Disentangling the origins of virophages and polintons

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Highlights

- Extensive horizontal gene transfer occurs in the eukaryote/giant virus/virophage/transposon system.
- Virophages and polintons have a shared but obscured ancestry.
- We outline the evidence for a virus first origin for both polintons and virophages.
- Future studies that target narrower questions about specific genes or particular lineages will reveal more than overarching theories
- Paleovirological techniques can be used to test alternative hypotheses and uncover plausible virophage-polinton intermediates

Abstract

Virophages and polintons are part of a complex system that also involves eukaryotes, giant viruses, as well as other viruses and transposable elements. Virophages are cosmopolitan, being found ranging from the Amazon River to Antarctic hypersaline lakes, while polintons are found in many single celled and multicellular eukaryotes. Virophages and polintons have a shared ancestry, but their exact origins are unknown and obscured by antiquity and extensive horizontal gene transfer. Paleovirology can help disentangle the complicated gene flow between these two, as well as their giant viral and eukaryotic hosts. We outline the evidence and theoretical support for polintons being descended from viruses and not vice versa. In order to disentangle the natural history of polintons and virophages, we suggest that there is much to be gained by embracing rigorous metagenomics and evolutionary analyses. Methods from paleovirology will play a pivotal role in unravelling ancient relationships, horizontal gene transfer and patterns of cross-species transmission.

Introduction

The inhabitants of the microscopic world of giant viruses, their eukaryotic hosts, virophages, and various transposable elements (TEs) interact in a tangled web of interrelatedness, parasitism and coevolution. Giant viruses, can reach up to 1500nm wide and harbour genomes over a megabase long. They are part of the nucleocytoplasmic large DNA viruses group (NCLDV), that share a compliment of six ‘core genes’ [1–3], and includes families such as the *Poxviridae* and *Phycodnaviridae*. Several of the largest NCLDVs contain genes once considered exclusively cellular which has been taken as evidence in favour of a reduced-cell explanation for their gigantism [4]. However, genome expansion from smaller viruses via horizontal gene transfer (HGT) and genomic duplication is more consistent with the phylogenetic and genomic evidence, such as the presence of recently acquired host translational machinery in the Klosneuviruses [5]. Furthermore Poxviruses, which are also NCLDVs, are known to adaptively expand their genomes rapidly by up to 10%, albeit only transiently [6].

Giant virus genomes have been colonised by TEs [7,8], and phylogenies reconstructed from different genes reveal conflicting topologies that indicating an extensive history of HGT. This complexity is reflective of their ecology, where giant viruses interact with both their hosts and associated satellite viruses known as virophages. All viruses are obligate parasites, but satellite viruses take parasitism a step further in that they require coinfection by a helper virus to replicate. The first virophage to be discovered, dubbed ‘Sputnik’, is associated with the giant virus *Acanthamoeba castellanii* mamavirus (ACMV) [9]. Like other giant viruses (and many NCLDVs), ACMV creates dense cytoplasmic structures known as viral factories. The term virophage is derived from bacteriophage, as Sputnik hijacks the replication machinery of these viral factories, rather than the native replication machinery of the host cell and has a negative impact on helper virus replication [9].

Virophages are dsDNA viruses but they do not appear to be closely related to other dsDNA viruses. Their morphogenesis genes, such as their pox-like A32 packaging proteins, place them in the diverse PRD1-adenovirus lineage [10], but some possess unrelated genes such as retroviral-like integrases, and they also contain genes of unknown function, some of which have no known homologues [11]. Interestingly however, there are remarkable similarities between virophages and a family of DNA transposable elements initially named mavericks but now more widely referred to as polintons [12]. Polintons are an atypical group of large TEs that are able to self-replicate using their own polymerase and integrase genes, hence the name 'Pol-Int-on' [13–15]. Although there are larger and smaller examples, most polintons are ~15-22 kbp in length [16,17] which is comparable to that of virophage genomes. While virophages and polintons are extremely diverse, both at the nucleotide level and in terms of their genomic content, they nonetheless share multiple genes indicative of shared ancestry [12,17,18].

The interactions between all the players in this tangled microscopic world are far from resolved, and the nature of the relationship between virophages and polintons is elusive. The clear indication of shared ancestry is set against a backdrop of frequent HGT that complicates attempts to identify distinct lineages, and the antiquity of their evolutionary interactions obscures the processes that shaped their combined natural histories [16,18,19]. However, because polintons are anciently integrated into host genomes, the relative evolutionary stability (in terms of mutation rate and frequency of HGT events) allows us to use techniques developed in paleovirology for the study of ancient viruses. As the field progresses and more data become available, we will be able to integrate observations from phylogenetic reconstruction and genomics. This will be informative for both virophages and polintons separately, but also in combined analyses that will reveal aspects of their shared evolutionary history. The synthesis of such analyses can additionally be contextualised with evidence from NCLDV evolutionary reconstruction and that of their eukaryotic hosts.

This paleovirological approach has previously been effective in resolving similarly complex evolutionary histories, such as that of a retrovirus with mammalian origins that has also integrated into farmed poultry, a herpesvirus, and poxvirus [20]. Similarly, a paleovirology approach was deployed to identify multiple gene transfer events between an unusual retroviral superantigen gene and three different herpesviruses, via two separate mammalian hosts [21]. In this paper, we review the prevailing evidence and opinions about the origins of

virophages and polintons with a paleovirological perspective. We consider the extent to which different aspects of their relatedness can be explained by their evolutionary interactions with giant viruses and eukaryotic hosts.

Virophages

Since the discovery of Sputnik, other virophage families have been discovered, primarily in metagenomic data [12,22–30]. Most virophages appear to have circular double stranded DNA genomes ranging from ~17-29 kbp, but the presence, order, and orientation of most genes are not well conserved between proposed families. Only a fraction of virophages have been experimentally validated and associated with a giant virus. Sputnik has been observed *in vivo*, and its presence in viral factories results in dysfunctional and abortive ACMV virions. This reduces the yield of ACMV by up to 70% and decreases host cell lysis threefold [9]. Although distantly related to Sputnik, mavirus similarly inhibits its host giant virus Cafeteria roenbergensis virus (CroV) and increases *C. roenbergensis* survival [12]. The exact mechanisms behind how Sputnik and mavirus hijack the viral factories are unknown, and since most virophages are only known from metagenomic data, it is not certain how many of them rescue the eukaryotic host. In contrast, the Zamilon virophage, a close relative of Sputnik, does not inhibit APMV reproduction or cell lysis [26]. It is conceivable that like the subviral satellite hepatitis D [31], a virophage could lead to increased morbidity and mortality in the eukaryotic host. It also is unclear how other players in the system, such as TEs, affect the outcome of the interaction between virophages, giant viruses, and eukaryotes.

Polintons

While giant viruses and virophages only infect single celled eukaryotes, polintons and polinton-like elements (PLEs) have been described in a diverse array of single celled and multicellular eukaryotes, from cnidarians to water moulds. Given how distantly related some of the hosts are, and the notable absence of polintons in mammals and terrestrial plants [16,17,32], their taxonomic distribution suggests frequent novel integrations, extensive HGT between different kingdoms/super groups and the repeated loss in descendant lineages of an ancient integration. Indeed, phylogenetic analysis indicates a mix of both vertical and horizontal transmission [16]. This pattern is not unlike that of endogenous retrovirus phylogenies, which contain both horizontally and vertically transmitted insertions [33]. As paleovirology focuses on such questions of gene flow, it could be used to quantify the relative

133 extent of these two processes via a statistical comparison of polinton and host phylogenies.

135 **Virophages $\rightleftharpoons$ Polintons**

136 While convergent evolution and HGT could explain some of their similarities, extensive
137 phylogenetic and genomic evidence linking virophages and polintons makes a compelling
138 case for the hypothesis of shared ancestry, for which there is broad consensus [19,32,34].
139 However, this leads to questions on the directionality of gene flow and whether the ancestor
140 of both entities was originally a TE that resembles contemporary polintons, or if a viral
141 ancestor gave rise to virophages and their genomic relatives. Conclusive evidence for either
142 scenario has yet to be unearthed, and arguments for both have been proposed (Figure 1). In
143 the TE-first scenario, virophages are the descendants of ‘escaped’ polintons that recombined
144 with an unknown virus to acquire a capsid (Figure 1A) [10]. An argument against this is that
145 because virophages are dependent on co-infection by giant viruses, while polintons are not,
146 the loss of dependency following integration is more readily explained than its arising *de novo*
147 in a DNA transposon [12,18]. Furthermore, there are seven genes in mavirus with similarity
148 to polinton genes, compared to only three potential homologs with other virophages like
149 Sputnik. This suggests that mavirus may be more closely related to polintons than it is to
150 other virophages, indicating that gene flow went from virus to TE, and that a virus is
151 ancestral to both groups [12,18,19].

Figure 1.
Two proposed scenarios for the origins of polintons. A) An unknown virus (grey icosahedron) infected a cell and recombined with pre-existing polintons. The polintons then escaped as virophages (purple icosahedron). B) A virophage (purple icosahedron) integrated into the genome of a cell and was then transmitted vertically, as a standard EVE, differentiating along with its hosts. In both cases polintons subsequently descend primarily vertically as do virophages.

Furthermore, there is experimental evidence of gene flow from a virophage to its host, which supports the virophage-first hypothesis. The spontaneous integration of mavirus into *C. roenbergensis* has been observed *in vitro*, where they remain quiescent until coinfection by CroV results in the production and eventual release of mavirus [35]. Unlike with normal mavirus infection, this does not seem to protect the individual cell initially infected with CroV, but it does protect the wider population from lysis, suggesting that endogenous virophages can act as antiviral defence systems in clonal protist populations [35].

This co-opting of endogenous viral elements (EVEs) for defence is known as EVE-derived immunity (EDI) and has been described in over a dozen cases in multicellular organisms [36]. As well as experimental observations, transcriptionally active genes of putative virophage origin, dubbed virophage like elements (VLEs), are incorporated in the genome of the algae *Bigeloviella natans* [37]. No known giant virus has been observed parasitizing *B. natans*, so any defensive function is speculative. Nevertheless, VLEs could be indicative of a historical confrontation with an ancient giant virus [37], and techniques from paleovirology are well-placed to investigating this. VLEs are in essence EVEs, and their presence gives us clues as to what sort of viruses ancestral *B. natans* had to contend with. It would be interesting to use functional paleovirology [38] to reconstitute virophages from VLEs to test their host giant virus range. Although they are the only two reported cases of virophage/VLE integration in eukaryotes, the cases of *B. natans* and *C. roenbergensis* provide evidence of an evolutionary scenario in which polintons arose from integrated virophages that were initially evolutionarily maintained as EDIs [35,37]. However, even if some polintons arose from EDIs, others could have randomly integrated without substantial effect on the host, and drifted or hitchhiked to fixation [39].

Paleovirological discoveries of additional VLEs combined with evolutionary analysis of these integrated viral sequences can differentiate these two scenarios. With this amount of HGT, the questions must be examined at the level of individual genes. Designating 'core genes' in virophages [10,19,40] will be a challenge, because the discovery of new taxa may whittle down their universality. Nonetheless, identifying widely conserved genes will facilitate answering specific questions, such as mapping the connections between polintons and virophages. For instance, integrase is going to be crucial as it is frequently found in both, even if we cannot include virophages where it is absent.

Networks and Trees

Different papers have presented phylogenetic trees with varying levels of disagreement, depending on which genes are used and which taxa are being sampled - even the monophyly of polintons and virophages is not consistent [12,19,29,32,41]. The matter is complicated further by the fact that virophages have open reading frames (ORFs) with no known non-giant virus/virophage homologues [11]. Because of this, despite a plausible evolutionary scenario for a 'virus first' model being demonstrated (supported with data and experimental evidence [37,42]), the sequence of events in this chicken-and-egg scenario is unknown.

Reconstructing the evolutionary history of virophages will be central to disentangling the complex history of HGT between virophages, giant viruses, polintons, and their hosts. Their relatively small size and ability to integrate mean that virophages could facilitate HGT between giant viruses and their host eukaryotes. Genes taken from a giant virus by a virophage that is subsequently donated to a eukaryote could be viewed as ‘vicarious EVEs’ [18]. Indeed, genes of likely NCLDV origin have been discovered in land plants, despite there being no known exogenous NCLDVs that infect them [43,44]. The large size of the giant virus genomes would make integration of the entire viral genome without disrupting cellular functions difficult, which may explain why no giant viral EVEs have been identified. Nevertheless, large viral integrations can and do occur, such as herpesviruses in primates [45–47], phycodnaviruses in algae [48,49], as well as endogenized phycodnavirus segments [50,51].

One way to make sense of the tangled relationships between NCLDVs, eukaryotes, virophages and bacteriophages is by drawing gene networks [10,19,34]. While these accurately capture a general pattern of HGT between these entities, they provide limited insight into the underlying biology, phylogenetic history, or direction of transfer. Furthermore, these similarity-based approaches do not distinguish on the basis of gene function. For example, many virophages contain genes not found in other virophage lineages such as retroviral-like integrase in maverick viruses. Similarly, Sputnik appears evolutionarily linked to bacterial TEs carrying TV-Pol family genes (*e.g.* IS605 or SCCmec elements) [52], and has a phage-like bacterial transposase that is unrelated to integrases from maverick virus, autonomous polintons, and some PLEs [9]. Thus, in addition to recombination, this indicates convergent evolution that gene network approaches cannot distinguish.

Origins Of Virophages: Kicking The Can Down The Road

If polintons are EVEs, descended from an anciently integrated virophage (Figure 1B), this does not answer where virophages came from. A third hypothesis addressing whether virophages or polintons came first puts hypothetical ‘polintoviruses’ at the origin of not just virophages and polintons, but also NCLDVs, bidnaviruses, and adenoviruses [10,53]. Based on the presence of ORFs that are predicted to form the double jelly roll fold seen in virophage capsid proteins, it has been suggested that many elements classified as non-viral transposable elements (*i.e.* polintons) are actually *bona fide* viruses [54]. In this hypothesis, tectiviruses (bacteriophages infecting gram-negative bacteria) came into eukaryotes with the

capture of mitochondria [10,40]. Tectiviruses acquired cysteine protease and integrase from already extant transposons, and subsequently became polintoviruses, capable of existing exogenously and endogenously, while those that lost their capsid gene became intracellular polintons [40].

The polintoviruses hypothesis also suggests that these integrated tectiviruses escaped the nucleus via the acquisition of DNA dependent RNA polymerase and an mRNA capping system, forking into cytoplasmic plasmids via genome reduction and into NCLDV's via the replacement of POLB, and a 10-100 fold (or more) genome expansion [40]. Additionally, polintoviruses differentiated into other exogenous viruses, some of which began parasitizing and swapping genes with some NCLDV's [40].

Possession of a coat-protein encoding gene, as well the ability to form virions are the defining features of a *bona fide* virus. Polintoviruses have never been observed, and thus far, there is no indication that their putative capsid ORFs are anything more than endogenized remnants. Nonetheless, it is likely that the ancestor of both polintons and virophages was a virus, and future work remains to demonstrate what it may have resembled and to what extent it is also the ancestor of other lineages.

In depth gene-by-gene paleovirological analysis will unravel the natural history of polintons and virophages

Regardless of the direction of transfer, polintons are endogenous relatives of virophages. As such, they can be studied under a paleovirology framework in the same manner as EVEs. Over the last several decades, retroviruses have been systematically investigated using robust phylogenetics and genome-wide statistical analyses. This has revealed insights ranging from uncovering ancient patterns of cross-species transmissions across vertebrates [33,55–57], to identifying the many ways that retroviral genes have been co-opted to serve a beneficial function [58–60]. Moreover, the relationship between extant virophages and polintons is analogous to that between contemporary and ancient retroviruses. For instance like polintons, the diversity and range of ancient retroviruses far exceeds that of their contemporary cousins [55]. Such paleovirological surveys have enabled us to re-evaluate our understanding of groups like lentiviruses and foamy viruses, as well as identify the broad and influential effect they have on host genomes [58,60–62]. By applying the same paleovirology principles, we

will be able to reach detailed and statistically robust conclusions about specific groups of polintons, virophages, and/or the genes contained within them.

Also central to the study of viruses in paleovirology is the adherence to certain standards of data analysis and rigorous evidence, since most research depends on third-party genomic data collected under different conditions and assembled to varying degrees of accuracy. The recognition that metagenomics and paleovirology have an overlapping set of methodologies and goals is giving rise to novel approaches in viral discovery and analysis [47,63]. Most virophages identified thus far have only bioinformatic representation, having been mined from large-scale metagenomic studies. While these sequences are invaluable, we must be cautious and apply the same level of scrutiny to these findings as we would with novel endogenous viruses. This includes offering supportive evidence for valid assembly as well as distinguishing between integrations and ‘free-living’ viruses.

Conclusions

There is unlikely to be a unifying theory that can at once explain the lack of synteny between virophages and giant viruses, between different lineages of virophages, and between virophages and polintons. Although a relationship between polintons and virophages is clear, there remains a large gap that separates the groups in terms of phylogenetic similarity, genomic organisation and gene content. Therefore while their ancestor was likely viral, the nature and identity of this virus remains to be determined. A more piecemeal approach that can explain parts of the puzzle with less uncertainty will inevitably develop as we identify new lineages of virophages and polintons. Moreover, increased taxonomic sampling will also reveal transitional viral fossils that could help disentangle the entwined natural histories of genes and genomes, by combining the information from phylogenetic reconstruction and analysis of genomic organisation.

The application of techniques that synthesise analyses of both EVEs and viral genomes will help reconstruct very ancient evolutionary events, and paleovirology is well equipped to deal with remarkably large timescales. For example, such hybrid approaches have been used to trace the origin of retroviruses to the seas of the early Palaeozoic Era more than 450 million years ago [62]. However, we must also be prepared to recognise that if the history of these entities extends far beyond such timescales - possibly even preceding the last universal cellular ancestor – then the answers to some (or all) of the questions posed here may be out of reach.

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