Genomic, proteomic, and phylogenetic analysis of spounaviruses indicates paraphyly of the order *Caudovirales*

Short title: Taxonomy of SPO1-like phages

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This paper is dedicated to Hans-Wolfgang Ackermann, a pioneer of prokaryotic virus electron microscopy and taxonomy, who died on February 12th, 2017, at the age of 80. He was involved in the early stages of this study, and his input is dearly missed.

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Abstract

Since the mid-20th century, prokaryotic double-stranded DNA viruses producing tailed particles (“tailed phages”) were grouped according to virion tail morphology. In the early 1980s, these viruses were classified into the families *Myoviridae*, *Siphoviridae*, and *Podoviridae*, later included in the order *Caudovirales*. However, recent massive sequencing of prokaryotic virus genomes revealed that caudovirads are extremely diverse. The official taxonomic framework does not adequately reflect caudovirad evolutionary relationships. Here, we reevaluate the classification of caudovirads using a particularly challenging group of viruses with large dsDNA genomes: SPO1-like viruses associated with the myovirid subfamily *Spounavirinae*. Our extensive genomic, proteomic, and phylogenetic analyses reveal that some of the currently established caudovirad taxa, especially at the family and subfamily rank, can no longer be supported. Spounavirins alone need to be elevated to family rank and divided into at least five major clades, a first step in an impending massive reorganization of caudovirad taxonomy.

53 ## Introduction

54 Prokaryotic virus taxonomy is the formal responsibility of the Bacterial and Archaeal Viruses
55 Subcommittee of the International Committee on Taxonomy of Viruses (ICTV). In recent
56 years, the Subcommittee has focused on classifying newly described double-stranded DNA
57 viruses producing tailed particles ("tailed phages") into species and genera included in
58 existing families in the order *Caudovirales* [1–5]. At the species rank, a similarity threshold of
59 95% nucleotide sequence identity is used for classification, i.e. viruses are classified into the
60 same species if they shared ≥95% identity over the entire length of their genomes.
61 Examination of wild populations of caudovirads infecting cyanobacteria demonstrated that
62 such a 95% threshold robustly captures formally delineable species using population genetic
63 metrics [6]. Extrapolation of such thresholds to viruses of the global surface oceans has
64 resulted in population-scale ecological understanding of thousands of new virus "species"
65 [7], which, however, do not have official status. At the genus rank, cohesive groups have
66 been defined for viruses sharing a significant genome similarity(≥60% nucleotide identity),
67 gene synteny, and a core gene set. This framework has helped to relatively rapidly establish
68 official low-rank taxonomic positions for newly isolated and sequenced viruses [1,2,5,8,9].
69 The currently available ranks in virus taxonomy (in ascending order *species*, *genus*,
70 *subfamily*, *family*, and *order*) limit the description of the full diversity of prokaryotic viruses.
71 This limitation is particularly acute in the case of the order *Caudovirales*, which represents
72 the most abundant and diverse group of viruses in the environment [10–12]. Indeed, the
73 diversity of caudovirads greatly surpasses that of other bacterial, archaeal and eukaryotic
74 double-stranded (ds)DNA viruses. A recent analysis of the dsDNA virosphere using a
75 bipartite network approach, whereby viral genomes are connected via shared gene families,
76 demonstrated that the global network of dsDNA viruses consists of at least 19 modules, 11

77 of which correspond to caudovirads [13]. The eight remaining modules encompass one or
78 more families of eukaryotic or archaeal viruses. Consequently, each of the caudovirad
79 modules could be considered to represent a separate family. Despite this remarkable
80 sequence diversity, all caudovirads are currently classified into three families—*Myoviridae,*
81 *Podoviridae*, and *Siphoviridae*. These families were historically established on the
82 morphological, not genomic, features of their members, forming an artificial classification
83 ceiling. In this study, the Subcommittee explored the diversity of the order *Caudovirales*
84 based on the example of a large group of Bacillus phage SPO1-related viruses (*Myoviridae*:
85 *Spounavirinae*), which forms a discrete caudovirad module [13,14].
86 The type virus of this group, Bacillus phage SPO1, was isolated in 1964 from soil in
87 Osaka, Japan, using *Bacillus subtilis* as a host [15]. Bacillus phage SPO1 has been extensively
88 studied ever since. Morphologically, the particle possesses an icosahedral head 87 nm in
89 diameter and a 140-nm long (18.6-nm wide) contractile tail of "stacked-disk" appearance
90 that is terminated by a complex baseplate structure [16,17]. The packaged genome is a
91 145.7-kb long, terminally redundant (13.1-kb) DNA molecule with thymine completely
92 replaced by 5-hydroxymethyluracil (HMU) [18–20]. This genome encodes at least 204
93 proteins and five tRNAs [21].
94 In 1995, Bacillus phage SPO1 was assigned to the species *Bacillus phage SP8*
95 (renamed *Bacillus phage SPO1* in 1996 and *Bacillus virus SPO1* in 2015), which in 1996 was
96 included into a monospecific myovirid genus currently called *Spo1virus* [1,22,23]. The
97 subfamily "*Spounavirinae*" was proposed in 2009 by Lavigne et al. to harbor Bacillus phage
98 SPO1, Staphylococcus phage Twort, Staphylococcus phage K, Staphylococcus phage G1,
99 Listeria phage P100, and Listeria phage A511 [4]. This subfamily became official in 2012 [24].
100 The unifying characteristics of members of this subfamily as described by Klumpp et al. are

101 that “(a) the[ir] host organisms are bacteria of the phylum Firmicutes; (b) [they] are strictly
102 virulent myovirids; (c) all… feature common morphological properties; (d) [their genomes]
103 consist of a terminally redundant, non-permuted dsDNA molecule of 127–157 kb in size; and
104 (e) [they] share considerable amino acid homology” [20]. The inclusion requirement of a
105 strictly lytic lifestyle became controversial when it was observed that a few related viruses
106 (Bacillus phages Bcp1, Bp8p-T, and Bp8p-C) can persist in host cultures without causing the
107 immediate lysis [25,26]. It remains unknown whether this persistence is due to virion
108 entrapment inside bacillus spores or some other kind of semi-stable virus-host relationship.
109 By 2015, the subfamily *Spounavirinae* had been expanded to include five genera
110 (*Kayvirus*, *P100virus*, *Silviavirus*, *Spo1virus*, and *Twortvirus*) and three unassigned or
111 “floating” species (*Enterococcus virus phiEC24C*, *Lactobacillus virus Lb338-1*, and
112 *Lactobacillus virus LP65*). It was already clear that *Bacillus virus SPO1* represented one of
113 two major lineages within the subfamily. Consequently, spounavirins were divided in the
114 Bacillus phage SPO1-like viruses, which have modified DNA (HMU) and which produce
115 particles possessing generally shorter tails; and, the Staphylococcus phage Twort-like
116 viruses, which feature non-modified DNA and produce particles with longer tails. Both
117 Bacillus phage SPO1-like and Staphylococcus phage Twort-like virus particles feature double-
118 ringed baseplates and visible capsomeres as a morphologic hallmark [20,27]. A third group,
119 represented by Bacillus phages sharing limited similarity with Bacillus phage SPO1 but
120 related to phage Bastille had already been proposed at that time but not yet officially
121 recognized [28]. In 2016, additional genera of Bacillus phage SPO1-like viruses (henceforth
122 “spouna-like viruses”) were established, but not all of these were immediately included in
123 the subfamily *Spounavirinae* [1,2].

124 In this study, we reevaluated the classification of spounavirins and spouna-like
125 viruses. To this end, a well-defined set of 93 viruses was analyzed using complementary DNA
126 and protein sequence analysis tools and phylogenetic methods. Our results indicate that the
127 subfamily *Spounavirinae* fails to adequately reflect the diversity of its current members, and
128 we therefore outline a better fitting classification scheme.
129

130 ## Results

131 **General overview**

132 To determine the phylogenetic relationship between 93 known and alleged spounavirins,
133 we employed genomic, proteomic and marker gene-based comparative strategies.
134 Regardless of the adopted phylogenetic approach applied, five separate, clear-cut clusters
135 were identified. We believe that they clearly have a common origin and ought to come
136 together under one caudoviral umbrella taxon. We propose to name this taxon
137 "*Herelleviridae,*" in honor of the 100th anniversary of the discovery of prokaryotic viruses by
138 Félix d'Hérelle (Table 1, Figs 1-3, S1 Table). The first cluster (here suggested to retain the
139 name *Spounavirinae*) groups *Bacillus*-infecting viruses that are similar to Bacillus phage
140 SPO1. The second cluster ("*Bastillevirinae,*" named after the type species *Bacillus virus*
141 *Bastille* [28]) includes *Bacillus*-infecting viruses that have only limited similarity to Bacillus
142 phage SPO1 and resemble Bacillus phage Bastille instead. The third cluster ("*Brockvirinae*,"
143 named in honor of Thomas D. Brock [1926–], an American microbiologist and educator
144 known for his discovery of hyperthermophiles, who worked on Streptococcus phages early
145 in his career) comprises currently unclassified viruses of enterococci that are similar to
146 Enterococcus phage ϕEF24C. The fourth cluster ("*Twortvirinae*," named in honor of
147 Frederick William Twort (1877–1950), the English bacteriologist who discovered prokaryotic

viruses in 1915) gathers staphylococci-infected viruses that are similar to Staphylococcus phage Twort, whereas the remaining cluster ("*Jasinskavirinae*," named in honor of Stanislawa Jasińska-Lewandowska (1921–1998), Polish scientist who was one of the first to study *Listeria* and their viruses) consists of viruses infecting *Listeria* that are similar to Listeria phage P100, the type isolate of the *P100virus* genus. The classification in five clusters left three viruses unassigned at this rank: Lactobacillus phage Lb338, Lactobacillus phage LP65, and Brochothrix phage A9.

These robust clusters can be further subdivided into smaller clades that correspond well with the currently accepted genera. The evidence supporting this suggested taxonomic re-classification is presented in the following sections.

158 **Table 1. Suggested new classification of the 93 spounavirins and spouna-like viruses in the new caudoviral family "*Herelleviridae*".**

Order	Family	Subfamily	Genus	Species[a]	Viruses
Caudovirales	*"Herelleviridae"*	*"Bastillevirinae"*	*Agatevirus*	*Bacillus virus Agate, Bacillus virus Bobb, Bacillus virus Bp8pC*	Bp8p-T
			B4virus	*Bacillus virus AvesoBmore, Bacillus virus B4, Bacillus virus Bigbertha, Bacillus virus Riley, Bacillus virus Spock, Bacillus virus Troll*	B5S
			Bastillevirus	*Bacillus virus Bastille, Bacillus virus CAM003, "Bacillus virus Evoli", "Bacillus virus HoodyT"*	
			Bc431virus	*Bacillus virus Bc431, Bacillus virus Bcp1, Bacillus virus BCP82, Bacillus virus JBP901*	
			Nit1virus	*Bacillus virus Grass, Bacillus virus NIT1, Bacillus virus SPG24*	
			Tsarbombavirus	*Bacillus virus BCP78, Bacillus virus*	BCU4

				TsarBomba	
			Wphvirus	*Bacillus virus BPS13*, *Bacillus virus Hakuna, Bacillus virus Megatron, Bacillus virus WPh, "Bacillus virus BPS10C"*	Eyuki
		"*Brockvirinae***"**	"*Kochikohdavirus*"	"*Enterococcus virus ECP3*", "*Enterococcus virus EF24C*", "*Enterococcus virus EFLK1*"	phiEFC24C-P2
			Unassigned	"*Enterococccus virus EFDG1*"	
		"*Jasinskavirinae***"**	*P100virus*	*Listeria virus A511*, *Listeria virus P100*	List-36, LMSP-25, AvB_LmoM_AG20, LP-125, LP-064, LP-083-2, LP-124, LP-125, LP-048, LMTA-34, LMTA-94, LMTA-148, LMTA-57, WIL-1
		Spounavirinae	*Cp51virus*	*Bacillus virus CP51, Bacillus virus JL, Bacillus virus Shanette*	
			Spo1virus	*Bacillus virus Camphawk*, *Bacillus*	

				virus SPO1	
			Unassigned	*"Bacillus virus Mater", "Bacillus virus Moonbeam", "Bacillus virus SIOphi"*	
		"Twortvirinae"	*Kayvirus*	*Staphylococcus virus G1, Staphylococcus virus G15, Staphylococcus virus JD7, Staphylococcus virus K, Staphylococcus virus MCE2014, Staphylococcus virus P108, Staphylococcus virus Rodi, Staphylococcus virus S253, Staphylococcus virus S25-4, Staphylococcus virus SA12, "Staphylococcus virus Sb1"*	676Z, A3R, A5W, Fi200W, IME-SA1, IME-SA118, IME-SA119, IME-SA2, ISP, MSA6, P4W, SA5, Staph1N, Team1
			Silviavirus	*Staphylococcus virus Remus, Staphylococcus virus SA11*	Romulus
			Sep1virus	*Staphylococcus virus IPLAC1C,*	

				Staphylococcus virus SEP1	
			Twortvirus	*Staphylococcus virus Twort*	
		Unassigned	Unassigned	*"Lactobacillus virus Lb338",* *"Lactobacillus virus LP65",* *"Brochothrix virus A9"*	

159 [a] The species listed here are representing the 93 genome dataset on which all analyses have been performed. Species ratified in 2017 and later
160 have not been included here.

161 **Genome-based analyses**

162 BLASTn analysis revealed that the genomes of several viruses were similar enough to
163 consider them strains of the same species (they shared >95% nucleotide identity, S1 Fig).
164 The *Staphylococcus* viruses fell into four distinct, yet closely related groups corresponding to
165 the established genera *Twortvirus, Sep1virus, Silviavirus*, and *Kayvirus* (S1 Fig). With the
166 exception of Enterococcus phage EFDG1, all *Enterococcus* viruses clustered as a clade
167 representing a new genus (here suggested to be named "*Kochikohdavirus*" after the place of
168 origin of the type virus of the clade, Enterococcus phage ϕEF24C; [29,30]). The *Bacillus*
169 viruses clustered into the established genera *Spo1virus*, *Cp51virus*, *Bastillevirus*, *Agatevirus*,
170 *B4virus*, *Bc431virus*, *Nit1virus*, *Tsarbombavirus*, and *Wphvirus*, with three species remaining
171 unassigned at the genus rank (Table 1). These results were also confirmed with VICTOR, a
172 genome-BLAST distance phylogeny (GBDP) method (S2 Fig) and the Dice score (S3 Fig) [31],
173 a tBLASTx-based measure that compares whole genome sequences at the amino acid level.

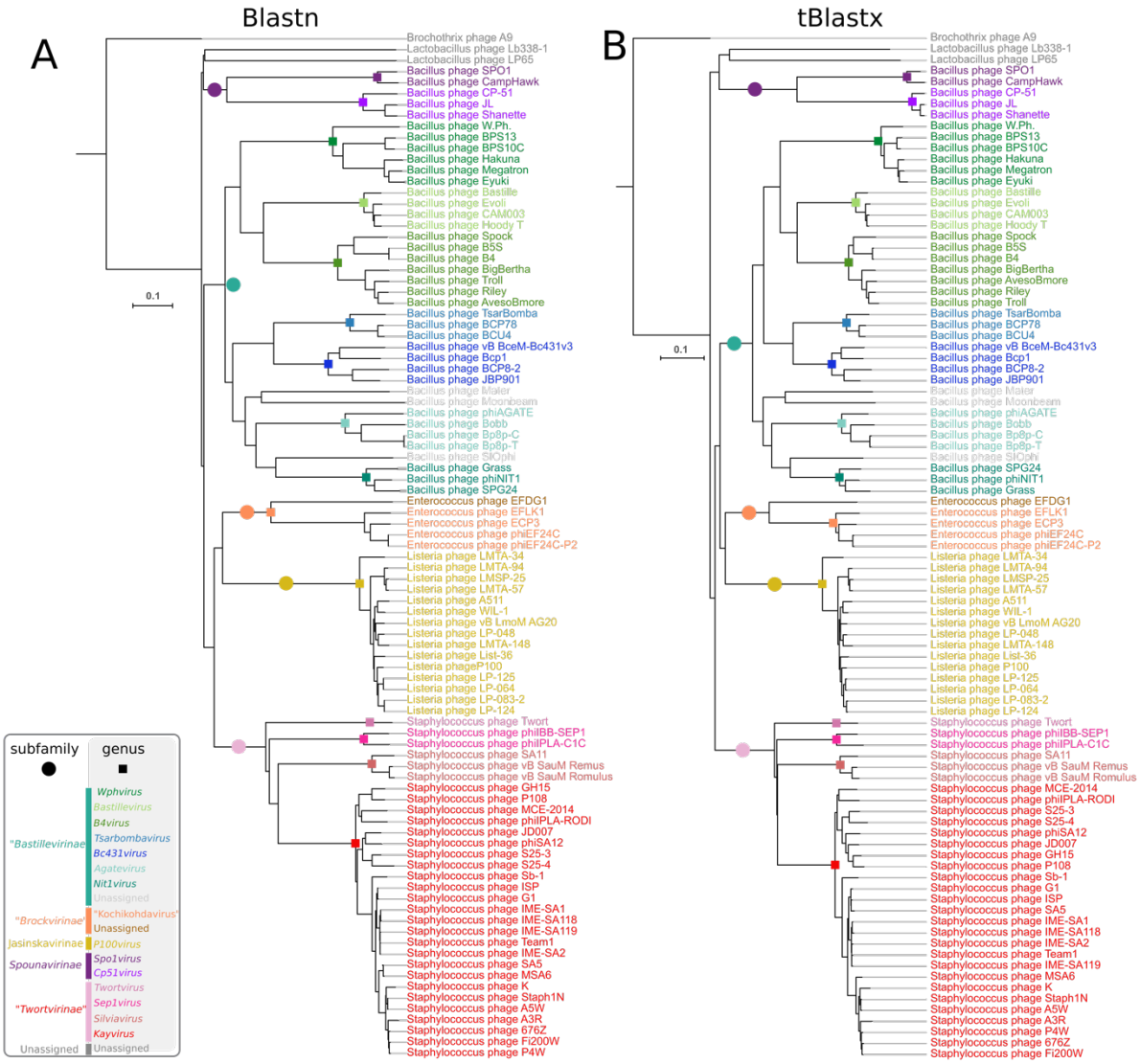


Fig 1: Genome-based clustering trees of 93 spounavirin and spouna-like viruses. Clustering was performed using nucleotide similarities (BLASTn, A) or translated nucleotide similarities (tBLASTx, B). Genomes were compared in a pairwise fashion using Gegenees, transformed into a distance matrix, clustered using R and visualized as trees using Itol. The trees were rooted at Brochotrix phage A9. Genera are delineated with colored squares and suggested subfamilies with colored circles.

The patterns coalesced at a higher taxonomic level when the genomes were analyzed using tBLASTx (S4 Fig). The *Enterococcus* viruses clustered into a single group sharing 41% genome identity, whereas the *Bacillus* viruses fell into two major groups, a group combining the genera *Spo1virus* and *Cp51virus*, and the remainder. All *Staphylococcus*

186 viruses clustered above ≈36% genome identity, whereas *Listeria* viruses grouped with more
187 than 79% genome identity. Overall, all these genomes were related at the level of at least
188 15% genome identity. *Lactobacillus* and *Brochothrix* viruses remained genomic orphans,
189 peripherally related to the remainder of the viruses in this assemblage.

190 **Predicted proteome-based analyses**

191 The virus proteomic tree shows four robust groupings mainly determined by the hosts that
192 the viruses infect, corresponding largely with the suggested subfamilies (Fig 2). Viruses that
193 infect *Bacillus* fell into two groups as described before, represented by the revised
194 *Spounavirinae* subfamily and the suggested new subfamily "*Bastillevirinae*." Similarly, the
195 *Listeria* and *Staphylococcus* viruses formed their own clusters, "*Jasinskavirinae*" and
196 "*Twortvirinae*", respectively. This clustering suggests that the major *Bacillus, Listeria,* and
197 *Staphylococcus* virus groups are represented, but that further representatives are required
198 from the under-sampled groups. The suggested "*Brockvirinae*" subfamily is under-sampled,
199 and the grouping observed in the tree is not as well-supported as the other clusters.
200

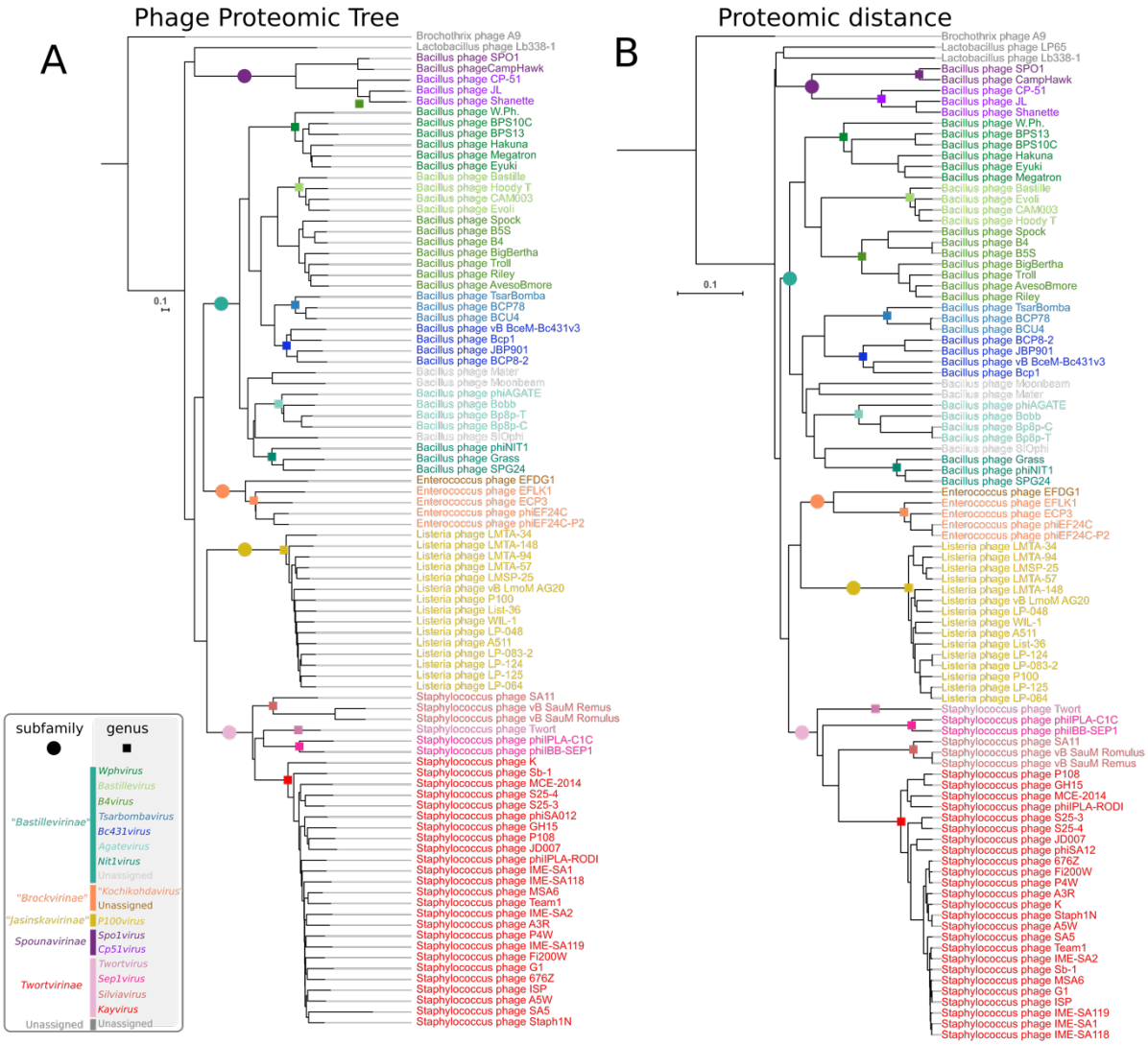


Fig 2: Predicted proteome-based clustering trees of 93 spounavirin and spouna-like viruses. Clustering was performed using the Phage Proteomics Tree approach (A) and proteomic distance (B). Distances were calculated pairwise between all sets of predicted proteomes, clustered with R and visualized using Itol. The trees were rooted at Brochotrix phage A9. Genera are delineated with colored squares and suggested subfamilies with colored circles.

Among 1,296 singleton proteins and 2,070 protein clusters defined using the orthologous protein clusters (OPC) approach, we identified 12 clusters common for all viruses (Table 2, S2 Table). Classification of the viral proteins using prokaryotic virus orthologous groups (pVOGs) showed that 38 pVOGs were shared between all 93 virus genomes (Table 2, S3 Table). This finding was in stark contrast with the results from core

214 genome analysis using Roary, which revealed only one core gene (the tail tube protein
215 gene). Upon closer inspection of the gene annotations, we found that these analyses might
216 have been confounded by the presence of introns and inteins in many of the core genes
217 (S5–S6 Figs). Indeed, many genes of spounavirins and related viruses are invaded by mobile
218 introns or inteins [32,33]. These gaps in coding sequences challenge gene prediction tools
219 and introduce additional bias in similarity-based cluster algorithms.
220
221 ***Table 2: Core genes with putative annotated functions identified in all 93 spounavirin and***
222 ***spouna-like virus genomes.***

Putative function of the core gene identified[a]	**pVOG[b] / OPC[b] ID**	**Identification method**
DnaB-like helicase	VOG0025, OPC6121	OPC, pVOG
Baseplate J-like protein	VOG4691, VOG4644, OPC6132	OPC, pVOG
Tail sheath protein	VOG0067, OPC6142	OPC, pVOG
Terminase large subunit	VOG0051, OPC6160	pVOG
Major capsid protein	VOG0061, OPC6148	OPC, pVOG
Prohead protease	VOG4568, OPC6150	pVOG
Portal protein	VOG4556, OPC6151	OPC, pVOG
DNA primase	VOG4551	pVOG
DNA polymerase I	VOG0668, OPC6097	OPC, pVOG
RNA polymerase	VOG0118	pVOG
Recombination exonuclease	VOG4575	pVOG

Recombination endonuclease	VOG0083	pVOG
Tail tape measure protein	VOG0069	pVOG
Tail tube protein	VOG0068, OPC6141	OPC, pVOG, Roary

223 [a] The full lists of orthologous proteins and pVOGs are available in S2 Table and S3 Table,
224 respectively.
225 [b] pVOG, prokaryotic virus orthologous group; OPC, orthologous protein clusters.
226
227 The pairwise comparison of the predicted proteome content of the viruses revealed
228 a very low overall relatedness at the protein level (S7 Fig S7). The majority of viruses shared
229 less than 10% of their proteins. However, at the suggested new subfamily rank, we observed
230 obvious virus groups sharing their proteomes. The *Enterococcus* viruses ("*Brockvirinae*")
231 shared over 35% of their protein content. The members of the *Bacillus* virus genera
232 *Spo1virus* and *Cp51virus* of the subfamily *Spounavirinae* (sensu stricto) had approximately
233 20% of their proteins in common, whereas the *Bacillus* virus genera *Bastillevirus*, *B4virus*,
234 *Bc431virus*, *Agatevirus*, *Nit1virus*, *Tsarbombavirus*, and *Wphvirus* ("*Bastillevirinae*") and the
235 *Staphylococcus* virus genera *Kayvirus*, *Silviavirus*, and *Twortvirus* ("*Twortvirinae*") shared
236 over 25% and over 30% of their predicted proteomes, respectively.
237 Genomic fluidity is a measure of the dissimilarity of genomes evaluated at the gene
238 level [34]. Accordingly, the genomic fluidity results followed those obtained using proteome
239 content analysis (S8 Fig). Despite a high genomic fluidity for most of these viruses, the newly
240 suggested subfamilies and genera were all supported.
241 The topology of the dendrogram obtained using the average amino acid identity
242 (AAI) approach also supported the suggested new taxonomic scheme (S8 Fig). The AAI was
243 greater than 35% within each subfamily and greater than 67% within each genus. The AAI of

244 all viruses analyzed in this study was not lower than 22%. The members of the genus
245 *Wphvirus* had the lowest AAI (76%) and the lowest AAI for a pair of proteomes (67%
246 between Bacillus phage W.Ph. and Bacillus phage Eyuki) but surprisingly they had a mid-
247 range genomic fluidity (0.15), suggesting that the protein sequences of wphviruses might
248 have evolved rapidly.

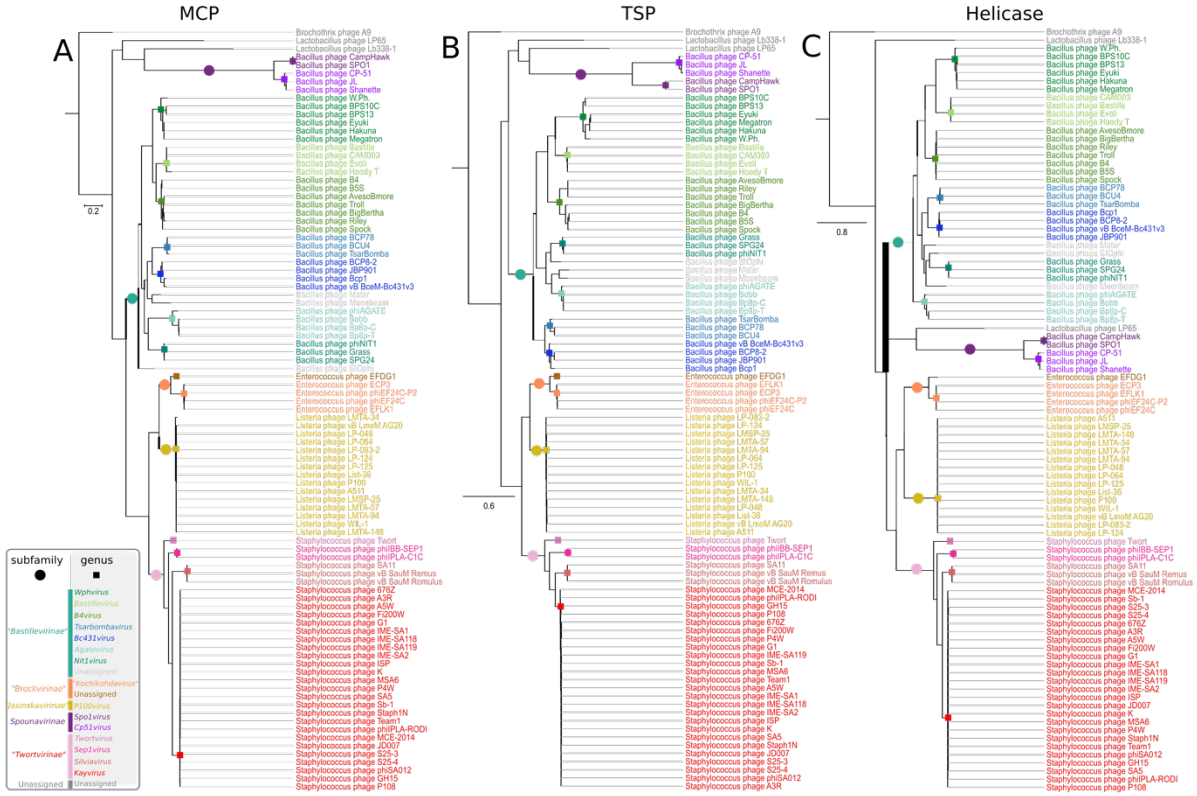
249 The pangenome of the spounavirins and spouna-like viruses as calculated using
250 Roary [35], consisting of 4,182 genes, was further analyzed by clustering the genomes based
251 on the presence or absence of the accessory genes (S9 Fig). The obtained tree supported the
252 current division of the viruses into approved genera and the suggested new subfamilies.

253 Many virus genomes are thought to be highly modular, with recombination and
254 horizontal gene transfer potentially resulting in "mosaic genomes" [36,37]. By clustering the
255 spounavirin and spouna-like virus genomes based solely on the gene order of their
256 genomes, we investigated whether the gene synteny was preserved (S10 Fig). The results
257 revealed that genomic rearrangements leave a measurable evolutionary signal in all
258 lineages, since the genomic architecture analysis clustered all viruses according the
259 suggested taxa with the potential exception of Bacillus phage Moonbeam [38]. However, we
260 did not observe the high modularity that may be expected with rampant mosaicism. The
261 lack of rampant mosaicism supports the recent findings by Bolduc et al. that at most about
262 10% of reference virus genomes have a high degree of mosaicism [14]. Thus, while the gene
263 order in viruses belonging to the newly suggested family "*Herelleviridae*" is not necessarily
264 strictly conserved, we observed a clear evolutionary pattern that is consistent with the
265 sequence-based approaches tested in this study.

266 **Single protein phylogenies**

267 The phylogenetic trees based on comparisons of the major capsid, tail sheath, and DnaB-like
268 helicase proteins are presented in Fig 3. All nine phylograms based on conserved proteins
269 (OPC) are depicted in the trees in S11 Fig. For nearly all single marker trees, the topologies
270 supported the suggested taxonomic scheme. Generally, each taxon is represented as a
271 separate branch on the dendrogram. Notable exceptions could be found in two trees based
272 on hypothetical proteins (OPCs 10357 and 10386). The first (10357) places revised the
273 subfamily *Spounavirinae* as a subclade of "*Bastillevirinae*" and the second (10386) shuffled
274 silviaviruses and kayviruses. This result may indicate that some degree of horizontal gene
275 transfer occurs between groups, which share common hosts.

276



278 **Fig 3: Single gene phylogenies of the major capsid protein (MCP, A), tail sheath protein**
279 **(TSP, B) and helicase (C) amino acid sequences of 93 spounavirin and spouna-like viruses.**
280 Amino acid sequences were aligned with Clustal Omega and trees were generated using

281 FastTree maximum likelihood with Shimidaira-Hasegawa tests. The scale bar represents the
282 number of substitutions per site. The trees were rooted at Brochotrix phage A9. Genera are
283 delineated with colored squares and suggested subfamilies with colored circles.
284

285

286 **Discussion**

287 Using the conventional definition of "tailed phage" families, *Myoviridae*, *Podoviridae*, and
288 *Siphoviridae* (order *Caudovirales*), researchers effectively classified caudovirads for decades
289 [39,40]. However, the classification of these viruses, defined by a traditional morphology-
290 based approach, has been contested with the advent of high-throughput sequencing. The
291 steadily increasing number of available genomes and debates on the impact of horizontal
292 gene transfer, which marked the late 1990s and early 2000s, resulted in a decade-long
293 moratorium on the introduction of any new taxonomy for prokaryotic viruses [41]. The
294 increasing discrepancy between the official taxonomic framework and the emerging high-
295 volume genomics and metagenomics research left ≈90% of prokaryotic viruses known from
296 genome sequences unclassified beyond the family rank, i.e. they were classified as orphan
297 species in a family. Consequently, prokaryotic virus diversity was vastly underappreciated,
298 and virus genome curation remained in disarray. Recently, rather than implementing a
299 'repeal and replace' strategy for prokaryotic virus taxonomy, the Committee has introduced
300 a holistic system, involving virus particle morphology, overall DNA and protein sequence
301 identities, and phylogeny—an approach used for classification of all other viruses [1,2].
302 Successful modern taxonomic approaches must scale up to accommodate the
303 increasing pace of prokaryotic virus discovery and be effective across the 4,470 putative
304 complete prokaryotic virus sequences currently deposited in GenBank and other databases
305 [42]. These scaling requirements have remained problematic, and although there are more

306 than 3,400 publicly available caudovirad genomes, only 873 have been officially classified by
307 the ICTV by now [43]. The remaining genomes are provisionally stashed within
308 "unclassified" bins attached to the order *Caudovirales* or its member families, either
309 because they still need to be classified in a species/genus/subfamily, or because they may
310 be unidentified isolates of already classified viruses.

311 The growth in the number of prokaryotic virus genome sequences now supports the
312 application of a range of genomic analyses for robust taxonomic classification [44,45].
313 Meanwhile, phylogenomic approaches are yielding to network-based approaches to better
314 reflect the evolution of viral genomes [9,14]. These network-based approaches help to
315 organize the viral sequence space into statistically-resolvable "viral clusters." These clusters
316 are approximately equivalent to ICTV-recognized genera and provide a taxonomic
317 description that better reflects evolutionary relationships. Given the high computational
318 demand of network-based approaches, however, the development of centralized resources
319 and authority-monitored cyberinfrastructure, such as the iVirus platform on Cyverse [46] or
320 the Joint Genome Institute Integrated Microbial Genomes Virus resource IMG/VR [47], will
321 have to assist the prokaryotic virus community with large dataset computation and
322 classification.

323 Based on the results of this study, we suggest that the group of spounavirin and
324 spouna-like viruses be removed from the family *Myoviridae* and be given a family rank.
325 Hence, we propose establishing the suggested family "*Herelleviridae*", in the order
326 *Caudovirales* next to a smaller *Myoviridae* and the established *Podoviridae* and *Siphoviridae*
327 families. The new family would contain five subfamilies: *Spounavirinae* (sensu stricto),
328 "*Bastillevirinae*", "*Twortvirinae*", "*Jasinkavirinae*", and "*Brockvirinae*", each comprising the
329 ICTV-established genera listed in Table 1 (with additional information in S1 Table).

330 This study represents the first example of a true taxonomic assessment from an
331 'ensemble of methods'. The *de facto* taxon splitting suggested here results from the
332 observed diversity of prokaryotic viruses. We are encouraged that the combination of
333 genome BLAST analyses, virus proteomic trees, core protein clusters, genomic synteny
334 (GOAT), and single gene phylogenies yields consistent and complementary results, showing
335 the robustness of the suggested taxa. In addition, the suggested genera correspond well
336 with the taxonomy of the hosts (*Bacillus, Listeria, Staphylococcus*, and *Enterococcus*)
337 indicating broader microbiological consistency. Moreover, only approximately 3% of viruses
338 are left as unassigned at the genus and subfamily rank at this time within this group. These
339 unassigned viruses may represent clades at the genus and subfamily rank that are still
340 under-sampled.
341 This work demonstrates the usefulness of genome-based classification at a higher
342 taxonomic rank and its ability to accommodate the complex viral diversity. Substitution of
343 the families *Podoviridae*, *Myoviridae*, and *Siphoviridae* with a set of new families which
344 more faithfully reflect the true genetic relationships of the viruses would clarify the
345 taxonomic situation. However, this change does not remove the historically established
346 virus morphotypes observed in the nature among caudovirads: myovirids forming particles
347 with contractile tails, siphovirids forming particles with long non-contractile tails, and
348 podovirids forming particles with short non-contractile ones. By disconnecting morphotype
349 and family classification of caudovirads, taxonomically related clades can be grouped across
350 morphotypes. This grouping includes the muviruses suggested to be classified in the family
351 "*Saltoviridae*" [48] and potentially the broad set of Escherichia phage lambda-related
352 viruses that are currently distributed among the families *Siphoviridae* and *Podoviridae* [49].

353 We believe that abolishment of the *Podoviridae*, *Myoviridae* and *Siphoviridae*

354 families will soon be followed by the “upgrade” of existing viral taxonomy with additional

355 taxon ranks required to accommodate the observed diversity in an orderly manner.

356

357 ## Materials and methods

358 **Creation of the dataset**

359 Genome sequences of known spounavirins and spouna-like viruses were retrieved from
360 GenBank or (preferably) RefSeq databases in based on literature data, ICTV and taxonomic
361 classifications provided by the National Center for Biotechnology Information (NCBI).
362 Records representing genomes of candidate spounavirins and spouna-like viruses were
363 retrieved by searching the same databases with the tBLASTx algorithm using terminase and
364 major capsid proteins of several type virus isolates as a query (i.e., Bacillus phage SPO1,
365 Staphylococcus phage Twort, Bacillus phage Bastille, Listeria phage A511, Enterococcus
366 phage ϕEF24C, and Lactobacillus phage LP65) [50,51]. Sequences were manually curated
367 and pre-clustered using CLANS (E-value cut-off 1e-10) to confirm their spounaviral affiliation
368 [52]. This search yielded a set of 93 complete virus genomes, which were used in the
369 following analyses (S1 Table).

370 The coding sequences in the genomes were re-annotated using PROKKA with the
371 settings --kingdom Viruses, --E-value 1e-6 [53]. All genome sequences are available from
372 NCBI (accession number information listed in S1 Table) or from Github
373 (github.com/evelienadri/herelleviridae).

374

375 **Genome-based analyses**

376 Gegenees [54] was used in BLASTn and tBLASTx modes (fragment length 200 bp; step length
377 100 bp) to analyze virus genome nucleotide similarities. Pairwise identities between all
378 genomes under study were determined using BLASTn and tBLASTx algorithms with default
379 parameters [55]. Symmetrical identity scores (% SI) were calculated for each pairwise
380 comparison using the formula
381
382 % SI = 2.0 x $\frac{HL\ x\ HI}{QL + SL}$ (1)
383
384 in which the HL is defined as the hit length of the BLAST hit, HI is defined as the percentage
385 hit identity, QL is defined as the query length, and SL is defined as the subject length.
386 Symmetrical identity scores were converted into distances using the formula
387 Distance = $\sqrt[2]{1.0\ -\ \%SI \div 100}$ (2)
388 The resulting distance matrix was hierarchically clustered (complete linkage) using the
389 hclust function of R [56]. Trees were visualized using Itol [57].
390 All pairwise comparisons of the nucleotide sequences using VICTOR, a Genome-
391 BLAST Distance Phylogeny (GBDP) method, were conducted under settings recommended
392 for prokaryotic viruses [58,59]. The resulting intergenomic distances (including 100
393 replicates each) were used to infer a balanced minimum evolution tree with branch support
394 via FASTME including subtree pruning and regrafting (SPR) post-processing [60] for each of
395 the formulas D0, D4, and D6, respectively. Trees were visualized with FigTree [61]. Taxon
396 demarcations at the species, genus and family rank were estimated with the OPTSIL
397 program [62], the recommended clustering thresholds [59], and an F value (fraction of links
398 required for cluster fusion) of 0.5 [58].
399

400 **Proteomic tree**

401 The Phage Proteomic Tree was constructed as described previously [63] and detailed at
402 https://github.com/linsalrob/PhageProteomicTree/tree/master/spounavirus. Briefly, the
403 protein sequences were extracted and clustered using BLASTp. These clusters were refined
404 by Smith-Waterman alignment using CLUSTALW version 2 [64]. Alignments were scored
405 using PROTDIST from the PHYLIP package [65]. Alignment scores were averaged and
406 weighted as described previously [63] resulting in the final tree.

407

408 **Core protein clusters**

409 Orthologous proteins were clustered using GET_HOMOLOGUES software, which utilizes
410 several independent clustering methods [66]. To capture as many evolutionary relationships
411 as possible, a greedy COGtriangles algorithm was applied with a 50% sequence identity
412 threshold, 50% coverage threshold, and an E-value cut-off equal to 1e-10 [67]. The results
413 were converted into an orthologue matrix with the "compare_clusters" script (part of the
414 GET_HOMOLOGUES suite) [65].

415 The orthologous protein clusters (OPCs) defined above were used to compute the
416 genomic fluidity for each pair of genomes. For two genomes i and j:

417 $$\text{Fluidity}(i,j) = \frac{Ui+Uj}{Mi+Mj} \quad (3)$$

418 with Ui being the number of genes of i not found in j and Mi being the number of genes in i
419 [34]. The resulting distance matrix was hierarchically clustered (complete linkage) using the
420 hclust function of R [56]. Trees were visualized using Itol [57].

421 Multiple alignments were generated for each OPC using Clustal Omega [68]. For each
422 cluster, the amino acid identity between all protein pairs inside a cluster were determined
423 using multiple alignment. For all genome pairs, the AAI [69] was then computed and
424 transformed into distance using the formula:

425 $$\text{Distance} = \frac{100-AAI}{100} \quad (4)$$

426 The resulting distance matrix was clustered and visualized as described above.

427 OPCs and multiple alignments for each cluster were used to determine a distance
428 similar to the distance used to generate the Phage Proteomic Tree. To estimate protein
429 distances, in this case, the dist function of the seqinR package [70]was preferred to
430 PROTDIST of the PHYLIP package [65] as the resulting distances are between 0 and 1.
431 Proteomic distances were then computed using the same formula as for the Phage
432 Proteomic Tree. The results were clustered and visualized as described above.

433 The Dice score is based on reciprocal BLAST searches between all pairs of genomes A
434 and B [31]. The total summed bitscoresof all tBLASTx hits with ≥30% identity, alignment
435 length ≥30 amino acids, and E-value ≤0.01 was converted to a distance DAB as follows:

436 $$\text{DAB} = 1-\frac{SAB+SBA}{SAA+SBB} \quad (5)$$

437 In which SAB SAB and SBA represent the summed bitscores between tBLASTx searches of A
438 versus B, and B versusu A, respectively, while SAA and SBB represent the summed tBLASTx
439 bitscores of the self-queries of A and B, respectively. The resulting distance matrix was
440 clustered with BionJ [71].
441

To investigate a genomic synteny-based classification signal, we developed a geneorder-based metric built on dynamic programming, the Gene Order Alignment Tool (GOAT, Schuller et al.: Python scripts are available on request, manuscript in preparation). GOAT first identified protein-coding genes in the 93 spounavirin and spouna-like virus genomes using Prodigal V2.6.3 in anonymous mode [72], and assigned them to the latest pVOGs [73]). pVOG alignments (9,518) were downloaded (http://dmk-brain.ecn.uiowa.edu/pVOGs/) and converted to profiles of hidden Markov models (HMM) using HMMbuild (HMMer 3.1b2, [74]). Proteins were assigned to pVOGs using HMMsearch (E-value <10-2) and used to generate a synteny profile of every genome. GOAT accounted for gene replacements and distant homology by using an all-vs-all similarity matrix between pVOG pairs based on HMM-HMM similarity (HH-suite 2.0.16) [75]). Distant HHsearch similarity scores between protein families were calculated as the average of reciprocal hits and used as substitution scores in the gene order alignment. The GOAT algorithm identified the optimal gene order alignment score between two virus genomes by implementing semi-global dynamic programming alignment based only on the order of pVOGs identified on every virus genome. To account for virus genomes being cut at arbitrary positions during sequence assembly, GOAT transmutes the gene order at all possible positions and in both sense and antisense directions in search of the optimal alignment score. The optimal GOAT alignment score GAB between every pair of virus genomes A and B, was converted to a distance DAB as follows:

$$\mathrm{DAB} = 1 - \frac{GAB + GBA}{GAA + GBB} \qquad (6)$$

463 in which GAB and GBA represent the optimal GOAT score between A and B, and B and A,
464 respectively, while GAA and GBB represent the GOAT scores of the self-alignments of A and
465 B, respectively. This pairwise distance matrix was clustered with BionJ [71].
466 Prokka re-annotated genomes were used to create pan-, core-, and accessory
467 genomes of all selected spounavirins and spouna-like viruses [53]. The annotations were
468 analyzed using Roary [35] with a 50% length BLASTp identity threshold for homologous
469 genes. Roary functions as follows: CD-HIT [76] was used to pre-cluster protein sequences
470 and perform an all-vs-all comparison of protein sequences with BLASTp to identify orthologs
471 and paralogs within the genomes. MCL [77] was then used to cluster the genomes based on
472 the presence and absence of the accessory genes. The resulting tree file was visualized using
473 FigTree v1.4.3 [61]. The tree was rooted in Brochothrix phage A9. The gene presence-
474 absence output table from Roary was then imported into R and using a custom R-script
475 (available from github.com/evelienadri/herelleviridae/tree/master). Pairwise shared gene
476 contents were calculated for each combination of genomes.
477

478 **Single gene phylogenies**

479 Based on the OPC and pVOG analyses, we chose nine well-annotated protein clusters
480 present in all 93 spounavirins and spouna-like viruses. Selected clusters included: DNA
481 helicases, major capsid proteins, tail sheath proteins, two different groups of baseplate
482 proteins, and four clusters with no known function. The members of these clusters were
483 aligned using Clustal Omega with default parameters [47]. Resulting alignments were
484 analyzed with ProtTest 3.4 [59] to determine a suitable protein evolution model (only
485 variations of models compatible with downstream software like JTT and WAG were
486 considered). Estimated models were used to generate phylograms with FastTree 2.1.7 [60].
487 The program implements the approximately maximum-likelihood method with Shimodaira-
488 Hasegawa tests to generate the tree and calculate support of the splits. This approach is
489 much faster than “traditional” maximum-likelihood methods with negligible accuracy loss
490 [59–61].
491

492 ## Figure legends

493 **Fig 1: Genome-based clustering trees of 93 spounavirin and spouna-like viruses.** Clustering
494 was performed using nucleotide similarities (BLASTn, A) or translated nucleotide similarities
495 (tBLASTx, B). Genomes were compared in a pairwise fashion using Gegenees, transformed
496 into a distance matrix, clustered using R and visualized as trees using Itol. The trees were
497 rooted at Brochotrix phage A9. Genera are delineated with colored squares and suggested
498 subfamilies with colored circles.

499

500 **Fig 2: Predicted proteome-based clustering trees of 93 spounavirin and spouna-like**
501 **viruses.** Clustering was performed using the Phage Proteomics Tree approach (A) and
502 proteomic distance (B). Distances were calculated pairwise between all sets of predicted
503 proteomes, clustered with R and visualized using Itol. The trees were rooted at Brochotrix
504 phage A9. Genera are delineated with colored squares and suggested subfamilies with
505 colored circles.

506

507 **Fig 3: Single gene phylogenies of the major capsid protein (MCP, A), tail sheath protein**
508 **(TSP, B) and helicase (C) amino acid sequences of 93 spounavirin and spouna-like viruses.**
509 Amino acid sequences were aligned with Clustal Omega and trees were generated using
510 FastTree maximum likelihood with Shimidaira-Hasegawa tests. The scale bar represents the
511 number of substitutions per site. The trees were rooted at Brochotrix phage A9. Genera are
512 delineated with colored squares and suggested subfamilies with colored circles.

513
514

515 ## Supporting information

516

517 **S1 Fig.** Heatmap of the blastn-based nucleotide similarities between pairs of genomes as

518 calculated with Gegenees at default parameters.

519 **S2 Fig.** Genome-blast Distance Phylogeny as calculated using VICTOR.

520 **S3 Fig.** Heatmap of the DICE coefficient calculated between each pair of genomes.

521 **S4 Fig.** Heatmap of the tblastx-based nucleotide similarities between pairs of genomes as

522 calculated with Gegenees at default parameters.

523 **S5 Fig.** Heatmap of the pairwise comparison of all genomes visualized as percentage of

524 shared orthologous proteins (OPCs) as calculated on original GenBank files.

525 **S6 Fig.** Heatmap of the pairwise comparison of all genomes visualized as percentage of

526 shared orthologous proteins (OPCs) as calculated on reannotated genomes.

527 **S7 Fig.** Heatmap of the pairwise comparison of all genomes visualized as percentage of

528 shared proteins as calculated with Roary on reannotated genome files.

529 **S8 Fig.** Clustering trees of genomic fluidity and amino acid identity calculated pairwise

530 between all genomes using orthologous protein clusters.

531 **S9 Fig.** Accessory genome clustering tree, calculated based on the presence and absence of

532 accessory genes in each genome.

533 **S10 Fig.** Heatmap and clustering tree calculated by the Gene Order Alignment Tool and

534 visualized as a distance matrix between all genome pairs.

535 **S11 Fig.** Maximum Likelihood trees of single gene phylogenies using protein clusters present

536 in all 93 genomes.

537 **S1 Table.** Overview of the 93 phage genomes used in this study.

538 **S2 Table.** Complete list of all orthologous proteins identified in the set of 93 spounavirin and

539 spouna-like virus genomes.

540 **S3 Table.** Complete list of prokaryotic virus orthologous groups identified in the set of 93
541 spounavirin and spouna-like virus genomes.
542

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556

557 Competing interests

558 All authors, except for MBPS, are members of the Bacterial and Archaeal Viruses
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561
562

563 **References**

564 1. Krupovic M, Dutilh BE, Adriaenssens EM, Wittmann J, Vogensen FK, Sullivan MB, et al.
565 Taxonomy of prokaryotic viruses: update from the ICTV bacterial and archaeal viruses
566 subcommittee. Arch Virol. Springer Vienna; 2016;161: 1095–1099.
567 doi:10.1007/s00705-015-2728-0
568 2. Adriaenssens EM, Krupovic M, Knezevic P, Ackermann H-W, Barylski J, Brister JR, et al.
569 Taxonomy of prokaryotic viruses: 2016 update from the ICTV bacterial and archaeal
570 viruses subcommittee. Arch Virol. Springer Vienna; 2017;162: 1153–1157.
571 doi:10.1007/s00705-016-3173-4
572 3. Lavigne R, Seto D, Mahadevan P, Ackermann H-W, Kropinski AM. Unifying classical
573 and molecular taxonomic classification: analysis of the Podoviridae using BLASTP-
574 based tools. Res Microbiol. 2008;159: 406–414. doi:10.1016/j.resmic.2008.03.005
575 4. Lavigne R, Darius P, Summer EJ, Seto D, Mahadevan P, Nilsson AS, et al. Classification
576 of Myoviridae bacteriophages using protein sequence similarity. BMC Microbiol.
577 2009;9: 224. doi:10.1186/1471-2180-9-224
578 5. Adriaenssens EM, Edwards R, Nash JHE, Mahadevan P, Seto D, Ackermann H-W, et al.
579 Integration of genomic and proteomic analyses in the classification of the
580 Siphoviridae family. Virology. 2015;477: 144–154. doi:10.1016/j.virol.2014.10.016
581 6. Gregory AC, Solonenko SA, Ignacio-Espinoza JC, LaButti K, Copeland A, Sudek S, et al.
582 Genomic differentiation among wild cyanophages despite widespread horizontal
583 gene transfer. BMC Genomics. BMC Genomics; 2016;17: 930. doi:10.1186/s12864-
584 016-3286-x
585 7. Brum JR, Ignacio-Espinoza JC, Roux S, Doulcier G, Acinas SG, Alberti A, et al. Patterns
586 and ecological drivers of ocean viral communities. Science (80-). 2015;348: 1261498–

587 1261498. doi:10.1126/science.1261498

588 8. Holmfeldt K, Solonenko N, Shah M, Corrier K, Riemann L, Verberkmoes NC, et al.
589 Twelve previously unknown phage genera are ubiquitous in global oceans. Proc Natl
590 Acad Sci U S A. 2013;110: 12798–12803. doi:10.1073/pnas.1305956110

591 9. Lima-Mendez G, Van Helden J, Toussaint A, Leplae R. Reticulate representation of
592 evolutionary and functional relationships between phage genomes. Mol Biol Evol.
593 2008;25: 762–777. doi:10.1093/molbev/msn023

594 10. Nishimura Y, Watai H, Honda T, Mihara T, Omae K, Roux S, et al. Environmental viral
595 genomes shed new light on virus-host interactions in the ocean. mSphere. 2017;2:
596 e00359-16. doi:10.1128/mSphere.00359-16

597 11. Paez-Espino D, Eloe-Fadrosh EA, Pavlopoulos GA, Thomas AD, Huntemann M,
598 Mikhailova N, et al. Uncovering Earth's virome. Nature. 2016;536: 425–430.
599 doi:10.1038/nature19094

600 12. Roux S, Brum JR, Dutilh BE, Sunagawa S, Duhaime MB, Loy A, et al. Ecogenomics and
601 potential biogeochemical impacts of globally abundant ocean viruses. Nature.
602 2016;537: 689–693. doi:10.1038/nature19366

603 13. Iranzo J, Krupovic M, Koonin E V. The double-stranded DNA virosphere as a modular
604 hierarchical network of gene sharing. MBio. 2016;7: e00978-16.
605 doi:10.1128/mBio.00978-16

606 14. Bolduc B, Jang H Bin, Doulcier G, You Z-Q, Roux S, Sullivan MB. vConTACT: an iVirus
607 tool to classify double-stranded DNA viruses that infect Archaea and Bacteria. PeerJ.
608 2017;5: e3243. doi:10.7717/peerj.3243

609 15. Okubo S, Strauss B, Stodolsky M. The possible role of recombination in the infection
610 of competent Bacillus subtilis by bacteriophage deoxyribonucleic acid. Virology.

611 1964;24: 552–562. doi:10.1016/0042-6822(64)90207-7

612 16. Parker ML, Eiserling FA. Bacteriophage SPO1 structure and morphogenesis I. Tail
613 structure and length regulation. J Virol. 1983;46: 239–249.

614 17. Duda RL, Hendrix RW, Huang WM, Conway JF. Shared architecture of bacteriophage
615 SPO1 and herpesvirus capsids. Curr Biol. 2006;16: R11–R13.
616 doi:10.1016/j.cub.2006.02.014

617 18. Hemphill HE, Whiteley HR. Bacteriophages of Bacillus subtilis. Bacteriol Rev. 1975;39:
618 257–315.

619 19. Okubo S, Yanagida T, Fujita DJ, Olsson-Wilhelm BM. The genetics of bacteriophage
620 SPO1. Biken J. 1972;15: 81–97.

621 20. Klumpp J, Lavigne R, Loessner MJ, Ackermann H-W. The SPO1-related bacteriophages.
622 Arch Virol. 2010;155: 1547–61. doi:10.1007/s00705-010-0783-0

623 21. Stewart CR, Casjens SR, Cresawn SG, Houtz JM, Smith AL, Ford ME, et al. The genome
624 of Bacillus subtilis bacteriophage SPO1. J Mol Biol. 2009;388: 48–70.
625 doi:10.1016/j.jmb.2009.03.009

626 22. Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, et al. Sixth
627 report of the International Committee on Taxonomy of Viruses. Arch Virol Suppl.
628 1995;10: 590.

629 23. Ackermann H-W, Elzanowski A, Fobo G, Stewart G. Relationships of tailed phages: a
630 survey of protein sequence identity. Arch Virol. 1995;140: 1871–1884.
631 doi:10.1007/BF01384350

632 24. Adams MJ, Carstens EB. Ratification vote on taxonomic proposals to the International
633 Committee on Taxonomy of Viruses (2012). Arch Virol. 2012;157: 1411–1422.
634 doi:10.1007/s00705-012-1299-6

635 25. Schuch R, Fischetti VA. The secret life of the anthrax agent Bacillus anthracis:
636 Bacteriophage-mediated ecological adaptations. PLoS One. 2009;4: e6532.
637 doi:10.1371/journal.pone.0006532
638 26. Yuan Y, Peng Q, Wu D, Kou Z, Wu Y, Liu P, et al. Effects of actin-like proteins encoded
639 by two Bacillus pumilus phages on unstable lysogeny, revealed by genomic analysis.
640 Appl Environ Microbiol. 2015;81: 339–350. doi:10.1128/AEM.02889-14
641 27. Klumpp J, Dorscht J, Lurz R, Bielmann R, Wieland M, Zimmer M, et al. The terminally
642 redundant, nonpermuted genome of Listeria bacteriophage A511: a model for the
643 SPO1-like myoviruses of gram-positive bacteria. J Bacteriol. 2008;190: 5753–65.
644 doi:10.1128/JB.00461-08
645 28. Barylski J, Nowicki G, Goździcka-Józefiak A. The discovery of phiAGATE, a novel phage
646 infecting Bacillus pumilus, leads to new insights into the phylogeny of the subfamily
647 Spounavirinae. Dąbrowska K, editor. PLoS One. 2014;9: e86632.
648 doi:10.1371/journal.pone.0086632
649 29. Uchiyama J, Rashel M, Maeda Y, Takemura I, Sugihara S, Akechi K, et al. Isolation and
650 characterization of a novel Enterococcus faecalis bacteriophage phiEF24C as a
651 therapeutic candidate. FEMS Microbiol Lett. 2008;278: 200–206. doi:10.1111/j.1574-
652 6968.2007.00996.x
653 30. Uchiyama J, Rashel M, Takemura I, Wakiguchi H, Matsuzaki S. In silico and in vivo
654 evaluation of bacteriophage phiEF24C, a candidate for treatment of Enterococcus
655 faecalis infections. Appl Environ Microbiol. 2008;74: 4149–4163.
656 doi:10.1128/AEM.02371-07
657 31. Mizuno CM, Rodriguez-Valera F, Kimes NE, Ghai R. Expanding the marine virosphere
658 using metagenomics. Rocha EPC, editor. PLoS Genet. 2013;9: e1003987.

659 doi:10.1371/journal.pgen.1003987

660 32. Goodrich-Blair H, Scarlato V, Gott JM, Xu M-Q, Shub DA. A self-splicing group I intron
661 in the DNA polymerase gene of bacillus subtilis bacteriophage SPO1. Cell. 1990;63:
662 417–424. doi:10.1016/0092-8674(90)90174-D

663 33. Lavigne R, Vandersteegen K. Group I introns in Staphylococcus bacteriophages.
664 Future Virol. 2013;8: 997–1005. doi:10.2217/fvl.13.84

665 34. Kislyuk AO, Haegeman B, Bergman NH, Weitz JS. Genomic fluidity: An integrative view
666 of gene diversity within microbial populations. BMC Genomics. 2011;12: 32.
667 doi:10.1186/1471-2164-12-32

668 35. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MTG, et al. Roary: rapid
669 large-scale prokaryote pan genome analysis. Bioinformatics. 2015;31: 3691–3693.
670 doi:10.1093/bioinformatics/btv421

671 36. Juhala RJ, Ford ME, Duda RL, Youlton A, Hatfull GF, Hendrix RW. Genomic sequences
672 of bacteriophages HK97 and HK022: pervasive genetic mosaicism in the lambdoid
673 bacteriophages. J Mol Biol. 2000;299: 27–51. doi:10.1006/jmbi.2000.3729

674 37. Krupovic M, Prangishvili D, Hendrix RW, Bamford DH. Genomics of bacterial and
675 archaeal viruses: Dynamics within the prokaryotic virosphere. Microbiol Mol Biol Rev.
676 2011;75: 610–635. doi:10.1128/MMBR.00011-11

677 38. Cadungog JN, Khatemi BE, Hernandez AC, Kuty Everett GF. Complete genome
678 sequence of Bacillus megaterium myophage Moonbeam. Genome Announc. 2015;3:
679 e01428-14. doi:10.1128/genomeA.01428-14

680 39. Ackermann H-W, DuBow MS. Viruses of prokaryotes. Boca Raton, FL, USA: CRC Press;
681 1987.

682 40. Ackermann H-W. Classification of bacteriophages. In: Calendar R, editor. The

683 Bacteriophages. 2nd ed. New York, NY, USA: Oxford University Press; 2006. pp. 8–17.
684 41. Tolstoy I, Kropinski AM, Brister JR. Bacteriophage taxonomy: an evolving discipline. In:
685 Azeredo J, Sillankorva S, editors. Methods in Molecular Biology: Bacteriophage
686 Therapy. Springer Nature; doi:Forthcoming
687 42. Karsch-Mizrachi I, Nakamura Y, Cochrane G. The International Nucleotide Sequence
688 Database Collaboration. Nucleic Acids Res. 2012;40: D33–D37.
689 doi:10.1093/nar/gkr1006
690 43. Davison AJ. Journal of General Virology – Introduction to "ICTV Virus Taxonomy
691 Profiles." J Gen Virol. 2017;98: 1–1. doi:10.1099/jgv.0.000686
692 44. Simmonds P, Adams MJ, Benkő M, Breitbart M, Brister JR, Carstens EB, et al.
693 Consensus statement: Virus taxonomy in the age of metagenomics. Nat Rev
694 Microbiol. 2017;15: 161–168. doi:10.1038/nrmicro.2016.177
695 45. Adams MJ, Lefkowitz EJ, King AMQ, Harrach B, Harrison RL, Knowles NJ, et al. 50 years
696 of the International Committee on Taxonomy of Viruses: progress and prospects.
697 Arch Virol. 2017;162: 1441–1446. doi:10.1007/s00705-016-3215-y
698 46. Bolduc B, Youens-Clark K, Roux S, Hurwitz BL, Sullivan MB. iVirus: facilitating new
699 insights in viral ecology with software and community data sets imbedded in a
700 cyberinfrastructure. ISME J. 2017;11: 7–14. doi:10.1038/ismej.2016.89
701 47. Paez-Espino D, Chen IMA, Palaniappan K, Ratner A, Chu K, Szeto E, et al. IMG/VR: A
702 database of cultured and uncultured DNA viruses and retroviruses. Nucleic Acids Res.
703 2017;45: D457–D465. doi:10.1093/nar/gkw1030
704 48. Hulo C, Masson P, Le Mercier P, Toussaint A. A structured annotation frame for the
705 transposable phages: A new proposed family "Saltoviridae" within the Caudovirales.
706 Virology. 2015;477: 155–163. doi:10.1016/j.virol.2014.10.009

707 49. Grose JH, Casjens SR. Understanding the enormous diversity of bacteriophages: The
708 tailed phages that infect the bacterial family Enterobacteriaceae. Virology. 2014;468–
709 470: 421–443. doi:10.1016/j.virol.2014.08.024
710 50. Brister JR, Ako-adjei D, Bao Y, Blinkova O. NCBI Viral Genomes Resource. Nucleic Acids
711 Res. 2015;43: D571–D577. doi:10.1093/nar/gku1207
712 51. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool.
713 J Mol Biol. 1990;215: 403–410. doi:10.1016/S0022-2836(05)80360-2
714 52. Frickey T, Lupas A. CLANS: A Java application for visualizing protein families based on
715 pairwise similarity. Bioinformatics. 2004;20: 3702–3704.
716 doi:10.1093/bioinformatics/bth444
717 53. Seemann T. Prokka: Rapid prokaryotic genome annotation. Bioinformatics. 2014;30:
718 2068–2069. doi:10.1093/bioinformatics/btu153
719 54. Ågren J, Sundström A, Håfström T, Segerman B. Gegenees: Fragmented alignment of
720 multiple genomes for determining phylogenomic distances and genetic signatures
721 unique for specified target groups. PLoS One. 2012;7: e39107.
722 doi:10.1371/journal.pone.0039107
723 55. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, et al. BLAST+:
724 architecture and applications. BMC Bioinformatics. 2009;10: 421. doi:10.1186/1471-
725 2105-10-421
726 56. Development Core Team R. R: A language and environment for statistical computing
727 [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2008. Available:
728 http://www.r-project.org
729 57. Letunic I, Bork P. Interactive Tree Of Life (iTOL): An online tool for phylogenetic tree
730 display and annotation. Bioinformatics. 2007;23: 127–128.

731 doi:10.1093/bioinformatics/btl529

732 58. Meier-Kolthoff JP, Hahnke RL, Petersen J, Scheuner C, Michael V, Fiebig A, et al.
733 Complete genome sequence of DSM 30083T, the type strain (U5/41T) of Escherichia
734 coli, and a proposal for delineating subspecies in microbial taxonomy. Stand Genomic
735 Sci. 2014;9: 2. doi:10.1186/1944-3277-9-2

736 59. Meier-Kolthoff JP, Göker M. VICTOR: genome-based phylogeny and classification of
737 prokaryotic viruses. Bioinformatics. 2017;33: 3396–3404.
738 doi:10.1093/bioinformatics/btx440

739 60. Lefort V, Desper R, Gascuel O. FastME 2.0: A comprehensive, accurate, and fast
740 distance-based phylogeny inference program. Mol Biol Evol. 2015;32: 2798–2800.
741 doi:10.1093/molbev/msv150

742 61. Rambaud. FigTree [Internet]. 2007 [cited 1 Aug 2015]. Available:
743 http://tree.bio.ed.ac.uk/software/figtree/

744 62. Göker M, García-Blázquez G, Voglmayr H, Tellería MT, Martín MP. Molecular
745 taxonomy of phytopathogenic fungi: A case study in Peronospora. PLoS One. 2009;4:
746 8–10. doi:10.1371/journal.pone.0006319

747 63. Rohwer F, Edwards R. The Phage Proteomic Tree: a genome-based taxonomy for
748 phage. J Bacteriol. Am Soc Microbiol; 2002;184: 4529–4535.
749 doi:10.1128/JB.184.16.4529

750 64. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al.
751 Clustal W and Clustal X version 2.0. Bioinformatics. 2007;23: 2947–2948.
752 doi:10.1093/bioinformatics/btm404

753 65. Felsenstein J. PHYLIP - Phylogeny inference package - v3.2. Cladistics. 1989;5: 164–
754 166. doi:10.1111/j.1096-0031.1989.tb00562.x

755 66. Contreras-Moreira B, Vinuesa P. GET_HOMOLOGUES, a versatile software package for
756 scalable and robust microbial pangenome analysis. Appl Environ Microbiol. 2013;79:
757 7696–7701. doi:10.1128/AEM.02411-13
758 67. Kristensen DM, Kannan L, Coleman MK, Wolf YI, Sorokin A, Koonin E V, et al. A low-
759 polynomial algorithm for assembling clusters of orthologous groups from
760 intergenomic symmetric best matches. Bioinformatics. 2010;26: 1481–7.
761 doi:10.1093/bioinformatics/btq229
762 68. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable generation
763 of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst
764 Biol. 2011;7: 539. doi:10.1038/msb.2011.75
765 69. Konstantinidis KT, Tiedje JM. Towards a genome-based taxonomy for prokaryotes. J
766 Bacteriol. 2005;187: 6258–6264. doi:10.1128/JB.187.18.6258-6264.2005
767 70. Charif D, Thioulouse J, Lobry JR, Perrière G. Online synonymous codon usage analyses
768 with the ade4 and seqinR packages. Bioinformatics. 2005;21: 545–547.
769 doi:10.1093/bioinformatics/bti037
770 71. Gascuel O. BIONJ: an improved version of the NJ algorithm based on a simple model
771 of sequence data. Mol Biol Evol. 1997;14: 685–695.
772 doi:10.1017/CBO9781107415324.004
773 72. Hyatt D, Chen GL, LoCascio PF, Land ML, Larimer FW, Hauser LJ. Prodigal: prokaryotic
774 gene recognition and translation initiation site identification. BMC Bioinformatics.
775 2010;11. doi:10.1186/1471-2105-11-119
776 73. Grazziotin AL, Koonin E V, Kristensen DM. Prokaryotic Virus Orthologous Groups
777 (pVOGs): a resource for comparative genomics and protein family annotation. Nucleic
778 Acids Res. 2017;45: D491–D498. doi:10.1093/nar/gkw975

74. Finn RD, Clements J, Eddy SR. HMMER web server: Interactive sequence similarity searching. Nucleic Acids Res. 2011;39: 29–37. doi:10.1093/nar/gkr367

75. Söding J, Biegert A, Lupas AN. The HHpred interactive server for protein homology detection and structure prediction. Nucleic Acids Res. 2005;33: W244-2488. doi:10.1093/nar/gki408

76. Fu L, Niu B, Zhu Z, Wu S, Li W. CD-HIT: Accelerated for clustering the next-generation sequencing data. Bioinformatics. 2012;28: 3150–3152. doi:10.1093/bioinformatics/bts565

77. Enright AJ, Van Dongen S, Ouzounis CA. An efficient algorithm for large-scale detection of protein families. Nucleic Acids Res. 2002;30: 1575–1584. doi:doi: 10.1093/nar/30.7.1575