Mung bean nuclease, indicating that the genome is a dsDNA molecule (Supplementary
285 Fig. 4). Genome sequencing and read assembly were performed as described above for pleolipoviruses and
286 yielded a 38,059 bp-long circular contig (GC% ~54%), which appears to represent a complete viral genome.
287 A total of 70 ORFs were predicted in the HFTV1 genome using Prodigal (Hyatt *et al.*, 2010), of which 28 (40%)
288 did not have any clear homologs in the public databases (Supplementary Table 3, Supplementary Fig. 5). Half
289 (35) of the gene products had sequence similarity (35-84% identity; Supplementary Table 3) to haloviruses:
290 15 to halophilic archaeal siphovirus HRTV-4 originating from a salt water sample from Margherita di Savoia,
291 Italy (Sencilo *et al.*, 2013), and 20 to uncultivated environmental haloviruses identified in the solar saltern of
292 Santa Pola, Spain (Garcia-Heredia *et al.*, 2012). The remaining 10% of the genes had closest homologs

293 encoded in cellular organisms. Namely, the most significant similarities were shared with archaea from the
294 order Halobacteriales (*Natrialba*, *Natronobacterium*, *Haloarcula*, *Halococcus* and *Haloterrigena*) and one to
295 *Cellulophaga baltica*, a marine bacterium from the order Flavobacteriales (Supplementary Fig. 5). All ORFs
296 but one are arranged in the same transcriptional direction (Fig. 6).

297 To determine the packaging mechanism employed by HFTV1, we analyzed the bias in distribution of the
298 1,657,094 sequencing reads along the HFTV1 genome using PhageTerm, a tool that relies on the detection
299 of biases in the number of sequencing reads observable at natural DNA termini compared with the rest of
300 the viral genome (Garneau *et al.*, 2017). The analysis revealed a pattern of sequencing read coverage
301 consistent with a circularly permuted, terminally redundant genome and headful packaging mechanism
302 initiated from a *pac* site, similar to that of bacteriophage P1 (Supplementary Fig. 6). Consistently, phylogeny
303 of the large subunit of the terminase (Supplementary Fig. 3B), an enzyme responsible for genome packaging
304 in bacterial and archaeal members of the order *Caudovirales*, revealed a relatively close relationship of HFTV1
305 to *Halorubrum* virus HRTV-4, a siphovirus for which the genome was also found to be circularly permuted
306 (Sencilo *et al.*, 2013).

307 The phylogenomic analysis using VICTOR (Meier-Kolthoff and Goker, 2017) confirmed the relationship of
308 HFTV1 with *Halorubrum* virus HRTV-4, and also revealed relationship to four uncultivated viruses, eHP-1,
309 eHP-15, eHP-19 and eHP-34 (Garcia-Heredia *et al.*, 2012), for which the hosts have not been previously
310 predicted (Supplementary Fig. 7A). The genomes of the latter viruses are generally collinear with those of
311 HFTV1 and HRTV-4. The highest sequence similarity between the genomes is observed within the genes
312 encoding for putative virion morphogenesis proteins, such as the major capsid protein, the large subunit of
313 the terminase and tail proteins (Fig. 6). Given this genomic conservation, we predict that the uncultivated
314 HFTV1-like viruses eHP-1, eHP-15, eHP-19 and eHP-34 infect halophilic archaea.

315 The genome of HFTV1 encodes several proteins putatively involved in DNA metabolism, namely a replicative
316 minichromosome maintenance (MCM) helicase (gp58), DNA polymerase sliding clamp protein PCNA (gp64),
317 DNA methyltransferase (gp61) and Rad52-like recombinase (gp50) (Fig. 6, Supplementary Table 3). The MCM

318 is the principal helicase responsible for unwinding of the dsDNA duplex during chromosomal replication in
319 archaea and eukaryotes (Bell and Botchan, 2013). MCM homologs have been previously identified in archaeal
320 viruses and plasmids with moderately-sized genomes (20-50 kb) (Krupovic *et al.*, 2018) and phylogenetic
321 analyses have suggested that mobile genetic elements have horizontally acquired the *mcm* genes from
322 cellular organisms on multiple independent occasions (Krupovic *et al.*, 2010b). The PCNA sliding clamp is
323 another key replication protein in archaea and eukaryotes and is known as a “molecular tool-belt” due to its
324 interaction with multiple other proteins involved in DNA replication and repair, including replicative DNA
325 polymerase, DNA ligase, replication factor C, Flap Endonuclease 1 (FEN1) and RNase H (Pan *et al.*, 2011).
326 Similar to MCM helicases, PCNA homologs have been previously identified in some haloarchaeal virus
327 genomes (Raymann *et al.*, 2014), whereas certain other archaeal viruses have been shown to specifically
328 recruit the host PCNA for the replication of their genomes (Gardner *et al.*, 2014). Thus, the virus-encoded
329 MCM and PCNA homologs are likely to orchestrate the replication of the HFTV1 genome.

330 Despite the synteny within the morphogenetic gene modules of HFTV1, HRTV-4, eHP-1, eHP-15, eHP-19, and
331 eHP-34, the genome replication modules of these viruses appear to be very different. Namely, among the
332 five viruses, only HFTV1 encodes both MCM helicase and PCNA. Notably, there is only one other known
333 archaeal halophilic virus, podovirus HSTV-1, which harbors genes for both proteins in its genome (Pietilä *et*
334 *al.*, 2013b; Raymann *et al.*, 2014). By contrast, the MCM helicase is encoded only by eHP-34, whereas HRTV-4,
335 the closest relative of HFTV1, as well as eHP-1, eHP-15 and eHP-19 do not encode either of the two replication
336 proteins. These observations reaffirm that virion formation and genome replication are uncoupled processes
337 and evolve independently (Krupovic and Bamford, 2010), as is also evident in the case of pleolipoviruses,
338 where viruses from the three genera encode non-homologous genome replication proteins (Krupovic *et al.*,
339 2018). Consequently, viral genomes are often mosaics of genes with different evolutionary histories (Juhala
340 *et al.*, 2000; Pope *et al.*, 2015; Iranzo *et al.*, 2016a; Yutin *et al.*, 2018).

341 Genetic mosaicism in tailed bacteriophage genomes is thought to be generated by illegitimate recombination
342 (Krupovic *et al.*, 2011a) or relaxed homologous recombination (De Paepe *et al.*, 2014). The former occurs at

343 essentially random positions within the genome, with nonviable recombinants being purged by natural
344 selection (Pedulla *et al.*, 2003). The latter process involves promiscuous phage-encoded recombinases, such
345 as phage λ recombinase Red β , which catalyze homologous recombination by annealing short and diverged
346 sequences (De Paepe *et al.*, 2014). Among the phage recombinases, the Rad52-like family is by far the largest
347 and most diversified (Lopes *et al.*, 2010). Interestingly, HFTV1 encodes a divergent member (gp50) of the
348 Rad52-like family of recombinases (Rad52, PDB profile 5JRB_A, HHpred probability of 94%), which might
349 facilitate genome remodeling in the replication modules of HFTV1-like viruses. Homologs of HFTV1 gp50 are
350 also encoded by HRTV-4, eHP-1, eHP-15, and eHP-34 as well as by several other uncultivated halophilic
351 viruses. Notably, the closest homologs of the HFTV1 PCNA are encoded by cellular organisms, suggesting that
352 the corresponding gene has been acquired by HFTV1 from halophilic archaea, rather than inherited from a
353 common ancestor shared with other viruses. Similarly, the closest homologs of the orthologous HFTV1 and
354 eHP-34 MCM helicases are encoded by halophilic archaea, whereas homologs from other viral groups are
355 more divergent. This suggests that the *pcna* and *mcm* genes have been acquired directly from the hosts in
356 different groups of archaeal viruses on several occasions, independently of each other.

357

358 **Narrow host range of haloarchaeal viruses from Lake Retba**

359 To determine the host range of viruses isolated from Lake Retba, we first tested their infectivity towards the
360 19 autochthonous haloarchaeal strains (Table 2). Despite the overall close genomic similarity, the
361 pleomorphic viruses HRPV10, HRPV11, and HRPV12 were found to have distinct host ranges. HRPV11
362 displayed the broadest host range, being able to infect four different *Halorubrum* strains isolated from Lake
363 Retba, whereas HRPV10 and HRPV12 each could infect only two different strains. *Halorubrum* sp. LR2-12 was
364 susceptible to all three pleomorphic viruses, albeit with highly different efficiencies of plating (EOP) (Table
365 2). Notably, *Haloferax* virus HFTV1 was found to infect hosts across the genus boundary. In addition to its
366 own isolation host, LR2-5, belonging to the genus *Haloferax* (Table 2), HFTV1 was able to infect *Halorubrum*
367 sp. LR1-23, albeit with an eight orders of magnitude lower efficiency (Table 2). Similarly, a previous cross-

368 infectivity study has shown that haloarchaeal siphoviruses generally display genus-restricted host ranges,
369 although some isolates were found to infect hosts belonging to two or three genera (Atanasova *et al.*, 2015c).

370 Next, we set out to explore the infectivity of the four viruses against *Haloferax* and *Halorubrum* strains
371 isolated from geographically remote locations (Israel, Italy, Slovenia, Spain, Thailand and Antarctica). Namely,
372 we tested 41 distinct *Halorubrum* strains originating from 10 different sampling sites and seven *Haloferax*
373 strains from five distinct locations (Supplementary Table 4). Among the 51 strains tested, only *Halorubrum*
374 sp. E200-4 isolated from Eilat, Israel was sensitive to pleomorphic virus HRPV11, albeit with a considerably
375 lower ($\sim 6 \times 10^{-3}$) EOP. This observation is consistent with the previous finding that most pleolipoviruses are
376 highly specific to their isolation hosts (Atanasova *et al.*, 2012; Atanasova *et al.*, 2015c), but also indicates that
377 occasional cross-infections that transcend site and time of isolation are possible. Similar patterns of infection,
378 whereby viruses preferentially infect hosts from the same site rather than hosts isolated from similar but
379 geographically remote sites, are also typical of bacterial virus-host systems from different ecological niches
380 (Vos *et al.*, 2009; Koskella *et al.*, 2011), including hypersaline environments (Villamor *et al.*, 2018). Thus, a
381 pronounced biogeographical pattern emerges in haloarchaeal virus-host interactions, possibly due to
382 increased diversification of the species composition of communities as a function of increasing geographic
383 and environmental distance (Weitz *et al.*, 2013). The specificity of viruses to autochthonous strains seemingly
384 contrasts the conclusions drawn from comparative (meta)genomic analysis of halophilic viral communities
385 which indicated that hypersaline viral communities should be considered as a genetic continuum across
386 continents (Roux *et al.*, 2016). Collectively, the results of the large-scale comparative genomics and local
387 infectivity studies suggest that the gene complements responsible for virion formation and adaptation to
388 environmental conditions are shared by haloarchaeal viruses across the globe, whereas the incessant
389 evolutionary arms race drives local adaptation of viruses and their hosts at a finer scale.

390 Haloarchaeal myovirus isolates appear to display a broader host range (Atanasova *et al.*, 2012; Atanasova *et*
391 *al.*, 2015c) than siphoviruses, such as HFTV1. This tendency appears to be general, because bacterial
392 myoviruses also display broader host range than siphoviruses and podoviruses (Wichels *et al.*, 1998). The

393 broader host range of archaeal myoviruses might be linked to the larger genomes and, accordingly,
394 functionally more diverse gene content (Krupovic *et al.*, 2018) including e.g. many auxiliary genes involved in
395 DNA and RNA metabolism (Sencilo *et al.*, 2013). For instance, HVTV-1 encodes an almost complete replisome
396 (Pietilä *et al.*, 2013c; Kazlauskas *et al.*, 2016), whereas HGTV-1 encodes an RNA ligase and lysyl-tRNA
397 synthetase and has 36 tRNA genes for all universal genetic code amino acids (Sencilo *et al.*, 2013).
398 Presumably, this extended gene baggage renders myoviruses more promiscuous and partly independent of
399 the corresponding cellular machineries compared to viruses with smaller genomes.

400

401 **Scarcity of haloarchaeal virus isolates in the environment**

402 The relative abundance of viruses in any particular sample or environment can be estimated by mapping the
403 sequence reads from a metavirome to the reference genomes and expressed as **Reads recruited Per Kb** of
404 genome per **Gb** of metagenome (RPKG). We used this approach to compare the relative abundance of the
405 four viruses described in this study to that of the previously reported cultivated and uncultivated
406 haloarchaeal viruses. To this end, we analyzed saltern viromes sequenced from Lake Retba (Roux *et al.*, 2016)
407 and South Bay Salt Works (Rodriguez-Brito *et al.*, 2010). Notably, the samples for the preparation of the Lake
408 Retba virome (Roux *et al.*, 2016) were collected during the same sampling trip as those used to isolate viruses
409 reported herein. However, none of the cultivated haloarchaeal viruses, including those described here, were
410 sufficiently similar to the sequences present in the available viromes. By contrast, uncultured viruses
411 predicted to infect *Haloquadratum walsbyi* recruited around 15,000 RPKG and formed a distinct clade in the
412 phylogenomic tree (Supplementary Fig. 7). Apart from these, other uncultured viruses with no identified host
413 and one virus predicted to infect nanohaloarchaea have recruitment of around 10 RPKG. The fact that all
414 currently cultured viruses recruit only negligible number of reads, even when the virome originates from the
415 same site as virus isolates is likely to reflect the still scarce and biased sampling of the (halo)archaeal virome.
416 At least in the case of the Lake Retba viruses and the corresponding virome, the two have been isolated at
417 the same time and thus temporal variation in virus diversity cannot explain this result. Given the low

418 abundance of *Halorubrum* spp. in salterns from warm environments (Garcia-Heredia *et al.*, 2012), the
419 currently used culture-based approaches appear to be biased towards isolation of viruses that represent a
420 rather minor fraction of the natural haloarchaeal virome. We note, however, that *Halorubrum* species
421 represent one of the dominant components of the haloarchaeal communities in the cold hypersaline
422 environments, such as Deep Lake in Antarctica (DeMaere *et al.*, 2013); thus, *Halorubrum* viruses might
423 specifically dominate the cold-adapted haloarchaeal viromes. To obtain further insights into the actual
424 diversity of haloarchaeal viruses and to initiate studies on the biology of ecologically relevant virus-host
425 systems, future work should focus on improving the cultivation protocols for the dominant inhabitants of the
426 hypersaline environments, such as *Haloquadratum* spp. (Oh *et al.*, 2010; Dyll-Smith *et al.*, 2011).
427 Nevertheless, further characterization of the “cultivable minority” component of the haloarchaeal virome,
428 as described in this study, provides important insights into the general mechanisms of haloarchaeal virus
429 evolution and might lead to the establishment of virus-host systems in genetically tractable haloarchaeal
430 hosts, such as *Haloferax*, for in-depth studies on virus-host interactions.

431 **Experimental Procedures**

432 **Sampling and growth conditions**

433 Samples were collected from Lake Retba, Senegal in May, 2011 (14°50'14" N, 17°14'55" W). The Lake Retba
434 sample 1 (LR1) was collected in the center of the lake, where salt was precipitated at the bottom. LR1 sample
435 contained grey water with grey sediment mixed with salt. The Lake Retba sample 2 (LR2) consists of purple
436 water with white salt sediment. The temperature, pH, and salinity of the water at the sampling site was
437 measured at the time of the sampling.

438 Isolation of microorganisms and viruses was carried out during the summer and autumn 2011. For isolation,
439 the liquid phase and the sediment (including precipitated salt) were separated by decanting. Water was
440 transferred to clean bottles. The sediments were dissolved by adding of 6% SW buffer (see below) until salts
441 dissolved at the room temperature (magnetic stirring). Liquid phase and the dissolved sediment were treated
442 as one sample.

443 Strains and viruses were aerobically grown in modified growth medium (MGM) (Nuttall and Dyall-Smith,
444 1993) at 37 °C. For plaque assay, different dilutions of virus sample were mixed with host culture (300 µl) and
445 melted top layer agar (3 ml) and plated on MGM plates. For plaque assay, the hosts were grown for 2-3 over
446 nights to obtain stationary phase culture. For making of MGM, 30% saltwater (SW) containing 240 g NaCl,
447 30 g MgCl₂ × 6H₂O, 35 g MgSO₄ × 7H₂O, 7 g KCl, 5 ml of 1 M CaCl₂ × 2H₂O, and 80 ml of 1 M Tris-HCl pH 7.2
448 (per liter of water) was prepared as described in the Halohandbook (Dyall-Smith, 2009). One litre of MGM
449 medium contained 5 g of peptone (Oxoid), and 1 g of Bacto yeast extract (Becton, Dickinson and Company).
450 Top layer, solid, and liquid medium contained 18% SW, 20% SW, and 23% SW, respectively. For the top layer
451 and solid media, 4 g or 14 g of Bacto agar (Becton, Dickinson and Company) was added, respectively.

452

453 **Isolation of microorganisms, 16S rRNA gene sequencing and phylogenetic tree**

454 To isolate strains from the samples, aliquots of samples (100 μ l) were directly plated on MGM plates and
455 grown at 37 °C in a covered box. A selection of colonies with different morphologies and colors were picked
456 and colony purified by streaking single colonies on solid media by three consecutive times. The archaeal
457 strains used in the study are listed in Table 2. The strains were identified based on their partial 16S rRNA gene
458 sequences, which were determined as described previously (Sime-Ngando *et al.*, 2011). The 16S rRNA genes
459 were amplified by PCR. The primers were either universal for both the bacteria and archaea, or specific for
460 the archaea (Eder *et al.*, 1999). The sequences of the universal prokaryotic forward primers were 5'-
461 AGAGTTTGATCCTGGCTCAG-3' (F27) and 5'-TCCGTGCCAGCAGCCGCGG -3' (F530), and those of the universal
462 prokaryotic reverse primers were 5'-ACGGHTACCTTGTTACGACTT-3' (1512uR) and 5'-
463 CGTATTACCGCGGCTGCTGG-3' (R518). The archaea-specific primers were 5'-TCYGGTTGATCCTGCC-3' (8aF)
464 and 5'-AGGAGGTGATCCAGCC-3' (AR1456). The reaction mixture (50 μ l of total volume) contained 1X Taq™
465 buffer (Promega, Madison, WI, USA), 1.5 mM MgCl₂, dNTPs at a concentration of 0.2 mM each, 1 U of Taq
466 polymerase, each primer at a concentration of 125 pmol, and 5 ng of template DNA. The amplification was
467 ended by an extension step for 10 min at 72°C. Negative and positive controls were included. Five μ l of PCR
468 products were loaded onto 0.8 % agarose gel in TAE 1X (Tris-acetic acid-EDTA buffer) and visualized under
469 UV light after ethidium bromide staining. PCR products obtained were cloned using TOPO TA cloning kit
470 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After blue-white selection, positive
471 clones were grown at 37°C overnight on 96-well tissue culture plates in the presence of kanamycin.

472 The clones were picked and suspended in TE followed by boiling at 96°C, and used as a template DNA for PCR
473 amplification using M13 primers targeting the cloning vector (5'-GTAAAACGACGGCCAG-3' and 5'-
474 CAGGAAACAGCTATGAC-3'). The selected clones were grown as previously to extract plasmid DNA using
475 Nucleospin Plasmid preparation Kit (Macherey-Nagel, EURL, France) and sent for Sanger sequencing using
476 M13 primers on both strands. For a first classification, we used the SILVA r128 rRNA classifier (Pruesse *et al.*,
477 2012). For the phylogenetic tree, sequences were aligned using MUSCLE (Edgar, 2004) and maximum

478 likelihood trees were constructed using the program FastTree2 (Price *et al.*, 2010). Bootstrapping (100
479 replicates) was performed using the Seqboot program in the PHYLIP package (Felsenstein, 1993).

480 The sequences are deposited in the NCBI data bank under the accession numbers MG462733-MG462751
481 (Table 2).

482

483 **Isolation of viruses**

484 Viruses were isolated either by direct plating or enrichment culture techniques. The pure cultures of LR1
485 host strains (Table 2) were used to isolate viruses from LR1 samples. Hosts of LR2 (Table 2) were used to
486 isolate viruses from LR2 samples. The LR1 and LR2 samples were first centrifuged at 13 000 rpm (Table-top
487 Eppendorf centrifuge) for 10 min at room temperature and the supernatant we used for virus isolation. To
488 remove microorganisms from the LR1 sediment sample, the sample was filtered (pore size 0.2 μm). For direct
489 plating, 100 μl of samples was mixed with dense host culture (300 μl) and melted top layer agar (3 ml) and
490 poured on a plate, which was incubated at 37 °C until a dense lawn of archaea was observed (typically two
491 to three days). For enrichments, 500 μl of samples was mixed with host culture (1 ml grown for 2-3 days) and
492 incubated in a shaker (200 rpm) 1-2 overnights. The enrichment samples (100 μl and 500 μl) were plated as
493 above. The obtained plaques were plaque purified. The plaque purification was carried out by growing the
494 viruses on their host strain to obtain separate plaques by using plaque assay. Plaques were picked by sterile
495 toothpick or Pasteur pipette, and a single plaque was resuspended in 0.5 ml of MGM liquid medium. The
496 plaque purification was repeated by using the single plaque as the starting material. The single plaque
497 purification was carried out by three consecutive times for each virus.

498 For all plaque assays and preparation of the virus stocks, appropriate virus dilution (100 μl) mixed with host
499 culture (300 μl) and melted top layer agar (3 ml) was plated. The plates were incubated for 2-3 days. The
500 virus stocks were prepared from semiconfluent plates. Top layer media from the semiconfluent plates were
501 collected by a sterile glass triangle into a sterile Erlenmeyer bottle and 2 ml of liquid medium was added per
502 each collected plate. The suspension was incubated for 1.5 hours at 37 °C. Cell debris and agar were removed

503 by centrifugation (Thermo Scientific F12 rotor, 8000 rpm, 20 min, 5 °C). The supernatant was put into a clean
504 bottle and it is referred as a virus stock. One semiconfluent plate produces approximately 3-3.5 ml of virus
505 stock. Stability of viruses (virus stocks stored at 4 °C) was monitored for four weeks by plaque assay. To test
506 the sensitivity of the viruses to organic solvents and detergents, viruses (virus stock in MGM) were incubated
507 in 20% (v/v) chloroform, 0.1% (v/v) Nonidet P-40, or 0.1% Triton X-100 for 15 min at 22°C. MGM was used as
508 a control. The infectivity of the viruses was determined by plaque assay and the experiments have been
509 repeated at least for two times.

510

511 **Virus purification and particle analysis**

512 The virus stocks (typically made of 200 plates producing 600-700 ml of virus stock or 400 plates producing
513 1200-1400 ml of virus stock) were treated with DNase I (70 µg/ml; 30 min at 37 °C; Sigma-Aldrich) prior the
514 purification. Viruses were precipitated from the virus stocks by using two-step polyethylene glycol (PEG)-
515 NaCl precipitation (Yamamoto *et al.*, 1970). First, the impurities were precipitated by using 4% (w/v) PEG
516 6000 (no NaCl added due to the high salinity of the virus stock). PEG was dissolved by magnetic stirring for
517 30 min at 4°C. After centrifugation (Thermo Scientific F12 rotor, 8000 rpm, 40 min, 5 °C), PEG was added to
518 the supernatant to obtain a final concentration of 11% (w/v). After dissolution of PEG and centrifugation (see
519 above), the obtained virus precipitate was dissolved in 18% SW buffer followed by removal of the aggregates
520 and undissolved components (Thermo Scientific F20 rotor, 7000 rpm, 10 min, 5 °C). Viruses were first purified
521 by rate zonal ultracentrifugation in sucrose by using linear 5-20% sucrose gradients (18% SW buffer; Sorvall
522 rotor AH629, 24 000 rpm, 15 °C). The running times were 2.5 h (HRPV10), 1 h 45 min (HRPV11), and 3 h
523 (HRPV12 and HFTV1). After rate zonal centrifugation, viruses were purified by equilibrium centrifugation in
524 CsCl gradients (mean ρ =1.30-1.35 g/ml in 18% SW; Sorvall rotor AH629, 20 000 rpm, 19 h, 20 °C), and
525 concentrated by differential centrifugation (Sorvall rotor T647.5, 32 000 rpm, 3-5 h, 15 °C). Virus purifications
526 were repeated at least three times for each virus. Protein concentrations were determined by Bradford assay
527 using bovine serum albumin as a standard (Bradford, 1976). The proteins were analyzed by using modified

528 tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (14% acrylamide in the separation gel;
529 (Schägger and von Jagow, 1987)). Gels were stained with Coomassie Brilliant Blue R 250 (Serva).

530 Viral lipids were isolated by chloroform-methanol extraction from the purified HRPV10, HRPV11, and HRPV12
531 virus particles and from the early-stationary-phase cells of *Haloarcula hispanica* (Juez *et al.*, 1986) and
532 *Halorubrum* sp. LR2-17, LR2-12, LR1-23 strains as previously described (Folch *et al.*, 1957; Kates *et al.*, 1972).
533 Extracted lipids were dissolved in chloroform-methanol (9:1) and analyzed on pre-activated thin layer
534 chromatography (TLC) silica plates, which were developed with chloroform–methanol–90% acetic acid
535 (65:4:35 [vol/vol/vol]). Lipids were visualized by ammonium molybdate staining (Arnold *et al.*, 2000). The
536 plate was quickly dipped into a solution containing 10% (v/v) H₂SO₄ and 5% (w/v) ammonium molybdate,
537 after which the excess liquid was dried, and the plate was incubated at 140 °C for around 15 min.

538 For transmission electron microscopy, 5 µl samples of the purified virus particles were adsorbed on copper
539 pioloform coated grids (Electron Microscopy Unit, HiLIFE Institute of Biotechnology, University of Helsinki).
540 The particles were negatively stained either with 3% (w/v) uranylacetate (pH 4.5) or 1% (w/v)
541 phosphotungstic acid (pH 7.0) , and visualized by JEOL 1400 transmission electron microscope (Electron
542 Microscopy Unit, HiLIFE Institute of Biotechnology, University of Helsinki) operating at 80 kV acceleration
543 voltage.

544

545 **Virus genome analysis, sequencing and annotation**

546 Nucleic acid was purified from the pure virus particles. The particles in 18% SW were diluted 1:4 in 20 mM
547 Tris-HCl, pH 7.2 and treated with 1% (w/v) sodium dodecyl sulphate and 100 µg/ml proteinase K (Thermo
548 Scientific) in the presence of 1 mM ethylenediaminetetraacetic acid (EDTA) for an hour at 37 °C. Nucleic acid
549 was extracted by phenol-ether extraction and followed by precipitation with NaCl and ethanol. Purified
550 nucleic acids were treated with RQ1 DNase (Promega), Exonuclease III (Fermentas), Mung bean nuclease
551 (MBN 0.025, 0.5, or 5.0 U/µg DNA; Promega) according to manufacturers' instructions. For MBN experiments,

552 phage ϕ X174 ssDNA genome and its dsDNA replicative form RFII (New England Biolabs) were used as
553 controls.

554 Libraries were prepared using TruSeq PCRfree library preparation. Samples were sequenced by Illumina
555 MiSeq 600 cycles (Illumina Inc., San Diego, CA) with 2x300 bp read length. The sequencing reads were
556 trimmed based on the quality scores (limit 0.05) from a base-caller algorithm available in the sequencing
557 files. The trimming was performed using the modified-Mott trimming algorithm implemented in the CLC
558 Genomics Workbench v7 (QIAGEN Bioinformatics) and the trimmed reads were subsequently assembled into
559 contigs using the same software with default parameters. Protein-coding genes were predicted using
560 Prodigal (Hyatt *et al.*, 2010), and tRNA genes using tRNAscan-SE (Lowe and Eddy, 1997). Additional
561 annotation of genes was done by comparing against the NCBI NR, COG (Tatusov *et al.*, 2003), and TIGRfam
562 (Haft *et al.*, 2001) databases, and also manually annotated using HHPRED server (Zimmermann *et al.*, 2018).
563 The sequences are deposited in the NCBI GenBank data bank under the accession numbers MG550110 -
564 MG550113.

565 All pairwise comparisons of the nucleotide sequences were conducted using the Genome-BLAST Distance
566 Phylogeny (GBDP) method (Meier-Kolthoff *et al.*, 2013) under settings recommended for prokaryotic viruses
567 (Meier-Kolthoff and Goker, 2017). All reference genomes were downloaded from
568 <https://www.ncbi.nlm.nih.gov/genome/browse/>

569 Genome phylogenies were constructed using VICTOR (Meier-Kolthoff and Goker, 2017), a Genome BLAST
570 Distance Phylogeny (GBDP) method which calculates intergenomic distances between pairs of viruses based
571 on pairwise comparison of nucleotide sequences. The resulting intergenomic distances (including 100
572 replicates each) were used to infer a balanced minimum evolution tree with branch support via FASTME
573 including SPR postprocessing (Lefort *et al.*, 2015) for the formula D0. The trees were rooted at the outgroup
574 and visualized with FigTree (Rambaut, 2006). For both single gene phylogenies, the sequences were aligned
575 using MUSCLE (Edgar, 2004). Maximum likelihood trees were constructed using the program FastTree2 (Price
576 *et al.*, 2010). Bootstrapping was performed using the Seqboot program in the PHYLIP package (Felsenstein,

577 1993). Comparisons among related viral genomes and reference genomes were performed using tBLASTx or
578 BLASTN (Edgar, 2010).

579

580 **Virus-host interaction studies**

581 Infectivity of HRPV10, HRPV11, HRPV12, and HFTV1 viruses was tested on 19 Lake Retba strains (Table 2) and
582 48 culture collection strains representing genus *Halorubrum* or *Haloferax* (Supplementary Table 3) by spot-
583 on-lawn assay. Undiluted and diluted (10^{-2}) virus stocks (10 μ l) were applied on the top layer agar inoculated
584 with the test strain. The virus host strain and MGM medium were used as positive and negative controls. All
585 positive results (growth inhibitions) were verified by plaque assay.

586

587 **Analysis of metaviromes**

588 Viromes were downloaded from Metavir 2 (Roux *et al.*, 2014). Only sequence matches longer than 50 bp
589 with *e*-value less than $1e^{-5}$ and more than 95% identity were considered. The recruitment of each genome
590 from the virome was calculated by dividing the number of hits by the length of the contig (in kb) and by the
591 size of the database (in Gb). This normalized measure is abbreviated as RPKG (**R**eads recruited **P**er **K**b of
592 genome per **G**b of metagenome).

593 In order to test the performance of the currently available tools for identifying archaeal viruses in
594 metagenomic dataset, we ran the VirSorter analysis (Roux *et al.*, 2015) against the RefSeq virus database. Of
595 the four genomes analyzed, only HFTV1 was considered by VirSorter to be of viral origin under the category
596 2 (“quite sure”), with three detected “phage hallmark genes”. None of the pleolipoviruses was recognized as
597 a virus, pointing to a need for improvement of the database of virus hallmark genes.

598

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610
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885 **Table and Figure legends**

886

887 **Table 1.** Viruses from Lake Retba

888

889 **Table 2.** Strains isolated from the Lake Retba samples

890

891 **Table 3.** Virus purification by PEG-NaCl precipitation, rate zonal, equilibrium and differential

892 ultracentrifugation

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894 rRNA gene sequences. Isolates from Lake Retba (LR) are highlighted in red. Sequences were aligned using

895 MUSCLE (Edgar, 2004) and the maximum likelihood tree was constructed using the FastTree2 program (Price

896 *et al.*, 2010). The numbers above the branches represent bootstrap support values from 100 replicates. The

897 scale bar represents the number of substitutions per site.

898

899 **Figure 2.** Transmission electron microscopy of purified viruses (A) HRPV10, (B) HRPV11, (C) HRPV12 (D)

900 HFTV1. (A-C) staining with uranyl acetate; (D) staining with phosphotungstic acid. HFTV1 particles devoid of

901 DNA are indicated by arrows. Bars, 100 nm.

902

903 **Figure 3.** Lipid and protein analysis of virions. (A-C) A thin-layer chromatogram of lipids extracted from virus

904 particles purified by PEG-NaCl precipitation, rate zonal (in sucrose) and equilibrium (in CsCl) centrifugation

905 and concentrated by differential centrifugation (A) HRPV10, (B) HRPV11, and (C) HRPV12 and their

906 corresponding host strains. The corresponding band of each lipid species are marked by 1-4. The major lipid

907 species of *Haloarcula hispanica* (Hh) are indicated on the right and their positions marked by the Roman

908 numerals as follows: PG, phosphatidylglycerol (I); PGP-Me, phosphatidylglycerophosphate methyl ester (II);

909 PGS, phosphatidylglycerosulfate (III); TGD, triglycosyl glycerodiether (IV). (D-G) SDS-PAGE analysis of the

910 purified viruses (D) HRPV10, (E) HRPV11, (F) HRPV12, (G) HFTV1. Molecular mass marker is shown (M, kDa).

911

912 **Figure 4.** Mung bean nuclease (MBN) analyses of (A) ϕ X174 single-stranded (ss) and double-stranded (ds)
 913 genomic DNA (B) HRPV10, (C) HRPV11, and (D) HRPV12. MBN amounts used are indicated as units (U) per
 914 1 μ g of DNA. All reactions contained 300 ng of DNA. Molecular mass marker (M) is indicated in kb.

915

916 **Figure 5.** HRPV10, HRPV11, HRPV12 and the members of the family *Pleolipoviridae*. (A) Phylogenomic tree
 917 was constructed using the Genome BLAST Distance Phylogeny (GBDP) strategy implemented in VICTOR
 918 (Meier-Kolthoff and Goker, 2017). The numbers above branches are GBDP pseudo-bootstrap support values
 919 from 100 replications. Clades corresponding to the genera *Alphapleolipovirus*, *Betapleolipovirus* and
 920 *Gammapleolipovirus* are colored light blue, beige, and grey, respectively. (B) Genomic comparison of
 921 pleolipoviruses depicted in panel A. Homologous genes are indicated with the same colors.

922

923 **Figure 6.** Genomic comparison of HFTV1, HRTV-4, eHP-4, eHP-15 and eHP-1. Open reading frames (ORFs)
 924 are depicted as arrows indicating the directionality of transcription. When possible, the predicted functions
 925 are indicated above the corresponding ORFs. Shading connecting the ORFs indicates the amino acid
 926 sequence identity between the corresponding protein products; the color key is provided at the bottom of
 927 the figure. Abbreviations: TerS and TerL, small and large subunits of the terminase, respectively; CBD,
 928 carbohydrate-binding domain; PAPS, phosphoadenosine phosphosulfate; MCM, minichromosome
 929 maintenance helicase; PCNA, proliferating cell nuclear antigen; MTase, methyltransferase.

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Table 1. Viruses from Lake Retba

Virus	Original host strain	Origin of the virus	Plaque morphology and diameter	Virus stock titer (pfu/ml)	Chloroform sensitivity ^a	Nonidet P-40 sensitivity ^b	Triton X-100 sensitivity ^c	Virion morphology
Halorubrum pleomorphic virus 10 (HRPV10)	<i>Halorubrum</i> sp. LR2-17	Sample LR2	Hazy, 3-5 mm	~1×10 ¹¹	Resistant	Sensitive, titer drops 11 logs	Sensitive, titer drops 11 logs	Pleomorphic
Halorubrum pleomorphic virus 11 (HRPV11)	<i>Halorubrum</i> sp. LR2-12	Sample LR2	Very hazy, 5-10 mm	~5×10 ¹¹	Sensitive, titer drops 2-3 logs	Sensitive, titer drops 8 logs	Sensitive, titer drops 11 logs	Pleomorphic
Halorubrum pleomorphic virus 12 (HRPV12)	<i>Halorubrum</i> sp. LR1-23	Sample LR1	Hazy, 5-8 mm	~1×10 ¹¹	Sensitive, titer drops ~1 log	Sensitive, titer drops 7 logs	Sensitive, titer drops 10 logs	Pleomorphic
Haloferax tailed virus 1 (HFTV1)	<i>Haloferax</i> sp. LR2-5	Sample LR2	Clear, 2-4 mm	~1×10 ¹²	Resistant	Resistant	Resistant	Icosahedral, long non-contractile tail

4 ^a assayed by plaque assay in the presence of 20% (v/v) chloroform

5 ^b assayed by plaque assay in the presence of 0.1 % (v/v) Nonidet P-40

6 ^c assayed by plaque assay in the presence of 0.1 % (v/v) Triton-X-100

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2 **Table 2. Strains isolated from the Lake Retba samples**

Strains	Origin ^a	16S rRNA sequence Acc. No; length (bp)	Efficiency of plating ^b			
			HRPV10	HRPV11	HRPV12	HFTV1
<i>Haloferax</i> sp. LR1-5	LR1	MG462733; 1443				
<i>Haloferax</i> sp. LR1-14	LR1	MG462735; 1529				
<i>Haloferax</i> sp. LR1-18	LR1	MG462745; 1443				
<i>Haloferax</i> sp. LR1-19	LR1	MG462736; 1443				
<i>Haloferax</i> sp. LR1-24	LR1	MG462739; 1443				
<i>Haloferax</i> sp. LR2-5	LR2	MG462740; 1443				1 (H)
<i>Haloferax</i> sp. LR2-16	LR2	MG462742; 1443				
<i>Halomicroarcula</i> sp. LR2-15	LR2	MG462749; 1442				
<i>Halorubrum</i> sp. LR1-6	LR1	MG462734; 1440				
<i>Halorubrum</i> sp. LR1-15	LR1	MG462744; 1440		~7×10 ⁻⁴		
<i>Halorubrum</i> sp. LR1-21	LR1	MG462746; 1440		~2×10 ⁻¹		
<i>Halorubrum</i> sp. LR1-22	LR1	MG462737; 1440				
<i>Halorubrum</i> sp. LR1-23	LR1	MG462738; 1440			1 (H)	~5×10 ⁻⁸
<i>Halorubrum</i> sp. LR2-4	LR2	MG462747; 1436				
<i>Halorubrum</i> sp. LR2-12	LR2	MG462741; 1441	~1×10 ⁻³	1 (H)	~2×10 ⁻⁴	
<i>Halorubrum</i> sp. LR2-13	LR2	MG462748; 1440		~9×10 ⁻³		
<i>Halorubrum</i> sp. LR2-17	LR2	MG462750; 1440	1 (H)			
<i>Halorubrum</i> sp. LR2-19	LR2	MG462751; 1440				
<i>Halorubrum</i> sp. LR2-20	LR2	MG462743; 1441				

3 a. LR1, Lake Retba sample 1; LR2, Lake Retba sample 2

4 b. The sensitivities of the archaeal strains to isolated viruses (Table 1) are shown as efficiency of plating (EOP)
5 measured as plaque forming units. For the original host (marked by H), the EOP was set to a value of 1. EOPs
6 on others strains are relative to the EOP of the original host.

7

1

2 **Table 3. Virus purification by PEG-NaCl precipitation, rate zonal (in sucrose), equilibrium (in CsCl) and**
 3 **differential ultracentrifugation**

Virus	Number of the infections purified viruses (total pfus) ^a	Recovery of the infectious purified viruses ^b (%)	Yield of the purified viruses in protein (total mg of protein) ^c	Specific infectivity of the purified viruses (pfu / mg of protein)
HRPV10	$\sim 2 \times 10^{13}$	~ 11	~ 0.8	$\sim 3 \times 10^{13}$
HRPV11	$\sim 7 \times 10^{13}$	~ 15	~ 1.5	$\sim 5 \times 10^{13}$
HRPV12	$\sim 8 \times 10^{12}$	~ 8	~ 0.4	$\sim 2 \times 10^{13}$
HFTV1	$\sim 3 \times 10^9$	~ 0.0005	~ 1.9	$\sim 2 \times 10^9$

4 ^a Total pfus (purified viruses) obtained from a liter of virus stock

5 ^b Calculated based on the total pfus in the starting material (virus stocks; see the virus stock titers in Table
 6 1) and the final sample (purified viruses)

7 ^c Total mg of protein (purified viruses) obtained from a liter of virus stock

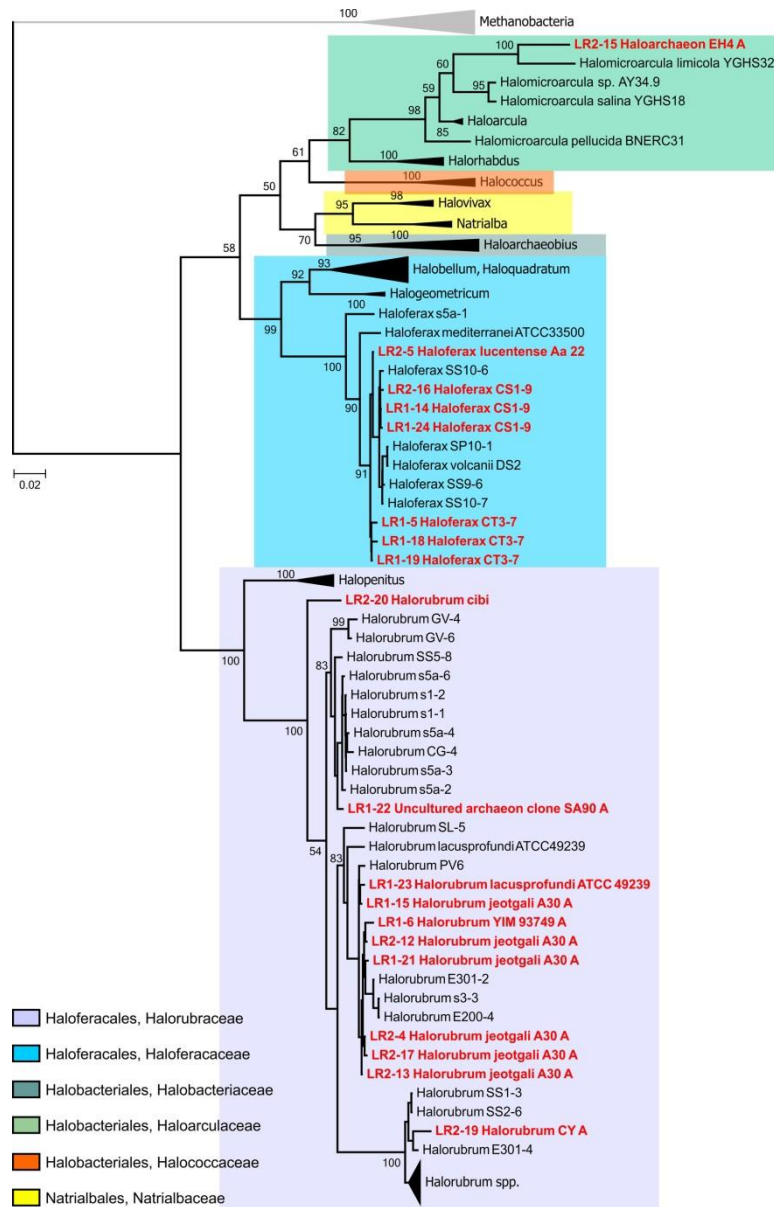


Figure 1. Maximum likelihood phylogenetic tree of the isolated Lake Retba strains based on the partial 16S rRNA gene sequences. Isolates from Lake Retba (LR) are highlighted in red. Sequences were aligned using MUSCLE (Edgar, 2004) and the maximum likelihood tree was constructed using the FastTree2 program (Price et al., 2010). The numbers above the branches represent bootstrap support values from 100 replicates. The scale bar represents the number of substitutions per site.

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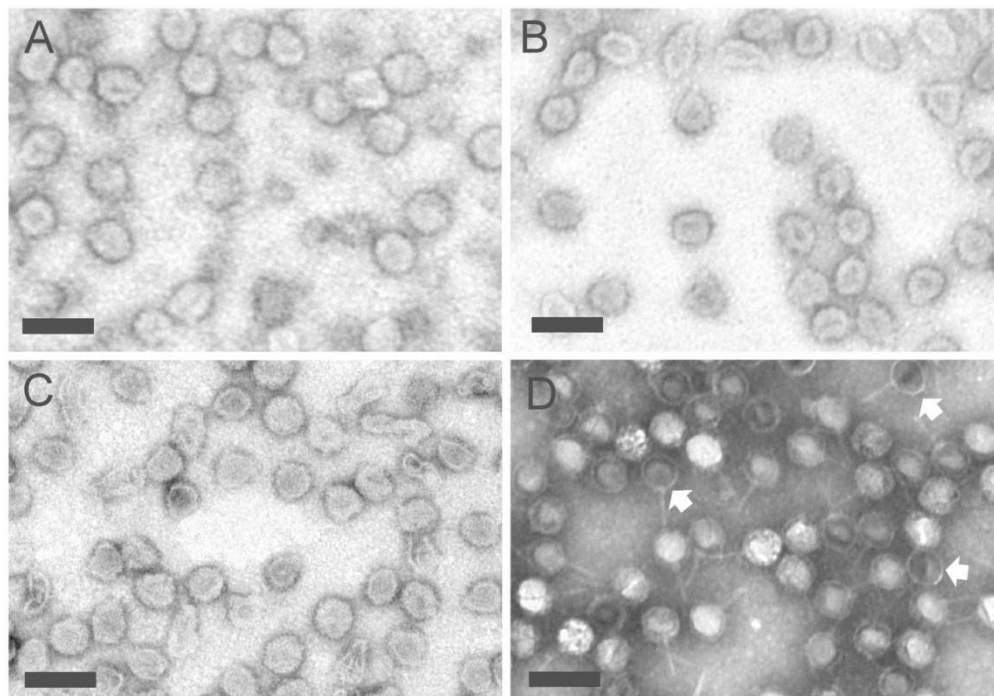


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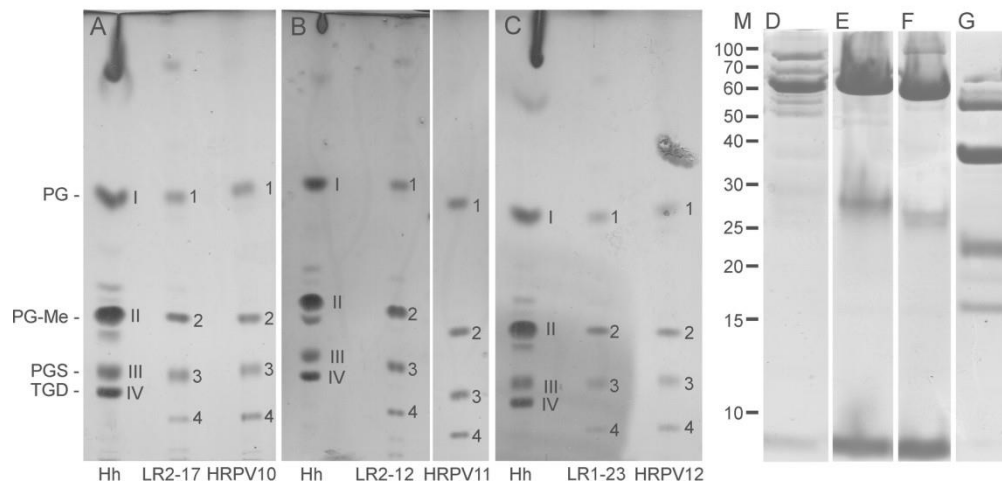


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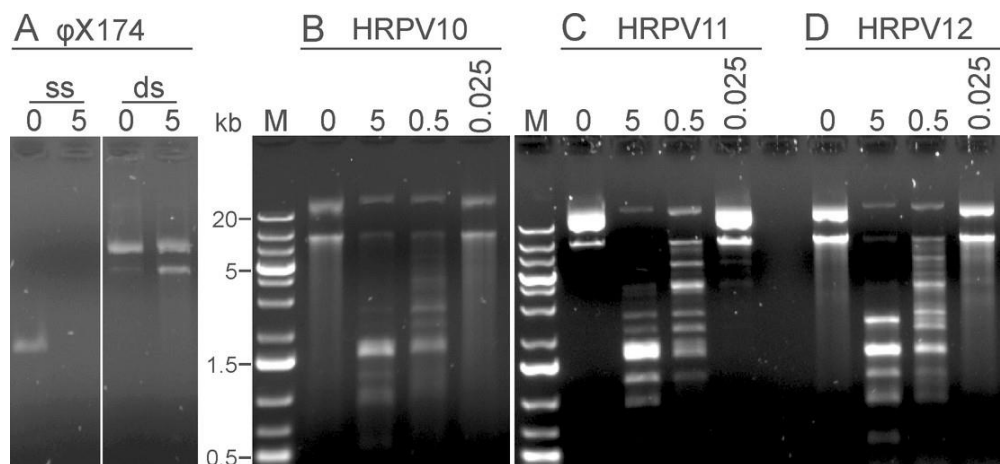


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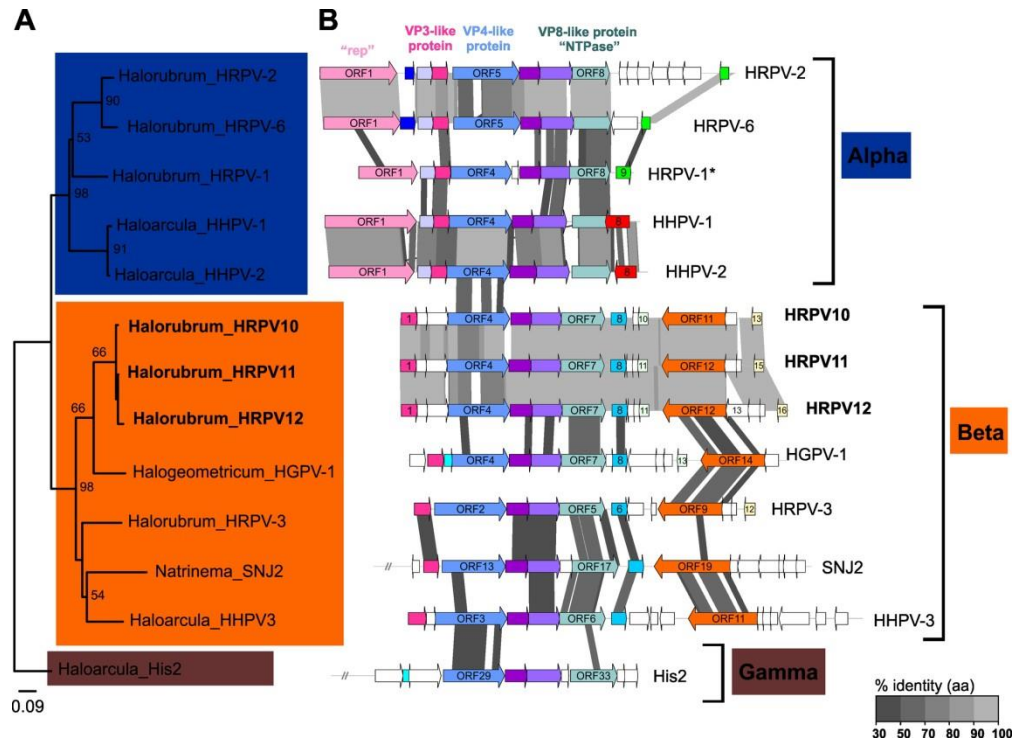


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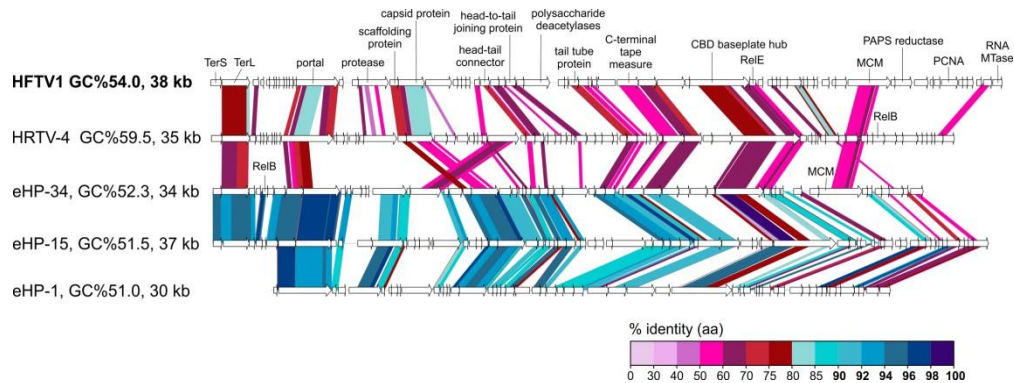


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