Phage diversity, genomics and phylogeny

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Abstract:

Recent advances in viral metagenomics have enabled the rapid discovery of an unprecedented catalogue of phages in many biomes. While it significantly expanded our understanding of how diverse phage sequences are, it also revealed that we have only scratched the surface in the discovery of novel viruses. Yet despite their remarkable diversity at the nucleotide sequence level, the structural proteins that make up their virion particles still show strong similarities and conservation. Phages are uniquely interconnected from an evolutionary perspective and undergo multiple events of genetic exchanges in response to the selective pressure of their hosts, which fuel their diversity. In this Review, we explore phage diversity at the structural, genomic and community level as well as the complex evolutionary relationships between phages, molded by the mosaicity of their genomes.

Phages are the most abundant and diverse biological entities on the planet. This opening statement has become a favorite of many viral ecologists. With an estimated 10^{31} on the planet[1], phages can even outnumber bacteria by approximately ten-fold in some ecosystems. They are found in every explored biome, from the human gastrointestinal tract to the global ocean, but also in startling places such as the oceanic basement[2] and a Middle Age fossilized stool specimen[3]. In aquatic environments, phages were shown to play major roles in biogeochemical cycling, by short-circuiting the flow of carbon through bacterial killing, known as the viral shunt[1]. They are also important modulators in the human gut, where they predominantly carry out a lysogenic lifestyle, which can affect their bacterial host's physiology and metabolism[4]. In addition to their ubiquity, phages exhibit a plethora of structural morphologies, with tailed dsDNA phages being the most represented in public databases as of 2019. Other seemingly less common phages can package their ssDNA, ssRNA or dsRNA genome into tailless virions. Despite their relatively small genomes, phages also show tremendous genomic diversity and complex evolutionary relationships that do not obey traditional hierarchical phylogeny, due to pervasive mosaicism.

Much of our knowledge of phage diversity has been redrawn following the advancements in large-scale viral metagenomics and culturing efforts. In recent years, scientists have discovered phages with a genome size nearly ten times larger than average[5]. Non-tailed dsDNA[6,7] and ssDNA[8,9] phages are increasingly identified and even perhaps dominant in some biomes. Thousands of viral sequences have been identified from metagenomic projects, yet most of them share no detectable homology with reference phages[10,11]. One feature that was certainly emphasised by metagenomics is the exceptional viral diversity at the genomic level.

This review will focus on phage (viruses infecting bacteria) diversity and will explore four levels of organization. First, we present their unique morphologies and the structural proteins that make up these viral particles. Second, we examine the genomic diversity and scarceness in gene content similarities. From these two analyses emerges a contradiction: even when no sequence homology exists between morphologically distinct phages, some viral proteins still show conservation at the structural level. Third, we evaluate viral diversity at the community level, by comparing phage abundance and composition in various ecosystems. Recent progress in viral metagenomics has broadened our view of phage abundance and diversity, especially in marine environments. Lastly, we explore gene exchanges between phages, which generate mosaicity and diversification, and illustrate that bacterial viruses are interconnected through a complex web.

Phage morphological and structural diversity

As indicated above, phage genomes are either composed of DNA or RNA and it may be double-stranded (ds) or single-stranded (ss). This genetic material is packaged into a capsid that can be polyhedral (*Microviridae*, *Corticoviridae*, *Tectiviridae*, *Leviviridae*, *Cystoviridae*), filamentous (*Inoviridae*), pleomorphic (*Plasmaviridae*) or connected to a tail (*Caudovirales*)[12] (Fig. 1). Up to date, most isolated phages are tailed and have dsDNA genomes[13]. Taxonomic classification of phage taxa is carried out by the International Committee on Taxonomy of Viruses (ICTV)[14]. While phage classification was historically based on characteristics such as genome type (DNA, RNA, ss, ds), viral morphology, and host range, it is currently undergoing a major overhaul, primarily using mostly genomic-based methods to classify them. For example, the 1999 ICTV report classified tailed phages into 3 families, 16 genera and 30 species, while the 2018 version grouped them into 5 families, 26 subfamilies, 363 genera and 1320 species (https://talk.ictvonline.org/taxonomy/p/taxonomy_releases). Comprehensive guidelines have been proposed for phage classification and it is expected that the list of virus taxa will significantly increase in the upcoming years[15].

Tailed bacteriophages

The large majority of phages described to date have a tailed morphology with dsDNA genome and belong to the *Caudovirales* order[13]. This viral order, while under re-classification[16] is currently comprised of five families: *Myoviridae, Siphoviridae, Podoviridae, Ackermannviridae* and *Herelleviridae*. The last two families were created only recently as network and meta-analyses indicated that they represented distinct clusters within the *Myoviridae* family[16,17]. A large variation in capsid size can be observed among members of *Caudovirales*, with diameters ranging from 45 nm to 185 nm, which is usually linked to genome size[18]. Most of the tailed phages (75%) have icosahedral capsid structures and around 15% have an elongated capsid aligned with the axis of the tail[13]. Interestingly, all members of *Caudovirales* share the same major capsid protein (MCP) fold (HK97-fold)[19]. The HK97-fold was identified following X-ray crystallography of the capsid of phage HK97. The capsid is connected to its tail through a connector complex often composed of a portal protein and two head completion/connector proteins. Structural studies have revealed that the portal complex is a dodecameric ring with a similar overall structure shared between most tailed phages despite low sequence similarity[20–22]. Capsid completion/connector proteins also form dodecameric rings as observed in the *Siphoviridae* SPP1 and HK97 phages. Homologs of these proteins are found in a variety of phages that have contractile and non-contractile tails[23]. Moreover, the head to tail connector protein gp4 of the *Podoviridae* P22 has a similar structure to the ones present in siphophages SPP1 and HK97, despite no observable trace of sequence similarity[24].

The tails of *Siphoviridae* are composed of a central tape measure protein surrounded by a tail tube and ends with a terminator protein[25]. Similar architecture is observed for phages of the *Myoviridae* family, where an additional layer, the protein sheath, enables contraction for the insertion of the tail tube into the bacterial host during infection[26]. Interestingly, the capsid-tail joining protein gpFII of the *Siphoviridae* phage λ has a similar tertiary fold to its tail tube protein gpV and adopts the same quaternary structure when assembled in the phage[27]. GpV also shares structural homologies with the tail tube of *Myoviridae* phages as well as some components of the bacterial type VI secretion system like the Hcp1 protein[28]. Moreover, the folds of proteins gpFII and gpV are similar to those of the baseplate hub of the myophages T4 and Mu, once again without any sequence homology[27]. These observations suggest that the tail tube-like fold adopted by the capsid-tail connector, the tail tube protein itself and the base plate is an important building block for members of *Siphoviridae* and *Myoviridae*. Members of the *Podoviridae* family, such as *E. coli* phage T7, have very short and non-contractile tails. A tube-like extension of the tail that penetrates both cell membranes was observed to be essential for genome delivery into the host[29].

Finally, receptor binding proteins (RBP) present at the tip of the tail or at the baseplate were characterized at the structural level in siphophages and showed high levels of structural similarity despite low sequence homology[30–32]. Moreover, RBP domains are interchangeable between different phages and are homologous with mammalian adenovirus[33]. Members of the *Ackermannviridae* family, formerly known as *Viunalikevirus*, have a myovirus-like morphology but they differ by their complex and unique adsorption structures. Short filaments with bulbous tips that resemble an umbrella and prong-like structures are attached to the baseplate[17].

Membrane-containing bacteriophages

Phages belonging to the *Tectiviridae* (phage PRD1) and *Corticoviridae* (phage PM2) families comprise icosahedral tailless virions that have an internal lipidic membrane and linear or circular dsDNA genomes, respectively. A hallmark characteristic of these two phage families is their trimeric major capsid protein, which is composed of a double β-barrel structure[34,35]. Structural analyses of the MCP of phage PRD1 revealed an N-terminal alpha helix, which interacts directly with the phage inner membrane and shares structural homologies with the MCP of adenoviruses[34]. Furthermore, the RBP present at the icosahedral vertices of phage PRD1 and PM2 capsids also shares N-terminal domains with human adenovirus[35–37]. Phage PRD1 does not have a tail to deliver its DNA to its Gram-negative host, but its membrane was observed to transform into a proteo-lipidic tube, which can pierce host envelopes[38]. Unlike *Corticoviridae* and *Tectiviridae* that have inner lipidic membranes, members of the *Cystoviridae* family including phage phi6 have lipidic membranes that surround their icosahedral capsids[39]. Finally, *Acholeplasma* virus L2 (AVL2 or also referred as MVL2) is currently the only classified member of the *Plasmaviridae* family. It infects the wall-less *Acholeplasma* bacterial species and new virions are released by membrane budding without causing cell lysis[40]. *Plasmaviridae* phages do not possess any capsid but their genomes are enclosed in a proteinaceous lipid vesicle that has a similar composition to the outer membrane of phi6[41].

Phages with small icosahedral capsids or filamentous morphology

Microviridae and its most studied member, phage phiX174, have small icosahedral capsids (26 nm) and ssDNA genomes (5,386 bp) (Fig. 1) [42]. The capsid is built on a protein fold that has a "jelly roll" β-barrel structure and has similarities with ssDNA eukaryotic viruses, including rhinoviruses[42]. They are currently classified in two subfamilies named *Bullavirinae* and *Gokushovirinae.* DNA delivery in the bacterial host relies on a protein, which oligomerizes to form a tube that crosses the host's periplasmic space by joining the outer and inner membranes[43]. Structural differences in proteins mediating host attachment have been observed for both subfamilies. *Bullavirinae* have pentameric major spike protein (MSP) complexes at the end of each capsid vertex[44], while *Gokushovirinae* have "mushroom-like" protrusions that extend along the threefold icosahedral axes of the capsid[44]. The MSP complexes in the *Bullavirinae* clade are also divergent, but as their structures are superimposable, they can be exchanged between phages[45].

Other small icosahedral viruses include members of the *Leviviridae* family, such as the phage MS2 that has a ssRNA genome (Fig. 1). The MS2 viral particle has only two proteins: a major capsid protein and a single copy of the maturation protein that interacts with the genomic RNA during packaging and with the host receptor during adsorption[46]. The MCP of phage MS2 can control replication by interacting with the initiation codon of the replicase-encoding gene, which switches the replication cycle to viral assembly[47]. Recent cryo-EM reconstruction of the viral capsid also revealed that the RNA genome is highly involved in virion assembly since it can adopt secondary structures that act as a scaffold[48]. Of note, this system of genome packing is radically different from the *Caudovirales*, in which an empty capsid is first assembled and then filled with the phage genome and the packaged capsid is then connected to its tail[49].

Finally, members of the family *Inoviridae* are dramatically different in terms of morphology and lifestyle (Fig. 1). Phage particles contained a dsDNA genome surrounded by thousands of copies of MCP that are assembled and then extruded from the host in a continuous manner[50]. The MCPs of filamentous phages are unique in their architecture, which consists of a long alpha-helix with an N-terminal signal peptide for membrane translocation[51]. The signal peptide is then cleaved before the proteins are assembled in a long cylindrical shell spiral with the C-terminal end interacting directly with the viral DNA[52].

Two sides of the same coin

From a morphological point of view, several diverse phages still share some commonalities. One example is the MCP fold, which is conserved at the structural level between all tailed phages but also extends to archaeal viruses and adenoviruses[53,54]. For the majority of these proteins where conservation is observed in their structure, no trace of homology can be detected, both at the amino acid and nucleic acid levels. This paradigm is explored further in Box 1, where convergent evolution or a common ancestor are discussed as possible explanation for structural similarities among viruses infecting the three domains of life.

Genomic diversity and viral metagenomics

Number of complete genomes

According to the NCBI, as of September 2019, there are 8,437 complete phage genomes divided into ten families (based on the ICTV classification at the time) and one unclassified group (Fig. 2a). More than half of them are members of the *Siphoviridae* family. This overrepresentation is due in large part to the isolation and genome sequencing of 1,537 siphophages infecting *Mycobacterium smegmatis* by the SEA-PHAGES program[55]. *Myoviridae* and *Podoviridae* represent 17 and 12% of the total phages, respectively, rendering *Caudovirales* (comprising also *Herelleviridae* and *Ackermannviridae*) the most abundant group of phages (> 85%) in public genomic databases. The disproportionate representation of tailed dsDNA phages will likely decrease in the near future with the discovery of new phages. For example, the genomic diversity within the *Microviridae* family was largely underestimated until 258 new ssDNA phages were detected in the gut of *Ciona robusta*[56]. In addition, the unclassified bacterial virus group within NCBI consists of phages discovered through metagenomic projects that have yet to be isolated or have been very recently propagated on a bacterial host. Part of this latter group includes 283 non-tailed dsDNA phages, infecting the ubiquitous marine *Vibrionaceae* bacterial family[7]. Recently, Roux and colleagues used a machine learning approach to mine microbial genomes and metagenomes for inoviruses[9]. They identified 10,295 inovirus-like sequences, from which 5,964 distinct species appeared to have been identified. This study alone represents a 100-fold expansion of the diversity previously described (57 genomes) within the *Inoviridae* family. The ever-increasing number of complete phage genomes in the NCBI database still represents only a small fraction of the actual phage genomic diversity, since half of them infect only seven host genera (*Mycobacterium*, *Streptococcus*, *Escherichia*, *Pseudomonas*, *Gordonia, Lactococcus,* and *Salmonella*). The total number of complete phage genomes available in public databases is also certainly far greater because of the numerous unidentified prophages in bacterial genomes[57].

Range in genome size

Phages have a wide range of genome sizes, with an average size of 62.5 ± 46.8 kb (Fig. 2b). Apparently, the smallest phage genome reported to date is that of *Leuconostoc* phage L5 with only 2,435 bp. At the other end of the spectrum, an increasing number of jumbo phages (> 200 kb) are being characterized and show unique genomic features[58]. Their large genome size allows jumbo phages to carry genes involved in replication and nucleotide metabolism that are absent in smaller phage genomes. The organization of these large viral genomes is also atypical because genes with associated functions do not show strong synteny and are instead, more dispersed[58]. A new group of phages with the largest genomes ever recorded to date, called Megaphages (> 540 kb), were just uncovered from human and animal gut metagenomes that are predicted to infect *Prevotella*[5]. These phages seem widespread in gut microbiomes, as they were identified in humans, baboons and pigs[5]. They were overlooked due to genome fragmentation and their use of an alternative genetic code, which consisted of a repurposed stop codon[5].

Contribution of viral metagenomics in exploring phage genomic diversity

Given the absence of a conserved genetic marker and the predicted large number in the biosphere[59], phage genomic diversity is difficult to comprehend. Phages infecting different hosts typically have little to no sequence similarity and phages that infect a single host may also exhibit considerable sequence differences[60–62]. For instance, a pairwise comparisons of 2,333 phages showed no detectable homology in 97% of cases, when measuring nucleotide distance and gene content[63]. Thanks to modern techniques that explore viral dark matter, such as viral metagenomics, we are starting to grasp the extent of phage global diversity. Viral metagenomics is defined here as the sequencing of the total nucleic acids from the viral fraction of a given environment. It overcomes the challenges of culture-based approaches and single marker genes by assessing the total viral nucleic acids (mostly dsDNA) isolated from any given environment. Before the arrival of next generation sequencing, the first viral metagenomics study was published in 2002 from surface seawater samples[64]. In recent years, the optimization of the steps required to obtain good-quality viral nucleic acids[65], the reducing costs of sequencing and an improved set of analytical tools[66] have allowed the construction of large-scale virome (viral sequences obtained from viral metagenomics) datasets from viral communities, mostly from marine and human gut samples. There are now at least 90 studies describing viromes from aquatic environments[67], 38 from the human gut and eight from soil[67]. Among them, three research consortia, *Tara* Oceans[68], the Pacific Ocean Virome[69] and the Malaspina oceanic research expedition[70], have performed viral metagenomics on marine samples from various depths and locations. This has led to the detailed characterization of ocean dsDNA viruses and their abundance patterns on local and global scales[71,72]. The first human gut virome was published in 2003 from a single healthy individual[73]. More studies on twins and their mothers[74], healthy adults[75,76] and patients with ulcerative colitis[77] have followed to describe longitudinal and inter-personal viral variations in health and diseases. In 2014, the mining of viral metagenomic libraries (viromes) also resulted in the discovery of the most abundant and widespread phage in the human gut, called crAssphage[78]. The results of these projects are summarized in the following sections.

Beyond viral metagenomics

A major inconvenience in describing viral communities with metagenomics is the lack of a fine enough resolution to reconstruct genomes of closely related sequences. This causes phage populations with high levels of microdiversity to be discarded from metagenomics assembly. The detection of this microdiversity is necessary to better understand phage-host interaction dynamics[79]. Single-virus genomics overcomes this obstacle by sorting individual phages prior to sequencing. Such approach led to the discovery of the most abundant marine phage[80], which is called vSAG 37-F6 and infects *Pelagibacter*[81]. Viral tagging may also provide additional insights into phage-host interactions, as reported for cyanophages infecting *Synechococcus*[82]. Although metagenomics does not specifically target viral DNA, a wealth of information can be still discovered about phage sequences[10]. Using an exhaustive collection of viral protein families manually identified as bait, over 125,000 viral genomes were detected from 3,042 metagenomes of diverse geographical origins[10]. This study was a major contribution to our understanding of viral diversity, as they expanded the number of viral genes by 16-fold. It also suggested that on a global scale, phage genomic diversity still remained widely uncharacterized, but the discovery rate in marine and human samples (the most studied biomes) was approaching saturation[10]. Yet, the percentage of unknown phages still consistently represents the majority of the sequences in the viral fraction of any given environmental sample, accounting sometimes for more than 90% of the reads[11,83]. Figure 3 outlines how omics and culturing efforts can be integrated to fully characterise entire phage communities.

Distribution and abundance

Phages in marine environments

Marine phages are thought to play major roles in modulating microbial communities, generating genetic diversity and influencing the nutrient cycle through bacterial mortality[84]. The critical role of marine phages can be attributed to their tremendous abundance and diversity. In a recent analysis combining 22 distinct marine surveys, 95% of viral abundance was observed to range from 10^5 to 10^7 virus like particles (VLPs) per ml, with a median virus-to-microbial cell ratio of 10:1[85]. Analyses of samples from six global ocean regions using quantitative transmission electron microscopy (qTEM)[6] revealed a dominance of non-tailed viruses in the samples (Fig. 4a) (79%) followed by *Myoviridae* (14%), *Podoviridae* (6%) and *Siphoviridae* (1%). Interestingly, the morphological distribution did not vary consistently with depth or oceanic region[6].

Comparative genomic analyses of more than 100 *Synechococcus*-infecting cyanophages collected over 15 years revealed genomic clusters and sub-clusters that exhibited clear temporal and/or spatial patterns of abundance[86]. Viral tagging metagenomics confirmed that phages infecting *Synechococcus* are clustered into at least 26 discrete populations with relative abundances ranging from 0.06 to 18.2%[82]. Possibly, the most abundant and well-distributed phages are those infecting *Pelagibacter*, a host dominating marine surface bacterioplankton communities[87]. Indeed, pelagiphages were among the most abundant phages in metagenomic datasets along longitudinal and depth gradients from all oceans[80]. The isolation and genome sequencing of 31 phages that infect *Cellulophaga baltica* (*Bacteroidetes*)[88] showed that cellulophage diversity was even higher than that observed for *Synechococcus* phages and comprised non-tailed dsDNA phages. Comparisons with existing metagenomic data also revealed that cellulophages are widespread in oceans, but in low numbers. More recently, a group of dsDNA non-tailed viruses called autolykiviruses, that were previously missed due to multiple methodological biases, were isolated[7]. Genomic sequencing of these new phages revealed that they were present in the genome of major bacterial phyla and in metagenomic datasets from the water column and sediments[7].

Taxonomic analyses of 24 Mediterranean metagenomes from diverse geographical and ecological biomes reported the dominance of *Caudovirales*, with *Myoviridae* accounting for 67%-96% of the viral reads detected (followed by *Podoviridae* and *Siphoviridae*), independently of the water depth[89]. The largest marine viral metagenomics study was recently published[11], which surveyed 145 samples from the *Tara* research expedition, including 41 samples from the polar circle. The authors identified 195,728 viral populations, 90% of which could not be taxonomically annotated, and found that *Caudovirales* dominated the known sequences. They confirmed that phages in the ocean form discrete populations and identified potential drivers of phage diversity, such as nitrate levels, photosynthetically active radiation and latitude.

In addition to exhibiting various morphological compositions, phage communities in the ocean have different replication strategies according to seasonal variations. In the western Antarctic Peninsula[90] and in the Canadian Arctic Shelf[91], prophages dominate in the spring while lytic infections prevail in the summer. This fluctuation can be explained by the Kill-the-Winner hypothesis, which states that a high bacterial abundance (caused by favorable growth conditions in the summer) is coupled with a high rate of lytic infections[92,93]. This model was further extended with the Piggyback-the-Winner model, in which the lysogenic lifestyle is instead privileged at high bacterial densities[94,95]. This was first observed in coral reefs, where the virus to host ratio was low despite heavy microbial density[94]. Following those dynamics, the abundant hosts have been killed by phages or became resistant lysogens, which in turn decreases phage titers when no more hosts are available for replication. According to a recent review[66], the new phages to occupy the niche are more likely to be descendants of a 'royal family', i.e. variants of the most abundant phages that overcame host resistance. The authors coined the term 'royal family model' to illustrate the persistence of dominant phages in aquatic ecosystems.

Phages form the soil

Compared to marine environments, soils are intrinsically diverse due in part to their wide compositional spectrum and spatial heterogeneity in terms of physicochemical properties. A recent meta-analysis of 24 soils indicated that viral abundance is highly variable and correlates with soil type, ranging from approximately 10^3 VLPs/g in desert soils to 10^9 VLPs/g in forest soils[67] (Fig. 4b). TEM observations of different soil types reported the predominance of non-tailed particles over tailed phages, and higher morphological diversity in forest soils compared to agricultural soils, in some cases[96,97]. Metagenomics were also used to assess the richness and evenness of viral communities in prairie, desert and rainforest soils[98]. Similar phage sequences were observed in all of these soils but were significantly different from the dominant types found in marine or faecal samples. Metagenomic analyses of different Antarctic soils revealed that tailed phages were dominant in all samples, with the presence of *Myoviridae* and *Siphoviridae* inversely correlated[99]. Of note*,* samples with low- and medium-diversity were completely dominated by *Siphoviridae* signatures. Abiotic factors like pH and the altitude of the sampling site appeared to be the main drivers of viral community composition[99].

Phages from the human gut

Phages are also highly abundant in the human gut microbiome with up to 10^8 VLPs/ml in faecal filtrates[100] (Fig. 4c). Of note, phage titer was higher in gut mucosal biopsies (10^9 per biopsy), possibly due to the affinity of host-associated phages to bind and accumulate in the mucosal secretion[101,102]. TEM visualizations demonstrated that *Caudovirales* dominate the gut, with striking inter-individual differences in the composition of morphologies and types[100]. Since most of the bacteria residing in the gut are difficult to culture, metagenomic sequencing is mainly used to assess the complexity and diversity of gut phage populations. Recent analyses confirmed that a large majority of contigs that could be identified belonged to the *Caudovirales* order, but members of the *Microviridae* family were also detected[75,103,104]. It should be mentioned that contigs with taxonomic attribution were low, which highlights the importance of the viral dark matter. The composition of the human gut virome seems also highly specific and stable over time. The differences among individuals are the main sources of variation, despite the fact that a core set of phages was found in 20–50% of individuals[75,76,103,104]. The viral community can also evolve considerably during the first years of life, leading to an increased abundance of *Microviridae*[8]. Finally, phage distribution is also dependent on individual health status. For example, patients with Crohn's disease and ulcerative colitis exhibit a distinct virome with a significantly increased number of *Caudovirales* phages compared to *Microviridae*[105].

Insights into evolutionary relationships between phages

Genetic mosaicism as the main actor in phage evolution

Defining clear evolutionary relationships is no easy task when it comes to phages. Ironically, what makes them so diversified and unique is perhaps one of the few features they have in common: the mosaicity of their genomes. Genetic mosaicism refers to phage genomes that share regions of high sequence similarity with abrupt transitions to adjacent regions with no detectable resemblance[106]. These regions are often the result of recombination between two non-identical ancestors. Such recombination events, called horizontal gene transfer (HGT), are major mediators of phage evolution, which complicate how we view their evolutionary relationships.

Horizontal gene transfer mechanisms at a glance

The molecular mechanisms leading to HGT have been well studied in model phages and consist of illegitimate, relaxed and homologous recombinations. Illegitimate recombination occurs randomly across the genome[107,108], disrupting genes and gene blocks, leaving most of the phage recombinants or chimeras to be eliminated by counterselection such as host barriers, including anti-phage systems. The mosaic joints (recombination sites) of the few "lucky" ones that emerge are not located randomly. They are rather positioned at gene or gene block boundaries as a result of natural selection favouring only phages whose biological functions remained undamaged[109]. Relaxed (also called homeologous) recombination takes place at sites of limited homology but that are somewhat related between genomes. In several phages such as lambda, Rad52-like recombinases are responsible for gene shuffling. Relaxed recombination efficiency depends on sequence identity and occurs more frequently than illegitimate recombination[110]. Homologous recombination, although hard to detect[111], is presumed to be the most frequent avenue for HGT and is promoted by the phage recombination machinery[112].

Temperate phages are the brokers in HGT

Genetic mosaicity has been studied most extensively with dsDNA phages and was first described in lambda[113]. In theory, all dsDNA phages are mosaic because they have access to a large common gene pool through HGT[106]. However, phages do not have equal accessibility to the entire reservoir, as it depends on the number of steps (genetic exchanges) required to bring any given sequence from that pool and a particular phage together. For gene exchange to occur, two phages need to infect the same host cell. One scenario involves two virulent phages exchanging genetic material while coinfecting the same cell. Co-infection appears to be prevalent in natural bacterial populations[114] and a bioinformatic analysis suggested that a possible chimera even occurred between a ssRNA and a ssDNA virus[115] during co-infection. Because temperate phages can integrate into the host genome and become prophages, they are thought to act as viral sequence reservoirs and likely play a central role in HGT[116]. When a prophage (functional or cryptic) behaves as a sequence donor, the infecting phage (virulent or temperate) becomes the recipient of a new gene or gene block allele, as demonstrated with a cryptic prophage in *Escherichia coli* infected by lambda[110] or with dairy phages[117]. Bioinformatics analyses support the idea that temperate phages (and prophages) undergo frequent HGTs, while mosaicism is still present but seems less crucial for virulent phages[118], which form clustered viral populations. Mavrich and colleagues showed that phages have two evolutionary modes with distinct rates of HGT[63]. Virulent phages typically fall into the low gene content flux category while temperate phages tend to be distributed in both low and high gene content flux categories. Another study (discussed also below) showed that if we represent phage relationships and gene exchanges as a big web, we find temperate phages at its center[119], connecting groups of virulent phages located on the periphery. Thus, temperate phages function as banks for HGT[119].

Evolutionary relationships between phages also differ by host

Along with lifestyle, the rate and differential manner in which phages appear to exchange genetic material depend on their hosts and which environments they thrive in[63] (Fig. 5). Additionally, groups of phages infecting the same host can either form discrete genotypic clusters, an uninterrupted genetic continuum or something in between[120]. For example, despite regular exchanges of photosynthesis genes by homologous recombination, cyanophage genomes still differentiate into stable discrete groups[86,121,122]. Virulent dairy phages infecting *Streptococcus thermophilus* have likely recombined with phages infecting other lactic acid bacteria species[123] and follow a high gene content flux despite their lytic lifestyle[124,125]. Mycobacteriophages fall into the "something in between" category as they are grouped in clusters and display an overall continuous spectrum of diversity. However, intra-cluster diversity and discreetness are highly variable and temperate mycobacteriophages evolve in both the low and high gene content flux[63,126]. More phages with other nucleic acid types (ssDNA and RNA) and that infect other bacteria still need to be characterized and sequenced. This will help to elucidate any possible universal patterns in viral evolutionary relationships, confirm the existence of discrete populations in nature, and verify whether or not they are the result of insufficiently sampled environments[127].

A network representation of phage phylogeny

Phage phylogeny has undergone several changes in the past two decades. Classification was initially based on morphology and traditional phylogenetic trees were used to visualize evolutionary relationships. With the rapid increase of viral metagenomics, a plethora of phage sequences were discovered without the determination of the virion morphology. It also became clear that no single gene or protein was found in all phage genomes, making it difficult to build a tree based on a single shared genomic feature[128]. In addition, phylogenetic trees cannot support the combinatorial nature of phage genomes[119]. Therefore, an alternative way to visualize phage phylogeny is to use networks, with nodes corresponding to phage genomes and edges representing similarities at the gene, protein or genome level. This was first shown by Lima-Mendez and colleagues in 2008, using a set of 306 phage genomes[119]. In their network, temperate phages were shown to be much more closely interconnected, whereas virulent phages were on the periphery, forming discrete clusters. The path from one virulent phage cluster to another had to pass through temperate phages in the center of the network. Gene-sharing networks were further explored on the complete dsDNA virosphere (eukaryotic and prokaryotic viruses)[129]. Supermodules were identified within the network that grouped phages according to their ICTV-based family, although some modules contained phages belonging to different families. Another advance in phage phylogeny is the development of vConTACT[16,130,131], a software that classifies viruses to build a network (Fig. 5). Already at its second version (vConTACT2[131]), this program extracts predicted proteins from each viral genome to build viral protein clusters, which is then used to calculate genome similarities between each pair of viruses. Genome pairs with a similarity score above a given threshold become linked by an edge and the viral cluster formation is performed by a program that can disentangle complex network relationships and delineate clusters. With this approach, the authors showed that viruses can be accurately clustered at the genus level and that the more the virosphere is sampled, the more robust the network will become.

Conclusion

Phage diversity operates differently depending on what aspect of phage biology is investigated. Nucleotide and gene content are extremely diverse in phage genomes, while protein structures are highly conserved among different phage families. We also highlighted recent work on viral metagenomics and how it contributed to the discovery of perhaps the most abundant phages in marine and gut environments. Viral metagenomics also expanded our knowledge of phage diversity in diverse ecosystems and the confines of this global diversity[132]. This ever-expanding catalogue of new phage sequences called for a reflection on how to best adapt the current viral taxonomy to properly classify phages discovered through metagenomics[133]. Phages are also interconnected from an evolutionary perspective and several factors drive higher or lower rates of gene exchanges. These complex phylogenetic relationships are more accurately represented by a network rather than a traditional tree, and the former may be better suited to define new phage genera, subfamilies and families[16].

We also want to emphasize the success of the SEA-PHAGE educational program, which contributed to the isolation, characterization and sequencing of the largest collection of phages infecting the same host. In an era when a plethora of new phages with completely new sequences are being discovered, this model highlights the importance of integrating phage research at the various teaching levels, which benefits both the students and the scientific community. As more phages are discovered, the better the community will be at catching more of them and elucidating the viral dark matter. Adding more phage sequences to reference databases will help identifying a larger diversity of viral sequences from metagenomes. Resolving the structure of more viral proteins will also provide additional insights into the existence of a common ancestor, as intermediate ancestors within the lineage may be uncovered. Finally, network-based phylogenies will be improved when more phage sequences will be added, as it will help clustering more accurately phage groups that are poorly-sampled at the moment[130].

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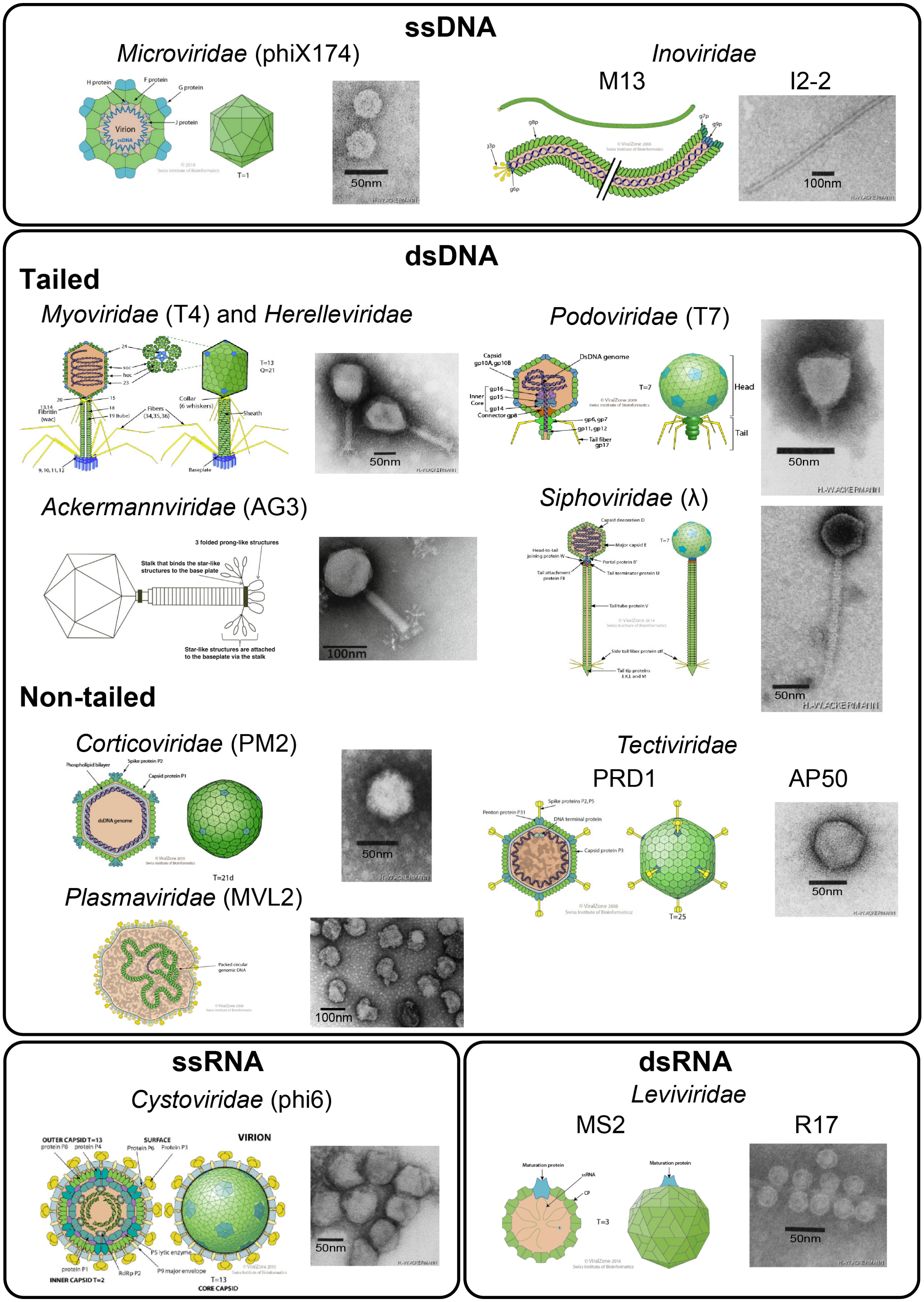

ssDNA
Microviridae (phiX174)
H protein
F protein
G protein
J protein
Virion
ssDNA
T=1
50nm
Inoviridae
M13
I2-2
g8p
g3p
g6p
g7p
g9p
100nm
dsDNA
Tailed
Myoviridae (T4) and Herelleviridae
24
soc
hoc
23
20
15
18
19 (tube)
13,14
Fibritin
(wac)
Fibers
(34,35,36)
9,10,11,12
T=13
Q=21
Collar
(6 whiskers)
Sheath
Baseplate
50nm
Podoviridae (T7)
Capsid
gp10A,gp10B
DsDNA genome
Inner
Core
gp16
gp15
gp14
Connector gp8
gp6,gp7
gp11,gp12
Tail fiber
gp17
T=7
Head
Tail
50nm
Ackermannviridae (AG3)
3 folded prong-like structures
Stalk that binds the star-like structures to the base plate
Star-like structures are attached to the baseplate via the stalk
100nm
Siphoviridae (λ)
Capsid decoration D
Major capsid E
Head-to-tail joining protein W
Portal protein B
Tail attachment protein FII
Tail terminator protein U
Tail tube protein V
Side tail fiber protein stf
Tail tip proteins
J,K,L and M
T=7
50nm
Non-tailed
Corticoviridae (PM2)
Phospholipid bilayer
Spike protein P2
Capsid protein P1
dsDNA genome
T=21d
50nm
Plasmaviridae (MVL2)
Packed circular genomic DNA
100nm
Tectiviridae
PRD1
AP50
Penton protein P31
Spike proteins P2,P5
DNA terminal protein
Capsid protein P3
T=25
50nm
ssRNA
Cystoviridae (phi6)
OUTER CAPSID T=13
protein P8
protein P4
SURFACE
Protein P6
Protein P3
VIRION
P5 lytic enzyme
protein P1
RdRp P2
P9 major envelope
INNER CAPSID T=2
T=13
CORE CAPSID
50nm
dsRNA
Leviviridae
MS2
R17
Maturation protein
ssRNA
CP
T=3
Maturation protein
50nm

Fig. 1 Phage classification based on morphology and genome nucleotide type composition. A schematic representation (SR) and an transmission electron micrograph (TEM) are shown for each morphology. *Microviridae* have an icosahedral capsid and small circular ssDNA genomes (SR and TEM of phiX174). The genome of filamentous phages of the *Inoviridae* family is composed of a circular supercoiled ssDNA molecule which is packed in a long filament (>500 nm) composed of thousands of MCP[13,134] (SR of M13 and TEM of I2-2). Most of the characterized phages are tailed with double-stranded DNA (dsDNA) genomes and belong to the *Caudovirales* order. To date, five families have been described for this order: *Myoviridae* (long contractile tails, SR and TEM of T4), *Podoviridae* (short non-contractile tails, SR and TEM of T7), *Ackermannviridae* (*Myoviridae* morphology with tail spikes at the base of the tail, SR and TEM of AG3) and *Siphoviridae* (long non-contractile tails, SR and EM of lambda). *Herelleviridae,* although an official family, shares the same morphology as *Myoviridae* and the two were merged in the figure. *Corticoviridae* have double-stranded circular DNA genome and capsids composed of an internal lipidic membrane surrounded by MCP (SR and TEM of PM2). *Tectiviridae* (SR of PRD1 and TEM of AP50) have an icosahedral capsid, which contains a linear double-stranded DNA genome and an internal lipidic membrane. Viruses belonging to the *Plasmaviridae* family (SR and TEM of MVL2) have a circular double-stranded DNA genome surrounded by lipidic envelope and no capsid. *Cystoviridae* have tri-segmented double-stranded RNA genomes contained in a spherical capsid (SR and TEM of phi6) with three structural layers: an outer lipidic membrane and a two layers inner capsid. *Leviviridae* have dsRNA genomes encoding only four proteins (MCP, replicase, maturation and lysis proteins) and capsids with icosahedral and spherical geometries (SR of MS2 and TEM of R17). Photo credit for EM goes to the late Prof. Dr. Hans-Wolfgang Ackermann (available at www.phage.ulaval.ca), with the exception of *Ackermannviridae* (from Adriaenssens et al. (2012) with permission) and *Plasmaviridae* (from Poddar et al. (1985) with permission).

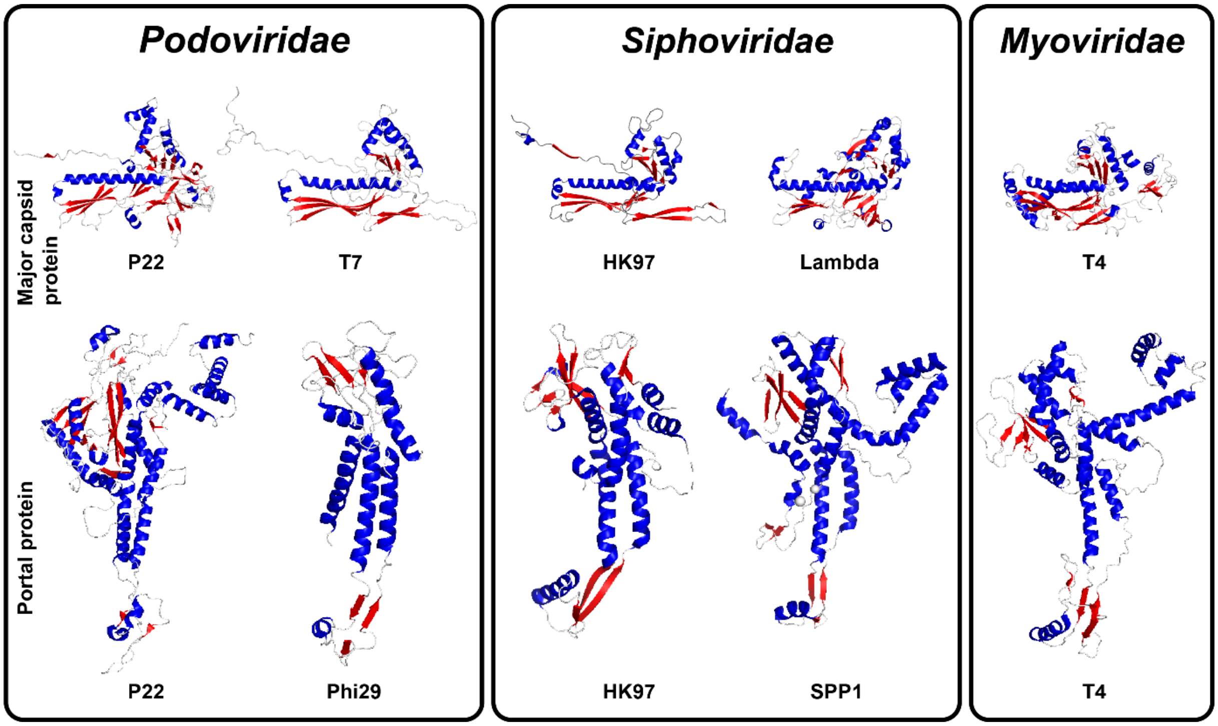
Box 1. Traces of a common origin

Despite extensive gene exchanges, which generate diversity, and the absence of homology at the nucleotide and amino acid levels for most phage pairs, we observed a finite and relatively small number of different virion structures. This raises the question as to whether these structural similarities can be explained by divergent or convergent evolution. A divergent evolution would indicate that viruses share a common ancestor and have diverged beyond detectable sequence homology, while maintaining the basic architecture of their structural proteins. A convergent evolution would suggest that viruses share no common ancestors, but rather have converged toward a structure that is particularly optimal to build a virion. While both can lead to a single common trait, the accumulation of similar structural characteristics seems to point toward the divergent evolution hypothesis and the existence of a common ancestor.

First, the *Tectiviridae* phage PRD1 MCP fold is highly similar to that of the archaeal virus STIV[135] and the mammalian adenovirus[34]. The MCP is a trimeric protein made of two eight-stranded jelly rolls (β-barrels). There are four different ways to fold such jelly rolls, but that one is only seen in these viruses[136]. Other features shared between PRD1 and adenovirus, include a linear dsDNA genome with inverted terminal repeats, the organization of the MCP on the capsid surface and the structure of spikes at the virion surface[137]. Other viruses are shown to have a PRD1-like structure, such as *Tectiviridae* infecting Gram-positive hosts (PRD1 infects Gram-negative hosts), *Corticoviridae*, eukaryotic and archaeal viruses[138]. This above suggests a common ancestor to PRD1-like viruses.

Second, a relationship also exists between tailed dsDNA phages, the archaeal virus HSTV-1[54] and herpesviruses[139]. The MCP of these viruses has a common fold, called the HK97 fold. Several other structural similarities exist in HK97-like viruses, such as the presence of a portal on one vertex of the capsid and their capsid assembly pathways[140]. A third case of similarities involves *Cystoviridae* phage phi6 and phi8 with eukaryotic viruses belonging to *Reoviridae* (blue tongue virus, BTV) and *Totiviridae*[141]. These dsRNA viruses share a similar inner coat protein[142] and have a segmented genome packaged in a double-shelled capsid[137].

Such structural resemblances between viruses infecting hosts spanning all three domains of life provide clues toward understanding the origin of viruses. Based on the previous examples of common ancestors, it has been proposed that viruses form polyphyletic lineages (PRD1-like, HK97-like and BTV-like) in contrast with the monophyletic origin of cellular life[143,144].



Tertiary structure of capsid or portal proteins protomers found in *Podoviridae*, *Siphoviridae* and *Myoviridae*. The HK97-like capsid protein structures were determined by X-ray diffraction for phages HK97 (PDB accession no. 10H6), T4 (PDB accession no. 1YUE) and lambda (PDB accession no. 3BQW) or cryo-EM for phages P22 (PDB accession no. 5UU5) and T7 (PDB accession no. 3J7W). The structure of the portal protein protomers was determined by X-ray diffraction for phages phi29 (PDB accession no. 1FOU), SPP1 (PDB accession no. 2JES), HK97 (PDB accession no. 3KDR) and P22 (PDB accession no. 3LJ4) or cryo-EM for phages T4 (PDB accession no. 3JA7). The coloring scheme used is based on secondary structures: red, β-strands; red, α-helices; grey, loops.

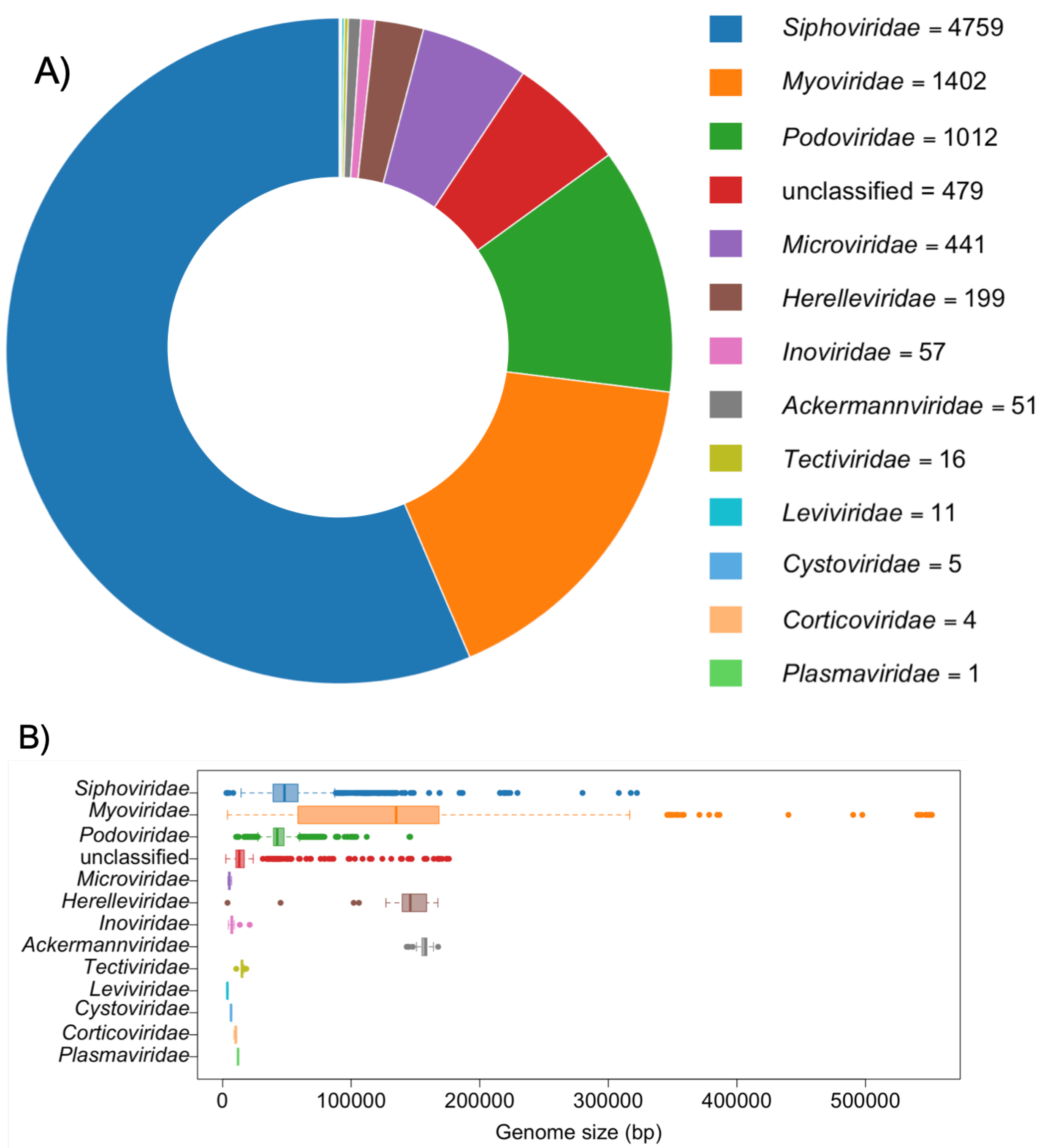


Fig. 2 Number of complete genomes (A) and genome size distribution (B) in each phage family as of September 2019 available in the NCBI Nucleotide database. The assignment of each phage to a family was done with the NCBI Taxonomy database. The unclassified group combines "unclassified *Caudovirales*", "unclassified dsDNA phages" and "unclassified bacterial viruses". This group is the fourth largest, emphasizing the increasing number of phages discovered through viral metagenomics for which no family can be assigned based on sequence information. Among the *Caudovirales* order, *Herelleviridae* and *Ackermannviridae* are the most homogenous families in terms of genome size. This is most likely because these two families were created after genomic analyses rather than morphological similarities.

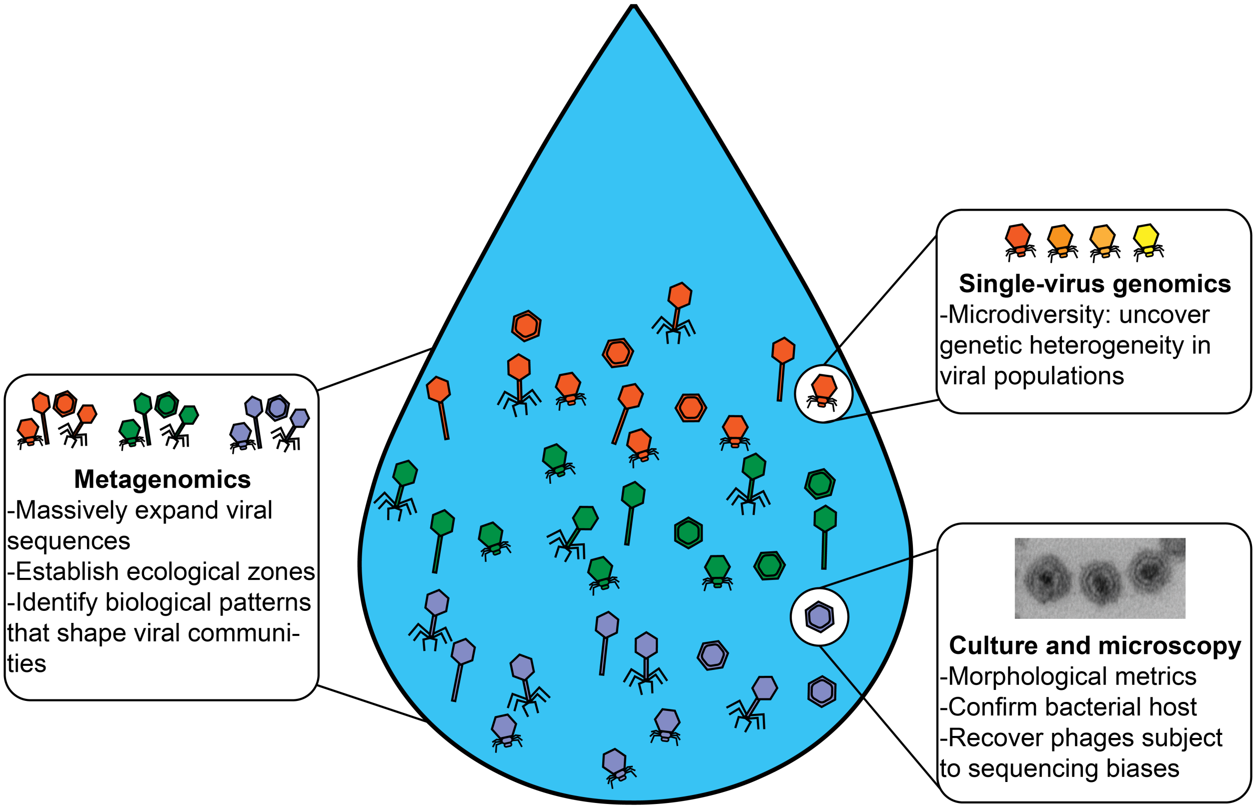

Metagenomics
-Massively expand viral sequences
-Establish ecological zones
-Identify biological patterns that shape viral communities
Single-virus genomics
-Microdiversity: uncover genetic heterogeneity in viral populations
Culture and microscopy
-Morphological metrics
-Confirm bacterial host
-Recover phages subject to sequencing biases

Fig. 3 Integrating metagenomics, single-virus genomics, culture and microscopy to explore the viral dark matter. Several techniques have been developed to characterise phage diversity in biological communities, mostly from marine samples[66]. We focus here on techniques that do not require previous knowledge and that *a priori* can characterize the entire community. Metagenomics deliver the largest diversity of phages, with up to thousands of viral populations being identified[11]. Single-virus genomics enables sequencing of individual virions[80]. This helps to reveal phage populations with high levels of microdiversity (represented here by different shades of orange in the podovirus), which normally impede genome assembly in metagenomics pipelines. Culturing techniques combined with observations through a transmission electron microscope permit the discovery of phages otherwise subject to sequencing biases.

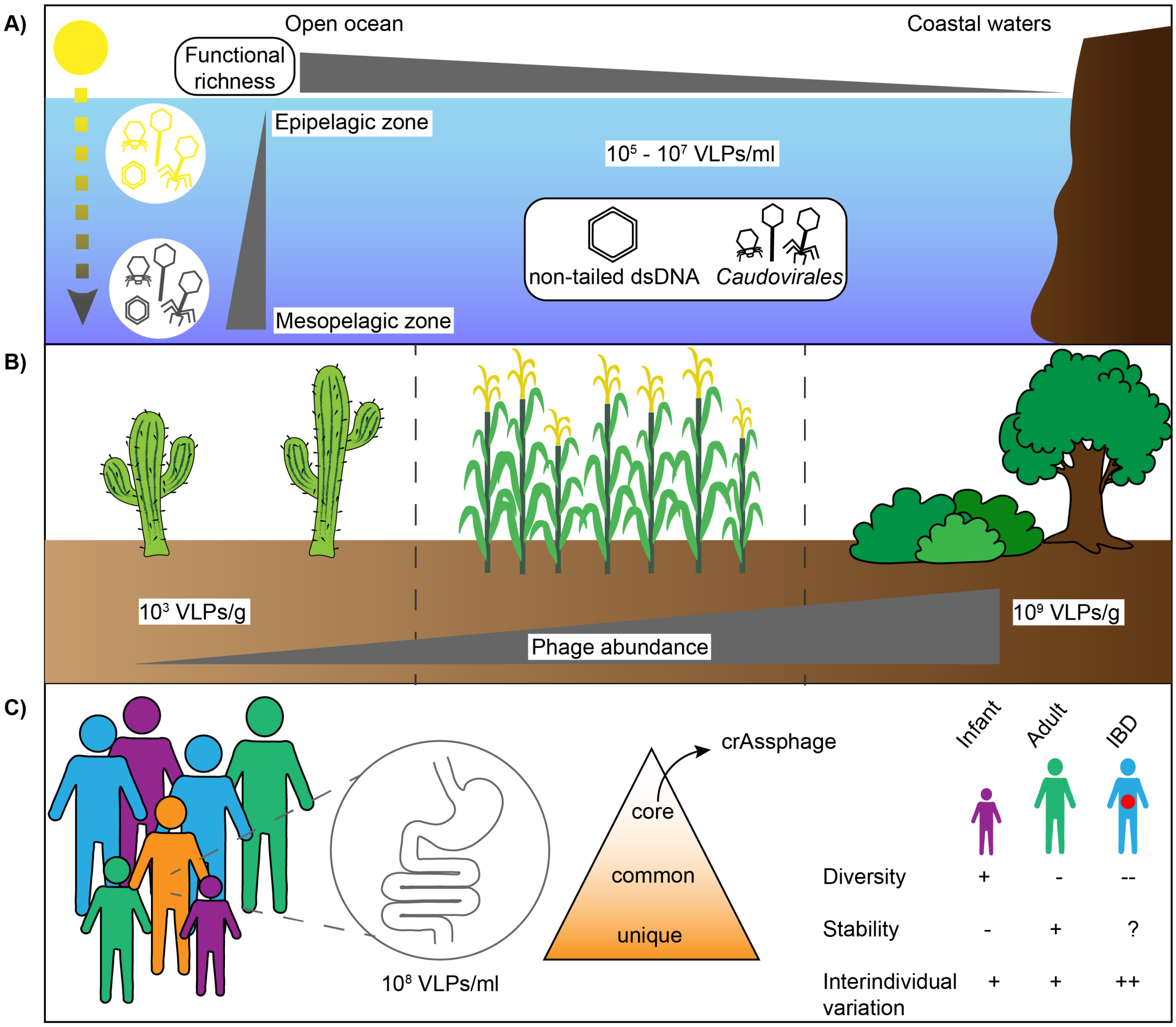


Fig. 4 Phage distribution and abundance in three ecosystems. A) Phages in the marine environment are extremely abundant with a virus to bacteria ratio often ranging from 1 to 100. qTEM of marine samples indicated that non-tailed phages are much more represented than tailed phages, which was also confirmed by metagenomic data[6,145,146]. Furthermore, phages from the mesopelagic zone were distinct from phages isolated from the epipelagic zone regarding gene content, life histories traits and temporal persistence[147]. Similarly, functional richness was observed to decrease from deep to surface water and with distance from the shore for surface water only[69]. **B)** Phage abundance in the soil is also highly variable and correlates with biomes types, pH and bacterial abundance. Indeed, viral abundance is the lowest in hot desert, intermediate in agricultural soils and the highest in forest and wetland soils[67]. Viral abundance also positively correlates with bacterial abundance in the soil and negatively correlated with pH, with phage counts decreasing at higher pH. **C)** The phage community in the human gut is mainly composed of members of the *Caudovirales* and *Microviridae* and a large majority of these phages remain unclassified[75,103,104]. Phage composition is essentially unique to individuals, with global metagenomic analysis indicating that some phages are globally distributed[75,76,103,104]. The phage community is also stable during time, but rapid changes are observed in early life[8]. Changes in the diversity and composition of the human virome were also reported to be related to the gut health status, particularly in the case of inflammatory bowel disease (IBD)[77,148].

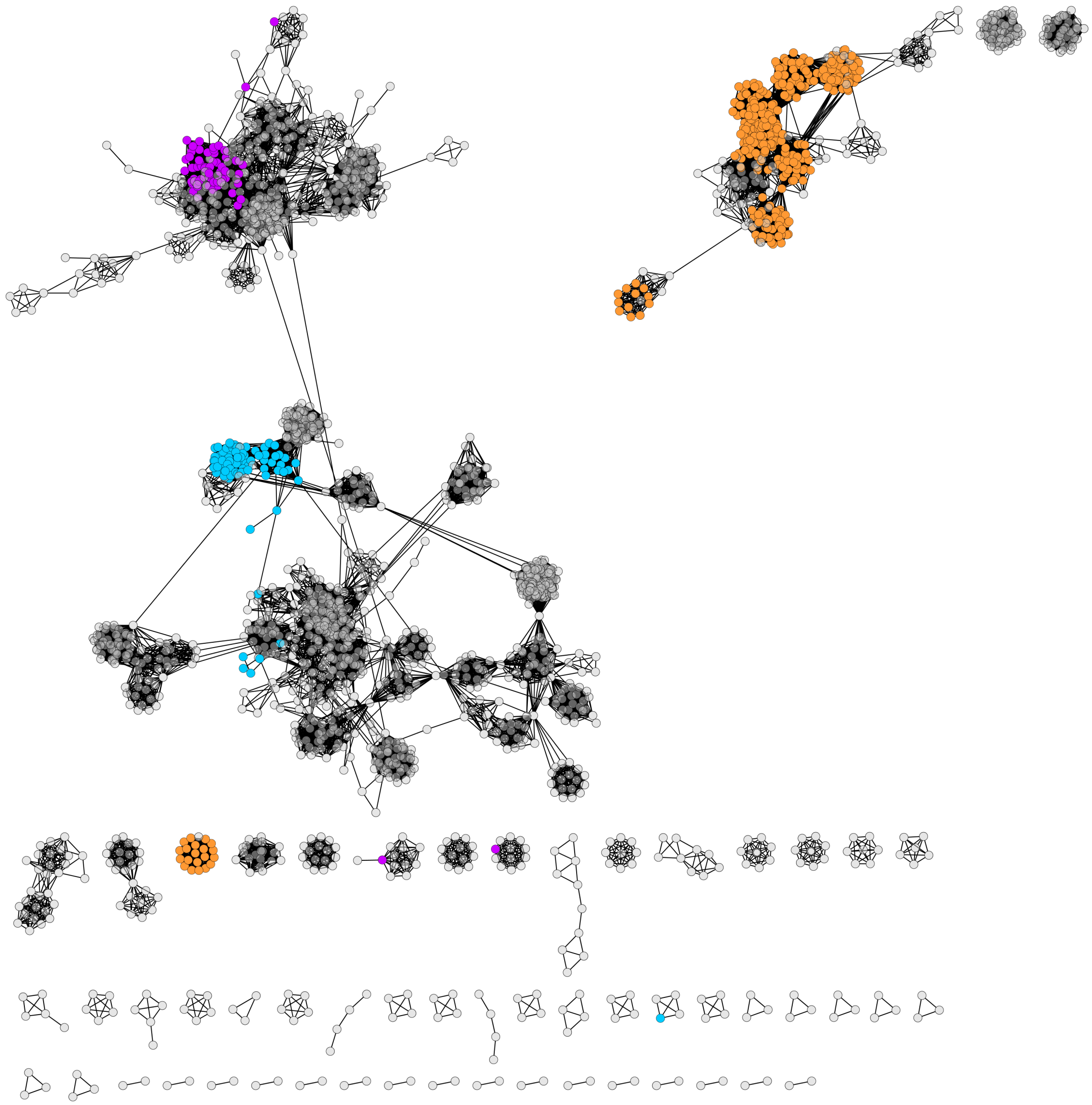


Fig. 5 A network representation of phage phylogeny. vConTACT2 was used to establish relationships between phages and visualization of the network was done with Cytoscape v3.7.1. This network comprises 2,617 RefSeq phage genomes, each represented by a node. An edge represents a connection between two nodes (genomes) based on the number of shared protein clusters. Purple, blue and orange nodes correspond to phages infecting *Streptococcus*, cyanobacteria (*Synechococcus* and *Prochlorococcus*), and *Mycobacterium*, respectively. Mycobacteriophages dominate the cluster on the upper-right side of the figure, which has no edges connecting the super-cluster on the left. *Streptococcus* phages are located in a densely connected area of the upper part of the super-cluster, in accordance with their high genetic flux. Cyanophages are more in periphery of the lower part of the super-cluster, consistent with a low genetic flux. Traditional phylogenetic trees are used with the assumption that phages follow a linear evolution. A network-based phylogeny improves our understanding of phage evolutionary relationships, as it gives information on horizontal gene transfers, which are pervasive in phages, and is therefore more representative.

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Glossary terms:

Lysogen: Bacterial cell containing a prophage.

Lysogenic cycle: Replication strategy where a phage enters a host cell, integrates its genome in the bacterial chromosome and replicate at the rate of cell division. Once integrated, a temperate phage is called a prophage. Upon an environmental stressor, the prophage can be induced, excised from the bacterial chromosome and enter the lytic cycle.

Lytic cycle: Replication strategy where a phage takes control of the host cell to replicate its genetic material, produce its structural components, self-assemble to form new virions and burst (lyse) the cell to release new viral particles.

Mosaicism: Observation that different regions (genes, gene blocks) of the phage genomes have distinct evolutionary histories, due to horizontal gene transfer events.

Temperate phage: Phage that can perform either a lytic or lysogenic mode of replication.

Virulent phage: Phage that can strictly undergo a lytic mode of replication.

Viral metagenomics: Sequencing genomes of the viral fraction in a sample.