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Pleolipoviridae, a newly proposed family comprising archaeal pleomorphic viruses with single-stranded or double-stranded DNA genomes

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Abstract Viruses infecting archaea show a variety of virion morphotypes, and they are currently classified into more than ten viral families or corresponding groups. A pleomorphic virus morphotype is very common among haloarchaeal viruses, and to date, several such viruses have been isolated. Here, we propose the classification of eight such viruses and formation of a new family, *Pleolipoviridae* (from the Greek *pleo* for more or many and *lipos* for lipid), containing three genera, *Alpha*-, *Beta*-, and *Gammapleolipovirus*. The proposal is currently under review by the International Committee on Taxonomy of Viruses (ICTV). The members of the proposed family *Pleolipoviridae* infect halophilic archaea and are nonlytic. They share structural and genomic features and differ from any other classified virus. The virion of pleolipoviruses is composed of a pleomorphic membrane vesicle enclosing the genome. All pleolipoviruses have two major structural protein species, internal membrane and spike proteins. Although the genomes of the pleolipoviruses are single- or double-stranded, linear or circular DNA molecules, they share the same genome organization and gene synteny and show significant similarity at the amino acid level. The canonical features common to all members of the proposed family *Pleolipoviridae* show that they are closely related and thus form a new viral family.

This article is related to an ongoing taxonomic proposal, submitted to the ICTV but not yet accepted at the time of submission. The taxonomy proposed here has not been endorsed by the ICTV Executive Committee, may differ from any new taxonomy that is ultimately approved by the ICTV, and is presented for discussion only but has no official standing.

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Introduction

Archaea and their viruses thrive in extreme environments, and most of archaeal viruses characterized so far infect extremophiles, either hyperhalophiles or hyperthermophiles [4–6, 29]. Viruses infecting archaea display diverse virion morphotypes, some of which are unique. Consequently, archaeal viruses have been classified into over ten viral families and one floating genus, *Salterprovirus*, by the International Committee on Taxonomy of Viruses (ICTV) [19, 29]. To date, about 140 archaeal viruses have been isolated, and most of these belong to the order *Caudovirales*, which is composed of three families of icosahedral tailed viruses [4–6, 19, 29]. In addition to these, spherical and linear, spindle-, bottle- and droplet-shaped, and pleomorphic viruses are known to infect archaea [4, 6, 29].

Archaeal viruses have revealed deep evolutionary relationships between viruses infecting organisms from all three domains of life. Structural studies have shown that tailless icosahedral viruses infecting archaea, bacteria, or eukaryotes share a common ancestor [1, 9, 10, 12, 13, 33]. Furthermore, icosahedral tailed viruses infecting archaea and bacteria have recently been shown to have the same

major capsid protein fold, pointing to a common origin [28]. Pleomorphic viruses infecting archaea and bacteria provide yet another example of viral relationships across domain barriers, as these viruses resemble each other at the virion level [16, 24–26]. The first isolate of pleomorphic, membrane-containing viruses infecting archaea, Haloru-brum pleomorphic virus 1 (HRPV-1), was discovered in 2009, and since then, several other isolates have been characterized [3, 21, 24, 34]. Comprehensive studies of the pleomorphic archaeal viruses have been performed, but these viruses have remained unclassified [3, 18, 21, 24–26, 34, 36]. We have proposed to classify these viruses into a new viral family designated as *Pleolipoviridae*, and here, we summarize the available information on their virion components, genomic data and relatedness.

Pleolipoviridae, a new family of eight archaeal pleomorphic viruses

To date, eight pleomorphic membrane-containing viruses infecting halophilic archaea of the phylum *Euryarchaeota* have been discovered (Table 1). These viruses originate from globally distant locations, and five of them, Haloru-brum pleomorphic viruses 1, 2, 3, and 6 (HRPV-1, HRPV-2, HRPV-3, and HRPV-6) as well as Halogeometricum pleomorphic virus 1 (HGPV-1), have been isolated using a host strain originating from the same sample (Fig. 1). Haloarcula hispanica pleomorphic viruses 1 and 2 (HHPV-1 and HHPV-2) and His2 have been isolated using a culture collection strain of *Haloarcula hispanica* [11, 21, 34]. These eight isolates share both genomic and structural features, showing that they are related. The distinguishing characteristics of this virus group are virion morphology and structural components, genome organization and gene synteny, and sequence similarity [7, 21, 24–26, 29, 34, 36]. When compared to the other known viruses, the only resemblance that haloarchaeal pleolipoviruses have is to pleomorphic viruses infecting bacterial mycoplasmas [2, 7, 16, 24, 29]. However, only one of these phages, L2, has been classified (in the family *Plasmaviridae*) [19]. This isolate shares no detectable sequence similarity to the pleomorphic archaeal viruses. Consequently, we propose the creation of a new family, *Pleolipoviridae*, to classify the pleomorphic archaeal viruses. The name *Pleolipoviridae* originates from the Greek *pleo* for more or many and *lipos* for lipid as the members of the proposed family have pleomorphic virions that are composed of a proteinaceous lipid vesicle enclosing the genome (Fig. 2 and 3). The current members of the proposed family are divided into three genera (see below). The members are referred to as pleolipoviruses, and the model virus system HRPV-1 is the best characterized one.

Table 1 Summary of the primary features of the *Pleolipoviridae* members

Virus isolate	Abbreviation	Origin	Isolation host	Genome	Genome accession number	Virion diameter (nm)	Identified structural proteins	References
Halorubrum virus HRPV-1	HRPV-1	Solar saltern, Trapani, Italy	*Halorubrum* sp. PV6	Circular ssDNA (7048 nt)	FJ685651	41.1 ± 2.2	VP3, VP4, VP8	[18, 24–26]
Halorubrum virus HRPV-2	HRPV-2	Solar saltern, Samut Sakhon, Thailand	*Halorubrum* sp. SS5-4	Circular ssDNA (10656 nt)	JN882264	54.0 ± 4.3	VP4, VP5	[3, 26, 36]
Halorubrum virus HRPV-3	HRPV-3	Experimental Dead Sea-Red Sea saltwater pond of Sedom, Israel	*Halorubrum* sp. SP3-3	Circular dsDNA (8770 bp)	JN882265	67.2 ± 5.2	VP1, VP2	[3, 26, 36]
Halorubrum virus HRPV-6	HRPV-6	Solar saltern, Samut Sakhon, Thailand	*Halorubrum* sp. SS7-4	Circular ssDNA (8549 nt)	JN882266	48.5 ± 2.7	VP4, VP5	[26, 36]
Halogeometricum virus HGPV-1	HGPV-1	Solar saltern, Cabo de Gata, Spain	*Halogeometricum* sp. CG-9	Circular dsDNA (9694 bp)	JN882267	55.5 ± 5.2	VP2, VP3, VP4	[3, 26, 36]
Haloarcula hispanica virus HHPV-1	HHPV-1	Solar saltern, Margherita di Savoia, Italy	*Haloarcula hispanica*	Circular dsDNA (8082 bp)	GU321093	51.7 ± 4.0	VP3, VP4	[26, 34]
Haloarcula hispanica virus HHPV-2	HHPV-2	Solar saltern, Hulu Island, Liaoning, China	*Haloarcula hispanica*	Circular ssDNA (8176 nt)	KF056323	~50	NA	[21]
His2 virus	His2	Hypersaline lake, Victoria, Australia	*Haloarcula hispanica*	Linear dsDNA (16067 bp)	AF191797	70.6 ± 3.6	VP27, VP28, VP29, VP32	[11, 26]

NA – not analyzed

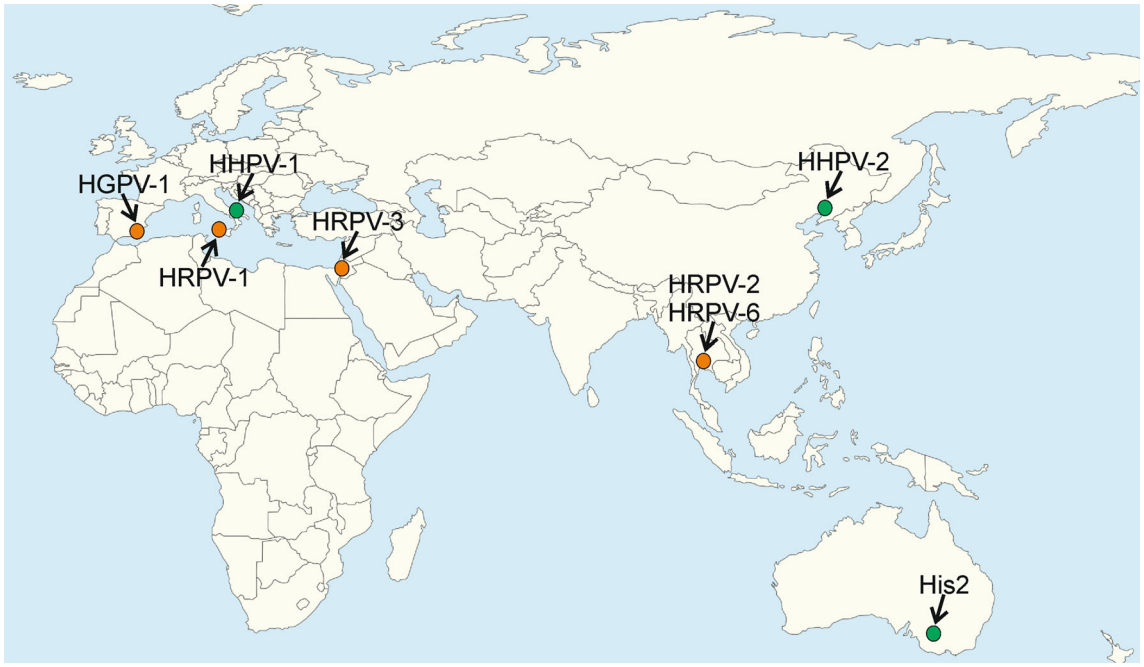

Fig. 1 Members of the proposed family *Pleolipoviridae* are globally distributed. Dots indicate the origin of virus isolates. Orange indicates that the virus was isolated using a host strain isolated from the same location, and green indicates that the virus was isolated using a culture collection strain of *Haloarcula hispanica* [3, 11, 21, 24, 26, 34]. Source of the map: Wikimedia Commons

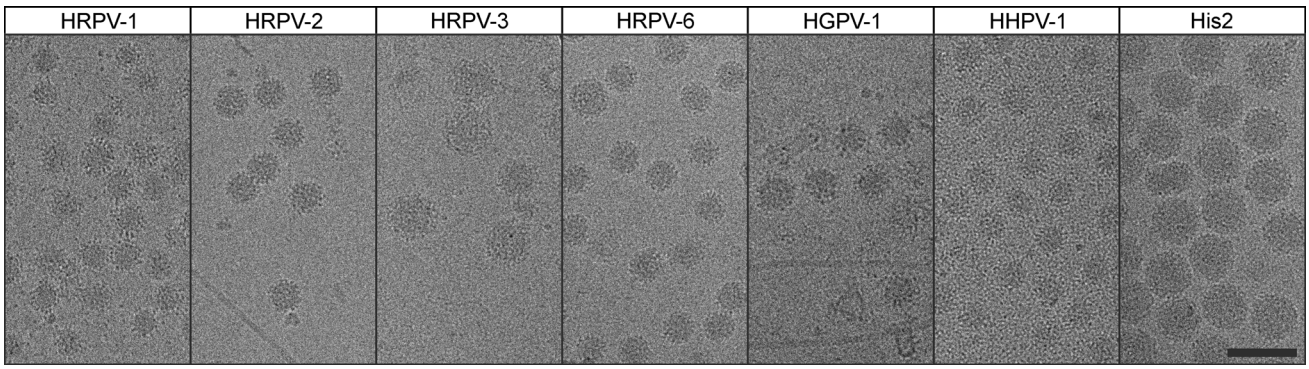

Fig. 2 Cryo-electron microscopy images of seven pleolipovirus isolates. Scale bar, 100 nm. Reproduced from reference 26 with permission

His2 virus is one of the members of the proposed family *Pleolipoviridae*. It has previously been suggested to be distantly related to the spindle-shaped virus His1 infecting *Haloarcula hispanica* [11]. Currently, His1 is classified as the type member of the floating genus *Salterprovirus*, and His2 has been listed as a virus that may be a member of the genus *Salterprovirus* [19]. However, His1 and His2 share no significant amino acid sequence similarity except for their putative DNA polymerases [11]. These protein-primed family B DNA polymerases of His1 and His2 have been independently acquired from archaeal transposon-like elements [20]. Cryo-electron microscopy (cryo-EM) studies revealed that His2 is not spindle-shaped like His1, but rather spherical in shape (Fig. 2) [17, 26]. In addition, the His2 virion has a canonical structural protein profile similar to those of the other isolates of the proposed family *Pleolipoviridae* (Fig. 3A), whereas the His1 virion protein pattern is unique [26, 27]. The genome synteny and amino acid sequence similarity also suggest a relationship between His2 and the other pleolipoviruses [24, 34, 36]. Thus, we propose that His2 should be classified as a member of the proposed family *Pleolipoviridae*.

In addition to the pleolipoviruses described above, three more haloarchaeal pleomorphic viruses, Halorubrum pleomorphic viruses 7 and 8 (HRPV-7 and HRPV-8) and Haloarcula pleomorphic virus 2 (HAPV-2) have recently been isolated [5]. These isolates display characteristics of pleolipoviruses: pleomorphic virion morphotypes observed

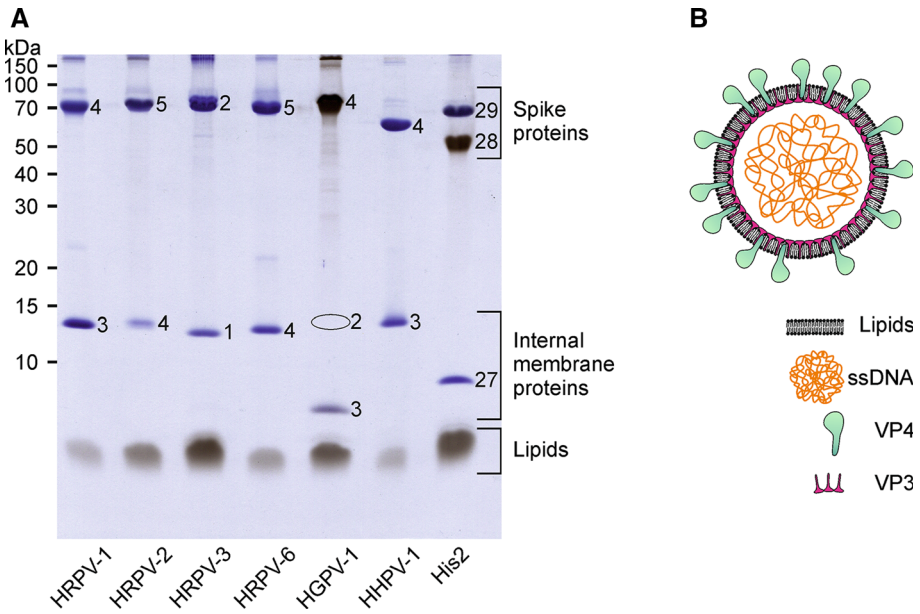

Fig. 3 Structural components of the seven pleolipoviruses. (A) Protein and lipid profile of the purified virions in a tricine-SDS-polyacrylamide gel stained with Coomassie blue and Sudan black B for proteins and lipids, respectively. Numbers on the left indicate the molecular masses of the markers. Numbers on the gel indicate the gene encoding the protein. The theoretical position of VP2 protein of HGPV-1 is indicated by a circle. Reproduced from reference 26 with permission. Protein and lipid profiles are not available for HHPV-2. (B) Schematic representation of the HRPV-1 virion. HRPV-1 is the model virus of the proposed family *Pleolipoviridae*. Genomes of the pleolipoviruses can be either ssDNA or dsDNA, linear or circular

by negative-staining transmission EM, pleolipovirus-like simple structural protein patterns, and hazy plaque morphologies. Moreover, infectivity is affected by the presence of chloroform (at least in the case of HRPV-7 and HAPV-2), suggesting that there is a membrane in the virions [5]. However, the genome sequences of these viruses are not available, and this precludes their further positioning within the pleolipovirus group. Thus, they are considered related virus isolates that may be members of the family *Pleolipoviridae*.

Pleolipoviruses have nonlytic life cycles

All studied pleolipoviruses infect hosts belonging to the family *Halobacteriaceae*. Furthermore, they have a very narrow host range; in most cases they were only able to infect the original isolation strain when representatives from several haloarchaeal genera were tested [3, 5, 11, 24, 34]. All current members of the proposed family *Pleolipoviridae* are nonlytic, and they form hazy plaques on the host lawn [21, 24, 26, 34]. In liquid cultures, progeny viruses are produced continuously, resulting in host growth retardation [24, 26, 34]. The nonlytic nature of the life cycle and the enveloped pleomorphic appearance of the virion imply that pleolipoviruses use budding as an exit mechanism. Accordingly, the most likely entry mechanism is the fusion of the virion envelope with the host cell membrane [24, 35].

Pleomorphic appearance of pleolipoviruses

Pleolipoviruses are sensitive to conditions of low salt concentration, confirming their halophilic nature [24–26]. Negative-stain transmission EM of the highly purified virions has suggested that the pleolipoviruses have a flexible virion structure that is not defined by a rigid protein capsid [21, 24–26, 34]. The pleomorphic appearance of the virions, which varies from spherical to elongated, does not resemble any of the previously described archaeal viruses [30, 32]. To avoid possible artifacts caused by negative staining, the virion morphology of the pleolipoviruses has also been studied using cryo-EM and cryo-electron tomography (cryo-ET). The cryo-electron micrographs show roughly spherical particles with decorating spikes on the virion surface (Fig. 2) [26]. It has been observed that the dimensions of the individual viruses vary. The smallest of the viruses is HRPV-1 (41.1 ± 2.2 nm), and the largest is His2 (70.6 ± 3.6 nm) [26]. The pleomorphicity of the viruses is thus obvious in the range of sizes that each virus exhibits. In addition, cryo-ET of HRPV-1 has shown that there is an apparent lack of longitudinal order in the surface spikes, emphasizing the pleomorphicity [26].

Virions of pleolipoviruses are simple and resemble membrane vesicles

In addition to their morphology, pleolipoviruses have a highly similar, simple structural protein profile (Fig. 3A). Although a protein profile is not available for HHPV-2, the high similarity of all of its predicted genes to those of HHPV-1 suggests that the protein profiles of these two viruses are essentially the same. The virions of pleolipoviruses are composed of two major structural protein species [24–26, 34]. The smaller-sized protein contains predicted transmembrane domains, and the larger-sized one has a C-terminal membrane anchor preceded by a predicted coiled-coil domain. Quantitative biochemical dissociation analysis has shown that the larger-sized proteins of pleolipoviruses are anchored to the membrane and that the smaller ones are in the membrane, facing the particle interior, where the genome resides (Fig. 3B) [25, 26]. There are no nucleoproteins associated with the genome. Thus, the two major protein species have been designated as the spike protein (VP4-like protein according to the HRPV-1 nomenclature; VP for virion protein) and the internal membrane protein (VP3-like protein according to the HRPV-1 nomenclature). HHPV-1, HRPV-1, HRPV-2, HRPV-3, and HRPV-6 have one of each, His2 has two spike proteins and HGPV-1 has two internal membrane proteins (VP2 and VP3; Fig. 3A) [26]. The internal membrane protein VP27 of His2 shares amino-acid-level sequence similarity only with the HGPV-1 protein VP3 and is functionally a VP3-like protein [26]. At the amino acid sequence level, VP3-like proteins are rather conserved in all pleolipoviruses, except in His2.

Cryo-electron tomography has shown that HRPV-1 spikes formed of protein VP4 are randomly distributed on the virion surface. Furthermore, the HRPV-1 internal membrane protein VP3 is mostly embedded in the envelope and does not form an ordered protein capsid or a thick matrix-like layer on the inner surface of the membrane [26]. In HRPV-1, one minor structural protein, VP8, has been identified. HRPV-1 VP8, with its putative counterparts in other pleolipoviruses, is predicted to be an NTPase [24, 36].

Some of the pleolipoviruses have modifications in their spike proteins. HRPV-1 VP4 is glycosylated [18, 25], and the major N-glycan is a pentasaccharide comprising glucose, glucuronic acid, mannose, sulphated glucuronic acid and a terminal 5-N-formyl-legionaminic acid residue [18]. This modification is involved in virus infectivity [18]. The spike proteins of His2 (VP28) and HGPV-1 (VP4) has been observed to be modified by unidentified lipid moieties [26].

Members of the proposed family *Pleolipoviridae* seem to acquire their lipid envelope from the host cell membrane, because the virions contain the same ratios of the major polar lipids as their host cells (the lipid profile is not available for HHPV-2) [24–26, 34]. Furthermore, it has been shown that the ratio of different lipids is the same in the viral and host membrane, indicating that the pleolipoviruses acquire their lipids unselectively from the host lipid pool [25, 34, 38]. Except for HGPV-1, the pleolipoviruses have three major phospholipids: phosphatidylglycerol (PG), phosphatidylglycerophosphate methyl ester (PGP-Me) and phosphatidylglycerosulfate (PGS) [24–26, 34]. The two major phospholipids of HGPV-1 and its host are PG and PGP-Me [26].

The pleolipoviral genomes are either single-stranded or double-stranded DNA molecules

All archaeal viruses characterized so far have a DNA genome, in contrast to known bacterial and eukaryotic viruses, which have either an RNA or DNA genome [6, 19, 29]. Until 2009, the genomic landscape of the studied archaeal viruses was limited to double-stranded (ds) DNA genomes. HRPV-1 was the first archaeal virus to be described containing a single-stranded (ss) DNA genome [24]. Since the isolation of HRPV-1, four more ssDNA viruses infecting archaea have been described [21, 23, 36]. Three of them are members of the proposed family *Pleolipoviridae*.

The genomes of the eight pleolipoviruses discussed here have been sequenced (Table 1). The nucleotide sequence similarity of the genomes to other sequences in the databases is very limited if the other pleolipoviruses are excluded. The genomes show collinear gene organization (Fig. 4), but the genomes of HRPV-1, HRPV-2, HRPV-6 and HHPV-2 are ssDNA molecules, whereas HHPV-1 and His2 have dsDNA genomes [11, 21, 24, 34, 36]. HRPV-3 and HGPV-1 contain dsDNA genomes, but with stretches of ssDNA [36]. His2 has a linear genome, and the other virus isolates have circular ones. The length of the circular genomes varies from 7,048 nt (HRPV-1) to 10,656 nt (HRPV-2), and the linear His2 genome is 16,067 bp in size (Table 1). The GC content of the genomes varies between 40 % (His2) and 64 % (HRPV-2). At the nucleotide sequence level, the genomes show similarity (60 % or higher) only along very short stretches. Exceptions to this are the HRPV-2 and HRPV-6 genomes as well as HHPV-1 and HHPV-2 genomes, which show considerable nucleotide sequence similarity. The set of canonical core genes of the pleolipoviruses consists of genes encoding the internal membrane and spike protein and three conserved predicted downstream genes, one of which is predicted to encode an NTPase (Fig. 4) [11, 24, 34, 36].

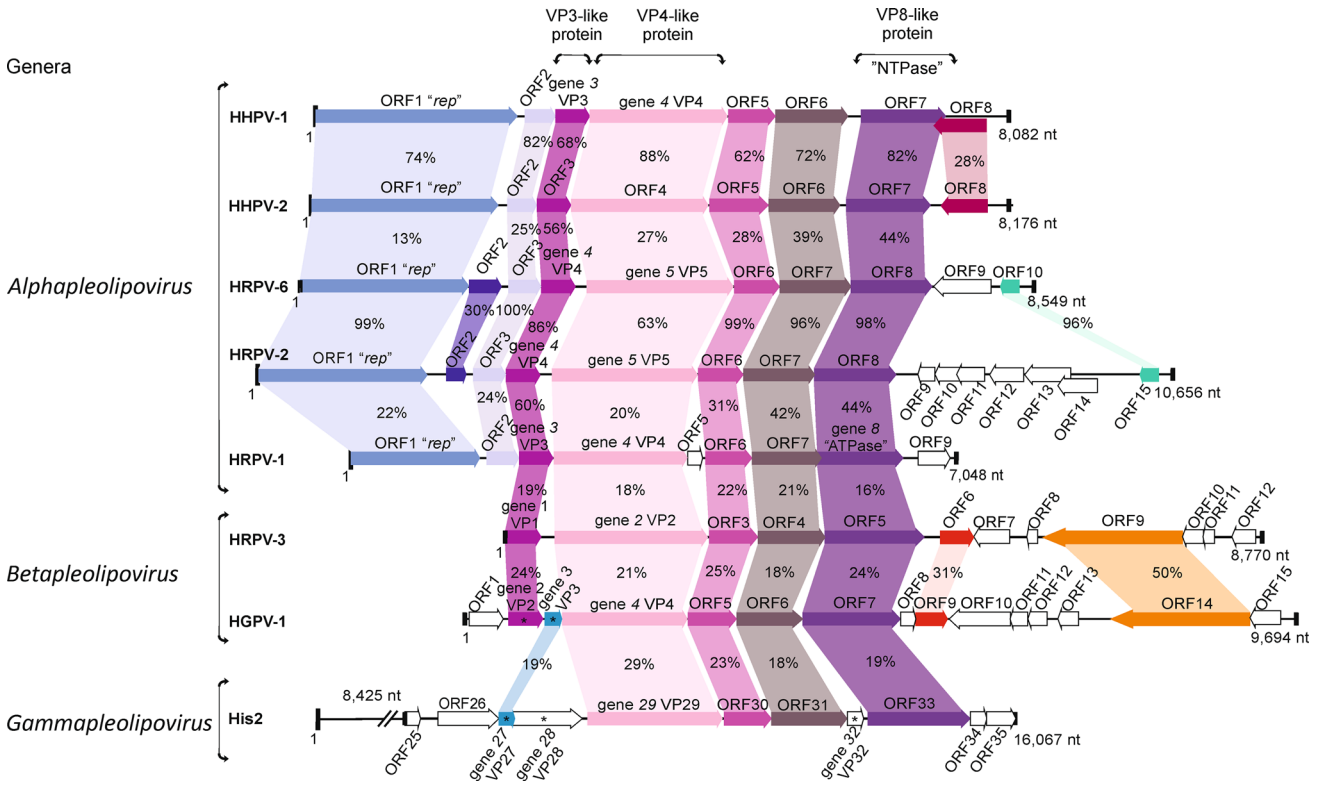

Fig. 4 A linear representation of the pleolipovirus genomes. The identities (%) between the amino acid sequences of two predicted (or identified) gene products are indicated. Based on their genome organization and the relatedness of their VP3-like proteins, the pleolipoviruses can be divided into three genera, which are indicated on the left

Among the proposed pleolipoviruses, the highest identity at the amino acid level can be found between the internal membrane VP3-like proteins (Fig. 4) [36]. One of the internal membrane proteins of HGPV-1 (VP3) shows similarity to the corresponding protein of His2 (VP27), and the other one (VP2) shows similarity to the internal membrane proteins of the other pleolipoviruses. In addition to the core genes, HRPV-1, HHPV-1, HHPV-2, HRPV-2 and HRPV-6 share a predicted gene encoding a putative rolling-circle replication initiation protein. The genomes of HRPV-3, HGPV-1 and His2 do not contain this putative gene but encode a protein homolog containing a C-terminal winged helix-turn-helix (wHTH) domain (HRPV-3 and HGPV-1) or a putative protein-primed family-B-type DNA polymerase (His2) [11, 36]. Thus, HRPV-1, HRPV-2, HRPV-6, HHPV-1 and HHPV-2 are proposed to use a rolling-circle replication mechanism [24, 34, 36]. The ends of the linear dsDNA genome of His2 contain inverted sequence repeats and terminal proteins and most likely replicate using protein priming, whereas the replication mechanisms of HRPV-3 and HGPV-1 remain unknown [11, 31, 36].

A total of fourteen putative pleolipovirus-like proviruses have been identified in the genomes of haloarchaeal strains from the genera *Haloarcula*, *Haloferax*, *Halomicrobium*, *Halopiger*, *Halorhabdus*, *Natrialba*, *Haloterrigena* and *Natronomonas* [15, 24, 34–36]. *Haloferax* plasmid pHK2 and *Halorubrum* plasmid pHRDV1 show gene synteny and significant amino acid sequence similarity to the pleolipovirus genomes. Thus, these plasmids are most likely proviruses related to the pleolipoviruses [14, 34]. Also, a metagenome from a hypersaline lake contained a sequence similar to those of the pleolipoviruses [37].

Archaeal pleolipoviruses and bacterial mycoplasmaviruses

Pleolipovirus-like morphology has also been observed among bacterial mycoplasmaviruses. The pleomorphic, enveloped phages L2 and L172, which infect *Acholeplasma laidlawii* cells, have circular dsDNA and ssDNA genomes, respectively [16, 22]. However, there is no detectable DNA homology between these viruses [16]. Both L2 and L172 acquire their lipids unselectively from the host cell membrane, as do pleolipoviruses [2]. Notably, the protein profile of L172 is very similar to that of the pleolipoviruses, as there are two major protein

Table 2 Taxonomic structure of the proposed family *Pleolipoviridae*

Genus	Species	Representive isolate[a]
Alphapleolipovirus	*Halorubrum virus HRPV-1* (type species)	Halorubrum pleomorphic virus 1 (HRPV-1)
	Halorubrum virus HRPV-2	Halorubrum pleomorphic virus 2 (HRPV-2)
	Halorubrum virus HRPV-6	Halorubrum pleomorphic virus 6 (HRPV-6)
	Haloarcula virus HHPV-1	Haloarcula hispanica pleomorphic virus 1 (HHPV-1)
	Haloarcula virus HHPV-2	Haloarcula hispanica pleomorphic virus 1 (HHPV-2)
Betapleolipovirus	*Halorubrum virus HRPV-3* (type species)	Halorubrum pleomorphic virus 3 (HRPV-3)
	Halogeometricum virus HGPV-1	Halogeometricum pleomorphic virus 1 (HGPV-1)
Gammapleolipovirus	*Haloarcula virus His2* (type species)	His2 virus (His2)

[a] The abbreviation of the virus name is given in parentheses

components, and their estimated masses are close to those of the major structural proteins of the pleolipoviruses [16, 24, 26]. As there is no sequence data available for L172, its classification is currently unclear. The protein profile of L2 differs from that of L172 and the pleolipoviruses [16, 26], and L2 shows no sequence similarity to the pleolipoviruses. Thus, the proposal of a new family for archaeal pleolipoviruses is in line with the current classification of L2 into the family *Plasmaviridae*.

Taxonomic structure of the proposed family *Pleolipoviridae*

We propose the following genus and species demarcation criteria for the family *Pleolipoviridae* (Table 2): (*i*) *Alphapleolipovirus*: A sequence comparison of viruses belonging to different species shows low sequence similarity over the whole genomic nucleotide sequence, but their genomes are collinear. All members encode a putative rolling-circle replication initiation protein. (*ii*) *Betapleolipovirus*: A sequence comparison of viruses belonging to different species shows very little sequence similarity, but their genomes are collinear. Members encode a conserved haloarchaeal protein containing a winged-helix DNA-binding domain. (*iii*) *Gammapleolipovirus*: This proposed genus currently includes only one member, Haloarcula virus His2, which has a gene encoding a putative protein-primed family-B-type DNA polymerase (Table 2).

Among the canonical pleolipoviral gene products, the VP3-like internal membrane protein shows the highest similarity at the amino acid sequence level [36]. The relatedness of the VP3-like proteins can also be used to divide the current members of the *Pleolipoviridae* into the three genera in the same way as with the above-proposed criteria based on gene content. The relatedness of *Alphapleolipovirus* members, which have either an ssDNA or dsDNA genome, can be further verified on the basis of VP3-like protein relatedness. In this case, the genome type is not an adequate criterion.

In conclusion, our recent data show that the eight sequenced pleolipoviruses infecting halophilic archaea share a conserved vesicle-like virion architecture. Based on this canonical virion architecture, pleolipoviruses differ from other known enveloped viruses, as there is no nucleoprotein or matrix protein typical of such viruses. Despite the different genome types, the pleolipoviruses share genome synteny. Accordingly, we propose a new viral family for these viruses, *Pleolipoviridae*. Subdivision of the family into three genera, *Alphapleolipovirus*, *Betapleolipovirus*, and *Gammapleolipovirus*, is proposed. Traditionally, the genome type has been an important criterion in virus classification [8, 19]. Thus, the proposed family *Pleolipoviridae* challenges this view by having both ssDNA and dsDNA viruses as well as both linear and circular genomes. This is most likely due to the replication strategies used resulting in different types of DNA molecules being encapsidated into a virion. We hope that further studies and comparisons will show whether the proposed archaeal virus family *Pleolipoviridae* and the phage family *Plasmaviridae* could form an order, for which we propose here the name *Pleolipovirales*.

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