



Evolution and Phylogeny of Large DNA Viruses, *Mimiviridae* and *Phycodnaviridae* Including Newly Characterized *Heterosigma akashiwo* Virus

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Nucleocytoplasmic DNA viruses are a large group of viruses that harbor double-stranded DNA genomes with sizes of several 100 kbp, challenging the traditional concept of viruses as small, simple ‘organisms at the edge of life.’ The most intriguing questions about them may be their origin and evolution, which have yielded the variety we see today. Specifically, the phyletic relationship between two giant dsDNA virus families that are presumed to be close, *Mimiviridae*, which infect *Acanthamoeba*, and *Phycodnaviridae*, which infect algae, is still obscure and needs to be clarified by in-depth analysis. Here, we studied *Mimiviridae*–*Phycodnaviridae* phylogeny including the newly identified *Heterosigma akashiwo* virus strain *HaV53*. Gene-to-gene comparison of *HaV53* with other giant dsDNA viruses showed that only a small proportion of *HaV53* genes show similarities with the others, revealing its uniqueness among *Phycodnaviridae*. Phylogenetic/genomic analysis of *Phycodnaviridae* including *HaV53* revealed that the family can be classified into four distinctive subfamilies, namely, Megaviridae (Mimivirus-like), *Chlorovirus*-type, and *Coccolitho/Phaeovirus*-type groups, and *HaV53* independent of the other three groups. Several orthologs found in specific subfamilies while absent from the others were identified, providing potential family marker genes. Finally, reconstruction of the evolutionary history of *Phycodnaviridae* and *Mimiviridae* revealed that these viruses are descended from a common ancestor with a small set of genes and reached their current diversity by differentially acquiring gene sets during the course of evolution. Our study illustrates the phylogeny and evolution of *Mimiviridae*–*Phycodnaviridae* and proposes classifications that better represent phyletic relationships among the family members.

Keywords: nucleocytoplasmic large DNA virus, *Heterosigma akashiwo* virus, *Phycodnaviridae*, *Mimiviridae*, evolution, phylogeny

INTRODUCTION

Nucleocytoplasmic large DNA viruses (NCLDVs) possess double-stranded DNA (dsDNA) genomes with sizes in some cases over 2 Mbp, and particle sizes that can exceed 1 μm. The discovery of NCLDVs challenges the traditional concept of a virus as an obligate pathogen with small, ‘filterable’ particle size and a simple genome, and expands the biological definition of a virus

(Sharma et al., 2016). Their large genomes suggest that their life cycles, i.e., infection processes in their hosts, are complex (Fischer and Sansom, 2002; Gazzarrini et al., 2003; Chen et al., 2005; Frohns et al., 2006; Claverie and Abergel, 2010; Hamacher et al., 2012; Kuznetsov et al., 2013; Mutsafi et al., 2013; Chelikani et al., 2014). Further discoveries of NCLDV that continue to set records for genome size have ignited discussion about their origin and evolution (Iyer et al., 2006; Suzan-Monti et al., 2006; Koonin and Yutin, 2010; Arslan et al., 2011; Colson et al., 2011; Abrahao et al., 2014; Abergel et al., 2015).

Heterosigma akashiwo virus (*HaV*) is an NCLDV isolated from a unicellular alga, *Heterosigma akashiwo*. *H. akashiwo* is a eukaryotic alga that is widely distributed in temperate, neritic waters, including off the coasts of North and South America, Eastern Asia, Oceania, and the Northern Atlantic region (Lackey and Lackey, 1963; Rojas de Mendiola, 1979; Rensel et al., 1989; Chang et al., 1990; Mackenzie, 1991; Honjo, 1993; Taylor, 1993; Tseng et al., 1993; O'Halloran et al., 2006). It is a member of class *Raphidophyceae*, and is a bloom forming species. Like *Aureococcus anophagefferens virus* (*AaV*) (Moniruzzaman et al., 2014), *Emiliania huxleyi virus* 86 (*EhV*) (Schroeder et al., 2002; Wilson et al., 2005), and *Phaeocystis globosa virus* (*PgV*) (Brussaard et al., 2004; Baudoux and Brussaard, 2005), *HaV* was originally isolated as a bloom terminating factor (Nagasaki and Yamaguchi, 1997) and is of ecological importance in controlling algal populations in nature. A *HaV* strain, *HaV01*, was characterized as a dsDNA virus with a genome size of ~290 kbp (Nagasaki et al., 2005), and we recently reported the complete genome sequence of *HaV* strain 53 (*HaV53*, submitted for publication).

Nucleocytoplasmic large DNA viruses infecting marine algae as natural hosts, including *HaV53*, have been collectively classified as a family, *Phycodnaviridae*, with prefix 'phycodnaviridae' meaning algae. The term 'algae' is rather broad and includes both multi- and unicellular, brown and green, aquatic photosynthetic organisms. Presumably reflecting the diversity of host species, several past studies have suggested that *Phycodnaviridae* consists of groups of viruses with different features. Notably, while classified as phycodnaviruses, *Chrysochromulina ericina virus* (*CeV*), *PgV* and *AaV* are similar in many respects to *Mimiviridae* (Brussaard et al., 2004; Monier et al., 2008; Moniruzzaman et al., 2014), giant NCLDV that infect *Acanthamoeba* (Raoult et al., 2004, 2007; Claverie et al., 2006, 2009; Raoult and Forterre, 2008), rather than to other phycodnaviruses. Several studies strongly suggest that these three viruses are closely related to *Mimivirus* (Yutin et al., 2009, 2013, 2014; Fischer et al., 2010; Thomas et al., 2011; Santini et al., 2013; Moniruzzaman et al., 2014; Legendre et al., 2015). The terms 'extended *Mimivirus*' and 'Megaviridae' have been used in several studies to encompass the *Mimivirus* lineages, smaller *Mimiviruses* (i.e., *Cafeteria roenbergensis virus*, *CroV*), and phycodnaviruses that share characteristic features with *Mimiviruses*, although 'Megaviridae' is yet to be adopted by the International Committee for Taxonomy of Viruses (ICTV) as a family classification. Collectively, the current family *Phycodnaviridae*, officially adopted by ICTV, includes viruses that may not necessarily be closely related evolutionarily or phylogenetically.

While the origin and evolutionary history of NCLDV in general are of great interest, the diversity of NCLDV imposes difficulties in collectively evaluating their phylogenetic relationships. Attempts to classify NCLDV and to infer their evolutionary history have involved comparisons of their sequences and genomic compositions. Orthologous genes in NCLDV can be identified by direct comparisons of viral genes, or using the established Nucleo-Cytoplasmic Virus Orthologous Groups (NCVOGs) database (Yutin et al., 2009). In this study, we analyzed the phylogenetic relatedness of *HaV53* to members of the *Phycodnaviridae* and *Mimiviridae*. Our results underscore the validity of demands for the reclassification of the current *Phycodnaviridae* family, in addition to providing insights into the evolution of *Mimiviridae* and *Phycodnaviridae* including *HaV53*.

MATERIALS AND METHODS

Sequence Information and Database

Viral genomes and encoded amino acid sequences were downloaded from <ftp://anonymous@ftp.ncbi.nlm.nih.gov/genomes/Viruses/>. The NCVOG database was downloaded from <ftp://ftp.ncbi.nlm.nih.gov/pub/wolf/COGs/NCVOG/>.

Amino acid sequences coded by genomes of following viruses were incorporated into the NCLDV database used in this study; *HaV53*, *Aureococcus anophagefferens virus* MM 2014 (*AaV*, NC024697), *Acanthocystis turfacea Chlorella virus* 1, (*AtCV*, NC008724), *Bathycoccus RCC1105 virus* (*BpV*, NC014765), *Haptolina ericina virus* *CeV*-01B (formerly *Chrysochromulina ericina virus*, *CeV*, KT820662), *Cafeteria roenbergensis virus* (*CroV*, NC014637), *Emiliania huxleyi virus*86 (*EhV*, NC007346), *Ectocarpus siliculosus virus*1 (*EsV*, NC002687), *Feldmannia species virus* (*FsV*, NC011183), *Megavirus chilensis* (*MegaV*, NC016072), *Acanthamoeba polyphaga mimivirus* (*MimiV*, NC014649), *Acanthamoeba polyphaga moumouvirus* (*MoumouV*, NC020104.1), *Micromonas RCC1109 virus* (*MpV*, NC014767), *Ostreococcus lucimarinus virus* 5 (*OIV*5, NC020852), *Ostreococcus tauri virus* 1, (*OtV*1, NC013288), *Ostreococcus tauri virus* 5 (*OtV*5, NC010191), *Paramecium bursaria Chlorella virus* 1 (*PBCV*1, NC000852), *Phaeocystis globosa virus* (*PgV*, NC021312), *Autographa californica nucleopolyhedrovirus* (*AcNPV*, NC 001623), *African swine fever virus* (*ASFV*, NC 001659), *Melanoplus sanguinipes entomopoxvirus* (*MsEV*, NC 001993), *Amsacta moorei entomopoxvirus* (*AEPV*, NC 002520), *Culex nigripalpus NPV* (*CnNPV*, NC 003084), *Heliothis virescens ascovirus* 3e (*HvaV*, NC_009233), *Infectious spleen and kidney necrosis virus* (*ISKNV*, NC 003494), *Mamestra configurata NPV-A* (*McNPV*, NC 003529), *Lymphocystis disease virus china* (*LDV*, NC 005902), *Spodoptera litura granulovirus* (*SIGV*, NC 009503), *Marseillevirus* (*MarV*, NC 013756), *Rodent herpesvirus Peru* (*RHV*, NC 015049), *Lausannevirus* (*LausV*, NC 015326), *Wiseana iridescent virus* (*WiV*, NC 015780), *Pithovirus sibericum* (*PithoV*, NC 023423), *Pandavirus dulces* (*PandraV*, NC_021858), *Mollivirus* (*MolliV*, NC 027867), and *Human herpesvirus 3* (*HHV*3, NC 001348).

For NCLDV CP (NCVOG0022), D5-like helicase primase (NCVOG0023), and DNA polymerase B (NCVOG0038) phylogenetic analyses (Supplementary Figure S2), the orthologs were determined by choosing best-hit target sequences obtained by BLASTP search (E -value $< 10^{-20}$) using the NCVOG orthologs as queries, and the databases were created from the amino acid sequences coded by the genomes of above mentioned viruses and of *Ambystoma tigrinum* virus (AtV, NC_005832), *Bovine papular stomatitis virus* (BpsV, NC_005337), *Fowlpox virus* (FpV, NC_002188), *Frog virus* (FrogV, NC_005946), *Invertebrate iridescent virus 6* (InvIV6, NC_003038), *Lymphocystis disease virus 1* (LDV1, NC_001824), *Molluscum contagiosum virus subtype 1* (McV, NC_001731), *Sheeppox virus* (ShpV, NC_004002), *Singapore grouper iridovirus* (SgiV, NC_006549), *Swinepox virus* (SwpV, NC_003389), *Trichoplusia ni ascovirus* (TnaV, NC_008518), *Vaccina virus* (VaccinaV, NC_006998), and Yaba monkey tumor virus (YmtV, NC_005179).

Software

BLAST+ (version 2.2.31) executables were downloaded from <ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/>. Databases for BLASTP and PSI-BLAST searches were constructed according to the provided manual. For phyletic studies, 18 viruses, namely AaV, AtCV, BpV, CeV, CroV, EhV86, EsV, FsV, HaV53, MegaV, MimiV, MoumouV, MpV1, OIV5, OtV1, OtV5, PBCV1, and PgV were selected. Proteins equal to or larger than 100 aa encoded by each virus were extracted and used as queries. When a single open reading frames (ORF) hit multiple target sequences in databases, the hit with the highest bit score was selected for further study. Similarly, when multiple ORFs in a viral genome hit the same target sequence in NCVOG, the ORF that hit with the highest bit score was selected for further study to identify a true ortholog rather than paralogs.

Multiple sequence alignments and phylogenetic reconstructions by neighbor-joining were performed in ClustalX version 2.1 (Larkin et al., 2007). Poorly conserved regions and positions including gaps were removed prior to phylogenetic analysis. Neighbor-joining phylogenetic inferences were conducted, and the confidence of the branching was assessed using 1,000 bootstrap resampling replicates of the analyzed dataset.

Pan-genome analysis was conducted using PGAP software using cut-off values of 20% identity and E -value $< 10^{-5}$ (Zhao et al., 2012). In this analysis, orthologs in each virus in the dataset were determined by all-to-all BLASTP search followed by MCL, and phyletic inference calculated by neighbor-joining based on the presence/absence matrix of the orthologs in each combination of the viruses (Zhao et al., 2012).

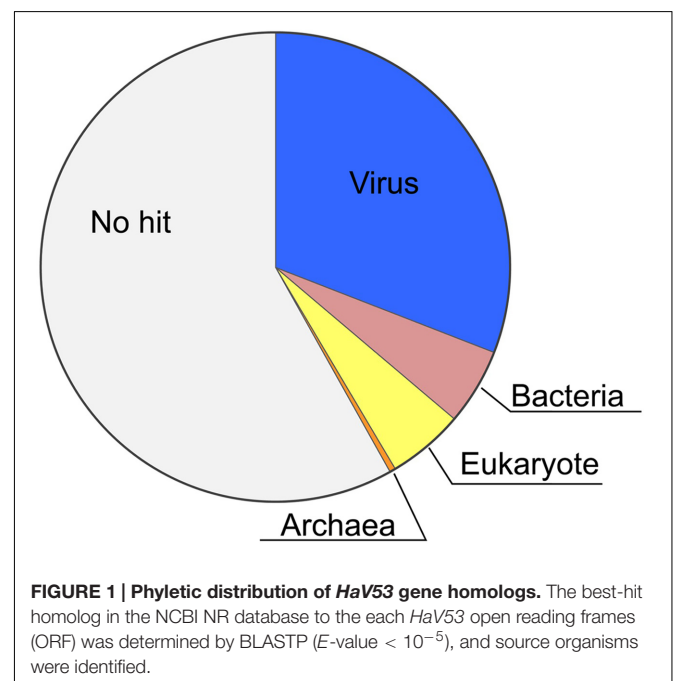
Gain and loss of gene families during evolution was mapped on a guide tree based on the concatenated sequence of nine preserved genes (Figure 4A) using COUNT software (Csuros, 2010; Kamneva and Ward, 2014). For each gene family, Wagner parsimony with gene gain penalties of 1 and 5 were used to infer the most parsimonious ancestral gene sets with different gain/loss pressures. We chose Wagner parsimony, rather than

other protocols, because it allows multiple gains with penalties and infers gene family expansion and contraction (Csuros, 2010). For both PGAP and COUNT analyses, we selected genes coding for proteins with 100 aa or more. The resulting trees from all the analyses were visualized using Geneious 9.0.5.

RESULTS

HaV53 Genes and NCLDV Orthologs

Recently, we completed sequencing of the HaV53 genome (GenBank accession number KX008963, (Ogura et al., 2016). To gain further insight into the potential functions of HaV53 gene products, we predicted the functions of HaV53 ORF using the NCVOG database (Yutin et al., 2009, 2014) and the NCBI NR protein database. HaV53 genes were annotated by searching the databases using BLASTP with E -value cutoff set to 10^{-5} (Supplementary Figure S1; Supplementary Table S1). All the search results were further supported by PSI-BLAST results (E -value $< 10^{-8}$, three iterations), confirming homology to the target sequences. Seventy-four HaV53 ORFs exhibited similarities to NCVOGs, while for 27, the best-hit proteins were from bacterial, archaeal, and eukaryotic genomes (Supplementary Table S1; Figure 1). The remaining HaV53 ORFs did not exhibit significant similarity to any protein in the databases (Figure 1). As expected, HaV53 possesses orthologs of four previously sequenced HaV01 genes, AGB-1, UKCH-2, NCLDV major capsid protein, and B-family DNA polymerase, with high sequence similarities (Supplementary Table S1). Like other NCLDVs, multiple occurrences of genes annotated with identical functions were also observed within the subset of HaV53 ORFs. To evaluate if these genes were originated from



insertion of multiple homologous genes from different source organisms, or due to duplication of a gene acquired from single horizontal gene transfer, we evaluated the homologies among the *HaV53* ORFs (paralogs) and compared their homologies to their potential orthologs found in NR database by BLASTP search (Table 1). When homologies among the *HaV53* paralogs and their orthologs in other organisms were compared, identities among *HaV53* paralogs were much higher than identities to orthologs, presumably suggesting that these redundancies were based on recent gene duplication rather than horizontal gene transfer from the species with the closest orthologs (Santini et al., 2013).

To certify that *HaV53* is indeed a phycodnavirus, we conducted phylogenetic analysis of DNA polymerase B, capsid protein, and D5-like helicase primase, and found that *HaV53* genes cluster with their orthologs from other phycodnaviruses, confirming that *HaV53* is a new member of the family (Supplementary Figure S2).

Similarity of *HaV53* to Other NCLDVs

To further evaluate the relatedness of *HaV53* and other NCLDVs, we first conducted a blanket comparison of all the

HaV53 ORFs with NCLDV genes. To this end, we constructed an NCLDV protein sequence database consisting of all the proteins encoded by representative, fully sequenced and annotated NCLDVs, including Megaviridae, *Phycodnaviridae*, *Marseilleviridae*, *Ascoviridae*, *Asfarviridae*, *Baculoviridae*, *Herpesviridae*, *Iridoviridae*, *Poxviridae*, Pandraviruses, and Pithovirus. First, we identified the NCLDV orthologs of each gene in *HaV53*, and identified the source viral species of the best-hit target genes (Figure 2). For comparison, the same analyses were conducted for genes carried by 17 members of *Phycodnaviridae* and proposed Megaviridae (Figure 2). Each virus showed a characteristic pattern in the distribution of best-hit sources. As expected, three lineages of mimiviruses, *MimiV* (lineage A), *MoumouV* (lineage B), and *MegaV* (lineage C), were found to be significantly related by this analysis; about 88, 92, and 92%, of *MimiV*, *MoumouV*, and *MegaV* genes, respectively, were most homologous to the other two mimivirus lineages. Among the proposed Megaviridae with smaller genome sizes, *AaV* and *CroV* exhibited similarities to both mimiviruses and smaller Megaviridae, while *PgV* and *CeV* showed significant relatedness to each other. *AaV*, *PgV*, and *CeV* are currently classified as *Phycodnaviridae*,

TABLE 1 | Redundant genes observed in *HaV53* open reading frames (ORFs).

Gene group	ID % to paralogs	Predicted function	Closest orthologs	ID % to orthologs
ORF1, ORF3, ORF118, ORF182	95.2 ~ 96.5	Transposases	Bacterial proteins	24.31 ~ 35.29
ORF9, ORF162	48.32	Glycosyltransferase,	Marseillevirus NCVOG2757	24.79 ~ 26.16
ORF28, ORF29	100	VV32-like packaging_ATPase	OtV1 NCVOG0249	48.89 ~ 49.07
ORF53, ORF183	95.35	Uncharacterized_protein	MpV1 NCVOG5117	18.05 ~ 38.51
ORF202, ORF214,	88.6	Outer membrane protein	Bacterial proteins	17.66 ~ 19.34
ORF203, 204, 205	70.3 ~ 78.9	Putative glutamine rich 2-like protein	Eukaryote proteins	23.00 ~ 24.75

Redundant genes, or paralogs, in *HaV53*, presumably products of gene duplications, were identified by all-to-all BLASTP using *HaV53* ORFs as query and database.

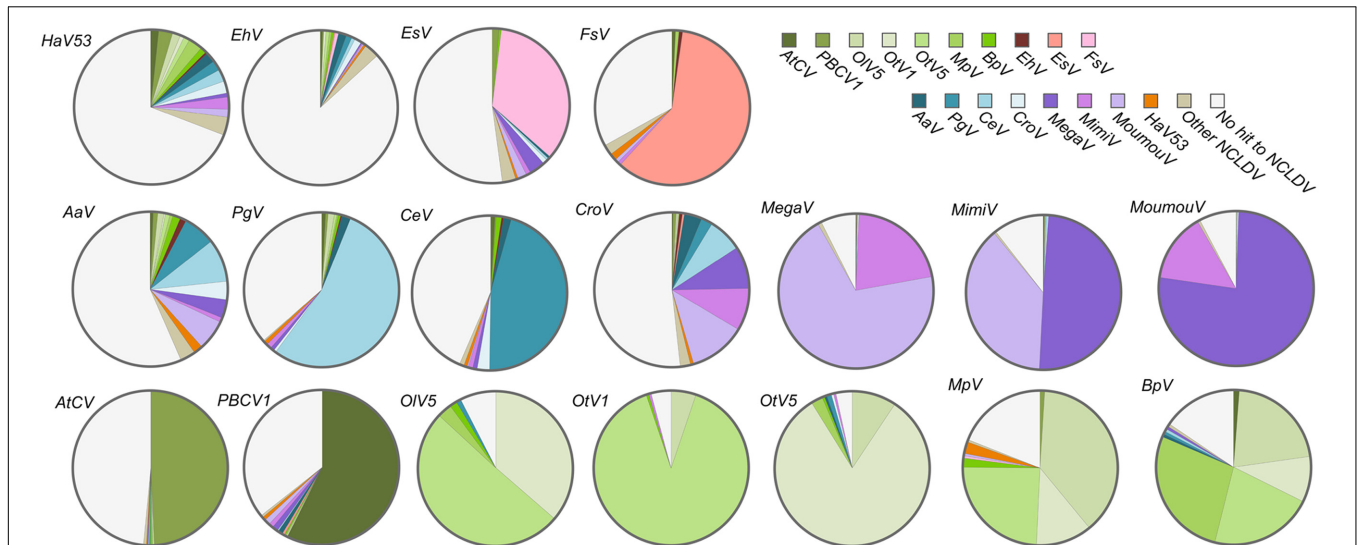


FIGURE 2 | Source viral species of the best-matching nucleocytoplasmic large DNA viruses (NCLDV) orthologs for genes from *HaV53*, Megaviridae, and *Phycodnaviridae*. The best-hit homologs in the NCLDV database, to viral ORFs were determined by BLASTP (E -value $< 10^{-5}$), and source NCLDVs were identified.

although they did not show strong similarities to the other members of the family. A group including chloroviruses, *OtV1/5*, *OIV5*, *MpV*, and *BpV* showed large proportions of orthologs identified from the group. *EhV* did not show significant similarities to any NCLDV, and contained the smallest proportion of genes (16.5%) showing similarities to NCLDVs. As expected, two phaeoviruses, *EsV* and *FsV*, showed significant similarities to each other. *HaV53* genes showed a similar degree of similarity to both Megaviridae and *Phycodnaviridae* minus Megaviridae, with 14.0 and 12.8%, respectively. These results indicate that members of *Phycodnaviridae* exhibit homologies to particular group of other

family members, or showed low homologies to others. These observation implies that *Phycodnaviridae* comprise several cluster of the members, which are not necessarily homologous to each other.

We next explored the presence/absence of all NCVOGs in the 18 viruses. We searched for NCVOG homologs for each viral ORF with size 100 aa or larger using BLASTP (E -value $< 10^{-5}$), then identified the target sequence that gave the highest bit score (Figure 3). When more than one viral ORF hit the same NCVOG, the ORF that gave the highest bit score was identified as the NCVOG ortholog. Nine NCVOGs were found in all the analyzed viruses including *HaV53*

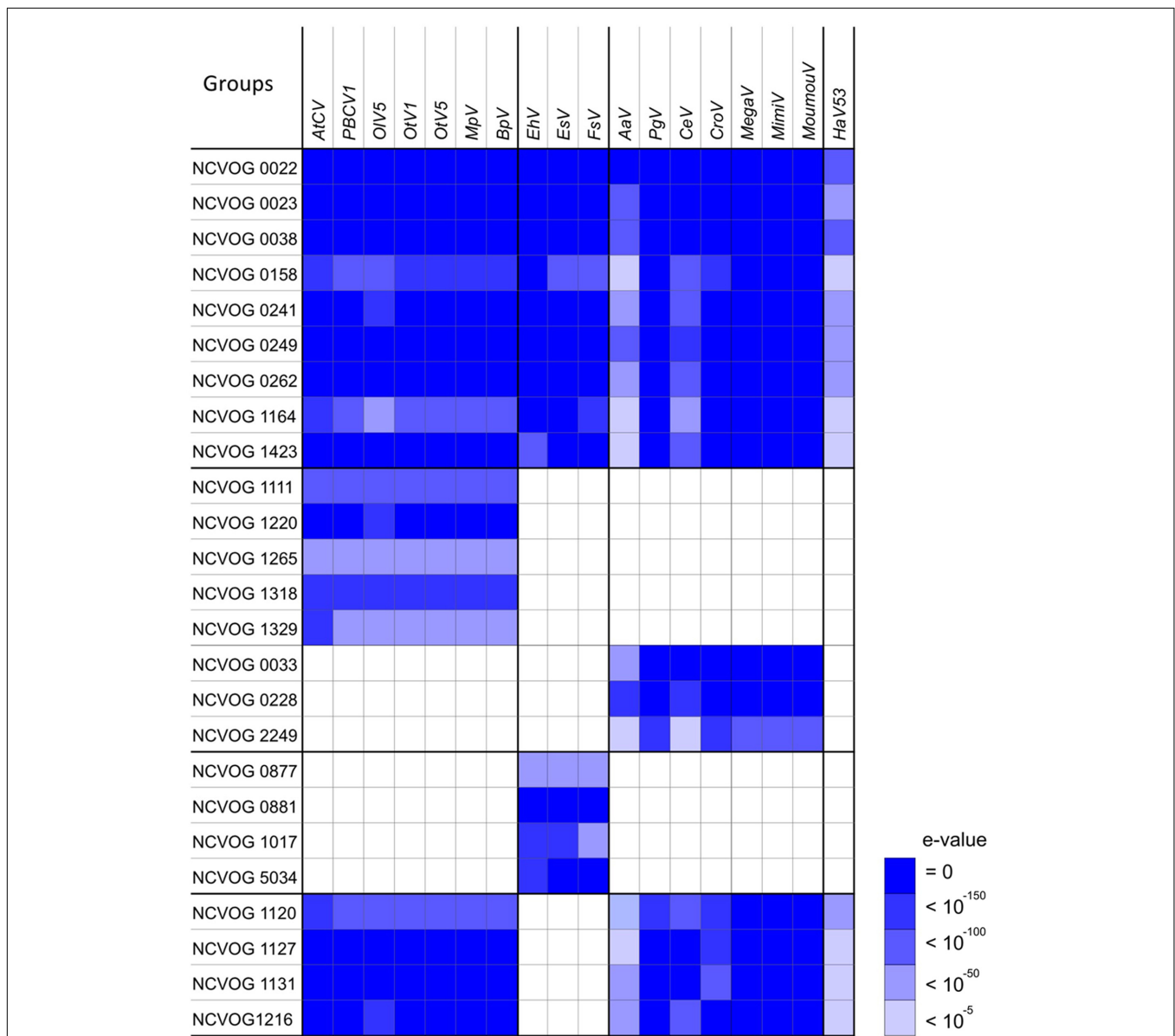


FIGURE 3 | Nucleo-Cytoplasmic Virus Orthologous Groups (NCVOG) orthologs in *Phycodnaviridae* and *Mimiviridae*. The NCVOG orthologs in each virus were identified by BLASTP search (E -value $< 10^{-5}$), and similarities between the query viral factors and NCVOGs are displayed. When several different viral ORFs hit an NCVOG, the viral factor with the highest bit score was chosen.

(**Figure 3**): NCLDV major capsid protein (NCVOG0022), D5-like helicase primase (NCVOG0023), DNA polymerase B (NCVOG0038), an uncharacterized protein (NCVOG0158), proliferating cell nuclear antigen protein (NCVOG0241), A32-type packaging ATPase (NCVOG0249), poxvirus late transcription factor VLTf3-like protein (NCVOG0262), A1L transcription factor/late transcription factor VLTf2 (NCVOG1164), and a protein with uncharacterized C-terminal domain conserved in iridovirus, phycodnavirus, and mimivirus (NCVOG1423). The homologies among the orthologs were further confirmed by reciprocal BLASTP searches. For three orthologs, NCVOG0038, NCVOG0249, and NCVOG0262, all combinations identified from the eighteen viruses showed significant similarities to each other (E -value $< 10^{-5}$), and all combinations for NCVOG1423 showed significant similarities except CeV and *EhV*. The other five orthologs showed similarities for some of the analyzed combinations (Supplementary Figures S3A–E). Among them, NCVOG0023 coded by Megaviridae and the rest of *Phycodnaviridae* did not show similarities, showing clear segregation between the groups (Supplementary Figure S3B). Significant similarities were observed between all the combinations of members of each group (Supplementary Figure S3B). The *HaV53* NCVOG0023 showed similarity with those of Megaviridae (Supplementary Figure S3B). At the same time, NCVOG0023 phylogenetic analysis revealed less clear segregation of *Phycodnaviridae* and *Mimiviridae* members from other NCLDVs (Supplementary Figure S1C), suggesting that NCVOG0023 may not be suitable to be used as a hallmark gene for classification.

Phylogenetic/Phylogenomic Positions of *HaV53* among *Phycodnaviridae* and Megaviridae Members

Based on the set of common orthologs identified above, we next analyzed phyletic relationships between the 18 viruses (**Figure 4**). As previously reported, three viruses currently classified as phycodnaviruses, AaV, PgV, and CeV, associate with mimivirus and *CroV* (Santini et al., 2013; Moniruzzaman et al., 2014), segregating from the remaining *Phycodnaviridae* and *HaV53*, with some ambiguity concerning the position of AaV (**Figure 4A**). The rest of *Phycodnaviridae* clustered into three distinct groups: one with *EhV*, *EsV*, and *FsV*; *HaV53*; and the seven remaining viruses including *PBCV* (**Figure 4A**).

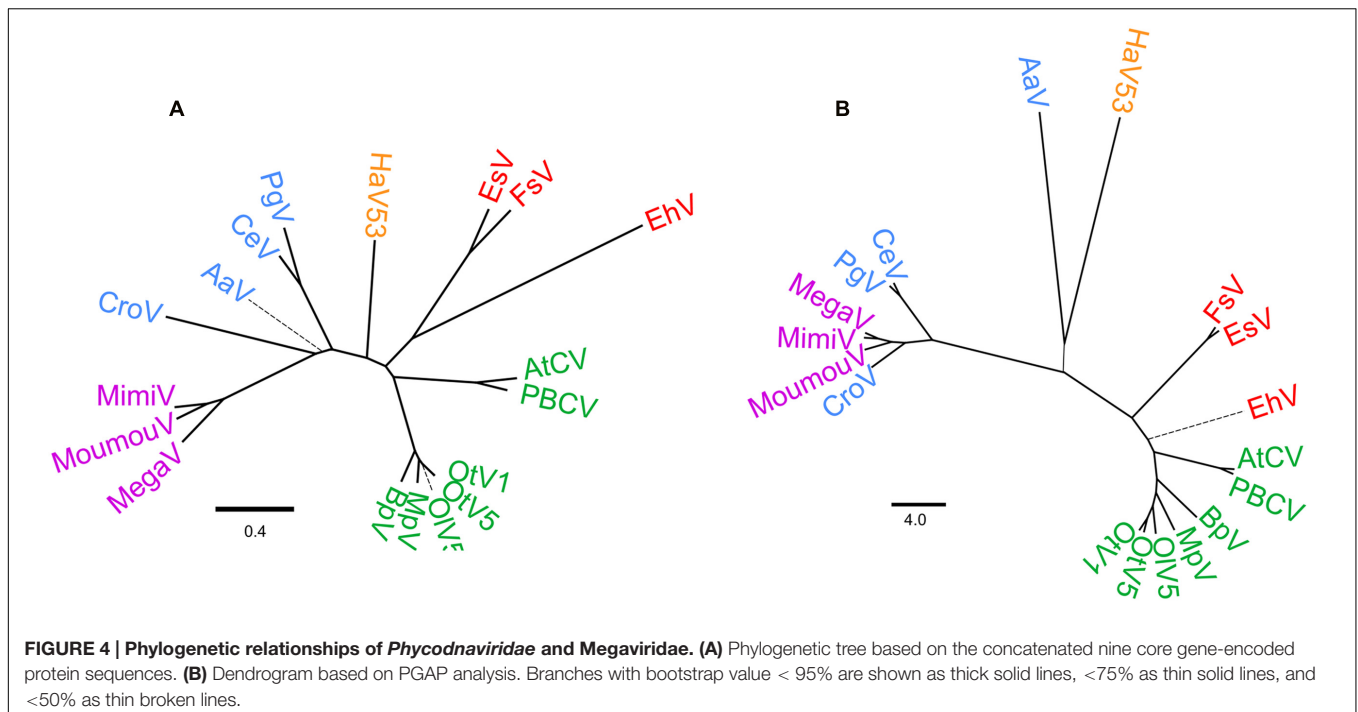
Further, we analyzed phyletic relations by pan-genome analysis (**Figure 4B**). This approach allows us to evaluate phylogenomic relationships among the dataset by *de novo* clustering, and is thus independent of the NCVOG database. Other than AaV and *HaV53* being clustered with low confidence, overall taxonomic/phylogenetic relationships were coherent with the results based on phylogenetic analysis (**Figures 4A,B**). Results of these two analyses revealed that current *Phycodnaviridae* can be categorized into four groups. The first group includes *AtCV*, *PBCV*, *OIV5*, *OtV1*, *OtV5*, *MpV*, and *BpV*. The second group consists of *EhV*, *EsV*, and *FsV*.

The third group includes *Phycodnaviridae* members that belong to Megaviridae, with some ambiguity concerning the position of AaV. *HaV53* appears to be independent of the other three groups, positioned between the *EhV* group and the Megaviridae-*phycodnaviruses*.

As shown in **Figure 3**, we also identified several NCVOGs that characteristically exist in specific viral groups deduced from the phylogenetic/pan-genomic analysis (**Figure 4**). For example, five NCVOGs with unknown functions (NCVOG1111, 1220, 1265, 1318, and 1329) were found in the *PBCV* group, but not in others. Also, four NCVOGs, unknown functions (NCVOG0877, 0881, 1017, and 5034) were found in *EhV*, *EsV*, and *FsV* exclusively, but not in other groups. In contrast, metalloproteinase WLM (NCVOG1120), transcription initiation factor IIB (NCVOG1127), and two uncharacterized proteins (NCVOG1131 and 1216) exist in all the analyzed viruses but *EhV*, *EsV*, and *FsV*. Some of the previously identified Megaviridae hallmark genes, DNA topoisomerase I (NCVOG0033), ATP-dependent protease (NCVOG0228), and DNA-directed RNA polymerase II subunit RPB7 (NCVOG2249), were found in the proposed Megaviridae including mimiviruses. Further, homologies among these orthologs were analyzed, and the orthologs that showed significant similarities between all combinations of species are summarized in **Table 2**.

One of the previously identified hallmark genes (Ogata et al., 2011), MutS7 (NCVOG0105), was not found in *MoumouV* by this analysis. However, *MoumouV* does possess MutS (YP_007354438.1), while it is categorized as NCVOG0199 by this method. The protein possesses MutS7 features including MutS domains II, III, and IV, and the orthologs in Megaviridae, including *MoumouV* NCVOG0199, exhibit significant similarities in all combinations, while being absent in other *Phycodnaviridae* (**Table 2**; Supplementary Figure S4A). Furthermore, asparagine synthetase (NCVOG0061) and polyA polymerase (NCVOG0575) genes were not found in AaV (Supplementary Figures S4B,C). With consistently higher e -values for other Megaviridae hallmark genes, AaV could be defined as an outlier of Megaviridae. In addition, NCVOG0061 orthologs were also found in several *PBCV*-type *Phycodnaviridae* members, and these displayed sequence similarities with those in Megaviridae members, casting questions on its status as a hallmark gene (Moniruzzaman et al., 2014) (Supplementary Figure S4B).

Finally, evolutionary scenarios of viruses were reconstructed using COUNT software (Csuros, 2010; Kamneva and Ward, 2014) with the core gene phylogenetic tree as the guide tree topology (**Figure 5**). By this program, the size of the common ancestor virus and subsequent evolutionary process can be inferred assuming different pressures for acquisitions and loss of genes, using different gene gain/loss penalties. When lower penalty for gene gains that gives the ancestor with only sixty-one NCVOG genes was chosen, *Mimiviridae* members were presumed to go through major gene gain when diverged from the smaller *Megaviridae* to reach to contemporary genome sizes. On the other hand, when high penalty for gene gain that gives the

**TABLE 2 | Group hallmark genes.**

Group	Nucleo-Cytoplasmic Virus Orthologous Groups (NCVOG)	Descriptions
PBCV-group	NCVOG1318	Uncharacterized protein
	NCVOG1329	Uncharacterized protein
EhV-group	NCVOG0881	Uncharacterized protein
	NCVOG1017	Uncharacterized protein
'Megaviridae'	NCVOG0033	DNA topoisomerase I
	NCVOG0228	ATP-dependent protease
	NCVOG0105	MutS (for MoumouV, NCVOG0199)

NCVOG specifically associated with the groups of viruses were identified (Figure 5), and the genes that showed significant homologies by comparisons of all the combinations viruses in the group are listed.

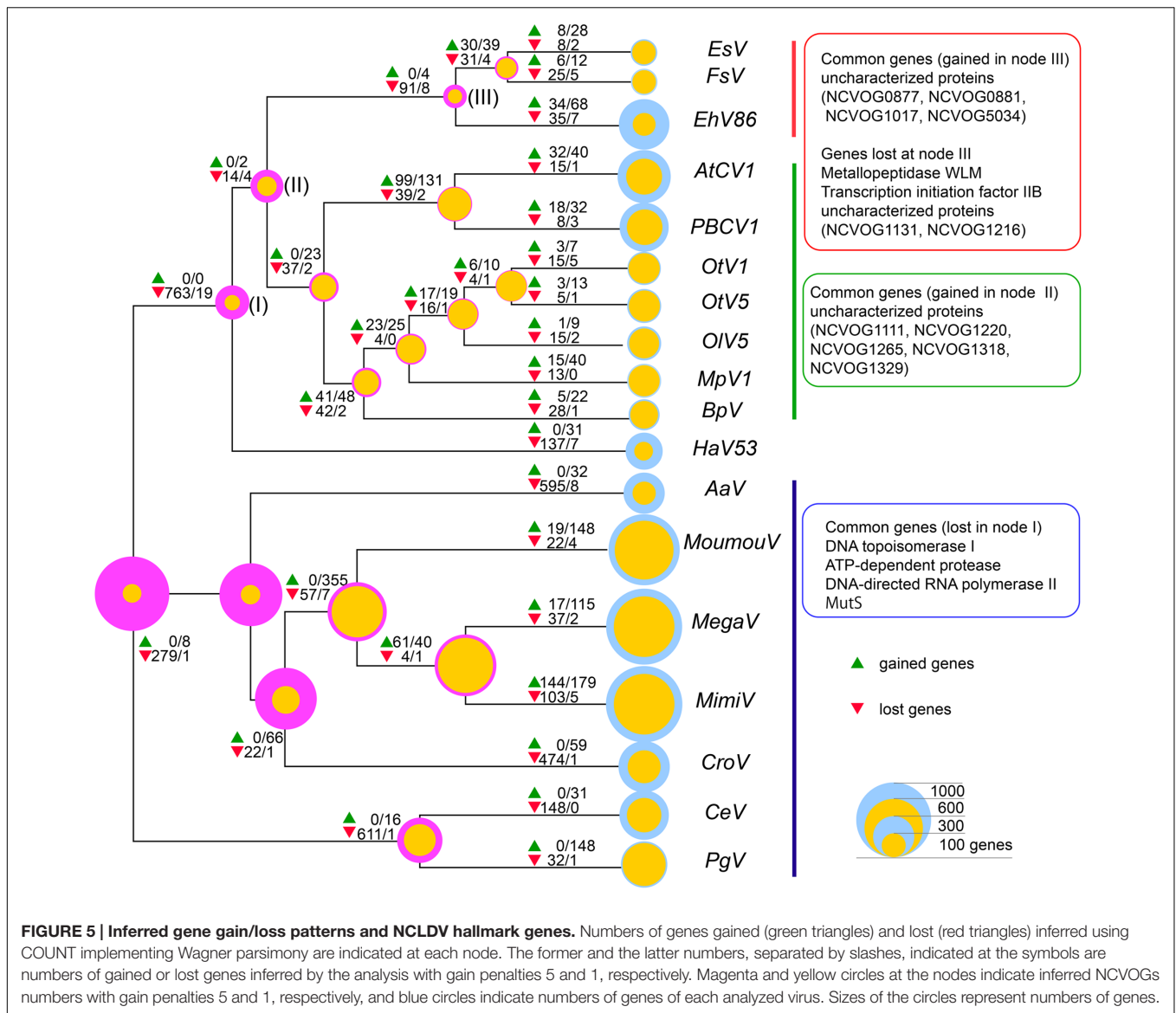
common ancestor with 996 NCVOGs was adopted, massive gene reduction at the timing of the divergence of the ancestor of *Phycodnaviridae* minus Megaviridae group from the ancestor of Megaviridae was inferred. The nodes where the hallmark genes identified in Figure 3 emerged or were lost were also predicted by the analysis (Figure 5). The inferred gene gain/loss ratio and estimated numbers of gene gains/losses per COG varied widely between clades as reported previously by analyzing evolutionally processes of closely related mimiviruses and phycodnaviruses (Filee, 2015).

DISCUSSION

The present study reveals that *HaV53*, the first raphidovirus isolated and characterized (Nagasaki and Yamaguchi, 1997;

Nagasaki et al., 2005), is a unique NCLDV in many respects. While ~31% of *HaV53* genes display homology to NCLDV proteins, ~11% show homology to bacterial and eukaryotic proteins, and ~58% show no homology to any proteins identified to date. That a significant percentage of *HaV53* genes do not show homology with proteins in the databases is typical of viruses belonging to a newly characterized lineage with no other sequenced representatives. Gene duplications followed by mutations or genetic drift and horizontal gene transfer from host to virus are presumable sources of unique *HaV53* genes. By a heuristic approach, several *HaV53* ORFs were identified as potential results of gene duplication (Table 1). On the other hand, possibility of horizontal gene transfer cannot be investigated, at this point, because of the scarcity of *H. akashiwo* genome/transcriptome information obtained to date.

HaV53 possesses 74 genes exhibiting homology to NCVOGs (Supplementary Table S1), while its composition is unique relative to other members of *Phycodnaviridae* or proposed Megaviridae (Figures 1 and 2). For example, *HaV53* does not possess the Megaviridae hallmark genes MutS7, DNA-directed RNA polymerases, polyA polymerase, and DNA topoisomerase I (Ogata et al., 2011; Santini et al., 2013; Moniruzzaman et al., 2014), supporting the conclusion that *HaV53* is not likely a member of the proposed family. Notably, *HaV53_ORF179* exhibits significant homology to bacterial asparagine synthetase A with an aminoacyl-transfer RNA synthetase domain (WP_003512022.1). Some mimiviruses characteristically possess aspartyl/asparaginyl-tRNA synthetase (Yutin et al., 2014). In the case of *HaV53_ORF179*, however, the motif corresponding to the anti-codon binding domain is missing, suggesting that the protein may not exhibit



tRNA synthase activity. Several orthologs that are shared among members of the *PBCV* and *EhV* groups are not found in *HaV53* (Figure 3). These data underscore the uniqueness of *HaV53* among *Phycodnaviridae* and proposed *Megaviridae*.

Importantly, *HaV53* does not possess DNA-directed RNA polymerase or polyA RNA polymerase, indicating that *HaV53* depends on its host's transcription machinery. On the other hand, as observed in many different NCLDVs, *HaV53* harbors several genes related to regulation of transcription, including transcription initiation factors, mRNA capping enzyme subunits, and ribonuclease III.

Among the viruses analyzed in this study, *AaV*, *EhV*, and *HaV53* possess higher proportions of unique genes that are not homologous to other NCLDV genes (Figures 2 and 5). This may be the main reason for the less well-defined phyletic positions of these three viruses in the results

of pan-genomic analysis (Figure 4B). In particular, *AaV* has been characterized as a *Megaviridae*-type phycodnavirus (Moniruzzaman et al., 2014). However, NCVOG orthologs commonly found in *Megaviridae*-type phycodnaviruses exhibit low homology to the corresponding genes in *AaV* (Figure 4). In addition, polyA polymerase (Supplementary Figure S4C) and asparagine synthetase (Moniruzzaman et al., 2014) are missing exclusively in *AaV*. These observations and Figure 4 show that *AaV* may be a non-standard member, or rather, outlier of the *Megaviridae*. *AaV* and *HaV53* clustered closely, though with low confidence, in our pan-genomic analysis. We also directly compared *AaV* and *HaV53* genes by all-to-all BLASTP, and consistent with the results presented in Figure 2, they did not exhibit particularly high homology to each other.

We observe a segregation of viruses currently considered to be phycodnaviruses into at least four groups. The proposed

Megaviridae-phycodnavirus group segregates from the rest. In addition, the *EhV* group clearly segregates from other *Phycodnaviridae*, consistent with the argument of Allen et al. (2006a; 2006b). *HaV53* does not show a strong association with any of the three groups, and thus presumably represents a novel, independent group. Accordingly, we found several orthologs that specifically associate with each group of *Phycodnaviridae* (Table 2). These group-specific genes can be utilized as hallmarks to classify *Phycodnaviridae* in the future.

Currently, there were two major scenarios for evolution of Giant dsDNA viruses; the ‘reduction model’ and the ‘expansion model.’ The ‘reduction model’ is based on the idea that the viruses presumably emerged from much more complex organisms with larger sizes of genome, and reached to current status by genome simplifications (Raoult et al., 2004; Martin et al., 2010; Boyer et al., 2011; Nasir and Caetano-Anollés, 2015). In the ‘expansion model,’ the viruses are presumed to descend from common ancestor virus with much smaller genomes, and reaching to contemporary sizes and diversity by progressively acquiring genes (Yutin et al., 2014). Assuming distinctive gene gain/loss penalty scores to yield ancestor virus with distinctive NCVOG numbers, two evolutionary paths resulting from the contrasting models were reproduced (Figure 5). When the ‘reduction model’ was reproduced with high gene gain penalty, the massive gene losses during the early stage of divergence of *Phycodnaviridae* minus Megaviridae from the rest (i.e., Megaviridae) were inferred [Figure 5, at node (I)]. On contrary, according to the ‘expansion model’ inferred by using lower gain penalty, major gene gains were observed after *Mimiviridae* diverged from smaller members of proposed Megaviridae. Comparative genome analyses of closely related members of *Phycodnaviridae* and *Mimiviridae* revealed specific patterns of gene gains and losses during the divergence of the lineages (Filee, 2015). Such future studies comprise of more distant viruses, possibly with more lineages, will provide insights into the overall evolutionally process of the Giant dsDNA viruses.

As *Phycodnaviridae* encompasses viruses infecting hosts of such vast diversity, they are expected to adopt varied strategies, and thus to develop genomes coding for distinctive genes with an array of functions during evolution. Our results, along with several previous observations, strongly

suggest that classification of *Phycodnaviridae* does not represent current similarity in their genetic components, viral life cycle, and evolutionary relatedness. Systematic reclassification of the family based on current knowledge may not only provide better taxonomy of viruses but also lead to a better representation and understanding of evolution of NCLDVs, which remain enigmatic biological entities.

AUTHOR CONTRIBUTIONS

FM conducted data analysis and participated in discussion and writing the paper. SU designed research, conducted data analysis, and wrote the paper.

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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.2016.01942/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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