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Mimivirus inaugurated in the 21st century the beginning of a reclassification of viruses

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Mimivirus and other giant viruses are visible by light microscopy and bona fide microbes that differ from other viruses and from cells that have a ribosome. They can be defined by: giant virion and genome sizes; their complexity, with the presence of DNA and mRNAs and dozens or hundreds of proteins in virions; the presence of translation-associated components; a mobilome including (pro)virophages (and a defence mechanism, named MIMIVIRE, against them) and transpovirons; their monophyly; the presence of the most archaic protein motifs they share with cellular organisms but not other viruses; a broader host range than other viruses. These features show that giant viruses are specific, autonomous, biological entities that warrant the creation of a new branch of microbes.

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The concept and definition of viruses during the 19th and 20th centuries

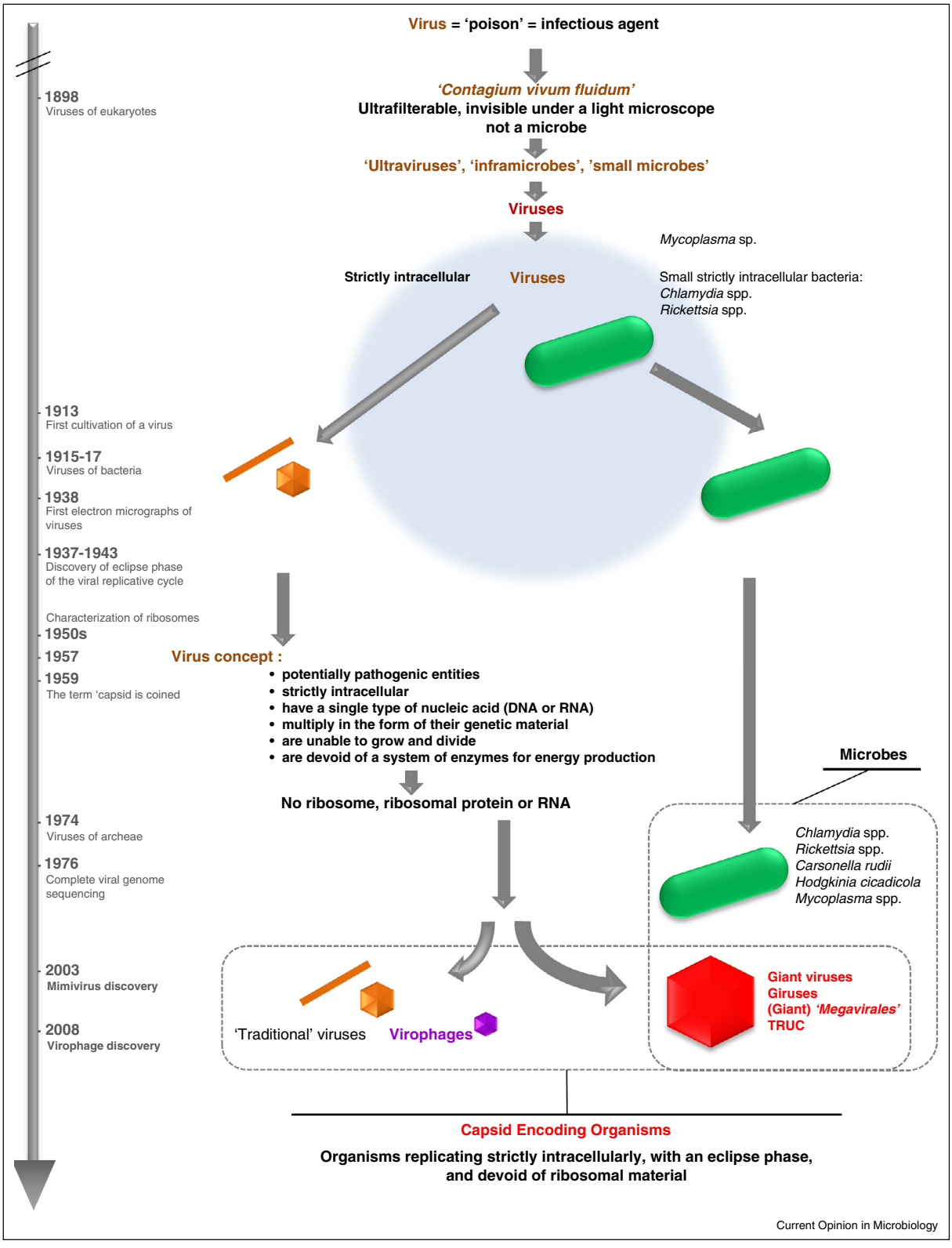
For a long time, the concept of 'virus' was muddled (Figure 1). The term 'virus' initially designated any infectious agent [1••]. During the 19th century, Pasteur and Roux considered the rabies agent as a microbe, although it was invisible under a light microscope [2]. Between 1886 and 1898, the foundations of virology were laid, with the discovery of causative agents of tobacco mosaic and foot-and-mouth diseases, which were ultrafilterable and invisible under light microscopy, in contrast to microbes [3–5]. Accordingly, these agents were named ultraviruses, or inframicrobes, and, eventually, viruses [1••]. During the 1910–1920s, viruses became increasingly established as small entities that need living cells to replicate; *Rickettsia* and *Chlamydia*, also intracellular parasites, definitively turned out not being viruses [6,7]. During the 1930–1940s, the first electron micrographs of virions were obtained [8] and the eclipse period of virus replication was discovered [9]. Then, during the 1950s, the virus concept was unravelled by A. Lwoff, based mainly on negative criteria [1••]. Lwoff defined viruses as potentially pathogenic strictly intracellular entities, which have either DNA or RNA, multiply in the form of their genetic material, are unable to grow and divide, and are devoid of energy production enzymes. Hence, viruses were considered as simple cell parasites consisting of a nucleic acid enclosed in a symmetric protein shell, the capsid [1••,7], and were, further, also shown to lack ribosomes [10].

Mimivirus challenges the definition of viruses

During the last 12 years, six new or putative families of giant viruses have been discovered through co-culture isolation, by inoculating environmental and human samples on amoebas. Mimivirus was the pioneer of this viral group [11••,12••]. Visibility under a light microscope and the Gram positivity of this virus, isolated in 1992 from cooling tower water, misled researchers into considering it as a bacterium. It was eventually revealed in 2003 to harbour a 0.5-μm-large icosahedral capsid and a 1.2-megabase pair (Mbp)-large genome with ≈1.000 genes [12••]. The discovery of Mimivirus led several groups to search for other giant viruses using amoeba co-culture. Subsequently, isolations of Marseillevirus [13], *Pandoravirus* spp. [14,15], *Pithovirus sibericum* [16], faustoviruses [17] and *Mollivirus sibericum* [18] confirmed the fruitfulness of this culture strategy. All these viruses were discovered in Marseille, France, by two different teams. Moreover, the first virophage (a Mimivirus-infecting virus) was also identified in this city. Strikingly, these viruses were isolated through strategies (co-culture on *Acanthamoeba polyphaga*, or *Vermamoeba vermiformis* for faustoviruses) implemented to grow microbes, and discovered by bacteriologists [19].

These giant amoeba viruses were linked through phylogenomics to other double-stranded DNA viruses including poxviruses, asfarviruses, asco-/irido-viruses, and phycodnaviruses, which were formerly the largest viral

Figure 1



Schematic of a brief history of virus naming and definition. The usage and significance of the term 'virus' changed over time. The definition of what a virus is evolved in different steps according to new discoveries and technologies. Giant amoeba viruses share numerous features with small intracellular microbes and stand apart from 'traditional' viruses, whose definition was mainly founded by Lwoff during the 1950s.

representatives and were shown in 2001 to share a set of 41 core conserved genes and grouped under the name of nucleocytoplasmic large DNA viruses (NCLDV) [12••,20••,21•]. Then maximum-likelihood reconstruction of the evolution of these viruses mapped a set of ≈50 genes on their putative ancestor [22••]. In 2012, it was proposed to classify giant amoeba viruses and NCLDV families in a new viral order, *Megavirales*, as these viruses have a common origin and virion architecture and share major biological characteristics, such as replication within viral factories [23••]. The term 'Girus' was also coined to designate these megaviruses, to underline their intermediate status between small parasitic prokaryotes and standard viruses [24]. Successive isolations of new *Megavirales* representatives continued to challenge previously established viral hallmark features and definitions. Simultaneously, the discovery of new giant viruses highlighted their diversity and ubiquity on earth and, for some, their presence in humans and, consistently, megaviruses related sequences were detected in environmental and human metagenomes [25,26]. The remarkable features of giant amoeba viruses challenged the virus paradigm and fuelled debates on the evolution, origin and the definition of viruses [12••,27••,28••]. In particular, their gene repertoire was greater than those of small bacteria and included homologs to cellular informational genes [12••,29••].

Why are giant viruses different from 'traditional' viruses?

Giant viruses display unique phenotypic and genotypic features that differentiate them from 'traditional' viruses and bring them close to some microbes, as these characteristics are considered as the hallmarks of cellular organisms (Figure 2).

Virion and genome size

Viruses have long been strictly understood as small infectious agents which are not visible under a light microscope and which can pass through 0.2 μm-pore filters [28••]. In contrast, *Megavirales* virions are ≈0.2–1.5 μm in size, *P. sibericum* being the largest currently [16,18]. This led Mimivirus and pandoravirus virions to be considered for a long time as a Gram-positive bacterium and parasitic endosymbionts, respectively [11••,15,19]. In addition, megaviruses display giant genomes at the scale of virions, from 105 (for an iridovirus) to 2474 kilo (k) bp (for *P. salinus*), whose size overlaps that of several cellular genomes [23••]. Particularly, giant virions that infect phagocytic protists have a diameter ≥200 nm and genomes >340 kbp that are predicted to encode for >400 proteins. It is notable that, when plotting the size of viral genomes available in the NCBI GenBank database, the curve comprises breaks around 350, 450, 600 and 1200 kbp (Supplementary Figure S1). The first break at 350 kbp indicates that there is a discontinuity in the genome size between 'traditional' viruses and giant amoeba viruses. The other breaks may suggest that the diversity of giant amoeba viruses is greater that currently apprehended.

Complexity

Giant viruses are more complex than 'traditional' viruses in terms of their nucleic acid and protein content. Thus, in contrast with most other viruses, megaviruses harbour both DNA and RNA, which includes messenger RNAs and transfer RNAs [23••,30]. In addition, proteomics identified dozens or hundreds of proteins inside giant virions, some of which are involved in transcription and translation, and a substantial proportion of which are hypothetical proteins [14,16–18,30]. These messenger RNAs and proteins may facilitate the first steps in the replicative cycle and make giant viruses far less dependent on their host for replication than other viruses.

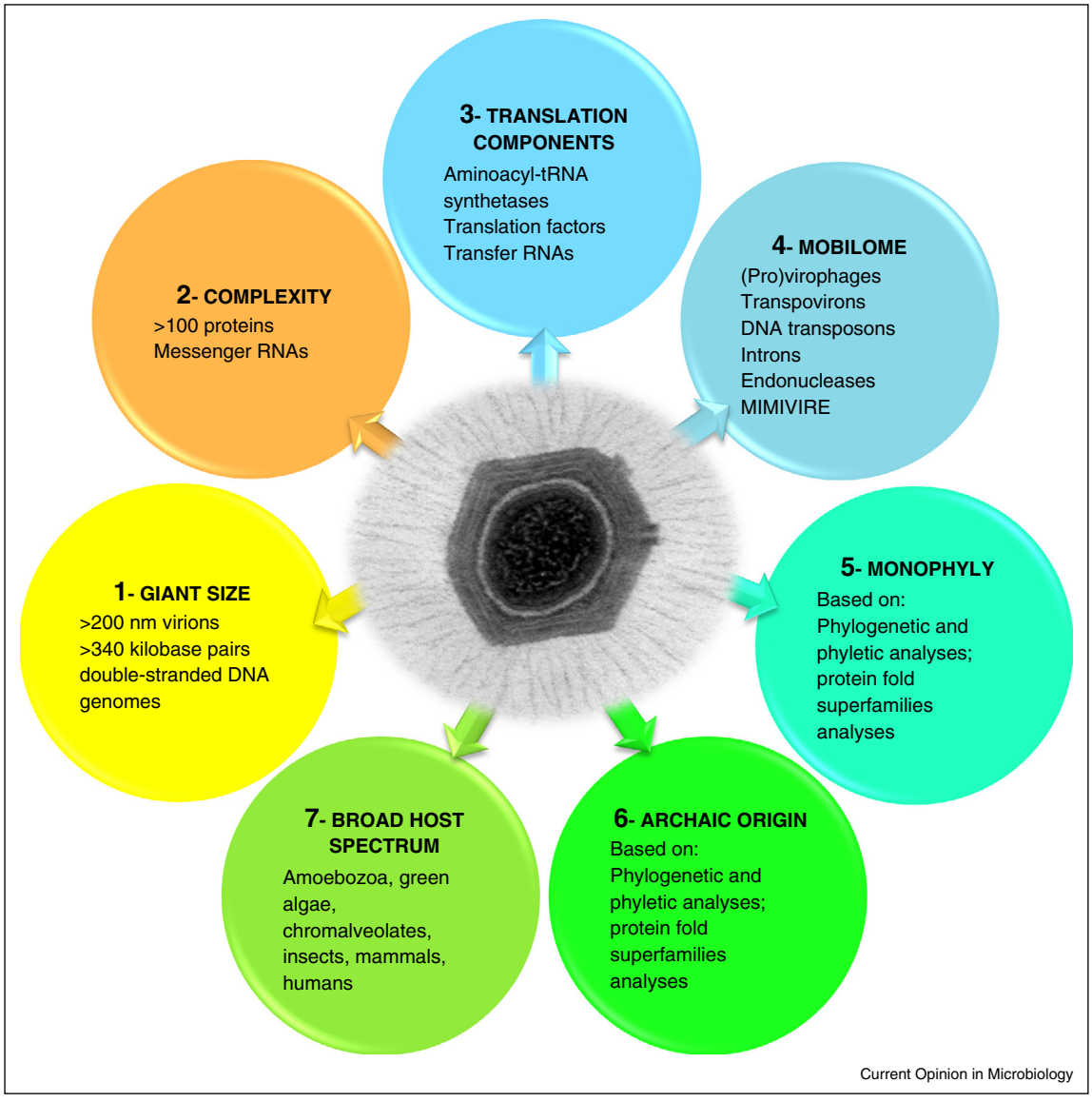
Presence of components of translation

The discovery of Mimivirus revealed the presence of translation factors including a peptide chain release factor eRF1, a GTP-binding elongation factor eF-Tu, two translation initiation factors, SUI1 and 4E, and four aminoacyl-tRNA synthetases, some of which were shown to be functional and expressed [12••,31]. Previously, only a gene encoding a translation elongation factor had been identified in phycodnaviruses [23••]. In addition, six transfer RNAs were detected [12••]. Genes encoding translation proteins and tRNA were then identified in the other giant amoeba viruses, with the exception of *P. sibericum* [16]. This is a very specific feature of these viruses, previously only observed in some phycodnaviruses, and some bacteriophages and herpesviruses for tRNA [12••].

Mobilome

Several group I and II introns were detected in conserved genes from giant viruses, whereas they are unusual in viruses [32]. Moreover, some megaviruses were revealed as having been themselves infected by other viruses, as are bacteria, archaea and eukaryotes [33••,34]. These virophages were shown to integrate into the mimivirus genomes as pro-virophages [35••]. In addition, transpovirons, a new class of transposable elements, were discovered in mimiviruses; they depend on these giant viruses for their replication and spread, and are analogous to virus-associated plasmids present in bacteria and archaea [35••]. Taken together, self-splicing introns, (pro)-virophages and transpovirons comprise a mobilome in mimiviruses. In addition, DNA transposable elements were detected in the *P. salinus* genome. Furthermore, amoeba mimiviruses were recently shown to harbour a defence system, named MIMIVIRE, which enables them to fight against infection by their virophages and is similar to CRISPR-mediated mechanisms of immunity against viruses deciphered during the past decade in bacteria and archaea [36••].

Figure 2



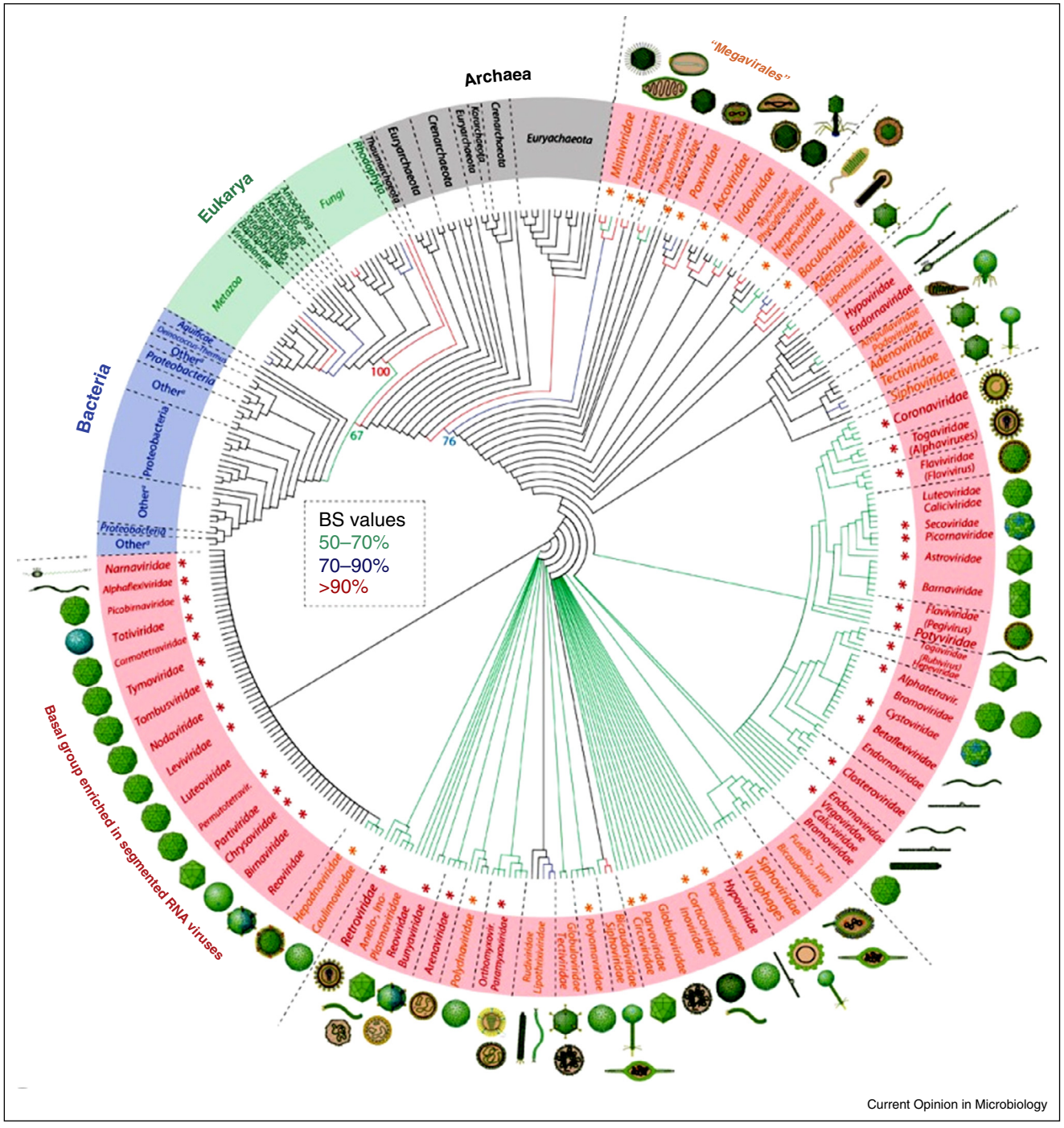
Main features specific to giant viruses compared to other viruses. The major differences between giant viruses and other viruses involve virion and genome sizes, complexity, presence of translation components, existence of a mobilome, monophyly, archaic origin and a broad host spectrum.

Monophyly

A major issue of controversy was whether or not these giant viruses comprise a new (i.e. fourth) branch in the tree of life, alongside *Bacteria*, *Archeaa* and *Eukarya* [12••,27••,29••,37]. From the onset, at the time of the Mimivirus analysis, it was put forward that it branched out near the origin of the *Eukarya* in a phylogeny based on seven conserved proteins [12••]. This observation was then strengthened by phylogenies of universal informational genes, including DNA-dependent RNA polymerase (RNAP) and DNA polymerase, which showed that *Megavirales* forms a strong monophylogenetic group apart from *Bacteria*, *Archaea* and *Eukaryotes* [29••,37]. These genes, particularly RNAP subunits 1/2, represent valuable markers to classify new *Megavirales* members and uncharacterised microbes [37]. The fourth branch encompassing giant viruses was not considered as an additional domain, as domains were defined by C. Woese based on ribosomal genes that are lacking in giant viruses. In addition to unique features exhibited by giant viruses, this led to this new branch of life being designated as a fourth TRUC, an acronym for Things Resisting Uncompleted Classification [38••]. The fourth branch of life hypothesis was criticised and considered artefactual by some teams, on the assumption that it relied on lateral gene transfers or convergent evolution [39,40]. It was also

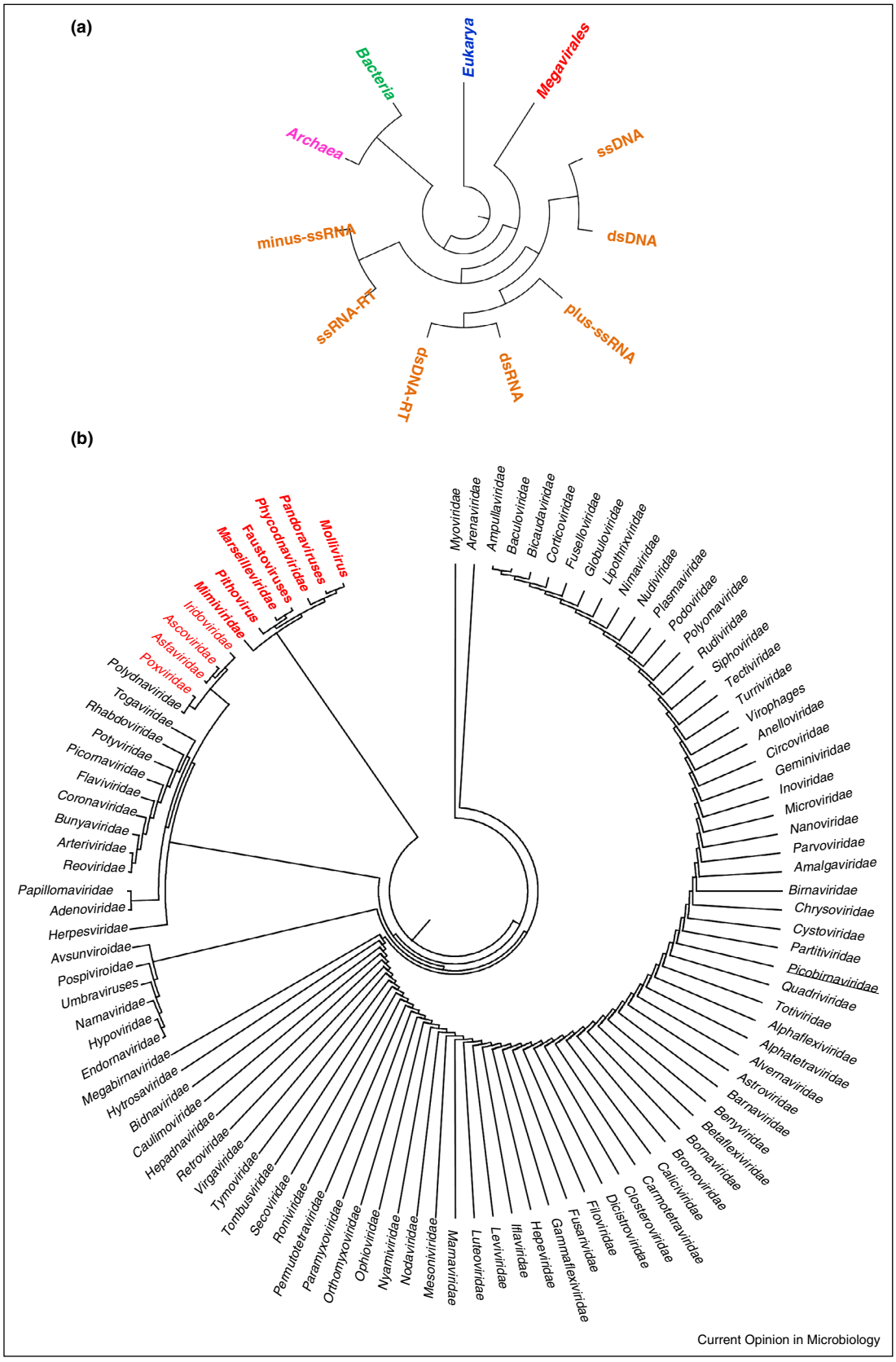
contested by E. Koonin and his team, whose interpretation of their phylogenomic analyses is that universal genes were gained by giant viruses from their eukaryotic hosts [41]. The view of J.M. Claverie and his team is more tempered and cautious [14,31]. In contrast, data from other teams argue for the existence of a fourth branch of life [42,43]. Thus, Wu *et al.* found some sequences in environmental metagenomes that existed in phylogeny reconstructions between the *Bacteria*, *Archaea*, and *Eukarya* branches, and may come from unknown viruses

Figure 3



Evolutionary relationships between viruses and cells based on proteomes tree. From [44], with permission. This tree of proteomes describes the evolution of 368 proteomes that were randomly sampled from cells and viruses and were distinguished by the abundance of 442 protein fold superfamilies shared between eukaryota, archeae, bacteria and viruses [44]. It shows that megaviruses are, among viruses, those that most deeply branch with cellular organisms.

Figure 4


(a)
Bacteria
Eukarya
Megavirales
Archaea
ssDNA
dsDNA
minus-ssRNA
plus-ssRNA
ssRNA-RT
dsDNA-RT
dsRNA
(b)
Mollivirus
Pandoraviruses
Phycodnaviridae
Faustoviruses
Marseilleviridae
Pithovirus
Mimiviridae
Iridoviridae
Ascoviridae
Asfaviridae
Poxviridae
Polydnaviridae
Togaviridae
Rhabdoviridae
Potyviridae
Picornaviridae
Flaviviridae
Coronaviridae
Bunyaviridae
Arteriviridae
Reoviridae
Papillomaviridae
Adenoviridae
Herpesviridae
Avsunviroidae
Pospiviroidae
Umbraviruses
Narnaviridae
Hypoviridae
Endornaviridae
Megabirnaviridae
Hytrosaviridae
Bidnaviridae
Caulimoviridae
Hepadnaviridae
Retroviridae
Virgaviridae
Tymoviridae
Tombusviridae
Secoviridae
Roniviridae
Permutotetraviridae
Paramyxoviridae
Orthomyxoviridae
Ophioviridae
Nyamiviridae
Nodaviridae
Mesoniviridae
Marnaviridae
Luteoviridae
Leviviridae
Iflaviridae
Hepeviridae
Gammaflexiviridae
Fusariviridae
Filoviridae
Dicistroviridae
Closteroviridae
Carmotetraviridae
Caliciviridae
Bromoviridae
Bornaviridae
Betaflexiviridae
Benyviridae
Barnaviridae
Astroviridae
Alvernaviridae
Alphatetraviridae
Alphaflexiviridae
Totiviridae
Quadriviridae
Picobirnaviridae
Partitiviridae
Cystoviridae
Chrysoviridae
Birnaviridae
Amalgaviridae
Parvoviridae
Nanoviridae
Microviridae
Inoviridae
Geminiviridae
Circoviridae
Anelloviridae
Virophages
Turriviridae
Tectiviridae
Siphoviridae
Rudiviridae
Podoviridae
Polyomaviridae
Plasmaviridae
Nudiviridae
Nimaviridae
Lipothrixviridae
Globuloviridae
Fuselloviridae
Corticoviridae
Bicaudaviridae
Baculoviridae
Ampullaviridae
Arenaviridae
Myoviridae
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[42]. In addition, Nasir and Caetano-Anolles showed, based on protein fold superfamilies (FSF), that giant viruses represent a distinct supergroup alongside *Archaea*, *Bacteria* and *Eukarya* [43,44••]. The same four branch topologies as obtained through phylogenies were generated through phyletic analyses of clusters of orthologous groups of proteins (COG) [29••,45]. In addition, such COG-based analyses show that megaviruses stand apart from other viruses (Supplementary Figure S2).

Archaic origin

Phylogenetic and phyletic analyses of informational genes and the study of FSF indicate that *Megavirales* members are, in evolutionary terms, very ancient in comparison with other viruses, and even with cells. The reconstructed *Megavirales* common ancestor was suspected to have an early origin, concomitant with eukaryogenesis [22••,46]. In addition, Nasir and Caetano-Anolles showed, based on protein FSF, that giant viruses coexisted with cellular ancestors, and phylogeny based on proteome trees showed that megaviruses are among the viruses that most deeply branched with cellular organisms (Figure 3) [43,44••]. In addition, FSF distribution among cellular organisms and viruses showed that giant viruses overlapped with many cellular organisms with parasitic and symbiotic lifestyles, such as *Mycoplasma* and *Proteobacteria*. The ten FSF identified as the most ancient in evolutionary terms (Nasir and Caetano-Anolles, personal data) were detected in megaviruses; in particular, the distribution among cellular organisms and viruses of the three most ancient FSF (namely, P-loop containing nucleoside triphosphate hydrolases, Ribonuclease H-like and DNA/RNA polymerases) and of another ancient FSF, a protein kinase-like, which are all found in >98% of megaviruses (Nasir and Caetano-Anolles, personal data), clearly showed that megaviruses are more similar to cells than to other viruses (Figure 4a).

Broad host spectrum

Compared to viruses from other orders or families, megaviruses infect a broad range of cellular hosts that belong to phylogenetically highly distant groups including invertebrates, mammals, amoebozoa, green algae, and chromalveolates [22••,47,48]. Mimiviruses, marseilleviruses and faustoviruses have been isolated or detected from different protists, insects, and mammals, including humans [26,48,49]. In addition, giant viruses that infect amoeba enter their host through phagocytosis, and Mimivirus was further shown to enter macrophages via a phagocytosis-like mechanism, thus acting like a bacteria [11••,13•,14,16–19,50]. This differs from entry mechanisms in 'traditional' viruses that involve specific interactions with cell receptors [50].

Other notable features

Other notable features of giant viruses include the presence of gene promoters in mimiviruses [51] and the presence of unique genes among viruses that are involved in DNA repair, protein folding, nucleotide synthesis, amino acid, lipid or polysaccharide metabolisms and protein modifications [12••,13•,14,16–18]. In addition, histone-like proteins are present in marseilleviruses [52]. Moreover, substantial proportions of ORFans are detected in the genome of giant viruses, ranging between 40 and 95% for those infecting amoeba; this suggests that giant viral genomes embed a large panel of unknown functions [14,16–18,53]. In addition, several phylogenies showed complex evolutionary histories, with genes being involved in horizontal transfers with other viruses and cellular organisms, and *Megavirales* genomes were described as mosaics [12••,13•,47]. The considerable level of mosaicism in giant amoeba viruses was linked to their sympatric lifestyle inside amoebas, where several microorganisms can multiply and exchange sequences [54]. Finally, the replicative cycle of *Megavirales* representatives mainly occurs in viral factories, which are the site of a massive production of virions and another particularity of these viruses [23••].

A different way to classify viruses than using the Linnean dichotomic system [7], replication strategy [55] or phylogeny [56] and that relies on Adansonian classification, which equally weights every feature [57], can be considered. Analyses by hierarchical clustering based on the presence/absence patterns of 23 phenotypic and genetic features for the 103 described viral families (http://www.ictvonline.org/virustaxonomy.asp) (Figure 4b; Supplementary Table S1), showed that giant viruses of phagocytic protists and phycodnaviruses comprise a separate group, apart from other smaller megaviruses and 'traditional' viruses. Thus, two subgroups can be delineated within *Megavirales*, one consisting of amoebal viruses and phycodnaviruses.

(Figure 4 Legend) Hierarchical clustering based on **(a)** the distribution of four protein fold superfamilies (FSF) including the three identified as the most ancient in evolution (namely, P-loop containing nucleoside triphosphate hydrolases, Ribonuclease H-like, and DNA/RNA polymerases) and another ancient FSF, protein kinase-like, and **(b)** the presence (1)/absence (0) patterns of genotypic and phenotypic features for a representative set of the 'traditional' virus families described by the International Committee on Taxonomy of Viruses (ICTV) (http://www.ictvonline.org/virustaxonomy.asp). Hierarchical clustering was performed using the Pearson correlation method and Mev Software (http://www.tm4.org/) and representation was built using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and MEGA6 (www.megasoftware.net) softwares. *Megavirales* representatives are indicated with a red font. In (a), *Megavirales* is apart from other viral groups and the closest to groups of cellular organisms, namely *Eukarya*, *Bacteria* and *Archeae*. In (b), families of giant amoeba viruses are apart from other *Megavirales* families and families of 'traditional' viruses.

Conclusion: *Megavirales* representatives are genuine microbes

The largest *Megavirales* representatives changed the virus paradigm as they do not fulfil several of the criteria that were established from the very onset of virology to define viruses and that fit almost all other viruses [1••,7,27••]. There is indeed a huge gap between them and 'traditional' viruses, and placing Mimivirus into the same basket than 'traditional' viruses as human immunodeficiency virus does not make scientific sense. Hence, taking into account *Megavirales*, there is no unifying view of the virus world, but a quantum discontinuity. Moreover, phylogenetic and phyletic analyses evidence that giant viruses comprise a fourth branch of life. This assumption is also bolstered by the complexity and gene content of these giant viruses and their high prevalence in the environment, which makes them difficult to ignore in biological terms. Furthermore, one critical issue is whether or not the largest *Megavirales* representatives are viruses, and the data summarised here show that they are, conspicuously, microbes and not of the same nature as 'traditional' viruses; they are TRUC. Taken together, these features make these giant viruses different, autonomous, biological entities.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mib.2015.12.010.

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