

An Update on Eukaryotic Viruses Revived from Ancient Permafrost

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Jean-Marie Alempic, Audrey Lartigue, Artemiy E Goncharov, Guido Grosse, Jens Strauss, et al.. An Update on Eukaryotic Viruses Revived from Ancient Permafrost. Viruses, 2023, 15 (2), pp.564. 10.3390/xxxxx . hal-04207147

HAL Id: hal-04207147 https://amu.hal.science/hal-04207147

Submitted on 14 Sep 2023 $\,$

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An update on eukaryotic viruses revived from ancient permafrost

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Abstract: One quarter of the Northern hemisphere is underlain by permanently frozen ground, re-20 ferred to as permafrost. Due to climate warming, irreversibly thawing permafrost is releasing or-21 ganic matter frozen for up to a million years, most of which decomposes into carbon dioxide and 22 methane, further enhancing the greenhouse effect. Part of this organic matter also consists of re-23 vived cellular microbes (prokaryotes, unicellular eukaryotes) as well as viruses that remained 24 dormant since prehistorical times. While the literature abounds on descriptions of the rich and di-25 verse prokaryotic microbiomes found in permafrost, no additional report about "live" viruses have 26 been published since the two original studies describing pithovirus (in 2014) and mollivirus (in 27 2015). This wrongly suggests that such occurrences are rare and that "zombie viruses" are not a 28 public health threat. To restore an appreciation closer to reality, we report the preliminary charac-29 terizations of 13 new viruses isolated from 7 different ancient Siberian permafrost samples, 1 from 30 the Lena river and 1 from Kamchatka cryosol. As expected from the host specificity imposed by our 31 protocol, these viruses belong to 5 different clades infecting Acanthamoeba spp. but not previously 32 revived from permafrost: pandoravirus, cedratvirus, megavirus, and pacmanvirus, in addition to a 33 new pithovirus strain. 34

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

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1. Introduction

Ongoing international modeling and monitoring studies keep confirming that the 38 continuous release of greenhouse gas (mostly CO₂) due to human activities since the in-39 dustrial revolution is causing significant climate change through global warming. It is 40 now widely acknowledged that an average temperature increase of 1.5°C relative to 1850-41 1900 would be exceeded during the 21st century, under all realistic circumstances [1] even 42 though the adequacy of present climate models to predict regional changes remains in 43 debate [2]. For instance, climate warming is particularly noticeable in the Arctic where 44 average temperatures appear to increase twice as fast as in temperate regions [3]. One of 45 the most visible consequences is the global thawing of permafrost at increasing depths [4, 46

Citation: Alempic, J.-M.; Lartigue, A.; Goncharov, A.E.; Grosse, G.; Strauss, J.; Tikhonov, A.N.; Fedorov, A.N.; Poirot, O.; Legendre, M.; Santini, S.; et al. An update on eukaryotic viruses revived from ancient permafrost. *Viruses* **2022**, *14*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s):

Received: date Accepted: date Published: date

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Copyright: © 2022 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/). 5] and the rapid erosion of permafrost bluffs [6], a phenomenon most visible in Siberia 47 where deep continuous permafrost underlays most of the North Eastern territories. 48

The thawing of permafrost has significant microbiological consequences. First, above 49 freezing temperatures, the return of liquid water triggers the metabolic reactivation of 50 numerous soil microorganisms (bacteria, archaea, protists, fungi)[7-11], exposing the or-51 ganic material previously trapped in permafrost to decomposition, releasing additional 52 CO2 and methane further contributing greenhouse gas to the atmosphere [5, 12, 13]. Yet, 53 a more immediate public health concern is the physical release of live bacteria (or archaea) 54 that have remained in cryptobiosis trapped in deep permafrost, isolated from the Earth's 55 surface for up to 2 million years [7, 14] (although a more consensual limit would be half a 56 million years [15]). On a shorter time scale, the periodical return of anthrax epidemics 57 devastating reindeer populations has been linked to the deeper thawing of the permafrost 58 active layer at the soil surface during exceptionally hot summers, allowing century-old 59 *Bacillus anthracis* spores from old animals burial grounds or carcasses to resurface [16-18] 60

One could imagine that very deep permafrost layers (i.e. million-year-old), such as 61 those extracted by open-pit mining, could release totally unknown pathogens [19]. Fi-62 nally, the abrupt thawing vertically operating along the whole wall of permafrost bluffs 63 (consisting of specific ice-rich deposits called "yedoma") such as seen in the Kolyma low-64 land or around the Yukon river Alaska, causes the simultaneous release of ancient micro-65 organisms from frozen soils dating from the whole Holocene to the late Pleistocene (i.e. 66 up to 120.000 years ago) [20]. Many culture-based and culture-independent studies (i.e. 67 barcoding and/or metagenomics) have documented the presence of a large diversity of 68 bacteria in ancient permafrost [7-9, 14, 21-25], a significant proportion of which are 69 thought to be alive, although estimates vary greatly with the depth (age) and soil proper-70 ties [14, 26, 27]. These bacterial populations include relatives of common contemporary 71 pathogens (Acinetobacter, Bacillus anthracis, Brucella, Campylobacter, Clostridia, Myco-72 plasma, various Enterobacteria, Mycobacteria, Streptococci, Staphylococci, Rickettsia) [8, 73 9, 21, 26, 28]. Fortunately, we can reasonably hope that an epidemic caused by a revived 74 prehistoric pathogenic bacterium could be quickly controlled by the modern antibiotics at 75 our disposal, as they target cellular structures (e.g. ribosomes) and metabolic pathways 76 (transcription, translation or cell wall synthesis) conserved during the evolution of all bac-77 terial phyla [29], even though bacteria carrying antibiotic-resistance genes appear to be 78 surprisingly prevalent in permafrost [23, 28, 30]. 79

The situation would be much more disastrous in the case of plant, animal, or human 80 diseases caused by the revival of an ancient unknown virus. As unfortunately well docu-81 mented by recent (and ongoing) pandemics [31, 32], each new virus, even related to 82 known families, almost always requires the development of highly specific medical re-83 sponses, such as new antivirals or vaccines. There is no equivalent to "broad spectrum 84 antibiotics" against viruses, because of the lack of universally conserved druggable pro-85 cesses across the different viral families [33, 34]. It is therefore legitimate to ponder the 86 risk of ancient viral particles remaining infectious and getting back into circulation by the 87 thawing of ancient permafrost layers. Focusing on eukaryote-infecting viruses should also 88 be a priority, as bacteriophages are no direct threat to plants, animals, or humans, even 89 though they might shape the microbial ecology of thawing permafrost [35]. 90

Our review of the literature shows that very few studies have been published on this 91 subject. To our knowledge, the first one was the isolation of Influenza RNA from one fro-92 zen biopsy of the lung of a victim buried in permafrost since 1918 [36] from which the 93 complete coding sequence of the hemagglutinin gene was obtained. Another one was the 94 detection of smallpox virus DNA in a 300-year-old Siberian mummy buried in permafrost 95 [37]. Probably for safety/regulatory reasons, there was not follow up studies attempting 96 to "revive" these viruses (fortunately). The first isolation of two fully infectious eukaryotic 97 viruses from 30,000-y old permafrost was thus performed in our laboratory and published 98 in 2014 and 2015 [38, 39]. A decisive advantage of our approach was to choose Acan-99

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2. Materials and Methods 2.1. Permafrost sampling

probably abundant in ancient permafrost.

The various on-site sampling protocols have been previously described in [28, 40] for 114samples #3 and #5 (collected in the spring 2015), in [41] for sample #4, in [42] for sample 115 #6, and [43, 44] for samples #7-9 (see Table 1). Liquid sample #2 and #4 were collected in 116 pre-sterilized 50 ml Falcon tube in august 2019, as well as sample #1 consisting of surface 117 soil without vegetation from the Shapina river bank collected on 07/15/2017 and since 118 maintained frozen at -20 C in the laboratory. 119

thamoeba spp. as a host, to act as a specific bait to potentially infectious viruses, thus elim-

inating 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 labor-

atory, but not yet published pending their complete genome assembly, annotation, or de-

tailed 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 vi-

ruses specific to many other untested eukaryotic hosts (protozoans or animals) remain

2.2. Sample preparation for culturing

About 1g of sample is resuspended in 40 mM Tris pH 7.5, from 2-10% V/V depending 121 on its nature (liquid, mud, solid soil) and vortexed at room temperature. After decanting 122 for 10 minutes, the supernatant is taken up, then centrifugated at 10,000 g for one hour. 123 The pellet is then resuspended in 40 mM Tris pH 7.5 with a cocktail of antibiotics (Ampi-124 cillin 100µg/mL, Chloramphenicol 34µg/mL, Kanamycin 20µg/mL). This preparation is 125 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/cm², the other 127 with A. castellanii cells previously adapted to Fongizone (1.25%). 128

2.3. Detection of virus infection

Morphological changes (rounding up, non-adherent cells, encystment, ...) of the A. 130 castellanii cells might eventually become detectable after 72 h, but might be due to a variety 131 of irrelevant causes (overconfluency, presence of a toxin, fungal proliferation, ...). Under 132 a light microscope, the areas exhibiting the most visible changes are spotted using a p1000 133 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 135 used to seed further T25 cell culture flasks of fresh A. castellanii cells. 136

2.4. Preliminary identification of infecting viruses

Potential viral infections are suggested by intracellular changes (presence of cyto-138 plasmic viral factories, nuclear deformation, lysis), or by the direct visualization of giant 139 virus particles. Using a set of in-house-designed family-specific primers (Table 2), a PCR 140test is performed using the Terra PCR Direct Polymerase Mix (Takara Bio Europe SAS, 141 Saint-Germain-en-Laye, France). Amplicons are then sequenced (Eurofins Genomics, 142 Ebersberg, Germany) to confirm the finding of new isolates. 143

2.5. Further characterization of new virus isolates

Positive subcultures are then reseeded and passaged in T25 then T75 cell culture 145 flasks (Nunc™ EasYFlasks™, Thermofisher scientific, Waltham, MA USA) until the den-146

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sity/quantity of viral particles allows their further characterization by Electron Microscopy (Figure 3). New viral isolates of particular interest are then eventually cloned and 148

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2.6. Viral genome sequencing

their whole genome sequenced.

Virus cloning, virus particles purification using a cesium chloride gradient, and DNA 151 extraction from approximately 5 x 10⁹ purified particles (using the Purelink Genomic ex-152 traction mini kit, ThermoFisher) have been previously described [45]. Sequence data was 153 generated from the Illumina HiSeq X platform provided by Novogene Europe (Cam-154 bridge, UK). Genome data assembly was performed in-house as previously described [45]. 155 The draft genome sequences listed in Table 3-6, were only minimally annotated using au-156 tomated BlastP similarity searches, pending further human-supervised validation and 157 analyses, and final submission to the EMBL/Genbank database. 158

2.7. 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)

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* 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	<i>P. salinus</i> (NC_022098) CDS_1260 Putative oxidoreductase	
cedratviruses	F: AAACCTAGGTTGCTAACTGTAGATCCTTG R: GGAACCAGCGTTACCGAGTGCATCTTC	<i>Cedratvirus A11</i> (NC_032108) BQ3484_149 Hypothetical protein	
Pithoviridae	F: GTGGTCCAAAACTGGAAGAACTA R: GCGTCAAGCTCAACATCAAGTTC	P. sibericum (NC_023423) pv_393 DNA/RNA helicase	
<i>Mimiviridae</i> (A, B, C lineages)	F: TGGAATAATGGTGATGGTATTGATGT R: ACTGGTACCTAATCCTTTGTAATATTT	<i>M. chilensis</i> (NC_016072) mg403 Topoisomerase 2	
pacmanviruses	F: GTCTCAATGGGCCACTTGAGCTG R: CCCGCTCTTGACCTCTGGGTTCC	Pacmanvirus A23 (LT706986) PACV_217 Major Capsid Protein	



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Figure 1. Morphological features guiding the early identification of newly isolated viruses (Nega-171 tive staining, Transmission Electron Microscopy). (A) The large ovoid particle (1,000 nm in length) 172 of Pandoraviruses with its characteristic apex ostiole (white arrowhead). (B) A mixture of Pandora-173 virus particles and Megavirus icosahedral particles exhibiting a "stargate" (white starfish-like struc-174ture crowning a vertex, white arrowhead). (C) The elongated particle of a Cedratvirus (1,500 nm in 175 length) exhibits two apex cork-like structures (white arrowheads). (D) The elongated particle of a 176 Pithovirus (1,900 nm in length) exhibits a single apex cork-like structure (white arrowhead). (E) The 177 large (770 nm in diameter) "hairy" icosahedral particle of a Megavirus, with its prominent "star-178 gate" (white arrowhead). (F) The smaller icosahedral particle (200 nm in diameter) typical of As-179 farviruses/Pacmanviruses. 180

3. Results

3.1. Pandoraviruses

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A majority (4/7) of the viruses that we isolated from ancient permafrost layers (sam-183 ple #5-9, Table 1) belong to the proposed family Pandoraviridae (not yet endorsed by 184 ICTV). One more was isolated from the Lena river (in August 2019, sample #2, Table 1) in 185 Yakutsk, the capital city of the Sakha (Yakutia) Republic, built on permafrost and known 186 as the coldest city on earth. Pandoraviruses remain the most enigmatic of giant viruses 187 infecting Acanthamoeba spp. with the largest known viral genomes (a linear DNA mole-188 cule up to 2.6 Mb) [48, 49], a puzzling bias in their nucleotidic 4-mer composition [50], the 189 largest proportion of protein-coding genes of unknown origins [49, 51], and the apparent 190 capacity to create such genes de novo from non-coding DNA segments at a relatively high 191 frequency [49, 51]. This study is the first to document their presence in ancient soils, in 192 contrast with their scarcity in metagenomic data [52]. Already visible under the light mi-193 croscope during the early phase of the isolation process, Pandoraviruses are easy to iden-194 tify by electron microscopy thanks to their typical ovoid particles (1 μ m in length and 0.5 195 µm in diameter) enclosed in a 70-nm thick electron-dense tegument, interrupted by an 196 ostiole-like apex (Figure 1.A). The replication cycle of Pandoraviruses in A. castellanii cells 197 lasts from 10 to 15 hours and is initiated by the internalization of individual particles via 198 phagocytic vacuoles. Eight to 10 hours after infection, the Acanthamoeba cells become 199 rounded, lose their adherence, and new particles appear at the periphery of the region 200 formerly occupied by the nucleus. The replicative cycle ends when the cells lyse releasing 201 about a hundred particles each. 202

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 205 gap-free sites in the multiple alignment of 17 RNA polymerases (RPB1) protein sequences using the 206 MAFFT online facility from the Osaka University server [53]. The inclusion of the permafrost iso-207 lates confirms the ancient branching of the Pandoraviridae in two separate clades. Accession num-208 bers 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" 211 [54, 55], a new clade (a new genus or a new subfamily) within the Pithoviridae family [56]. 212 One was cultivated from the same Lena river water sample previously cited (Sample #2, 213 Table 1), one from surface cryosol in Kamchatka collected during the summer (Sample #1, 214 Table 1), and one from mud flowing into the Kolyma river at Duvanny yar, resulting from 215 the thawing of permafrost layers of mixed ages (Sample #4, Table 1). One additional mem-216 ber of the Pithoviridae was isolated from a 27.000-y old permafrost sample containing a 217 large amount of mammoth wool (Sample #7, Table 1). It is worth recalling that the proto-218 type of this family was previously isolated from an ancient permafrost layer of more than 219 30,000-y BP [56]. Other members of this family are the most abundant in a recent meta-220 genomic study of various Siberian permafrost samples focusing on eukaryotic viruses 221 [52]. 222

Even though cedratviruses and pithoviruses produce large ovoid particles like those 223 of Pandoraviruses, they are easyly distinguishable thanks to several morphological fea-224 tures. Their particles are usually more elongated (up to 2µm), have a much thinner wall, 225

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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). 227

Cedratvirus/Pithoviruses are very different from pandoraviruses, with much smaller 228 circular DNA genomes of only 460 to 600 kbp, although a non-isolated member appear to 229 reach 1.6 Mb [52]. After entering Acanthamoeba cells by phagocytosis, the particles are 230 stripped from their corks, allowing the viral genome to be delivered through the apical 231 opening into the cytoplasm after membrane fusion. This is then followed by an eclipse 232 phase and the formation of a cytoplasmic electron-lucent viral factory, where a complex 233 morphogenesis occurs. After ~12 h of infection, mature viral particles are released by cell 234 lysis. In contrast with Pandoravirus infections, the cell nucleus maintains its shape 235 throughout the entire Pithovirus replication cycle, a trademark of fully cytoplasmic vi-236 ruses carrying a functional transcription apparatus in their particles [57]. 237

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

Virus	Accession #	Base pairs	Amplicon identity	
<i>Cedratvirus A11</i> (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 240rescued from ancient permafrost. It was isolated from the highly productive sample 241 (dated > 27.000-y BP) exhibiting fossil mammoth wool (sample #7, Table 1) together with 242 two other viruses: Pithovirus mammoth and Pandoravirus mammoth. The prototype of 243 the Mimiviridae family, Mimivirus, was isolated in 2003 [58]. Its landmark discovery re-244 vealed the existence of giant viruses with particles visible by light microscopy and ge-245 nomic size (>1 Mb) exceeding that of many parasitic bacteria [59]. Initially thought to be 246 exclusively associated to amoebal hosts, the Mimiviridae family has diversified enor-247 mously, and now includes many divergent members infecting a variety of microalgae [60-248 62] and heterotrophic protozoans [63, 64]. Several large-scale metagenomic studies have 249 suggested that the Mimiviridae family (now comprising several subgroups) constitutes 250the largest part of the eukaryotic virome in both freshwater [65] and marine aquatic envi-251 ronments [66], with hundreds of reported Metagenomic Assembled Genomes (MAG) [67]. 252 A recent metagenomic study revealed the presence of Mimiviridae-related DNA in sev-253 eral ancient permafrost layers [52]. The Mimiviridae particles exhibit the icosahedral 254 shape encountered in many smaller viruses, except that their diameter is around 0.5 µm, 255 to which an external layer of dense fibrils (up to 125 nm thick) is added, making them 256 easily visible by light microscopy. In electron microscopy, the internal nucleoid appears 257 as a dark electron-dense sphere, surrounded by a lipidic membrane. Strikingly, one ver-258 tice exhibits a well-defined starfish-like structure, called the "stargate", the opening of 259 which delivers the nucleoid inside the cytoplasm of the infected cells after membrane fu-260 sion [68]. These features (icosahedral symmetry, large size, fibrils, and stargate) are unique 261 to Mimiviridae members, making their identification straightforward (Figure 1.E). The 262 replication cycle of the first megavirus genus member was fully described previously [69]. 263 As other giant viruses infecting Acanthamoeba spp., their particles penetrate by phagocy-264 tosis. Six hours p.i. infected cells start rounding and losing adherence. New particles are 265 then produced in very large cytoplasmic viral factories, leaving the cell nucleus intact. 266

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They are then released in large quantities (burst size ≈ 500) through cell lysis after 12-17 267 hours (depending on the virus and host strain)[69]. Those parameters can be strongly af-268 fected by the presence of the ubiquitous virophages, a smal satellite virus, parasitising the 269 various Mimiviridae family members [70, 71]. 270

Virus Accession # Amplicon identity **Base pairs** 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) NC_020104 Moumouvirus 1,021,348 83% (1248/1497) Moumouvirus australiensis MG807320 1,098,002 82% (1244/1497) NC_014649 1,181,549 77% (1158/1497) Mimivirus

Table 5. Identification of Megavirus mammoth among previously isolated Megavirinae members. 271

The PCR results indicate that Megavirus mammoth is a member of the clade C 273 Megamimivirinae (i.e. the Megavirus genus) (Table 5). In addition, it encodes all the trade-274 mark proteins of the genus: the MutS-like DNA mismatch DNA repair enzyme (ORF 570, 275 99% identical residues), the glutamine-dependent asparagine synthetase (ORF 434, 98% 276 identical residues), and the 5 amino-acyl tRNA ligase: Ileu AARS (ORF 383, 99% ID), Asp 277 AARS (ORF 771, 100% ID), Met AARS (ORF 798, 99% ID), Arg AARS (ORF 834, 99% ID), 278 Cys AARS (ORF 837, 98% ID), Trp AARS (ORF 876, 96% ID), and Tyr AARS (ORF 944, 279 97% ID). 280

3.4. Pacmanvirus lupus

Pacmanviruses are recently discovered Acanthamoeba-infecting viruses distantly re-282 lated to the African swine fever virus, until then the only known members of the Asfarvir-283 idae family that infects pigs [72]. We now report the isolation of a third member of this 284 newly defined group from the frozen intestinal remains of a Siberian wolf (*Canis lupus*) 285 preserved in a permafrost layer dated >27,000-y BP. At variance with the other truly giant 286 viruses (i.e. associated with unusually large particles), their icosahedral particles are about 287 220 nm in diameter (Figure 1.F), hence not individually discernable under the light micro-288 scope (Nomarski optic). However, DAPI (4', 6-diamidino-2-phenylindole) fluorescence 289 staining can be used to reveal cytoplasmic viral DNA replication in Acanthamoeba cultures 290 suspected to undergo viral infection. In culture of A. castellanii, Pacmanvirus lupus repli-291 cated slowly, producing a low yield of particles, without causing massive cell lysis. 292

Pacmanvirus lupus genome consists of a double-stranded DNA linear molecule of 293 407,705 bp, comparable in size to that of the previously studied members of this group 294 (Table 6). However, out of its 506 predicted protein-coding genes, only 241 (47.6%) exhibit 295 homologs in the two previously sequenced pacmanvirus genomes, and 221 (43.7%) are 296 ORFans. Thus, if *P. lupus* appears definitely closer to pacmanviruses than to any other 297 known viruses, its evolutionary distance is larger than usually observed within a subfam-298 ily or a genus. This large global discrepancy in gene content, is reflected in the low level 299 of similarity observed between various core genes of P. lupus and their homologs in other 300 pacmanviruses and closest relatives (Table 7). The asfarviruses appear even more distant 301 with half the number of genes and half the genome size, calling into question the initial 302 classification of pacmanviruses as truly related to family Asfarviridae (Table 7) [72]. Yet, in 303 a phylogenetic tree built on these sequences, P. lupus appears unambiguously clustered 304 with the other Pacmanviruses, justifying its given name (Figure 3). 305

Table 6. Identification of Pacmanvirus lupus (strain Tums2) among previously isolated pac-306 manviruses. 307

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	Virus	Accession #	Base pairs	Amplicon identity
	Pacmanvirus A23	NC_034383	395,405	100% (470/470)
	Pacmanvirus S19	MZ440852	418,588	93% (439/470)
			407 705	<85% (399/468)
Pacman	Pacmanvirus lupus (strain Tums2)	-	407,705	+ 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	79%	80%	49%	42%	41%
(RPB1) ORF 302	(1124/1415)	(1130/1415)	(707/1434)	(598/1429)	(596/1457)
RNA polymerase	85%	85%	55%	44%	43%
(RPB2) ORF 33	(1093/1289)	(1104/1301)	(681/1241)	(528/1211)	(526/1228)
DNA polymerase	65%	65%	37%	27.5%	33%
(PolB) ORF 265	(1036/1591)	(1032/1591)	(513/1385)	(344/1250)	(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 based310on 1300 gap-free sites. Although P. lupus is well clustered with other pacmanviruses, this clade (to-
gether 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 Uni-
versity server [53]. Accession numbers are indicated following the isolate name when available.310

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Following initial reports published more than 5 years ago [38, 39], this study confirms 318 the capacity of large DNA viruses infecting Acanthamoeba to remain infectious after more 319 than 48,500 years spent in deep permafrost. Our results extend our previous findings to 3 320 additional virus families or groups: 4 new members of the Pandoraviridae, one member of 321 the Mimiviridae, and one pacmanvirus (Table 1). One additional pithovirus was also re-322 vived from a particularly productive sample dated 27,000-y BP (sample#7, Table 1) exhib-323 iting mammoth wool. Given these viruses' diversity both in their particle structure and 324 replication mode, one can reasonably infer that many other eukaryotic viruses infecting a 325 variety of hosts much beyond Acanthamoeba spp. may also remain infectious in similar 326 conditions. Genomic traces of such viruses are detected in a recent large-scale meta-327 genomic study of ancient permafrost [52] as well as in Arctic lake sediments [73]. They 328 include well documented human and vertebrate pathogens such as poxviruses, herpesvi-329 ruses, and asfarviruses, although in lower proportions than protozoan infecting viruses. 330

In our recent metagenomic study [52], Pandoraviruses are notably absent while they 331 constitute the large majority of the viruses revived from permafrost and cryosols. Such a 332 discrepancy might originate from the fact that the extraction of genomic DNA from their 333 sturdy particles requires a much harsher treatment than for most other viruses. Their 334 abundance in environmental viromes might thus be much larger than the small fraction 335 they contribute to the DNA pool. Such DNA extraction bias may apply to many other 336 microbes, and is a serious limitation to the validity of metagenomic approaches for quan-337 titative population studies. 338

The types of viruses revived in our study are indeed the results of even stronger bi-339 ases. First, the only viruses we can expect to detect are those infecting species of Acan-340 thamoeba. Second, because we rely on "sick" amoeba to point out potentially virus-repli-341 cating cultures, we strongly limit ourselves to the detection of lytic viruses. Third, we fa-342 vor the identification of "giant" viruses, given the important role given to light micros-343 copy in the early detection of viral replication. It is thus likely that many small, non-lytic 344 viruses do escape our scrutiny, as well as those infecting many other protozoa that can 345 survive in ancient permafrost [10]. 346

However, we believe that the use of *Acanthamoeba* cells as a virus bait is nevertheless 347 a good choice for several reasons. First, Acanthamoeba spp. are free-living amoebae that are 348 ubiquitous in natural environments, such as soils and fresh, brackish, and marine waters, 349 but are also in dust particles, pools, water taps, sink drains, flowerpots, aquariums, sew-350 age, as well as medical settings hydrotherapy baths, dental irrigation equipment, humid-351 ifiers, cooling systems, ventilators, and intensive care units [74]. The detection of their 352 virus may thus provide a useful test for the presence of any other live viruses in a given 353 setting. Second, if many Acanthamoeba species can be conveniently propagated in axenic 354 culture conditions, they remain "self-cleaning" thanks to phagocytosis, and are capable of 355 tolerating heavy contamination by bacteria (that they eat) as well as high doses of antibi-356 otics and antifungals. The third, but not the least, advantage is that of biological security. 357 When we use Acanthamoeba spp. cultures to investigate the presence of infectious unknown 358 viruses in prehistorical permafrost (in particular from paleontological sites, such as RHS 359 [43, 44]), we are using its billion years of evolutionary distance with human and other 360 mammals as the best possible protection against an accidental infection of laboratory 361 workers or the spread of a dreadful virus once infecting Pleistocene mammals to their 362 contemporary relatives. The biohazard associated with reviving prehistorical amoeba-in-363 fecting viruses is thus totally negligible, compared to the search for "paleoviruses" di-364 rectly from permafrost-preserved remains of mammoths, woolly rhinoceros, or prehis-365 toric horses, as it is now pursued in the Vector laboratory (Novosibirsk, Russia) [75], for-366 tunately a BSL4 facility. Without the need of embarking on such a risky project, we believe 367 our results with Acanthamoeba-infecting viruses can be extrapolated to many other DNA 368 viruses capable of infecting humans or animals. It is thus likely that ancient permafrost 369 (eventually much older than 50,000 years, our limit solely dictated by the validity range 370 of radiocarbon dating) will release these unknown viruses upon thawing. How long these 371

viruses could remain infectious once exposed to outdoor conditions (UV light, oxygen, 372 heat), and how likely they will be to encounter and infect a suitable host in the interval, is 373 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 375 the Arctic in the wake of industrial ventures. 376

Author Contributions: Conceptualization and funding, C.A. and J.-M.C..; resources (samples),377A.E.G., G.G, J.S., A.N.T.; virus isolation and characterization: J.-M.A., A.L.; bioinformatic analysis,378O.P., M.L., S.S., J.-M.C.; writing, J.-M.C..; All authors have read and agreed to the published version379of the manuscript.380

Funding: This work was supported by the Agence Nationale de la Recherche grant (ANR-10-INBS-
09-08) to J-MC and the CNRS Projet de Recherche Conjoint (PRC) grant (PRC1484-2018) to CA, as
well as recurrent CNRS funding to the IGS laboratory. GG and JS were funded by a European Re-
search Council starting grant (PETA-CARB, #338335) and the Helmholtz Association of German
Research Centres (HGF) Impulse and Networking Fund (ERC-0013).381

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

Acknowledgments: We thank our volunteer collaborator, Alexander Morawitz, for collecting the388Kamchatka soil samples and Dr. Lyubov Shmakova (Soil Cryology Lab, Pushchino, Russia) for her389generous gift of Siberian permafrost samples from which, unfortunately, no viruses were rescued390in the present study. We acknowledge the computing support of the PACA BioInfo platform. We391thank M. Ulrich (DFG project #UL426/1-1) and P. Konstantinov for helping with fieldwork at the392Yukechi site, as well as the Alfred Wegener Institute and Melnikov Permafrost Institute logistics for393field support and sample acquisition.394

Conflicts of Interest: The authors declare no conflict of interest.

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