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Welcome to pandoraviruses at the ‘Fourth TRUC’ club

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Nucleocytoplasmic large DNA viruses, or representatives of the proposed order *Megavirales*, belong to families of giant viruses that infect a broad range of eukaryotic hosts. Megaviruses have been previously described to comprise a fourth monophylogenetic TRUC (things resisting uncompleted classification) together with cellular domains in the universal tree of life. Recently described pandoraviruses have large (1.9–2.5 MB) and highly divergent genomes. In the present study, we updated the classification of pandoraviruses and other reported giant viruses. Phylogenetic trees were constructed based on six informational genes. Hierarchical clustering was performed based on a set of informational genes from *Megavirales* members and cellular organisms. Homologous sequences were selected from cellular organisms using TimeTree software, comprising comprehensive, and representative sets of members from *Bacteria*, *Archaea*, and *Eukarya*. Phylogenetic analyses based on three conserved core genes clustered pandoraviruses with phycodnaviruses, exhibiting their close relatedness. Additionally, hierarchical clustering analyses based on informational genes grouped pandoraviruses with *Megavirales* members as a super group distinct from cellular organisms. Thus, the analyses based on core conserved genes revealed that pandoraviruses are new genuine members of the ‘Fourth TRUC’ club, encompassing distinct life forms compared with cellular organisms.

Keywords: giant virus, *Pandoravirus*, nucleocytoplasmic large DNA viruses, *Megavirales*, informational genes, domains of life, TRUC, phylogeny

Introduction

Defining microbes has been a long journey. During the 18th century, Pasteur first described these organisms as microscopic organisms invisible to the naked eye, but visible under the microscope (Raoult, 2013). At the beginning of the 20th century, Chatton classified these microbes as eukaryotes and prokaryotes based on the presence or absence of a nucleus, and C. Woese subsequently classified these organisms into three domains of life, *Archaea*, *Bacteria*, and *Eukarya*, based on ribosomal DNA (Woese et al., 1990; Raoult, 2013). Although recently revealed as the most abundant biological entities in the biosphere, infecting cellular organisms from these three domains, viruses have been excluded from this classification of the living world because these organisms

lack ribosomal DNA (Suttle, 2005; Raoult and Forterre, 2008; Raoult, 2013, 2015). Indeed, viruses were defined as invisible under a light microscope, and these organisms were initially referred to as inframicrobes. However, giant viruses of amoeba, first described in 2003 with the discovery of Mimivirus as a *bona fide* microbe, have suggested that this paradigm should be revisited (Raoult et al., 2004). Mimivirus, which was isolated through co-culturing with *Acanthamoeba polyphaga*, was initially considered to be a small bacterium due to its large size (750 nm in diameter) and Gram-positive staining (Raoult et al., 2007). Mimivirus has a large genome comprising 1.2 megabase pairs (Mbp) that was until recently the largest amongst viruses and even larger than those of small bacteria, such as *Mycoplasma genitalium* (Raoult et al., 2004). Moreover, this microbe has a repertoire of almost 1,000 genes encoding unexpected proteins, previously considered to be a trademark of cellular organisms, including components of the translation apparatus. Subsequently, several dozens of giant viruses of amoeba have been identified and classified in the family *Mimiviridae*, founded by Mimivirus (La Scola et al., 2008; Fischer et al., 2010; Arslan et al., 2011; Yoosuf et al., 2012; Santini et al., 2013; Yutin et al., 2013), or family *Marseilleviridae*, founded by Marseillevirus (Boyer et al., 2009; Thomas et al., 2011; Pagnier et al., 2013; Aherfi et al., 2014). These viruses are commonly observed in the biosphere, consistent with the frequent isolation of these organisms from environmental water, and sediment/soil samples in several countries and the concurrent detection of sequences related to the genomes of these microbes in various environmental metagenomes (Ghedini and Claverie, 2005; Monier et al., 2008; Kristensen et al., 2010; Williamson et al., 2012). Moreover, mimiviruses and marseilleviruses have been recently identified in human samples, associated with pneumonia and adenitis, respectively, (Popgeorgiev et al., 2013; Saadi et al., 2013). Giant viruses of amoeba have been associated with nucleocytoplasmic large DNA viruses (NCLDV), a monophyletic group of viruses first identified in 2001, encompassing asfarviruses, poxviruses, phycodnaviruses, ascoviruses, and iridoviruses (Iyer et al., 2001). The reclassification of all NCLDV families in a new viral order, called the *Megavirales*, has recently been proposed (Colson et al., 2012, 2013). Phylogenetic and phyletic analyses have revealed a common origin for these viruses dating back to the early stage of eukaryotic evolution (Yutin et al., 2009; Boyer et al., 2010; Koonin and Yutin, 2010; Yutin and Koonin, 2012).

Subsequent to the identification of Mimivirus, the idea of a fourth domain of life emerged based on the phylogeny of the conserved genes shared by this virus and members from *Eukarya*, *Archaea*, and *Bacteria* (Raoult et al., 2004). Subsequently, in 2010, phylogenetic and phyletic analyses of informational genes involved in nucleotide metabolism and DNA processing and shared by cellular organisms and giant viruses suggested that the *Megavirales* members comprise a fourth branch of life with an origin as ancient as that of the three cellular branches (Boyer et al., 2010). This four branch topology was criticized based on the argument that the single-matrix evolutionary models used by Boyer et al. (2010) for their phylogeny reconstructions were not adequate with

respect to the substantial compositional heterogeneity bias and homoplasy detected in the informational genes used (Williams et al., 2011). Accordingly, alternative trees were proposed by Williams et al. (2011) using models presented as more appropriate, but these phylogeny reconstructions did not show a monophyly for *Eukarya*. Besides, it was proposed that the informational genes present in *Megavirales* members might have been acquired from eukaryotic hosts through horizontal gene transfer (Moreira et al., 2005; Moreira and Brochier-Armanet, 2008; Yutin et al., 2014). In contrast, other findings have strengthened the fourth branch of life assumption. Thus, phylogeny reconstructions describing the evolution of proteomes and conserved protein domain structures of cellular organisms and giant viruses suggested that megaviruses comprise a fourth super group that is distinct from the *eukaryotic*, *archaeal*, and *bacterial* groups in the universal tree of life (Nasir et al., 2012). In addition, RNA polymerase (RNAP) beta subunit homologs were detected in the Global Ocean Sampling (GOS) expedition metagenomic database, representing novel branches, apart from those encompassing *Bacteria*, *Archaea*, and *Eukarya* and large viruses (only poxviruses being analyzed) and were considered to be possibly derived from unknown viruses (Wu et al., 2011). Moreover, we recently extended our 2010 study using DNA-dependent RNA polymerase subunit 2 (RNAP2) from a comprehensive, representative, and unbiased set of species from cellular organisms selected by using TimeTree, and from the *Megavirales* representatives, and the results strongly supported a four branch topology (Sharma et al., 2014). The name 'TRUC' (an acronym for "Things Resisting Uncompleted Classification") corresponds to a new classification that has been recently created to accommodate the division of currently known microbes in four branches, i.e., bacteria, archaea, eukaryotes, and giant viruses (Raoult, 2013). The redefinition of microbes as TRUCs emphasizes that the three domains of life topology is based on ribosomal DNA and is thus associated with cellular organisms, while neglecting giant viruses.

In 2013, two new giant viruses, *Pandoravirus dulcis*, and *P. salinus*, were isolated from *Acanthamoeba* present in the mud of a pond in Australia and a marine sediment layer in Chile, respectively (Philippe et al., 2013). These viruses became the record holders of size of virions (ovoids in shape and $\approx 1 \mu\text{m}$ long) and contained the largest genome sizes (1.9 and 2.5 Mbp, respectively) identified to date. The genome from another *Pandoravirus* strain named *Pandoravirus inopinatum* has been recently sequenced. It is 2.2 Mbp large, being the second largest viral genome known to date, and is predicted to encode 1,902 proteins (Antwerpen et al., 2015). *P. inopinatum* was first found as an endocytobiont of *Acanthamoeba* strains isolated from the contact lens storage cases of a patient presenting keratitis, demonstrating that humans are exposed to these giant viruses (Scheid et al., 2014; Antwerpen et al., 2015). Notably, similar to Mimivirus, which has long been considered a bacterium instead of a virus (Raoult et al., 2007), *Pandoravirus*-like particles have been initially classified in 2008 as "extraordinary" endocytobionts of *Acanthamoeba* sp. and were described subsequently in greater details (Scheid et al., 2008, 2010, 2014; Scheid,

2014; Antwerpen et al., 2015). Noteworthy, *Pandoravirus*-related sequences have been recently detected in metagenomes generated from various soil samples worldwide (Kerepesi and Grolmusz, 2015).

Pandoraviruses have been identified as highly divergent viral entities. Indeed, only 16% of the predicted genes from *P. salinus* have significant matches in the NCBI GenBank sequence database, and more than half of these genes belong to families of paralogs (Philippe et al., 2013). In addition, only 14 of the 31 class I-III core genes of *Megavirales* members and 17 of the 49 inferred *Megavirales* ancestral genes have been identified in pandoraviral genomes (Philippe et al., 2013; Yutin and Koonin, 2013). Moreover, the size and shape of pandoraviruses are unique among viruses, and no capsid protein-encoding gene has been identified (Philippe et al., 2013). In the present study, we aimed to demonstrate that the giant pandoraviruses are members of the fourth TRUC.

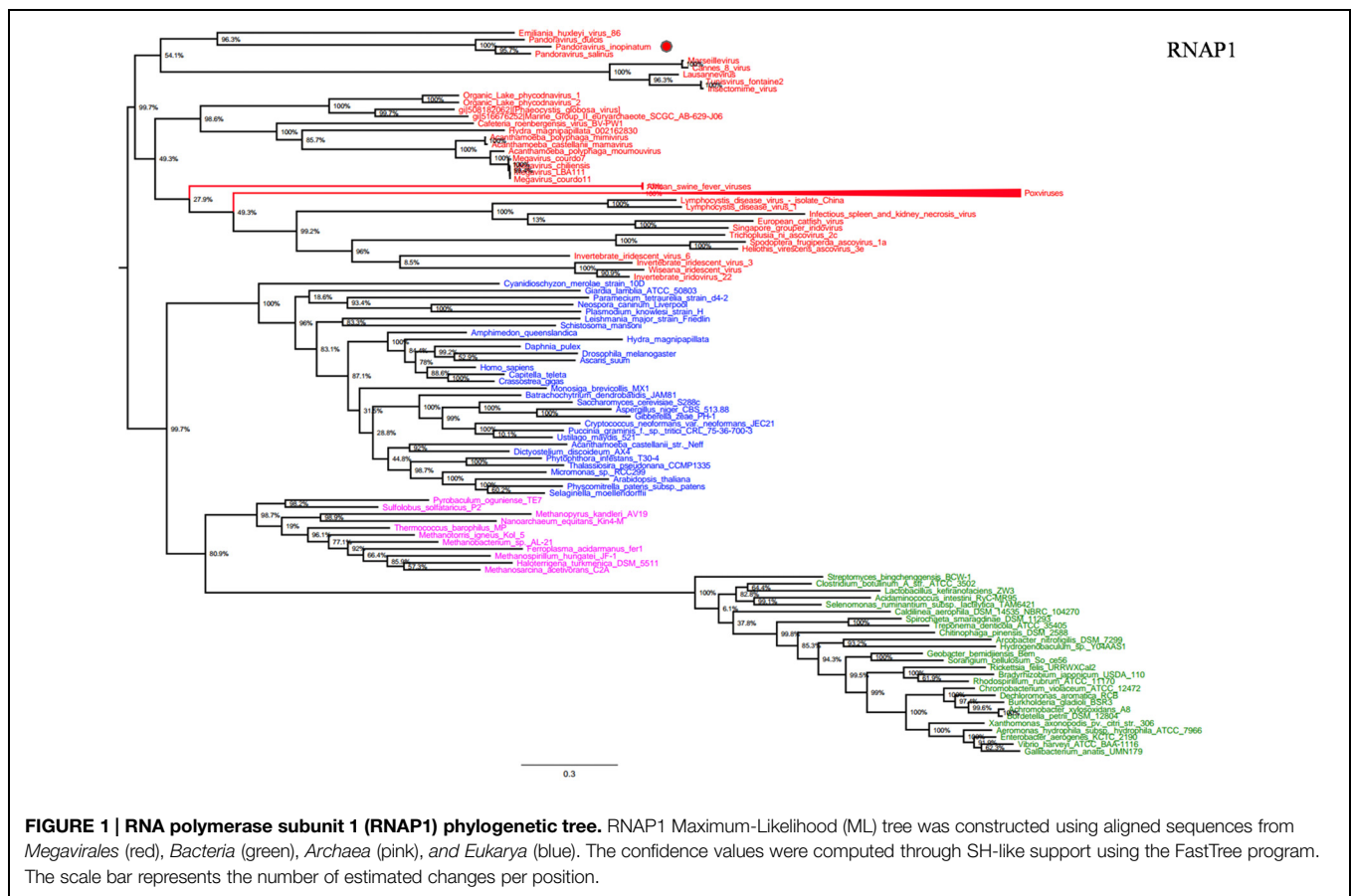
Materials and methods

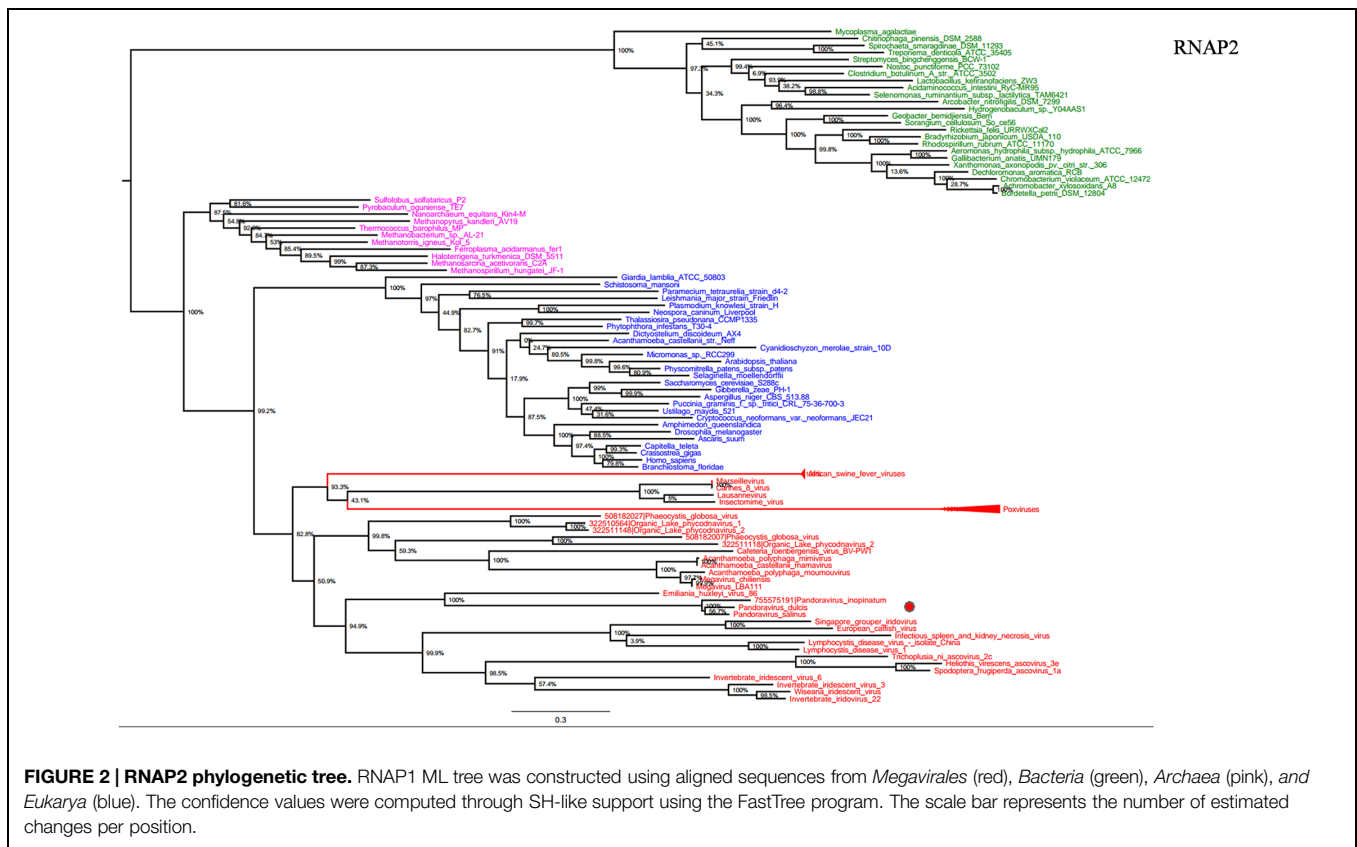
Viral Genes Used for the Analyses

In the present study, we used the approach previously described by Boyer et al. (2010). The genes considered herein were identified from clusters of orthologous groups of proteins (COGs) function categories associated with nucleotide transport and

metabolism and information storage and processing (F, J, A, K, L, and B). Notably, these clusters included the genes encoding ribonucleotide reductase (RNR) and thymidylate synthase (TS), which are both key enzymes involved in the RNA-DNA shift; DNA polymerase family B (DNAPol); topoisomerase II A (TopoIIA); the Flap endonuclease (FEN); the processing factor Proliferating Cell Nuclear Antigen (PCNA); the RNAP2; the transcription factor II B (TFIIB); four amino-acyl tRNA synthetases; and the putative elongation factor EF-1. The presence of these genes was assessed in the genomes of pandoraviruses. Despite having tremendous gene contents, pandoraviruses lack many ancestral genes and have been described to share only 17 of the 49 conserved genes assigned to the putative common ancestor of the *Megavirales* members using maximum likelihood (ML) evolutionary reconstruction (Yutin et al., 2009) and 5 of the 12 genes involved in DNA processing. Therefore, the gene markers used in the present study included RNAP1, RNAP2, DNAPol, RNR, tyrosyl-tRNA synthetase, and TFIIB. The viral orthologous sequences were collected using the OrthoMCL procedure (Li et al., 2003) with gene repertoires from 317 DNA viral genomes encoding proteomes comprising more than 100 protein sequences directly downloaded from the NCBI GenBank sequence database¹. Recently available sequences from *P. inopinatum* were collected from the NCBI GenBank protein

¹<http://ftp.ncbi.nih.gov/genomes/Viruses/>





sequence database². Comparative analysis of the gene repertoires from the three *Pandoravirus* isolates was performed using OrthoMCL and through the strategy of reciprocal best BLASTp hits (using as thresholds an *e*-value <1e-3, an amino acid identity >30% and a sequence coverage >70%) to identify the sets of *bona fide* orthologous genes (Jordan et al., 2002; Li et al., 2003).

Search for Homologous Sequences in Cellular Life Forms

Protein BLAST searches were performed using Standalone Blast 2.2.27 (Altschul et al., 1990) and giant viral protein sequences as queries against the NCBI GenBank non-redundant protein sequence database (nr) to collect homologous sequences from the members of the three cellular domains of life (*Bacteria*, *Archaea*, and *Eukarya*). The number of target sequences was limited to 20,000 per query.

Sequence Selection Criteria for Cellular Domains

Conserved genes might possess multiple homologs in sequence databases, and random selection among these genes for phylogeny reconstruction might result in the selection of strongly biased non-representative sequence sets. Hence, we selected homologous sequences from various species corresponding to representatives from different phyla of *Bacteria*, *Archaea*, and

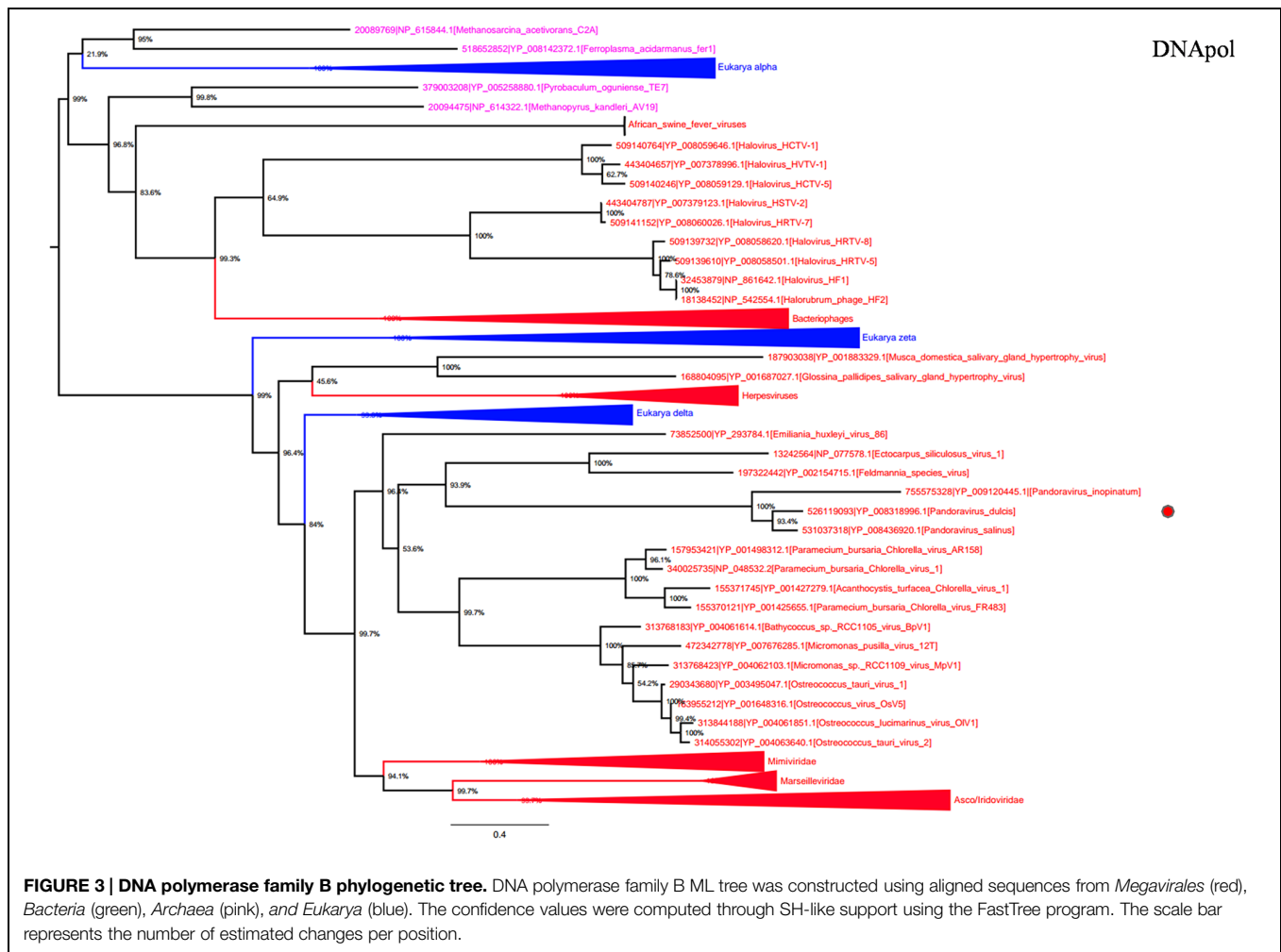
Eukarya using TimeTree, as previously described (Sharma et al., 2014). TimeTree is a public knowledge-base of divergence times among organisms estimated from molecular data in published studies (Hedges et al., 2006). This resource facilitated here the selection of sequences from species that diverged approximately 500 million years ago, which allowed obtaining an appropriate set of members from the three cellular domains of life through a good equilibrium between comprehensiveness and representativeness. In addition, the genomes of most of these cellular organisms are available and have been annotated. The Taxon filter program was subsequently used to filter out taxon and gi identification numbers from the results of the BLAST analysis, which facilitated the downloading of selected protein sequences directly from the NCBI GenBank non-redundant database. Partial or identical sequences were removed through clustering using the CD-HIT suite program (Huang et al., 2010).

Multiple Sequence Alignments and Phylogeny Reconstructions

The obtained sequences were aligned using the Muscle program (Edgar, 2004). Alignment quality was manually analyzed, and phylogenetic reconstructions were performed using ML inference, including the WAG model, and confidence values were calculated using the Shimodaira-Hasegawa (SH) test through the FastTree program (Price et al., 2010). Phylogenetic trees were constructed using the FigTree software program³.

²<http://www.ncbi.nlm.nih.gov/genbank/>

³<http://tree.bio.ed.ac.uk/software/figtree/>



Phyletic Pattern Analysis with Clusters of Orthologous Groups of Protein

Clusters of orthologous groups of proteins corresponding only to the selected functional COG categories [J, A, K, L, B, and F], encoding proteins involved in information storage and processing and nucleotide transport and metabolism, were used for the analysis. BLASTp searches for the selected COG categories, with *e*-values $<1e-3$, were performed against the members from *Bacteria*, *Archaea*, *Eukarya*, and *Megavirales*. Using the BLASTp output results, presence (1) and absence (0) matrices were constructed. A hierarchical clustering dendrogram was constructed using the Pearson distance method with R package 'amap'⁴.

Results

A comprehensive search for the 12 informational genes used in a previous study to delineate a fourth TRUC showed that only four genes were conserved in pandoraviruses (Boyer et al., 2010).

These genes encode RNAP1 and RNAP2, a DNAPol, a RNR, and a tyrosyl-tRNA synthetase.

Phylogenetic Analyses

Informational Genes Showing Vertical Evolution

RNA polymerase is a multi-subunit enzyme that uses a DNA template for the synthesis of RNA to complete transcription (Werner and Grohmann, 2011). Both RNAP 1 and 2 subunits are universal informational genes conserved in all cellular life forms and *Megavirales* members (Sharma et al., 2014). Phylogenetic analyses based on RNAP1/2 showed that *P. salinus*, *P. dulcis*, and *P. inopinatum* were clustered with *Emiliana huxleyi* virus, a coccolithovirus from the family *Phycodnaviridae* (Figures 1 and 2). Notably, newly sequenced mimiviruses or marseilleviruses, including LBA111 virus (Saadi et al., 2013), Insectomime virus (Boughalmi et al., 2013), and Tunisvirus (Aherfi et al., 2014), were identified as new *bona fide* members of the 'Fourth TRUC' club. Similarly, two hidden *Megavirales* members, recently misclassified as *Hydra magnipapillata*, which is a freshwater cnidarian, and a Marine Group II euryarchaeota, which is a marine archaea, were also identified as members of the 'Fourth

⁴<http://www.r-project.org/>

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of the fourth TRUC. Beside this most parsimonious explanation, other evolutionary scenarios including homoplasy, and divergent evolution could not be ruled out.

Otherwise, *Pandoravirus* genomes harbor several genes involved in translation, a feature shared with other giant viruses. Notably, amino-acyl tRNA synthetases are conserved in some *Megavirales* members including pandoraviruses. Tyrosyl-tRNA synthetase is present in *P. salinus* and *P. dulcis* but absent in *P. inopinatum*. Phylogeny reconstruction based on this protein showed that pandoraviruses were clustered with *Acanthamoeba castellanii* str. Neff, as observed previously (Philippe et al., 2013), suggesting that these viruses have acquired this gene from their hosts, whereas family *Mimiviridae* forms a separate monophyletic cluster (Figure 5). We did not find a clustering between an *A. castellanii* gene and a megaviral gene in phylogenies built here apart from tyrosyl-tRNA from this amoeba and from pandoraviruses. Moreover, phylogeny based on tyrosyl-tRNA synthetase provided a complex evolutionary scenario, as *Eukarya* and *Archaea* members were scattered in the tree, suggesting that horizontal gene transfers occurred several times within different phyla.

Phyletic Pattern Analyses

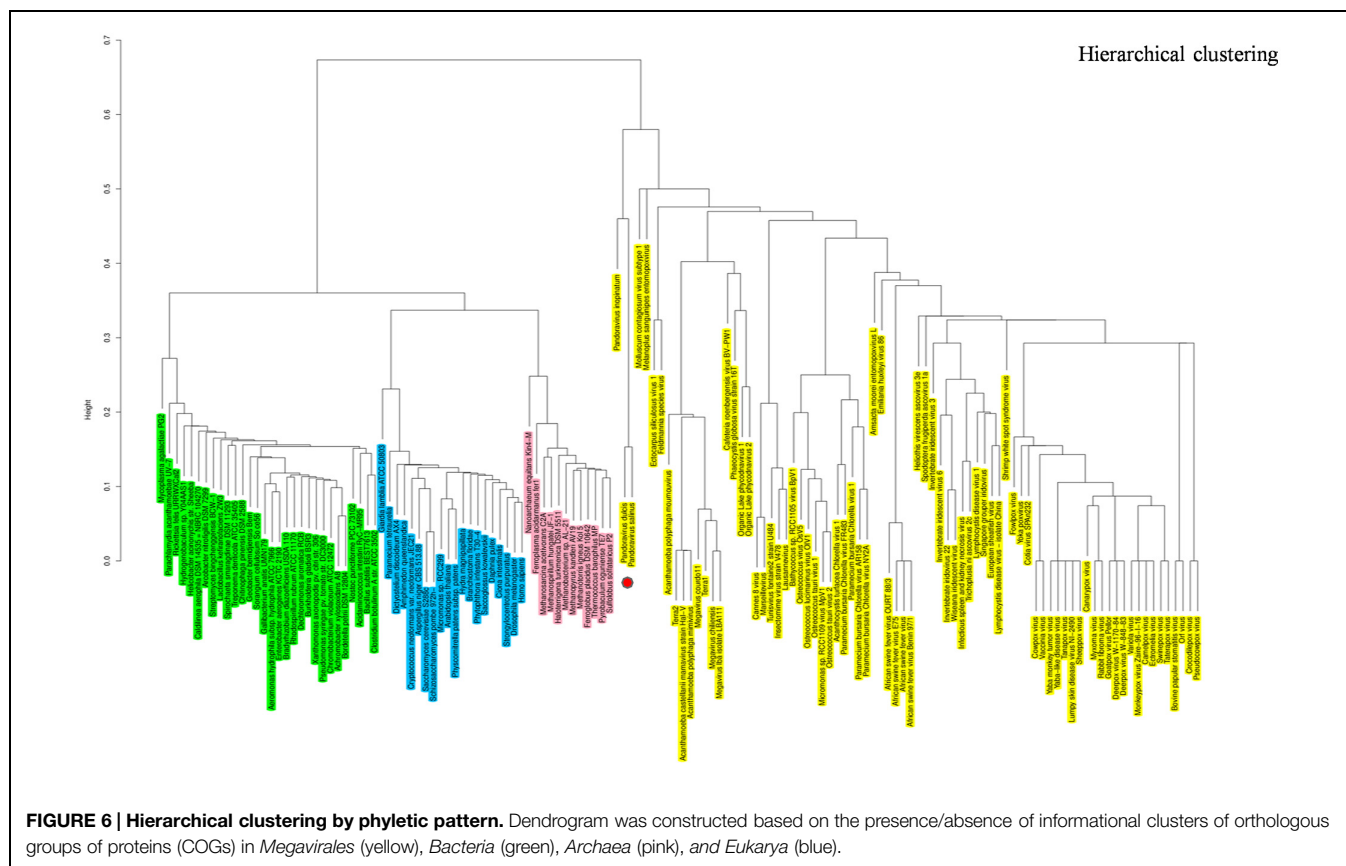
The hierarchical clustering dendrogram based on the presence/absence matrix using 737 COGs comprised of informational genes sharply delineated four clades. Pandoraviruses were

embedded in the *Megavirales* branch. This finding is consistent with those from phylogenetic analyses, indicating that pandoraviruses are new genuine *Megavirales* members (Figure 6). Six COGs were present in *P. salinus* and *P. dulcis* and absent in other megaviruses. These COGs are widespread throughout the genomes of the members of the three cellular TRUCs, and only three of these genomes harbor less than two of these COGs and are considered to be short-sized genomes [491–1,485 kilobase pairs (kbp)]. Conversely, 70 COGs were absent from the *P. salinus* and *P. dulcis* genomes but present in at least one other *Megavirales* member, and these COGs were more abundant among mimiviruses, and secondarily, marseilleviruses, and phycodnaviruses. In addition, these COGs are widely represented in cellular organisms, the lowest number being 24. Moreover, the genome of *P. inopinatum* encodes 32 COGs among which 14 are shared with *P. dulcis* and 10 with *P. salinus*. Consistently, phyletic analyses considering similar gene contents and sizes showed that pandoraviruses clustered with *Megavirales*.

Comparative analysis of *Pandoravirus* genomes

Comparative analyses performed using OrthoMCL with gene repertoires from *P. salinus* (2,556 predicted genes), *P. dulcis* (1,502 predicted genes; Philippe et al., 2013) and *P. inopinatum* (1,902 predicted genes; Antwerpen et al., 2015) identified 1,103 COGs, out of which 681 were core genes, being shared by all





three pandoraviruses. In addition, *P. inopinatum* was found to share 179 genes with *P. salinus* and 73 with *P. dulcis*. Besides, the number of pairs of genes involved in reciprocal best BLASTp hits was 562 for *P. salinus* and *P. inopinatum*, 510 for *P. dulcis* and *P. inopinatum*, and 668 for *P. salinus* and *P. dulcis*; while 375 of these *bona fide* orthologs shared by *P. salinus* and *P. dulcis* were also shared by these two viruses and *P. inopinatum*.

Discussion

Herein, we used the informational genes shared by Megavirales members and cellular organisms to demonstrate that *P. salinus*, *P. dulcis* and *P. inopinatum* are three new genuine members of the fourth TRUC. Thus, these giant viruses, the largest identified so far, were clustered with other megaviruses based on both phylogenetic and phyletic analyses conducted using a previously published strategy, which showed that Megavirales members comprise a monophyletic group apart from Bacteria, Archaea, and Eukarya (Boyer et al., 2010). In addition, in the present study, we used relevant criteria for the selection of cellular organisms, as recently reported (Sharma et al., 2014), to obtain a better understanding of the origin and evolution of Megavirales members.

RNA polymerase 1 and 2 subunits are universal genes, conserved in giant viruses and cellular organisms. Moreover,

these genes are refractory to lateral gene transfer and, in viruses, these genes are resistant to recombination with the host genome (Case et al., 2007; Adékambi et al., 2009). The ubiquity of RNAP facilitated the construction of a phylogenetic tree comprising all studied organisms, confirming that pandoraviruses, and other giant viruses, comprised a fourth branch in the tree of life (Boyer et al., 2010; Sharma et al., 2014). Moreover, the phylogenies of other informational genes (including DNAPol and TFIIB) with vertical evolution and poor evidence of recombination established a clear evolutionary picture, in which pandoraviruses, and other giant viruses, are new members of a 'Fourth TRUC' club. Phylogenetic analyses based on these genes and phyletic analysis using a set of informational COGs revealed that Megavirales members comprise a monophyletic super phylum, consistent with previous findings (Boyer et al., 2010; Colson et al., 2011). It has been suggested that Megavirales representatives evolved a majority of their genes via horizontal gene transfer and therefore do not represent a fourth TRUC (Williams et al., 2011). In contrast, phylogenomic analyses using conserved structural domains have also revealed large to medium DNA viruses as ancient biological entities distinct from Bacteria, Archaea, and Eukarya (Nasir et al., 2012). We also conducted a phylogenetic analysis based on RNR small subunit, which plays an important role in nucleotide metabolism, and an aminoacyl-tRNA synthetase, and the results suggested complex evolutionary histories with multiple horizontal gene transfers among life forms from different TRUC, as previously

reported (Wolf et al., 1999; Moreira et al., 2005; Lundin et al., 2010).

Previous studies have suggested that *Megavirales* members originate from an ancestral virus, and phylogenetic analyses based on certain informational genes represent these organisms apart from *Bacteria*, *Archaea*, and *Eukarya* (Yutin et al., 2009; Boyer et al., 2010; Koonin and Yutin, 2010; Colson et al., 2012; Yutin and Koonin, 2013; Sharma et al., 2014). The finding that phylogenetic reconstructions, based on RNAP1 and RNAP2 genes, display the same topologies, and are consistent with the ribosomal tree of life, suggests that both genes followed the same monophylogenetic evolutionary history. The phylogenetic and phyletic analyses performed herein obtained the same results in favor of *Megavirales* as a fourth super-group, supporting the results of previous studies, and provide insight into the vertical and horizontal evolution of pandoraviruses.

Pandoraviruses are mosaic organisms, similar to other microbes, and they are highly divergent from other *Megavirales* members. A small proportion of the pandoravirus gene complement is indeed shared with other known giant viruses, and these organisms even lack a majority of the *Megavirales* core genes (Philippe et al., 2013), while only five of the 12 informational genes previously identified by Boyer et al. (2010) were detected in the *Pandoravirus* genome. Notwithstanding, some genes allow the construction of phylogenies showing *Megavirales* as a monophyletic group and suggest that early representatives may have harbored these *bona fide* ancient genes, then lost genes and acquired new ones from their hosts and other sources. Moreover, phylogenetic analyses based on genes with vertical evolution and phyletic analysis of the presence/absence of conserved informational COGs strongly suggest that pandoraviruses are new

genuine members of the 'Fourth TRUC' club. Interestingly, pandoraviruses were found to be taxonomically the closest to coccolithoviruses, which belong to the family *Phycodnaviridae*. This is congruent with results from previous studies obtained using genes from *P. salinus* and *P. dulcis* (Philippe et al., 2013; Yutin and Koonin, 2013). It has been further hypothesized that pandoraviruses were highly derived phycodnaviruses that might have experienced considerable genome expansion from smaller *Megavirales* representatives (Yutin and Koonin, 2013).

Conclusion

Phylogenetic and phyletic analyses using informational genes suggest that pandoraviruses are new *bona fide* members of the 'Fourth TRUC' club, in addition to newly sequenced *Megavirales* representatives. The monophylogeny of *Megavirales* suggests a common origin and evolution from ancestral DNA viruses.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2015.00423/abstract>

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