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An update on eukaryotic viruses revived from ancient permafrost

Jean-Marie Alempic [1#], Audrey Lartigue [1#], Artemiy E Goncharov [2], Guido Grosse [3,4], Jens Strauss [3], Alexey N. Tikhonov [5], Alexander N. Fedorov [6], Olivier Poirot [1], Matthieu Legendre [1], Sébastien Santini [1], Chantal Abergel [1] , and Jean-Michel Claverie [1,*]

[1] IGS, Information Génomique & Structurale (UMR7256), Institut de Microbiologie de la Méditerranée (FR 3489), Institut Microbiologie, Bioénergies et Biotechnologie, and Institut Origines, CNRS, Aix Marseille University, Marseille, 13288, France
[2] Department of Molecular Microbiology, Institute of Experimental Medicine, Saint Petersburg, Russia, Department of Epidemiology, Parasitology and Disinfectology, Northwestern State Medical Mechnikov University, Saint Petersburg, 195067, Russia
[3] Permafrost Research Section, Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, 14473, Potsdam, Germany
[4] Institute of Geosciences, University of Potsdam, 14478 Potsdam, Germany.
[5] Laboratory of theriology, Zoological Institute of Russian Academy of Science, St. Petersburg, 199034, Russia.
[6] Melnikov Permafrost Institute, 677010 Yakutsk, Russia

# These authors contributed equally.
* Correspondence: Jean-Michel.Claverie@univ-amu.fr; Tel.: +33 413946777

Abstract: One quarter of the Northern hemisphere is underlain by permanently frozen ground, referred to as permafrost. Due to climate warming, irreversibly thawing permafrost is releasing organic matter frozen for up to a million years, most of which decomposes into carbon dioxide and methane, further enhancing the greenhouse effect. Part of this organic matter also consists of revived cellular microbes (prokaryotes, unicellular eukaryotes) as well as viruses that remained dormant since prehistorical times. While the literature abounds on descriptions of the rich and diverse prokaryotic microbiomes found in permafrost, no additional report about "live" viruses have been published since the two original studies describing pithovirus (in 2014) and mollivirus (in 2015). This wrongly suggests that such occurrences are rare and that "zombie viruses" are not a public health threat. To restore an appreciation closer to reality, we report the preliminary characterizations of 13 new viruses isolated from 7 different ancient Siberian permafrost samples, 1 from the Lena river and 1 from Kamchatka cryosol. As expected from the host specificity imposed by our protocol, these viruses belong to 5 different clades infecting *Acanthamoeba spp*. but not previously revived from permafrost: pandoravirus, cedratvirus, megavirus, and pacmanvirus, in addition to a new pithovirus strain.

Keywords: Permafrost; Acanthamoeba; Giant Virus; Pleistocene, Siberia, Kamchatka

1. Introduction

Ongoing international modeling and monitoring studies keep confirming that the continuous release of greenhouse gas (mostly CO_2) due to human activities since the industrial revolution is causing significant climate change through global warming. It is now widely acknowledged that an average temperature increase of 1.5°C relative to 1850–1900 would be exceeded during the 21st century, under all realistic circumstances [1] even though the adequacy of present climate models to predict regional changes remains in debate [2]. For instance, climate warming is particularly noticeable in the Arctic where

average temperatures appear to increase twice as fast as in temperate regions [3]. One of the most visible consequences is the global thawing of permafrost at increasing depths [4, 5] and the rapid erosion of permafrost bluffs [6], a phenomenon most visible in Siberia where deep continuous permafrost underlays most of the North Eastern territories.

The thawing of permafrost has significant microbiological consequences. First, above freezing temperatures, the return of liquid water triggers the metabolic reactivation of numerous soil microorganisms (bacteria, archaea, protists, fungi)[7-11], exposing the organic material previously trapped in permafrost to decomposition, releasing additional CO2 and methane further contributing greenhouse gas to the atmosphere [5, 12, 13]. Yet, a more immediate public health concern is the physical release of live bacteria (or archaea) that have remained in cryptobiosis trapped in deep permafrost, isolated from the Earth's surface for up to 2 million years [7, 14] (although a more consensual limit would be half a million years [15]). On a shorter time scale, the periodical return of anthrax epidemics devastating reindeer populations has been linked to the deeper thawing of the permafrost active layer at the soil surface during exceptionally hot summers, allowing century-old *Bacillus anthracis* spores from old animals burial grounds or carcasses to resurface [16-18]

One could imagine that very deep permafrost layers (i.e. million-year-old), such as those extracted by open-pit mining, could release totally unknown pathogens [19]. Finally, the abrupt thawing vertically operating along the whole wall of permafrost bluffs (consisting of specific ice-rich deposits called "yedoma") such as seen in the Kolyma lowland or around the Yukon river Alaska, causes the simultaneous release of ancient microorganisms from frozen soils dating from the whole Holocene to the late Pleistocene (i.e. up to 120.000 years ago) [20]. Many culture-based and culture-independent studies (i.e. barcoding and/or metagenomics) have documented the presence of a large diversity of bacteria in ancient permafrost [7-9, 14, 21-25], a significant proportion of which are thought to be alive, although estimates vary greatly with the depth (age) and soil properties [14, 26, 27]. These bacterial populations include relatives of common contemporary pathogens (Acinetobacter, Bacillus anthracis, Brucella, Campylobacter, Clostridia, Mycoplasma, various Enterobacteria, Mycobacteria, Streptococci, Staphylococci, Rickettsia) [8, 9, 21, 26, 28]. Fortunately, we can reasonably hope that an epidemic caused by a revived prehistoric pathogenic bacterium could be quickly controlled by the modern antibiotics at our disposal, as they target cellular structures (e.g. ribosomes) and metabolic pathways (transcription, translation or cell wall synthesis) conserved during the evolution of all bacterial phyla [29], even though bacteria carrying antibiotic-resistance genes appear to be surprisingly prevalent in permafrost [23, 28, 30].

The situation would be much more disastrous in the case of plant, animal, or human diseases caused by the revival of an ancient unknown virus. As unfortunately well documented by recent (and ongoing) pandemics [31, 32], each new virus, even related to known families, almost always requires the development of highly specific medical responses, such as new antivirals or vaccines. There is no equivalent to "broad spectrum antibiotics" against viruses, because of the lack of universally conserved druggable processes across the different viral families [33, 34]. It is therefore legitimate to ponder the risk of ancient viral particles remaining infectious and getting back into circulation by the thawing of ancient permafrost layers. Focusing on eukaryote-infecting viruses should also be a priority, as bacteriophages are no direct threat to plants, animals, or humans, even though they might shape the microbial ecology of thawing permafrost [35].

Our review of the literature shows that very few studies have been published on this subject. To our knowledge, the first one was the isolation of Influenza RNA from one frozen biopsy of the lung of a victim buried in permafrost since 1918 [36] from which the complete coding sequence of the hemagglutinin gene was obtained. Another one was the detection of smallpox virus DNA in a 300-year-old Siberian mummy buried in permafrost [37]. Probably for safety/regulatory reasons, there was not follow up studies attempting to "revive" these viruses (fortunately). The first isolation of two fully infectious eukaryotic viruses from 30,000-y old permafrost was thus performed in our laboratory and published in 2014 and 2015 [38, 39]. A decisive advantage of our approach was to choose *Acanthamoeba spp.* as a host, to act as a specific bait to potentially infectious viruses, thus eliminating any risk for crops, animals or humans. However, no other isolation of a permafrost virus has been published since, which might suggest that these were lucky shots and that the abundance of viruses remaining infectious in permafrost is very low. This in fact is wrong, as numerous other *Acanthamoeba*-infecting viruses have been isolated in our laboratory, but not yet published pending their complete genome assembly, annotation, or detailed analysis. In the present article we provide an update on thirteen of them, most of which remain at a preliminary stage of characterization. These isolates will be available for collaborative studies upon formal request through a material transfer agreement. The ease with which these new viruses were isolated suggests that infectious particles of viruses specific to many other untested eukaryotic hosts (protozoans or animals) remain probably abundant in ancient permafrost.

2. Materials and Methods

Permafrost sampling

The various on-site sampling protocols have been previously described in [28, 40] for samples #3 and #5 (collected in the spring 2015), in [41] for sample #4, in [42] for sample #6, and [43, 44] for samples #7-9 (see Table 1). Liquid sample #2 and #4 were collected in pre-sterilized 50 ml Falcon tube in august 2019, as well as sample #1 consisting of surface soil without vegetation from the Shapina river bank collected on 07/15/2017 and since maintained frozen at -20 C in the laboratory.

Sample preparation for culturing

About 1g of sample is resuspended in 40 mM Tris pH 7.5, from 2-10% V/V depending on its nature (liquid, mud, solid soil) and vortexed at room temperature. After decanting for 10 minutes, the supernatant is taken up, then centrifugated at 10,000 g for one hour. The pellet is then resuspended in 40 mM Tris pH 7.5 with a cocktail of antibiotics (Ampicillin 100µg/mL, Chloramphenicol 34µg/mL, Kanamycin 20µg/mL). This preparation is then deposited one drop at a time onto two 15 cm-diameter Petri dishes (Sarsted 82.1184.500) one previously seeded with *Acanthamoeba castellanii* at 500 cells/cm^2, the other with *A. castellanii* cells previously adapted to Fongizone (1.25%).

Detection of virus infection

Morphological changes (rounding up, non-adherent cells, encystment, …) of the *A. castellanii* cells might eventually become detectable after 72 h, but might be due to a variety of irrelevant causes (overconfluency, presence of a toxin, fungal proliferation, …). Under a light microscope, the areas exhibiting the most visible changes are spotted using a p1000 pipetman. This 1 mL volume is then centrifugated (13,000 g for 30 minutes), the pellet resuspended in 100 µL and scrutinized under a light microscope. This subsample is also used to seed further T25 cell culture flasks of fresh *A. castellanii* cells.

Preliminary identification of infecting viruses

Potential viral infections are suggested by intracellular changes (presence of cytoplasmic viral factories, nuclear deformation, lysis), or by the direct visualization of giant virus particles. Using a set of in-house-designed family-specific primers (Table 2), a PCR test is performed using the Terra PCR Direct Polymerase Mix (Takara Bio Europe SAS, Saint-Germain-en-Laye, France). Amplicons are then sequenced (Eurofins Genomics, Ebersberg, Germany) to confirm the finding of new isolates.

Further characterization of new virus isolates

Positive subcultures are then reseeded and passaged in T25 then T75 cell culture flasks (Nunc™ EasYFlasks™, Thermofisher scientific, Waltham, MA USA) until the density/quantity of viral particles allows their further characterization by Electron Microscopy (Figure 3). New viral isolates of particular interest are then eventually cloned and their whole genome sequenced.

Viral genome sequencing

Virus cloning, virus particles purification using a cesium chloride gradient, and DNA extraction from approximately 5 x 10^9 purified particles (using the Purelink Genomic extraction mini kit, ThermoFisher) have been previously described [45]. Sequence data was generated from the Illumina HiSeq X platform provided by Novogene Europe (Cambridge, UK). Genome data assembly was performed in-house as previously described [45]. The draft genome sequences listed in Table 3-6, were only minimally annotated using automated BlastP similarity searches, pending further human-supervised validation and analyses, and final submission to the EMBL/Genbank database.

Design of virus-specific PCR primers

Clusters of protein-coding genes common to all known members of a viral family or clade were identified using Orthofinder [46]. The protein sequence alignments of these clusters were converted into nucleotide alignments using Pal2nal [47], and sorted using the "most unrelated pair criteria". These alignments were thus visually inspected to select the variable regions flanked by strictly conserved sequences suitable as PCR primers. The primers and their genes of origin are listed in Table 2.

Table 1. Samples and virus description

Sample #	GPS coordinates	Description	Isolated virus
1	N 55°06′54′′ E 159°57′48′′	Surface soil, Shapina river bank, Kamchatka Modern	*Cedratvirus kamchatka* (strain P5)
2	N 62°06′23;92′′ E 129°48′35′′	Lena river, Yakustk Modern	*Cedratvirus lena* (strain DY0) *Pandoravirus lena* (Strain DY0)
3	N 61° 45' 39.1'' E 130° 28' 28.78''	Talik, -6.5 m below a lake, Yukechi Alas [40] Isolation: >53 y BP	*Pandoravirus talik* (strain Y4)
4	N 68°38′21.1′′ E 159°3′20.67′′	Melting ice wedge Duvanny yar [20, 41] Mixed ages	*Cedratvirus duvanny* (strain DY1) *Pandoravirus duvanny* (Strain DY1)
5	N 61° 45' 39.1'' E 130° 28' 28.78''	-16 m below a lake, Yukechi Alas [40] Isolation: >48,500 y BP	*Pandoravirus yedoma* (strain Y2)
6	N 74°13′00′′ E 141°03′48′′	Woolly mammoth stomach content, Maly Lyakhovsky Island [42] Isolation: >28,600 y BP	*Pandoravirus mammoth* (strain Mm38)
7	N 70°43′25″ E 135°25′47″	Soil with mammoth wool RHS paleolithic site, Yana river left bank [43, 44] Isolation: >27,000 BP	*Megavirus mammoth* (strain Yana14) *Pithovirus mammoth* (strain Yana14) *Pandoravirus mammoth* (strain Yana14)
8*	N 70°43′25″ E 135°25′47″	Fossil wolf (Canis lupus) intestinal content, RHS paleolithic site [43, 44] Isolation: >27,000 y BP	*Pandoravirus lupus* (strain Tums1)
9*	N 70°43′25″ E 135°25′47″	Fossil wolf (Canis lupus) intestinal content, RHS paleolithic site [43, 44] Isolation: >27,000 y BP	*Pacmanvirus lupus* (strain Tums2)

* Same location, but different frozen remains.

Table 2. PCR primers used to identify the newly isolated viruses

Virus family or subgroups	Primer sequences (forward & reverse)	Prototype gene #
Pandoraviridae	F: TCGTGGATCGACATTGGCGTGCAGTT R: CTGGTAGGTGACGGCAAAGTT	*P. salinus* (NC_022098) CDS_1260 Putative oxidoreductase
cedratviruses	F: AAACCTAGGTTGCTAACTGTAGATCCTTG R: GGAACCAGCGTTACCGAGTGCATCTTC	*Cedratvirus A11* (NC_032108) BQ3484_149 Hypothetical protein
Pithoviridae	F: GTGGTCCAAAACTGGAAGAACTA R: GCGTCAAGCTCAACATCAAGTTC	*P. sibericum* (NC_023423) pv_393 DNA/RNA helicase
Mimiviridae (A, B, C lineages)	F: TGGAATAATGGTGATGGTATTGATGT R: ACTGGTACCTAATCCTTTGTAATATTT	*M. chilensis* (NC_016072) mg403 Topoisomerase 2
pacmanviruses	F: GTCTCAATGGGCCACTTGAGCTG R: CCCGCTCTTGACCTCTGGGTTCC	*Pacmanvirus A23* (LT706986) PACV_217 Major Capsid Protein

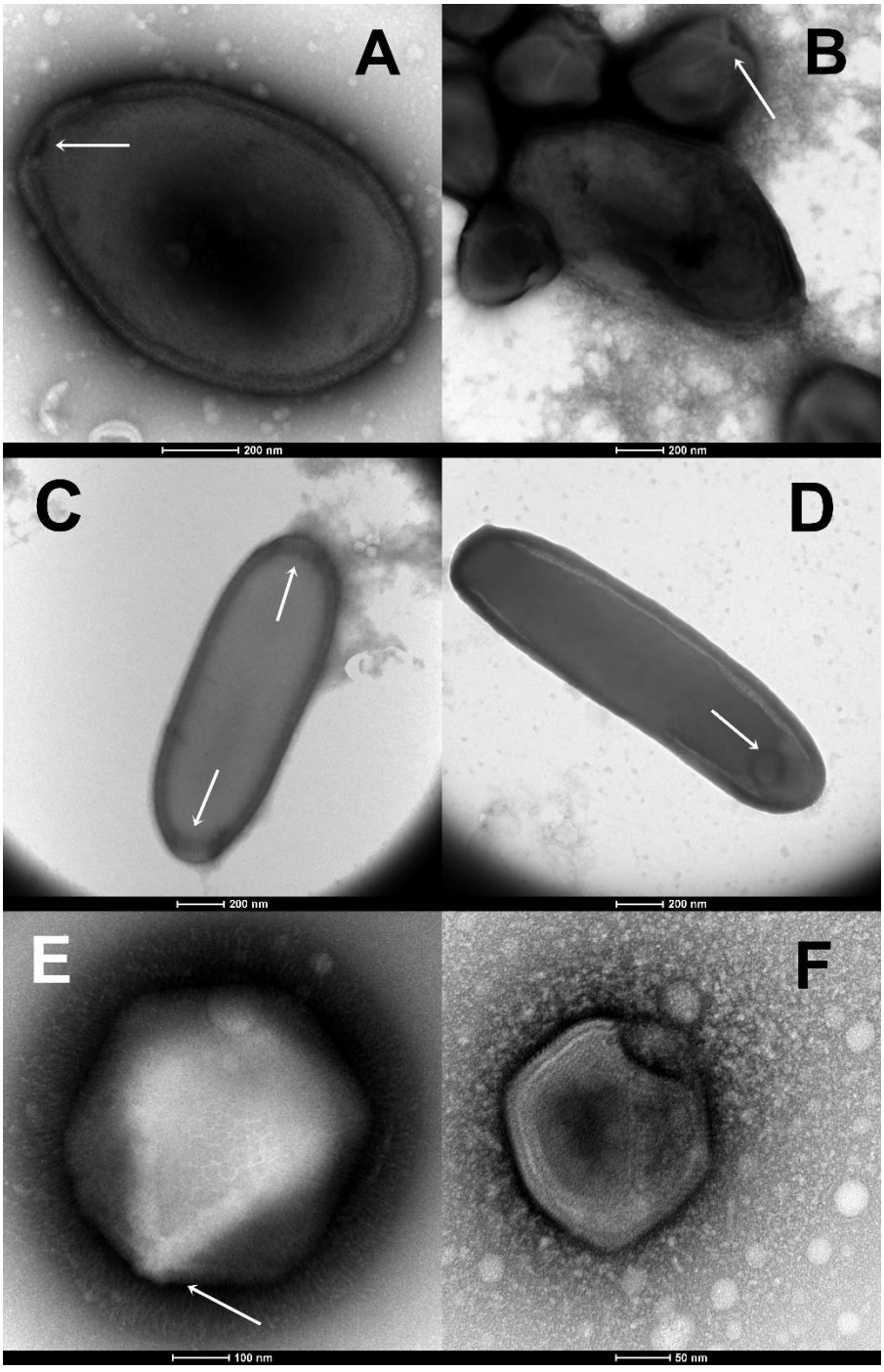

Figure 1. Morphological features guiding the early identification of newly isolated viruses (Negative staining, Transmission Electron Microscopy). (**A**) The large ovoid particle (1,000 nm in length) of Pandoraviruses with its characteristic apex ostiole (white arrowhead). (**B**) A mixture of Pandoravirus particles and Megavirus icosahedral particles exhibiting a "stargate" (white starfish-like structure crowning a vertex, white arrowhead). (**C**) The elongated particle of a Cedratvirus (1,500 nm in length) exhibits two apex cork-like structures (white arrowheads). (**D**) The elongated particle of a Pithovirus (1,900 nm in length) exhibits a single apex cork-like structure (white arrowhead). (**E**) The large (770 nm in diameter) "hairy" icosahedral particle of a Megavirus, with its prominent "stargate" (whaite arrowhead). (**F**) The smaller icosahedral particle (200 nm in diameter) typical of Asfarviruses/Pacmanviruses.

3. Results

3.1. Pandoraviruses

A majority (4/7) of the viruses that we isolated from ancient permafrost layers (sample #5-9, Table 1) belong to the proposed family Pandoraviridae (not yet endorsed by ICTV). One more was isolated from the Lena river (in August 2019, sample #2, Table 1) in Yakutsk, the capital city of the Sakha (Yakutia) Republic, built on permafrost and known as the coldest city on earth. Pandoraviruses remain the most enigmatic of giant viruses infecting *Acanthamoeba spp.* with the largest known viral genomes (a linear DNA molecule up to 2.6 Mb) [48, 49], a puzzling bias in their nucleotidic 4-mer composition [50], the largest proportion of protein-coding genes of unknown origins [49, 51], and the apparent capacity to create such genes *de novo* from non-coding DNA segments at a relatively high frequency [49, 51]. This study is the first to document their presence in ancient soils, in contrast with their scarcity in metagenomic data [52]. Already visible under the light microscope during the early phase of the isolation process, Pandoraviruses are easy to identify by electron microscopy thanks to their typical ovoid particles (1 μm in length and 0.5 μm in diameter) enclosed in a 70-nm thick electron-dense tegument, interrupted by an ostiole-like apex (Figure 1.A). The replication cycle of Pandoraviruses in *A. castellanii* cells lasts from 10 to 15 hours and is initiated by the internalization of individual particles via phagocytic vacuoles. Eight to 10 hours after infection, the *Acanthamoeba* cells become rounded, lose their adherence, and new particles appear at the periphery of the region formerly occupied by the nucleus. The replicative cycle ends when the cells lyse releasing about a hundred particles each.

Table 3. PCR identification of previous and new pandoravirus isolates

Virus	Accession #	Base pairs (contigs)	Amplicon identity
P. salinus (prototype)	NC_022098	2,473,870	100% (1203/1203)
Pandoravirus_celtis	MK174290	2,028,440	93% (1128/1203)
Pandoravirus_quercus	NC_037667	2,077,288	93% (1125/1203)
Pandoravirus_inopinatum	NC_026440	2,243,109	93% (1133/1203)
Pandoravirus_dulcis	NC_021858	1,908,524	92% (1122/1203)
Pandoravirus_neocaledonia	NC_037666	2,003,191	86% (1045/1203)
Pandoravirus_macleodensis	NC_037665	1,838,258	86% (1039/1203)
Pandoravirus lena (strain DY0)	-	2,030,260 (6)	93% (1131/1203)
Pandoravirus duvanny (strain DY1)	-	unassembled	92% (1114/1203)
Pandoravirus talik (strain Y4)	-	1,817,546 (1)	92% (1114/1203)
Pandoravirus mammoth (strain Yana14)	-	not yet sequenced	91% (1104/1203)
Pandoravirus_yedoma (strain Y2)	-	not yet sequenced	91% (1095/1203)
Pandoravirus lupus (strain Tums1)	-	not yet sequenced	91% (1092/1203)
Pandoravirus mammoth (strain Mm38)	-	1,776,082 (2)	86% (1040/1203)

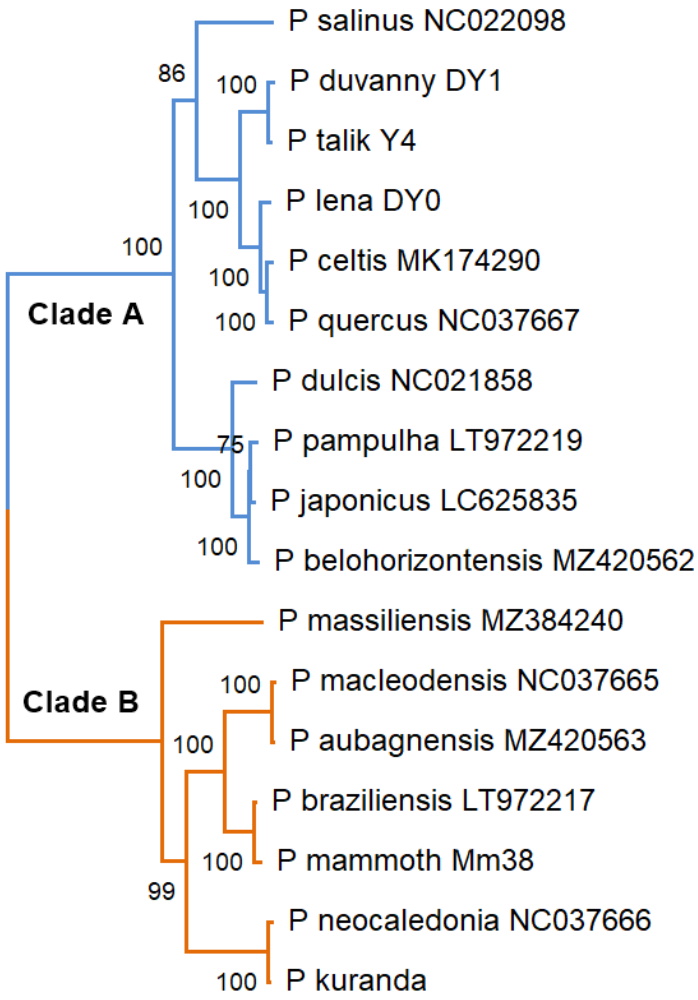

Figure 2. Neighbor-joining tree of the available pandoravirus isolates. The tree was built from 2052 gap-free sites in the multiple alignment of 17 RNA polymerases (RPB1) protein sequences using the MAFFT online facility from the Osaka University server [53]. The inclusion of the permafrost isolates confirms the ancient branching of the Pandoraviridae in two separate clades. Accession numbers are indicated following the isolate name when available.

3.2. *Cedratviruses and pithoviruses*

Three of the newly isolated viruses belong to the recently proposed "cedratviruses" [54, 55], a new clade (a new genus or a new subfamily) within the *Pithoviridae* family [56]. One was cultivated from the same Lena river water sample previously cited (Sample #2, Table 1), one from surface cryosol in Kamchatka collected during the summer (Sample #1, Table 1), and one from mud flowing into the Kolyma river at Duvanny yar, resulting from the thawing of permafrost layers of mixed ages (Sample #4, Table 1). One additional member of the *Pithoviridae* was isolated from a 27.000-y old permafrost sample containing a large amount of mammoth wool (Sample #7, Table 1). It is worth recalling that the prototype of this family was previously isolated from an ancient permafrost layer of more than 30,000-y BP [56]. Other members of this family are the most abundant in a recent metagenomic study of various Siberian permafrost samples focusing on eukaryotic viruses [52].

Even though cedratviruses and pithoviruses produce large ovoid particles like those of Pandoraviruses, they are easyly distinguishable thanks to several morphological features. Their particles are usually more elongated (up to 2µm), have a much thinner wall, and exhibit unique characteristic cork-like structures at their extremity (often two on each side for cedravirus particles) [54, 55](Figure 1.C, 1.D).

Cedratvirus/Pithoviruses are very different from pandoraviruses, with much smaller circular DNA genomes of only 460 to 600 kbp, although a non-isolated member appear to reach 1.6 Mb [52]. After entering *Acanthamoeba* cells by phagocytosis, the particles are stripped from their corks, allowing the viral genome to be delivered through the apical opening into the cytoplasm after membrane fusion. This is then followed by an eclipse phase and the formation of a cytoplasmic electron-lucent viral factory, where a complex morphogenesis occurs. After ~12 h of infection, mature viral particles are released by cell lysis. In contrast with Pandoravirus infections, the cell nucleus maintains its shape throughout the entire Pithovirus replication cycle, a trademark of fully cytoplasmic viruses carrying a functional transcription apparatus in their particles [57].

Table 4. PCR Identification of previous and newly isolated Cedratvirus and Pithovirus species

Virus	Accession #	Base pairs	Amplicon identity
Cedratvirus A11 (prototype)	NC_032108	589,068	100% (1239/1239)
Cedratvirus lausannensis	LT907979	575,161	94% (1176/1239)
Cedratvirus kamchatka	MN873693	466,767	88% (1091/1239)
Cedratvirus lena (strain DY0)	-	465,544	87% (1090/1239)
Cedratvirus duvanny (strain DY1)	-	472,117	87% (1087/1239)
Pithovirus sibericum (P1084-T)	NC_023423	610,033	100% (593/593)
Pithovirus mammoth (strain Yana14)	-	610,309	97% (581/593)

3.3 *Megavirus mammoth*

Megavirus mammoth (strain Yana14) is the first *Mimiviridae* family member ever rescued from ancient permafrost. It was isolated from the highly productive sample (dated > 27.000-y BP) exhibiting fossil mammoth wool (sample #7, Table 1) together with two other viruses: *Pithovirus mammoth* and *Pandoravirus mammoth*. The prototype of the *Mimiviridae* family, Mimivirus, was isolated in 2003 [58]. Its landmark discovery revealed the existence of giant viruses with particles visible by light microscopy and genomic size (>1 Mb) exceeding that of many parasitic bacteria [59]. Initially thought to be exclusively associated to amoebal hosts, the *Mimiviridae* family has diversified enormously, and now includes many divergent members infecting a variety of microalgae [60-62] and heterotrophic protozoans [63, 64]. Several large-scale metagenomic studies have suggested that the *Mimiviridae* family (now comprising several subgroups) constitutes the largest part of the eukaryotic virome in both freshwater [65] and marine aquatic environments [66], with hundreds of reported Metagenomic Assembled Genomes (MAG) [67]. A recent metagenomic study revealed the presence of *Mimiviridae*-related DNA in several ancient permafrost layers [52]. The *Mimiviridae* particles exhibit the icosahedral shape encountered in many smaller viruses, except that their diameter is around 0.5 µm, to which an external layer of dense fibrils (up to 125 nm thick) is added, making them easily visible by light microscopy. In electron microscopy, the internal nucleoid appears as a dark electron-dense

sphere, surrounded by a lipidic membrane. Strikingly, one vertice exhibits a well-defined starfish-like structure, called the "stargate", the opening of which delivers the nucleoid inside the cytoplasm of the infected cells after membrane fusion [68]. These features (icosahedral symmetry, large size, fibrils, and stargate) are unique to *Mimiviridae* members, making their identification straightforward (Figure 1.E). The replication cycle of the first megavirus genus member was fully described previously [69]. As other giant viruses infecting *Acanthamoeba spp.*, their particles penetrate by phagocytosis. Six hours p.i. infected cells start rounding and losing adherence. New particles are then produced in very large cytoplasmic viral factories, leaving the cell nucleus intact. They are then released in large quantities (burst size ≈ 500) through cell lysis after 12-17 hours (depending on the virus and host strain)[69]. Those parameters can be strongly affected by the presence of the ubiquitous virophages, a smal satellite virus, parasitising the various *Mimiviridae* family members [70, 71]

Table 5. Identification of *Megavirus mammoth* among previously isolated *Megavirinae* members

Virus	Accession #	Base pairs	Amplicon identity
Megavirus chilensis	NC_016072	1,259,197	100% (1497/1497)
Megavirus vitis	MG807319	1,242,360	99% (1493/1497)
Megavirus mammoth (strain Yana14)	-	1,260,651	99% (1493/1497)
Megavirus powai lake	KU877344	1,208,707	93% (1396/1497)
Megavirus baoshan	MH046811	1,224,839	92% (1379/1497)
Moumouvirus	NC_020104	1,021,348	83% (1248/1497)
Moumouvirus australiensis	MG807320	1,098,002	82% (1244/1497)
Mimivirus	NC_014649	1,181,549	77% (1158/1497)

The PCR results indicate that *Megavirus mammoth* is a member of the clade C *Megamimivirinae* (i.e. the Megavirus genus) (Table 5). In addition, it encodes all the trademark proteins of the genus: the MutS-like DNA mismatch DNA repair enzyme (ORF 570, 99% identical residues), the glutamine-dependent asparagine synthetase (ORF 434, 98% identical residues), and the 5 amino-acyl tRNA ligase: Ileu AARS (ORF 383, 99% ID), Asp AARS (ORF 771, 100% ID), Met AARS (ORF 798, 99% ID), Arg AARS (ORF 834, 99% ID), Cys AARS (ORF 837, 98% ID), Trp AARS (ORF 876, 96% ID), and Tyr AARS (ORF 944, 97% ID).

3.4 Pacmanvirus lupus

Pacmanviruses are recently discovered *Acanthamoeba*-infecting viruses distantly related to the African swine fever virus, until then the only known members of the *Asfarviridae* family that infects pigs [72]. We now report the isolation of a third member of this newly defined group from the frozen intestinal remains of a Siberian wolf (*Canis lupus*) preserved in a permafrost layer dated >27,000-y BP. At variance with the other truly giant viruses (i.e. associated with unusually large particles), their icosahedral particles are about 220 nm in diameter (Figure 1.F), hence not individually discernable under the light microscope (Nomarski optic). However, DAPI (4', 6-diamidino-2-phenylindole) fluorescence staining can be used to reveal cytoplasmic viral DNA replication in *Acanthamoeba* cultures suspected to undergo viral infection. In culture of *A. castellanii*, *Pacmanvirus lupus* replicated slowly, producing a low yield of particles, without causing massive cell lysis.

Pacmanvirus lupus genome consists of a double-stranded DNA linear molecule of 407,705 bp, comparable in size to that of the previously studied members of this group (Table 6). However, out of its 506 predicted protein-coding genes, only 241 (47.6%) exhibit homologs in the two previously sequenced pacmanvirus genomes, and 221 (43.7%) are ORFans. Thus, if *P. lupus* appears definitely closer to pacmanviruses than to any other known viruses, its evolutionary distance is larger than usually observed within a subfamily or a genus. This large global discrepancy in gene content, is reflected in the low level of similarity observed between various core genes of *P. lupus* and their homologs in other pacmanviruses and closest relatives (Table 7). The asfarviruses appear even more distant with half the number of genes and half the genome size, calling into question the initial classification of pacmanviruses as truly related to family *Asfarviridae* (Table 7) [72]. Yet, in a phylogenetic tree built on these sequences, *P. lupus* appears unambiguously clustered with the other Pacmanviruses, justifying its given name (Figure 3).

Table 6. Identification of *Pacmanvirus lupus* (strain Tums2) among previously isolated pacmanviruses.

Virus	**Accession #**	**Base pairs**	**Amplicon identity**
Pacmanvirus A23	NC_034383	395,405	100% (470/470)
Pacmanvirus S19	MZ440852	418,588	93% (439/470)
Pacmanvirus lupus (strain Tums2)	-	407,705	<85% (399/468) + two large insertions

Table 7. Closest virus relatives of *Pacmanvirus lupus*

Pacmanvirus lupus Predicted protein	*Pacmanvirus A23* NC_034383 %ID (aa)	*Pacmanvirus S19* MZ440852 %ID (aa)	Faustovirus KJ614390 %ID (aa)	Kaumoebavirus NC_034249 %ID (aa)	Asfarviruses NC_044958 %ID (aa)
RNA polymerase (RPB1) ORF 302	79% (1124/1415)	80% (1130/1415)	49% (707/1434)	42% (598/1429)	41% (596/1457)
RNA polymerase (RPB2) ORF 33	85% (1093/1289)	85% (1104/1301)	55% (681/1241)	44% (528/1211)	43% (526/1228)
DNA polymerase (PolB) ORF 265	65% (1036/1591)	65% (1032/1591)	37% (513/1385)	27.5% (344/1250)	33% (382/1163)
Genome size					
407,705 bp	395,405 bp	418,588 bp	457-491 kb	351-363 kb	172-191 kb

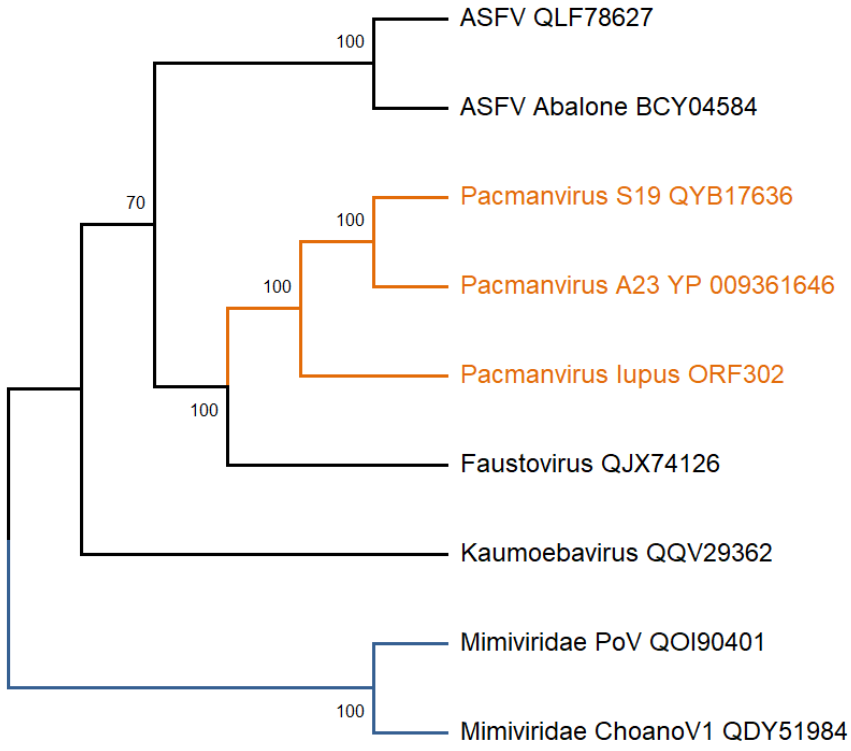

Figure 3. Neighbor-joining tree of the closest *Pacmanvirus lupus* relatives (using RPB1 homologs, Table 7). The closest *Mimiviridae* RPB1 sequences are used as an outgroup. The alignment is based on 1300 gap-free sites. Although *P. lupus* is well clustered with other pacmanviruses, this clade (together with faustovirus) clearly appears as a sister group rather than *bona fide* members within the *Asfarviridae* (ASFV) family. The tree was built from 1300 gap-free sites in the multiple alignment of 9 RNA polymerases (RPB1) protein sequences using the MAFFT online facility from the Osaka University server [53]. Accession numbers are indicated following the isolate name when available

4. Discussion

Following initial reports published more than 5 years ago [38, 39], this study confirms the capacity of large DNA viruses infecting *Acanthamoeba* to remain infectious after more than 48,500 years spent in deep permafrost. Our results extend our previous findings to 3 additional virus families or groups: 4 new members of the *Pandoraviridae*, one member of the *Mimiviridae*, and one pacmanvirus (Table 1). One additional pithovirus was also revived from a particularly productive sample dated 27,000-y BP (sample#7, Table 1) exhibiting mammoth wool. Given these viruses' diversity both in their particle structure and replication mode, one can reasonably infer that many other eukaryotic viruses infecting a variety of hosts much beyond *Acanthamoeba spp.* may also remain infectious in similar conditions. Genomic traces of such viruses are detected in a recent large-scale metagenomic study of ancient permafrost [52] as well as in Arctic lake sediments [73]. They include well documented human and vertebrate pathogens such as poxviruses, herpesviruses, and asfarviruses, although in lower proportions than protozoan infecting viruses.

In our recent metagenomic study [52], Pandoraviruses are notably absent while they constitute the large majority of the viruses revived from permafrost and cryosols. Such a discrepancy might originate from the fact that the extraction of genomic DNA from their sturdy particles requires a much harsher treatment than for most other viruses. Their abundance in environmental viromes might thus be much larger than the small fraction they contribute to the DNA pool.

Such DNA extraction bias may apply to many other microbes, and is a serious limitation to the validity of metagenomic approaches for quantitative population studies.

The types of viruses revived in our study are indeed the results of even stronger biases. First, the only viruses we can expect to detect are those infecting species of *Acanthamoeba*. Second, because we rely on "sick" amoeba to point out potentially virus-replicating cultures, we strongly limit ourselves to the detection of lytic viruses. Third, we favor the identification of "giant" viruses, given the important role given to light microscopy in the early detection of viral replication. It is thus likely that many small, non-lytic viruses do escape our scrutiny, as well as those infecting many other protozoa that can survive in ancient permafrost [10].

However, we believe that the use of *Acanthamoeba* cells as a virus bait is nevertheless a good choice for several reasons. First, *Acanthamoeba* spp. are free-living amoebae that are ubiquitous in natural environments, such as soils and fresh, brackish, and marine waters, but are also in dust particles, pools, water taps, sink drains, flowerpots, aquariums, sewage, as well as medical settings hydrotherapy baths, dental irrigation equipment, humidifiers, cooling systems, ventilators, and intensive care units [74]. The detection of their virus may thus provide a useful test for the presence of any other live viruses in a given setting. Second, if many *Acanthamoeba* species can be conveniently propagated in axenic culture conditions, they remain "self-cleaning" thanks to phagocytosis, and are capable of tolerating heavy contamination by bacteria (that they eat) as well as high doses of antibiotics and antifungals. The third, but not the least, advantage is that of biological security. When we use *Acanthamoeba spp.* cultures to investigate the presence of infectious unknown viruses in prehistorical permafrost (in particular from paleontological sites, such as RHS [43, 44]), we are using its billion years of evolutionary distance with human and other mammals as the best possible protection against an accidental infection of laboratory workers or the spread of a dreadful virus once infecting Pleistocene mammals to their contemporary relatives. The biohazard associated with reviving prehistorical amoeba-infecting viruses is thus totally negligible, compared to the search for "paleoviruses" directly from permafrost-preserved remains of mammoths, woolly rhinoceros, or prehistoric horses, as it is now pursued in the Vector laboratory (Novosibirsk, Russia) [75], fortunately a BSL4 facility. Without the need of embarking on such a risky project, we believe our results with *Acanthamoeba*-infecting viruses can be extrapolated to many other DNA viruses capable of infecting humans or animals. It is thus likely that ancient permafrost (eventually much older than 50,000 years, our limit solely dictated by the validity range of radiocarbon dating) will release these unknown viruses upon thawing. How long these viruses could remain infectious once exposed to outdoor conditions (UV light, oxygen, heat), and how likely they will be to encounter and infect a suitable host in the interval, is yet impossible to estimate. But the risk is bound to increase in the context of global warming when permafrost thawing will keep accelerating, and more people will be populating the Arctic in the wake of industrial ventures.

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Data Availability Statement: The newly isolated viruses described here are available for collaborative studies upon formal request through a material transfer agreement.

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