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Current Opinion in Virology

The global virome: not as big as we thought?

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Viruses likely infect all organisms, serving to unknown extent as genetic vectors in complex networks of organisms. Environmental virologists have revealed that these abundant nanoscale entities are global players with critical roles in every ecosystem investigated. Curiously, novel genes dominate viral genomes and metagenomes, which has led to the suggestion that viruses represent the largest reservoir of unexplored genetic material on Earth with literature estimates, extrapolating from 14 mycobacteriophage genomes, suggesting that two *billion* phage-encoded ORFs remain to be discovered. Here we examine (meta)genomic data available in the decade since this provocative assertion, and use 'protein clusters' to evaluate whether sampling technologies have advanced to the point that we may be able to sample 'all' of viral diversity in nature.

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Viral abundances and the Virus–Host Ratio: Viral-like particles from stain-based counts are routinely ~10-fold more abundant than microbes which suggests there are about 10^{31} viruses on Earth [1]. These particles are of great importance ecologically as they impact microbes, which fuel planet Earth [2], through mortality, horizontal gene transfer and modulating global biogeochemical cycles [3]. Evolutionarily, intriguing hypotheses now exist suggesting that some RNA viral groups may predate eukaryotic supergroups [4], while others may represent a fourth domain of life [5] and still others have contributed to the evolutionary trajectory of global photosystems [6]. Pragmatically, viruses in nature are incredibly hard to study with even basic, stain-based counts first, including 'fake viral particles' [7] and missing some viral types [8] that may be a substantive fraction of those in nature [9,10], and second, not linking viruses to their hosts (but see emerging methods [11–13]).

In spite of these 'count' challenges, host range assays and emerging theory also support the conclusion that multiple viruses infect any given bacterium [14], and genomics is beginning to delineate how different viruses infecting a particular bacterium might be. For example, one study [15••] showed that at least 12 viral *genera* infect *Cellulophaga baltica* strains and re-evaluates available genomes to show that this is also the case for non-marine heterotrophic hosts (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*), but not for marine cyanobacteria where only three cyanophage genera infect marine *Prochlorococcus* and *Synechococcus*. If more than one virus infecting a bacterium is constant throughout the microbial realm, and if there is not significant overlap of viruses from one host to another (an open question), then it is easy to suggest that viral genetic diversity should be high — perhaps balanced by smaller genome sizes to be on par with that of their bacterial hosts.

Again, problematically, estimating viral diversity is complicated due to the lack of a universal genetic marker. Estimates from metagenomes using assembly-based methods suggest that there are between 532 (Arctic Ocean) to 129 000 (British Columbia) viral types in 10–150 L of seawater [16]. These highly variable estimates makes global extrapolations challenging, but the underlying data also have issues (reviewed in Ref. [17]) that could drastically impact an estimate of viral richness that relates back to a single time point and sample.

Regardless, viruses are clearly abundant and their high rates of mutation (though little data for environmentally relevant viruses exist) leave us expecting that new genomic technologies will reveal extensive sequence diversity as the global virome is sampled. Such metagenomic data should greatly impact the ever-widening scope of viral evolutionary studies. As a discovery tool, metagenome-derived sequences can be phylogenetically analyzed using core gene sets, analogous to 16S analysis, to document novel clades in well-studied lineages [18]. Additionally, metagenomics can recover novel, full virus genome sequences [19–21] which provide opportunities to test spatio-temporal evolutionary theories by sampling naturally occurring communities over space and time [22].

Rampant mosaicism and the homogeneity of viral genomes: To counter this expectation of extensive sequence diversity, horizontal gene transfer (HGT) may homogenize viral diversity. In fact, *Caudovirales* evolutionary theory suggests that rampant mosaicism blurs taxonomic boundaries, a hypothesis predominantly derived from

observations in siphovirus genomes [23]. Broadly, phage genomes are thought to evolve through accumulations of HGT events involving transcriptionally autonomous genetic units (called 'morons') that introduce variation and only remain if offering a fitness advantage. Beyond 'morons' other mobile elements impact phage genomes ranging from promoter stem loops (PeSLs, transcriptionally autonomous with consensus-flanking sequences [24]) to more traditionally characterized elements such as homing endonucleases [25], introns [26], and transposons [27], and there are likely more to be discovered. Additionally, identical phage gene sequences occur globally and phage infection can cross biomes; this has led to the hypothesis that phages have high dispersal rates and access to a global gene pool [28].

With so many mechanisms seeking to homogenize genomes, it is hard to imagine that any vertical evolutionary signal remains in phage genomes. However, HGT has not blurred all evolutionary phage lineages. Again, among *Caudovirales*, T4-like phage genomes [29,30] and metagenomic contigs [31•] are predominantly syntenic with much variation captured in niche-defining hypervariable islands similar to those observed in microbes [32,33]. Further, phylogenomic approaches show that most (~80%) T4-like phage core genes are vertically inherited [34•], suggesting that at least some genes resist HGT. Outside T4-like phages, though not as formally tested, similar vertically inherited core gene sets are emerging for T7-like cyanophages [35], and seem probable for the nearly invariant, small ssDNA [19] and RNA [20] viral genomes assembled from metagenomes.

Finally, since delineating an ecologically and evolutionarily meaningful unit to 'count' is fundamental for studies in nature, virology could learn from parallel research efforts in microbes. Specifically, there has been significant interest in the role of HGT and whether it blurs microbial species boundaries. The current view is that ecological and genetic species can be defined (reviewed in Ref. [36••]) and arise when new alleles (mutations) or genes (HGT) sweep through a population in conjunction with ecological differentiation, where subsequent HGT events would help to maintain cohesion rather than disperse it [37]. Notably, there are many parallel and competing microbial species concepts, but such careful and meticulous empirical and theoretical work on microbes in nature is constrained predominantly to study of an r-selected, copiotroph (*Vibrio* sp.). Thus application of these evolutionary principles may not be straightforward for other microbes featuring different lifestyles. Similarly, variable lifestyles were recently observed in marine phages [12••], with evolutionary implications for rates of HGT being more important when comparing across viruses that span temperate and lytic lifestyles (due to increased accessibility of prophage genomic DNA).

The protein cluster as an organizational tool to explore viral sequence space

Viral metagenomes are dominated by the 'unknown', so a critical advance has been to organize unknown viral sequence space into protein clusters (PCs, [38••,39,40]), again taking the nod from our microbiology colleagues [41]. The PCs approach is valuable as a universal metric for comparing diversity of viral assemblages, and as a community resource will provide each first, a 'handle' to apply OTU-based ecological theory, independent of known function, using new and expanding community tools (e.g., QIIME, http://qiime.org/), and second, an informatics structure to allow propagation of functional data to novel 'unknown' proteins. Finally, PCs serve as a metric to estimate how well viral sequence space has been sampled given the genomic and metagenomic data that have been accumulated in the decade since Rohwer's estimate of two billion viral-encoded proteins [42•].

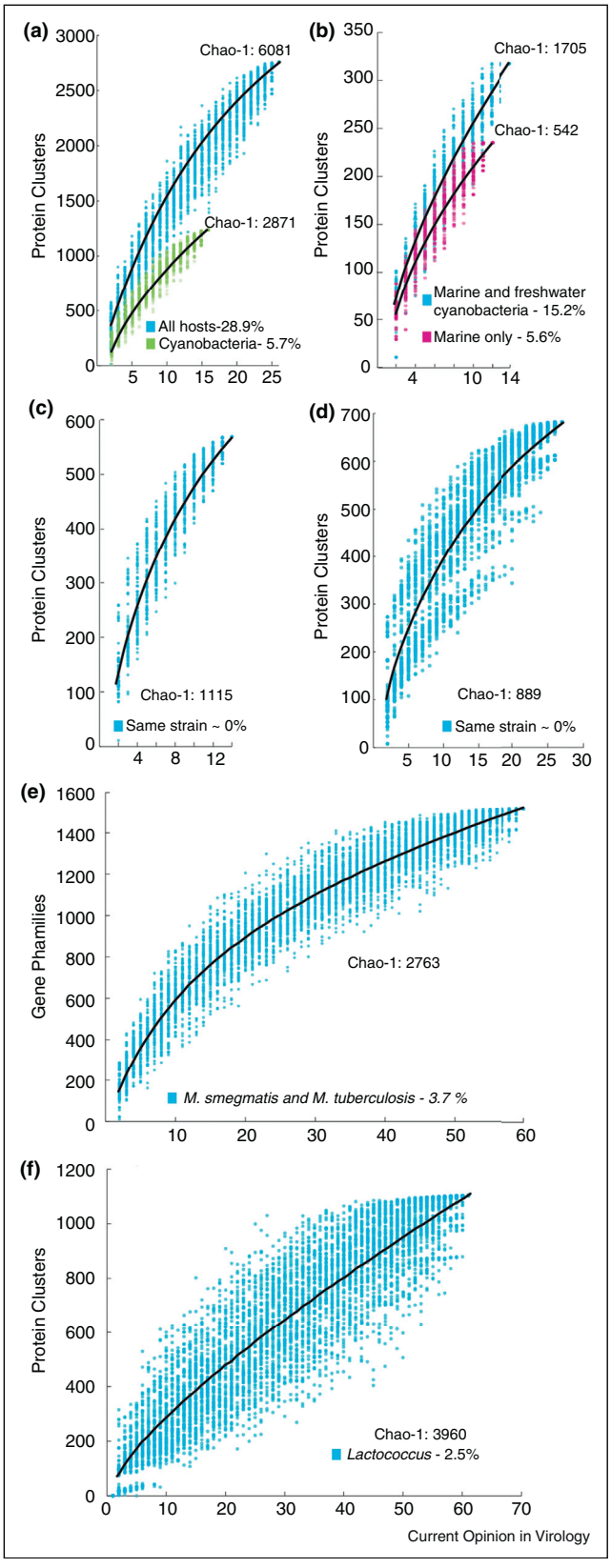
An alternative organization scheme to PCs is Phage Orthologous Groups [43]. POGs are grounded in the evolutionary concept of the conservation of ortholog function (as opposed to the relative evolutionary freedom of duplicated paralogs), and leverage phylogeny and heuristics to define sets of genes thought to share function across different organisms. These heuristics (e.g., reciprocal best blast hit) are required for scaling analyses to modern datasets and are relatively accurate compared to more sophisticated phylogenetic approaches [43]. However, the assumption that orthologs are more likely to share function than paralogs should be considered for each new research question [44].

Pragmatically, PCs and POGs are complementary approaches, with PCs perhaps most valuable for monitoring the expansion of sequence space and POGs best utilized to document phage-specific functional space expansions [45] that might be used to test 'phageness' of metagenomes — thus providing a new dimension in viral metagenomic analyses. Looking forward, as computational approaches scale, protein structure is a valuable trait in viral evolution and taxonomy [46] that will enable detection of functional similarity across evolutionarily distant lineages using information beyond the primary sequence used by PCs and POGs.

Using phage genomes to estimate global viral sequence diversity

Given that the most abundant hosts available in nature are bacteria, it is thought that the most abundant viruses infect bacteria-termed phages. Deeply genome-sequenced phage groups include the cyanophages (T4-likes [29], T7-likes [35], siphoviruses [47]), the mycobacteriophages (siphoviruses and myoviruses [48]), phages that infect *Staphylococcus* [49], *Pseudomonas* [50] and *Lactococcus* [51]. Here, by sequentially evaluating each

Figure 1



new gene in a genome against the genes already observed, we calculate 'collectors curves' or 'rarefaction curves' of PCs to document how well sampled the 'flexible' or 'pan' genome is in each phage group.

These analyses revealed a pan genome estimate that ranged from a few hundred to a few thousand PCs per phage–host system examined (Figure 1). While highly controversial, the number of microbial species is thought to be ~6 million globally [52], and we calculate here that the viral pan genome associated with any particular group of bacteria is a few hundred to a few thousand PCs. Assuming that viruses do not infect or share genes across host species, then the global virome should be on the order of 0.6–6 billion protein clusters — very similar to Rohwer's decade-old estimate of two billion proteins [42•]. A violation in either of those assumptions of host range and shared genes would reduce this total.

Using metagenomes to estimate global viral sequence diversity

Metagenomics is often used to survey nature beyond that can be observed through culture-based observations. The Pacific Ocean Virome (POV) dataset represents the largest viral metagenomic survey to date and is prepared in a quantitative manner using a highly efficient iron-chloride concentration method [53], as well as purification steps [40] and linker amplified library construction [54] that have well understood biases. The POV dataset is comprised 32 metagenomes obtained from four regions in the Pacific Ocean that represent a relatively wide range of ecological characteristics from the coastal-to-open and surface-to-deep oceans [38••]. Its value for ecological study is already apparent having helped enumerate the most abundant ocean viruses observed to date (pelagiphages [55]).

Notably, the initial description of the POV dataset (Figures 3 and 4 in [38••]) shows a non-linear relationship between sampling effort and PC accumulation, which suggests that sampling is approaching saturation in the Pacific Ocean pelagic viral sequence space (Figure 2). In fact, ~422 K PCs were observed in the POV dataset [38••], with extrapolation suggesting a total of 516 K to

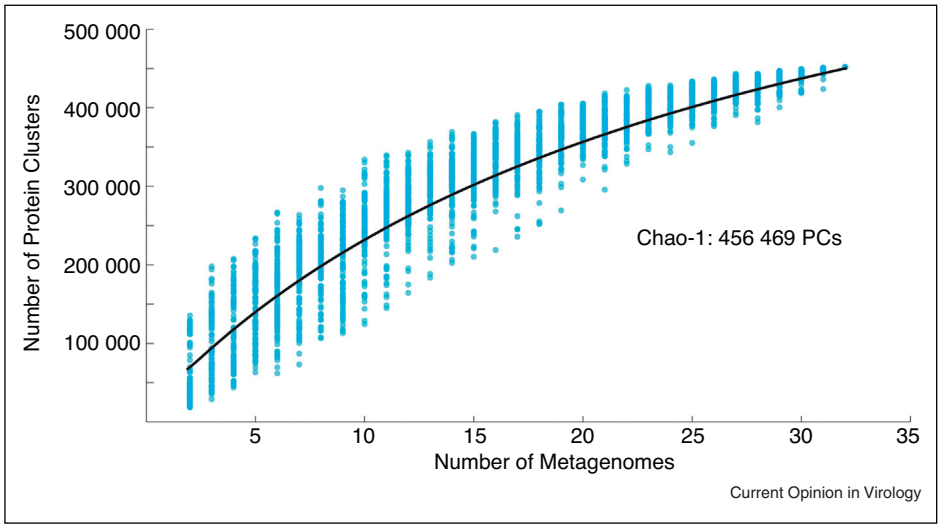
(Figure 1 Legend) Viral pan-genomes seem an endless source of novel genes. Genomic comparisons of A.T4-like Myoviruses (26 genomes), B. T7-like cyanopodoviruses (14 genomes), C. *Pseudomonas* phages (14 genomes), D. *Staphylococcus* phages (27 genomes), E. *Mycobacterium* phages (60 genomes) and F, *Lactococcus* phages (61 genomes). Each dot corresponds to the number of protein clusters when *k* members are sampled (from $k = 2$ to n). Only 1000 randomizations of the process are shown. Trend lines correspond to the average of 10 000 repetitions. Chao-1 is calculated as: observed + $[(\text{Singletons})^2]/(2 \times \text{Doubles})$. Microbial % in each panel corresponds to the 16S gene divergence of the host strains used for isolating the phages. The multiple trajectories in panel D reflect the fact that multiple phage groups are pooled in these analyses.

Table 1

Richness estimation from metagenomics. Four functions [58] were used to fit the accumulation curve observed in Figure 2

	Function	POV metagenome	
		Asymptote (95% CI)	*R*-Squared
Michaelis–Menten	$Y = (a \times x)/(1 + (b \times x))$	1 313 853 (963 153–3 232 913)	0.8181
Negative exponential	$Y = a \times (1 - \exp((-b) \times x)$	516 600 (508 400–524 700)	0.999
Rational	$Y = (a \times (b \times x))/(1 + (c \times x))$	691 957 (678 691–710 620)	0.9981
Hyperbolic	$Y = (a \times x)/(b + x)$	737 200 (680 400–793 900)	0.9894

Figure 2



Exploration of viral protein sequence space by means of PCs suggests near saturation. Protein cluster rarefaction curve of the number of protein clusters that metagenomic comparison totals calculated as described in Figure 1.

1.3 M PCs depending upon the model used for fitting the accumulation curve (Table 1). This suggests that between 32 and 82% of the possible pelagic Pacific Ocean virus proteins have been sampled. Further, the oceans are thought to harbor about 33% of the total microbial species on Earth [52]. Assuming the same is true for viruses then this suggests that there are likely to be no more than 3.9 M PCs (the 1.3 M maximum × 3-fold more species) in the global virome — or nearly three orders of magnitude less than the two billion previously estimated [42•].

Conclusions

There undoubtedly remains much diversity to be discovered in the world of viruses. However, the large discrepancy between genome-derived and metagenome-observed PC diversity requires consideration of its underlying causes. First, ascertainment bias is high as only a fraction of the phage–host systems and environments that occur in nature are sequenced. Even in the relatively well-sampled oceans, pelagic Pacific Ocean waters do not represent the diversity of possible ocean environments. As well, recent studies show that firstly, cultures do not represent the dominant morphotypes in the ocean [9], and secondly, roughly half of the viruses in the oceans (RNA viruses, [10]) and the larger-genome giant viruses [56] would not be captured using the methods that generated the POV dataset. Notably, RNA viruses are abundant, but their genomes appear small and of low diversity; in contrast, giant viruses are much less abundant, but their genomes are large and quite diverse. With so little data on these viral groups their contribution to the global virome is a big unknown. Second, the Chao1 non-parametric richness estimator is flawed when the shape of the 'rare tail' in the rank abundance curve is not well described — so much so that it is not reliable for comparisons across samples [57]. This is likely a significant problem for the genomic data, but less so for the metagenomic data examined here. Third, metagenomic PC observations are reliant upon open reading frame predictions, which are not very good in viruses (smaller ORFs, overlapping ORFs, smaller genome sizes limit algorithm 'training') and could lead to underestimations. These are unlikely to be order of magnitude underestimations but may be a factor. Fourth,

viral genomic content and host ranges may be more commonly shared across hosts than is currently recognized. This would decrease global virome estimates by an unknown factor, and new methods should enable culture-independent [11,13] and higher-throughput [12••] linkages to be made between viruses and hosts. Obtaining a better estimate of the global virome will clearly require filling knowledge gaps across all of these factors. However, it seems likely that viral sequence space, while large, is unlikely to approach the two billion genes estimated from 14 genomes a decade ago.

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