Thesis for the Degree of

Master of Education

Purification and genetic analysis of a new *Prorocentrum minimum* infecting virus

by

Ju Hee Jeong

The Graduate School of Education

Pukyong National University

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Purification and genetic analysis of a new *Prorocentrum minimum* infecting virus

(해양 미세조류 *Prorocentrum minimum*에 감염하는 신종 바이러스의 순수분리를 통한 유전자 분석)

Advisor : Prof. Tae Jin Choi

by

Ju Hee Jeong

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Abstract

One of the biggest problems in sequencing the whole genome of a marine algal virus is purifying the virus. Most of the host cultured in laboratory has a concentration of 10^{3}~10^{4} particle/cell and hence when the host is inoculated with virus, the virus titer is not sufficient enough for molecular analysis. There is additional losses during the process of filtration and

concentration. Therefore, it is difficult to obtain high concentration of pure viral DNA for molecular analysis. In this study, various purification and concentration methods have been carried out to overcome the problems. Pre-filtration and mini-size hollow fiber ultracentrifugation was conducted to obtain large quantities of *Prorocentrum minimum* DNA Virus 01 (PminDNAV01). The virus suspension were treated with chloroform, DNaseI and RNaseA to make sure that all bacteria have been removed. Next generation sequencing was carried out with total DNA extrated from PminDNAV01 infected genome and the viral genome sequences were assembled using previously reported algal virus sequences.

Although, whole genome sequence of the virus could not been obtained, partial genome of the virus has been successfully recovered. Two contigs, PMV53(52,425bp) and PMV93 (46,021bp), were obtained after assembly of sequences. There are 19 ORFs of PMV53 and 7 ORFs of PMV93 that showed homology with proteins in GenBank database. According to the GenBank database, PminDNAV01 is closely related with nucleo-cytoplasmic large DNA viruses (NCLDVs). The improved virus purification process and whole genome shot-gun sequencing using next generation sequencing could reveal new viral genomes of a novel algal-infecting virus, PminDNAV01.

INTRODUCTION

For years, viruses had been known to exist in seawater (Bergh *et al.*, 1989; Proctor and Fuhrman, 1990), but 22 years ago, they caused great excitement because of their unexpected high abundance (concentrations typically ranging from 10^5 to 10^8 $m\ell^{-1}$) (Bergh *et al.*, 1989; Fuhrman, 1999; Proctor, 1997). Furthermore, viruses were shown to be infectious to the dominant organisms in the ocean (Suttle *et al.*, 1990; Suttle, 2005).

Most of these particles are presumably bacteriophage, but viruses infecting algae have also been demonstrated to be important agents of phytoplankton mortality in marine ecosystems (Bratbak *et al.*, 1993; Suttle *et al.*, 1990; Suttle, 1994).

Viral impacts on blooms of *Emiliania huxleyi*, *Heterosigma akashiwo*, and *Heterocapsa circularisquama* suggest that viruses may prevent and terminate phytoplankton bloom dynamics including harmful algal blooms (HABs) and toxic species (Suttle, 2000; Tarutani *et al.*, 2000; Nagasaki *et al.*, 2004; Wommack and Colwell, 2000).

Dinoflagellates are single-celled, aquatic organisms with two dissimilar flagellate. These species are considered to be one of the most abundant and diverse groups of phytoplankton as the net primary producers (Graham and Wilcox, 2000).

The host strain, *Prorocentrum minimum* is a small, marine dinoflagellate and has caused serious damage almost every year in Korea and other countries (Heil *et al.*, 2005; Lee and Kim, 2008; Nakamura and Hirata, 2006). In addition, its blooms have been associated with shrimp and fish die-offs in the southern coastal waters of Korea (Lee and Kim, 2008).

Virus and VLPs (virus like particles) have been observed in approximately 50 different marine eukaryotic algae, including microalgae and macroalgae, and have been isolated and characterized within the last two decades. Table 1 shows a list of viruses infecting eukaryotic microalgae that have been characterized by year 2011 (Nagasaki and Bratbak, 2010).

Table 1. Viruses infecting algae (2011)

Name	Host	Size (nm)	Genome Type	References
Chlorella virus (e.g., HVCV)	*Chlorella*-like alga (symbiont of *Hidea viridis*)	170-180	dsDNA, 200kb	Van Etten *et al.*, 1981; 1982; 1991
Chlorella virus (e.g., PBCV-1, NY-2A, R158)	*Chlorella* NC64A (symbiont of *Paramecium bursaria*)	150-190	dsDNA, 331-369kbp	Van Etten *et al.*, 1983, 1991, 2002; Van Etten and Meints, 1999; Yamada *et al.*, 1999, 2006; Fitzgerald *et al.*, 2007a
Chlorella virus (e.g., MT325, FR483)	*Chlorella* Pbi (symbiont of *Paramecium bursaria*)	140-150	dsDNA, 314-321kbp	Reisser *et al.*, 1988a, b; Van Etten *et al,*. 1991; Yamada *et al.*, 2006; Fitzgerald *et al.*, 2007b
EsV	*Ectocarpus siliculatus*	130-150	dsDNA, 336kbp	Müller 1991; Lanka *et al.* 1993; Müller *et al.* 1996, 1998; Van Etten *et al. 2002*
MpV	*Micromonas pusilla*	115	dsDNA, 200kbp	Cottrell and Suttle, 1991; 1995; Mayer and Taylor, 1979; Waters and Chan, 1982
FsV	*Feldmannia species*	150	dsDNA, 158kbp & 178kbp	Henry and Meints 1992; Müller *et al.* 1998; Meints *et al.* 2008
FlexV	*Feldmannia simplex*	120-150	dsDNA, 170kbp	Friess-Klebl *et al.*1994; Müller *et al.* 1998
CbV	*Chrysochromlina brevifilum*	145-170	dsDNA	Suttle and Chan, 1995
CroV (BV-PW1)	*Cafeteria roenbergensis* (reported as Bodo sp.)	230-300	dsDNA, 730kbp	Garza and Suttle 1995; Suttle pers. comm.
EfasV	*Ectocarpus fasciculatus*	135-140	dsDNA, 340kbp	Müller *et al.* 1996, 1998
PpV	*Phaeocystis pouchetii*	130-160	dsDNA, 485kbp	Jacobsen *et al.*, 1996; Bratbak *et al.*, 1998 Yan *et al.*, 2005
FirrV	*Feldmannia irresguralis*	140-170	dsDNA, 180kbp	Kapp *et al.* 1997; Müller *et al.* 1998
HaV	*Heterosigma akashiwo*	202	dsDNA, 294kbp	Nagasaki and Yamaguchi, 1997; Nagasaki *et al.*, 1999; 2005b; Tarutani *et al.*, 2000; Tomaru *et al.*, 2004b
HincV	*Hincksia hinckiae*	140-170	dsDNA, 220kbp	Kapp et al. 1997; Müller *et al.* 1998
MclaV	*Myriotrichia clavaeformis*	170-180	dsDNA, 340kbp	Kapp et al. 1997; Müller *et al.* 1998
AaV(BtV)	*Aureococcus anophagefferens*	140	dsDNA	Garry *et al.*, 1998; Gastrich *et al.*, 2004 Gobler *et al.*, 2004, 2007; Rowe *et al.*, 2008
PlitV	*Pilayella littoralis*	161	dsDNA, 280kbp	Maier *et al.* 1998; Müller *et al.* 1998
CeV	*Chrysochromlina ericina*	160	dsDNA, 510kbp	Sandaa *et al.*, 2001; Thyrhaug *et al.*, 2003; Monier *et al.*, 2008
HaNIV	*Heterosigma akashiwo*	30	-	Lawrence *et al.*,2001
HcV	*Heterocapsa circularisquama*	197±8	dsDNA, 356kbp	Tarutani *et al.*, 2001
PoV	*Pyramimonas orientalis*	180-220	dsDNA, 560kbp	Sandaa *et al.*, 2001
EhV	*Emiliania huxleyi*	170-200	dsDNA, 410-415kbp	Castberg *et al.*, 2002; Wilson *et al.*, 2002, 2005; Schroeder *et al.*, 2003; Thyrhaug *et al.*, 2003; Allen *et al.*, 2006
Mimivirus	*Acanthamoeba polyphaga*	750	dsDNA, 1.2Mbp	La Scora *et al.* 2003; Raoult *et al.* 2004

Table 1. continued

Name	Host	Size (nm)	Genome Type	References
Ols1	*Heterosigma akashiwo*	30 & 80	dsDNA, 20 & 130kbp	Juneau *et al.*, 2003; Lawrence *et al.*, 2006; Lawrence (unpublished)
RsRNAV	*Rhizosolenia setigera*	32±2	ssRNA, 11.2kbp	Nagsaki *et al.*, 2003
HcRNAV	*Heterocapsa circularisquama*	30	ssRNA, 4.4kbp	Tomaru *et al.*, 2004a; Nagasaki *et al.*,,. 2004a, 2005a, 2006; Mizumoto *et al.*, 2007
MpRV	*Micromonas pusilla*	50-60	dsRNA, 24.6kbp in total (segmented)	Brussaard *et al.*, 2004b; Attoui *et al.*, 2006
PgV Group I	*Phaeocystis globosa*	150	dsDNA, 466kbp	Brussaard *et al.*, 2004a; 2007; Baudoux and Brussaard, 2005
PgV Group II	*Phaeocystis globosa*	100	dsDNA, 177kbp	Brussaard *et al.*, 2004a; 2007 Baudoux and Brussaard, 2005
RsRNAV	*Rhizosolenia setigera*	32	ssRNA, 8.9kb	Nagasaki *et al.*, 2004b; Shirai *et al.*, 2006
Chlorella virus (e.g.,ATCV-1, ATCV-2)	*Chlorella* SAG 3.83 (symbiont of *Acanthocystis turfacea*)	140-190	dsDNA, 288kbp	Bubeck and Pfizner, 2005; Fitzgerald *et al.*, 2007c
CsalDNAV	*Chaetoceros salsugineum*	38±3	(ss+ds)DNA, 6kb & 1kbp	Nagasaki *et al.*, 2005c; Tomaru *et al.*, 2011
CspNIV	*Chaetoceros* cf. *gracilis*	25	-	Bettarel *et al.*, 2005
SssRNAV	*Aurantiochytrium sp.* NIBH N1-27 (reported as *Schizochtrium* sp. NIBH N1-27)	25±2	ssRNA, 10.2kbp	Takao *et al.*. 2005, 2006
MpVN1	*Micromonas pusilla*	110-130	DNA	Zingone *et al.*, 2006
MpVN2	*Micromonas pusilla*	110-130	DNA	Zingone *et al.*, 2006
PgV-102P	*Phaeocystis globosa*	98	dsDNA, 176kbp	Wilson *et al.*, 2006
SmDNAV	*Sicyoidochytrium minutum*	140	dsDNA, 250kbp	Takao *et al.* 2007
CdebDNAV	*Chaetoceros debilis*	30	ssDNA fragmented?	Tomaru *et al.*, 2008
CtenRNV	*Chaetoceros tenuissimus*	31±2	ssRNA, 8.9 & 4.3kb	Shirai *et al.*, 2008
OtV5	*Ostreococcus tauri*		dsDNA, 186kbp	Derelle *et al.*, 2008
CsfrRNAV	*Chaetoceros socialis* f. *radians*	22±1	ssRNA, 9.5kb	Tomaru *et al.*, 2009
CwNIV	*Chaetoceros* cf. wighamii	25±1.4		Eissler *et al.*, 2009
TampV	*Teleaulax amphioxeia*	203		Nagasaki *et al.*, 2009
OtV-1	*Ostreococcus tauri*	100-120	dsDNA, 191kbp	Derelle *et al.*, 2008; Weynberg *et al.*, 2009
ClorDNAV	*Chaetoceros lorenzianus* Grunow	32±3	(ss+ds)DNA 5.8kbp & 1kbp	Tomaru *et al.*, 2011
Csp05DNAV	*Chaetoceros* sp. (strain TG07-C28)	32±2	ssDNA	Toyoda *et al.*. 2011

Until now about 50 species of algae viruses have been researched in Japan, United States and Europe. Table 2 shows the 14 viruses that genome have been completely sequenced and most of them were chlorella virus which have high virus titer (Wilson and Schroeder, 2010). Most of the molecular biological researches on algal viruses were carried out by partial sequencing. Axenic culture and isolation of virus are needed to sequence a viral genome. This is because a non-axenic culture can cause bacterial sequence contamination.

Another major problem in sequencing algae virus complete genome is that scientists have to obtain large quantity of the virus to isolate high concentration of viral DNA (Wilson and Schroeder, 2010). However, it is difficult to obtain high concentration of pure viral DNA. This is because virus titer could be reduced during the process of filtration and concentration. Besides, DNA extraction protocol can also cause loss of virus.

The objective of this study is to determine the complete genome of a new *Prorocentrum minimum* infecting virus, PminDNAV01. Hence, to minimize the loss of virus during the purification steps, effective purification procedures have been developed to obtain large quantity of pure PminDNAV01 viral DNA sufficient enough for genetic analysis.

Table 2. Algal viruses whose completed genome sequences have been determined (NCBI, 2011)

Virus	Family	Accession	Size	Reference
DNA Virus				
Emiliania huxleyi virus 86	Phycodnaviridae	NC_007346	4.7,339	Wilson *et al.* 2005
Paramecium bursaria Chlorella virus NY2A	Phycodnaviridae	NC_009898	368,683	Fitzgerald *et al.* 2007a
Paramecium bursaria Chlorella virus AR158	Phycodnaviridae	NC_009899	344,691	Fitzgerald *et al.* 2007a
Paramecium bursaria Chlorella virus 1	Phycodnaviridae	NC_000852	335,593	Li *et al.* 1997
Paramecium bursaria Chlorella virus FR483	Phycodnaviridae	NC_008603	321,240	Fitzgerald *et al.* 2007b
Paramecium bursaria Chlorella virus MT325	Phycodnaviridae	DQ491001	314,335	Fitzgerald *et al.* 2007b
Acanthocystis turfacea Chlorella virus 1	Phycodnaviridae	NC_008724	288,047	Fitzgerald *et al.* 2007c
Ostreococcus virus OsV5	Phycodnaviridae	NC_010191	185,373	Derelle *et al* 2008
Feldmannia species virus	Phycodnaviridae	NC_011183	154,641	Schroeder *et al.* 2009
RNA Virus				
Micromonas pusilla reovirus	Reoviridae	NC_008171 NC_008181	25,563	Attoui *et al.* 2006
Chaetoceros tenuissimus RNA virus	Unclassified	AB375474	9,431	Shirai *et al.* 2008
Heterosigma akashiwo RNA virus SOG263	Marnaviridae	NC_005281	8,587	Lang *et al.* 2004
Chaetoceros salsugineum Nuc Incl virus	Unclassified	NC_007193	6,000	Nagasaki *et al.* 2005b
Heterocapsa circularisquama RNA virus	Unclassified	NC_007518	4,375	Nagasaki *et al.* 2005a

MATERIALS AND METHODS

1. Host and virus

The host strain of *Prorocentrum minimum* NF-F-PMI-1d was provided by the National Fisheries Research and Development Institute (NFRDI) in Korea. This strain had already been identified by PCR amplification using rDNA primer sets in NFRDI. The host strain was cultured in modified f/2 medium (Table 3) under 12L:12D cycle of 80 to 90 μmol photons with cool white fluorescent illumination at 20℃.

The virus, PminDNAV01 was isolated in Jaran Bay, Namhae, Korea by virus lab in Pukyong National University, Busan, Korea (07/28/2009). Size of the virus was 175±10㎚ in diameter (average ± standard deviation; n=30) and the shape was icosahedral. There was no outer membrane or tail-like structure. After inoculation of PminDNAV01 to the host, host cells were completely lysed 96 hpi (Kim, 2011).

Table 3. Composition of the modified f/2 medium

Filtered seawater		1L
add	FRITZ F/2 ALGAE FOOD PART A	132㎕
	FRITZ F/2 ALGAE FOOD PART B	132㎕
	$Na_2SiO_3 \cdot 9H_2O$	1㎖

FRITZ F/2 ALGAE FOOD PART A (FRITZ INDUSTRIES, INC.)	
Iron (Fe)	1.3%
Manganese (Mn)	0.034%
Cobalt (Co)	0.002%
Zinc (Zn)	0.0037%
Copper (Cu)	0.00017%
Molybdate (Mo)	0.0009%

FRITZ F/2 ALGAE FOOD PART B (FRITZ INDUSTRIES, INC.)	
Nitrogen (N)	6.0%
Phosphate (P_2O_5)	2.0%
Vitamine B_1	0.07%
Vitamine B_{12}	0.0002%
Biotin	0.0002%

Ingredients : Ferric Chloride, EDTA, Cobalt Chloride, Zinc Sulfate, Copper Sulfate, Manganese Chloride, Sodium Molybdate, Sodium Nitrite, Monosodium Phosphate, Thiamine Hydrochloride (Vit B_1), Vitamin B_{12}, Biotin

$Na_2SiO_3 \cdot 9H_2O$ (Sigma-Adrich®)
30 g/L × dH_2O

2. Next generation sequencing

For next generation sequencing, 500㎖ of exponentially growing cultures of *Prorocentrum minimum* NF-F-PMI-1d strain were inoculated with 20㎖ of the virus, PminDNAV01 suspension. Control cultures were established by adding 20㎖ of f/2 medium was added, served as control. Around 80 hpi (before the virus burst out of the host cell), inoculated host cells were harvested by centrifugation at 4000rpm for 40 min. Extraction by modified CTAB method on the harvested host cells were carried out to obtain the total genomic DNA (Minas *et al.* 2011; Winnepenninckx *et al.* 1993).

The total genomic DNA was sent to NICEM at Seoul National University for analysis of whole genole sequencing by next genration sequencing. The DNA sample was dyed with PicoGreen (Quant-iT™ PicoGreen dsDNA Reagent and Kits, invitrogen™) and the concentration of the DNA was checked by using victor3 (PerkinElmer Inc.). Library of the total genomic DNA was prepared by TruSeq® DNA Sample Prep Kits (illumina, INC.). Quality of the library was checked by using Agilent DNA 1000 chip (Agilent Technologies) and analysed by Agilent 2100 Bioanalyzer (Agilent Technologies). The sample was then sequenced by HiSeq™ 2000 (illumina, INC.).

3. Virus preparation and DNA extraction

A 20ℓ of exponentially growing *Prorocentrum minimum* culture was inoculated by 4ℓ of virus, PminDNAV01 lysate and lysed. The 24ℓ lysate was sequentially passed through 3.0㎛ (hydrophobic PTFE membrane capsule filter, SYNOPEX), 0.45㎛ (PES membrane capsule filter, SYNOPEX) and 0.2㎛ (PES membrane capsule filter, SYNOPEX) capsule filters to remove cell debris. Then the filtrate was concentrated by 30 kDa cut-off tangential flow ultra-filtration system (SYNOPEX) to a final volume of 240㎖ (100X concentration).

Polyethylene glycol 6000 (PEG #6000; Tokyo Chemical Industry Co., Ltd.) was added to the filtrate and totally dissolved to a final concentration of 10% (wt/vol), and the suspension was stored overnight at 4℃ in the dark. After centrifugation at 67,800xg at 4℃ for 2.5 hrs, the viral pellet was resuspended with 10㎖ of DEPC-treated distilled water and centrifuged again at 222,000xg at 4℃ for 4.5 hrs to collect the virus particles. They were resuspended in 1㎖ of DEPC-treated distilled water, and an equal volume of chloroform was added. After vortexing, the suspension was centrifuged at 2,200xg for 20 min at room temperature to remove the chloroform. The water phase (viral suspension) was transferred to a new tube (Kensuke *et al.* 2012).

The viral suspension was then incubated with 50U/㎖ of DNaseI (Takara Bio Inc.) and 0.1㎍/㎕ of RNaseA (GENET BIO,

Korea) at 37℃ for 1 hrs.

A discontinuous sucrose density gradient was prepared by layering successive decreasing sucrose gradients solution from 10-40% in polyallomer tubes. The viral suspension was put on top of the prepared sucrose gradients solution. The tube was then centrifuged at 210,000xg at 4℃ for 2.5 hrs. The viral band was collected by a side puncture and distilled water was added to a final volume of 10㎖. The viral suspension was then centrifuged again with same conditions as above for 3hrs. The viral pellet was resuspended with 1㎖ of DEPC-treated distilled water. Twenty microliter of the viral suspension was used for SDS-PAGE and the remaining suspension was used for PminDNAV01 nucleic acid extraction.

The nucleic acid of PminDNAV01 was extracted by modified CTAB methods (Minas *et al.* 2011; Winnepenninckx *et al.* 1993) and the resultant pellet was dissolved in 100㎕ of DEPC-treated distilled water (Fig. 1).

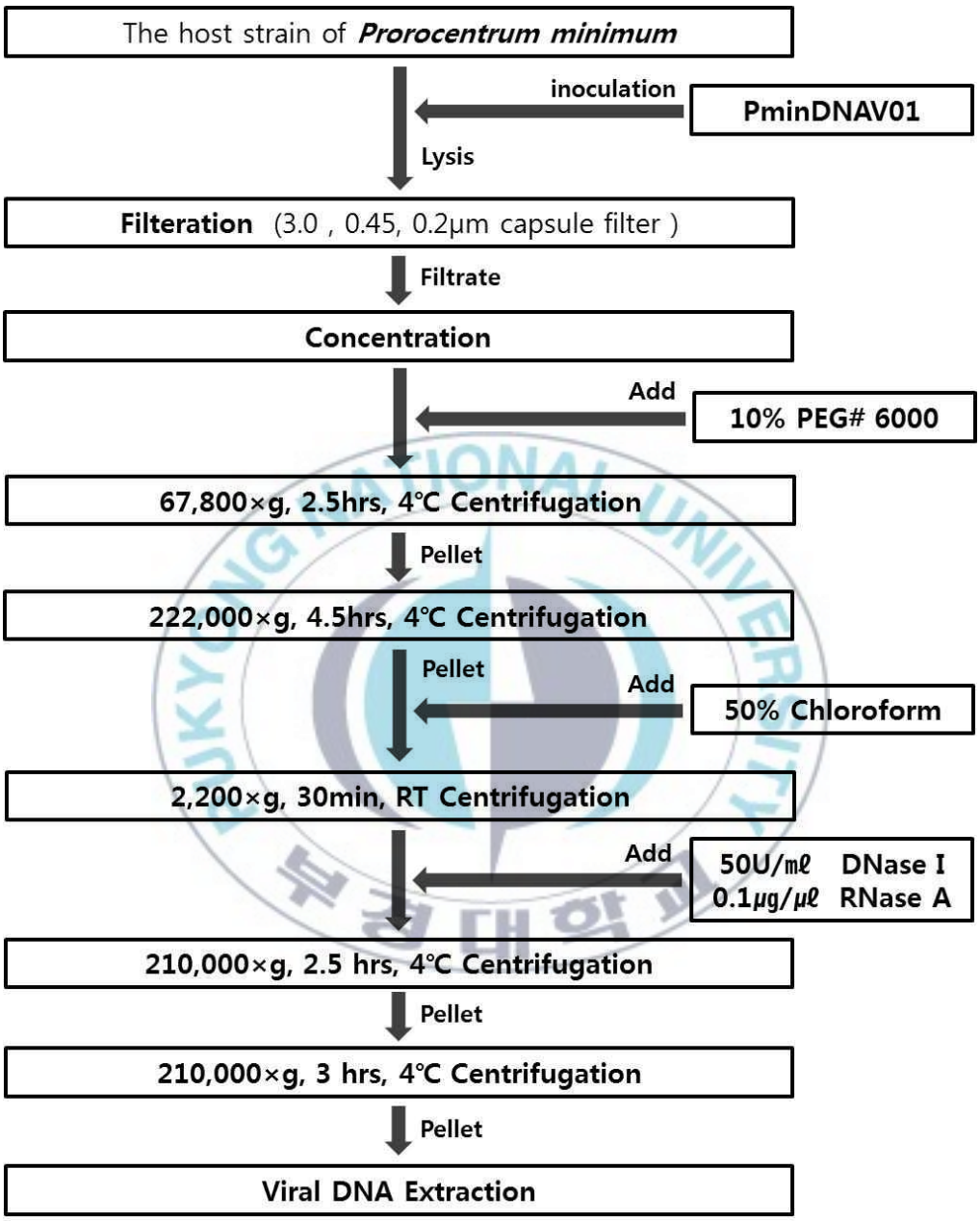

Figure 1. Summarized scheme of algal virus concentration and purification procedure

3.1. PCR of major capsid protein gene, DNA polymerase gene

In order to amplify the major capsid protein gene, specific PCR primers, mcp Fwd. and mcp Rev. designed by Larsen *et al.* (2008) were used. For amplification of the DNA polymerase gene, 2 sets of primers have been used, AVS1-AVS2 and AVS1-AVS3 designed by Chen and Suttle (1995). Sequences and Tm (melting temperature) of the 5 primers are shown in Table 4.

PCR were performed in a final volume of 30㎕ containing 100pmol of each primer, 2㎕ of concentrated PminDNAV01 genomic DNA and 2×Prime Taq Premix (GENET BIO, Korea).

The PCR program for the amplification of the major capsid protein gene was initial denaturation at 94℃ for 5 min followed by 35 cycles at 94℃ for 30 sec, 49℃ for 1 min, 72℃ for 1 min and final extension at 72℃, 7 min.

The PCR program for the amplification of the DNA polymerase gene was initial denaturation at 94℃ for 5 min followed by 35 cycles at 94℃ for 30 sec, 55℃ for 1 min, 72℃ for 1 min and final extension at 72℃, 7 min.

The amplified products were analyzed by using electrophoresis on 1% agarose gel (SeaKem® LE Agarose; Lonza) and photographed under UV trans-illumination after staining with ethidium bromide (0.5 ㎍/㎖$^{-1}$)

Table 4. Sequences and Tm of major capsid protein gene, DNA polymerase gene PCR primers

Primer	Sequence	Tm (℃)	Reference
major capsid protein gene			
mcp Fwd	5′- GGY GGY CAR CGY ATT GA - 3′	51.8℃	Larsen *et al.* 2008
mcp Rev	5′- TGI ARY TGY AYI AGG TA - 3′	48.9℃	Larsen *et al.* 2008
DNA polymerase gene			
AVS1	5' - GAR GGI GCI ACI GTI YTI GAY GC - 3'	68.3℃	Chen and Suttle. 1995
AVS2	5' - GCI GCR TAI CKY TTY TTI SWR TA - 3'	58.2℃	Chen and Suttle. 1995
AVS3	5' - SWR TCI GTR TCI CCR TA - 3'	49.1℃	Chen and Suttle. 1995

3.2. SDS-PAGE

20㎕ of PminDNAV01 suspension was mixed with four volume of denaturing sample buffer (62.5mM Tris-HCl, 5% 2-mercaptoethanol, 2% sodium dodecyl sulfate [SDS], 20% glycerol, 0.005% bromophenol blue) and boiled for 5 min. The proteins were then separated according to the different size of protein by sodium dodecyl sulfate polyacrylamide gel eletrophoresis (SDS-PAGE) on 12 % separation gel at 50 V for 1 hrs and 100 V for 2 hrs. The proteins were visualized using Coomassie brilliant blue stain. Prestained protein ladder (Thermo SCIENTIFIC) ranging from 10 kDa to 170 kDa was used for size calibration.

3.3. PCR of 16S rRNA for confirmation of bacteria contamination

16S rRNA primers, 1492RF-27RF, EUB338RF-Bac514RF and Bac514RF-Bac785RF were used to amplify the viral DNA sample. Sequences and Tm (melting temperature) of the 5 primers are shown in Table 5.

The PCR program for the amplification of 16S rRNA was initial denaturation at 94℃ for 5 min followed by 35 cycles at 94℃ for 30sec, 58℃ for 1 min, 72℃ for 1 min and then final extension at 72℃, 7 min.

Table 5. Sequences and Tm of 16S rRNA PCR primers

Primer	Sequence	Tm (℃)	Reference
16S rRNA			
1492RF	5' - ACC GYT ACC TTG TTA CGA CTT - 3'	59.1℃	Adachi *et al.* 2002
27RF	5' - CTG AGC CAK GAT CAA ACT CT - 3'	59.4℃	Adachi *et al.* 2002
EUB338RF	5′ - GCT GCC TCC DDG TAG GAG T - 3'	64.4℃	Adachi *et al.* 2002
Bac514RF	5' - ATT ACC GCG GCT GCT GGC ACG - 3'	76.7℃	Adachi *et al.* 2002
Bac785RF	5' - CTA CCA GGG TAT CTA ATC C - 3'	52.4℃	Adachi *et al.* 2002

RESULT

1. Host and Virus

Prorocentrum minimum NF-F-PMI-1d cultures and the PminDNAV01 observed by optical microscopes. In 4 days *Prorocentrum minimum* NF-F-PMI-1d was completely lysed after inoculated with PminDNAV01.

Different from previous researches, large scale culture of 20L of host cell was inoculated with 4L of PminDNAV01 lysate in this study. In small scale culture, inoculated host cells showed complete lysis at 96 hpi but in this study, it took around 1 week post-inoculation for the host cell to reach complete lysis (Fig. 2).

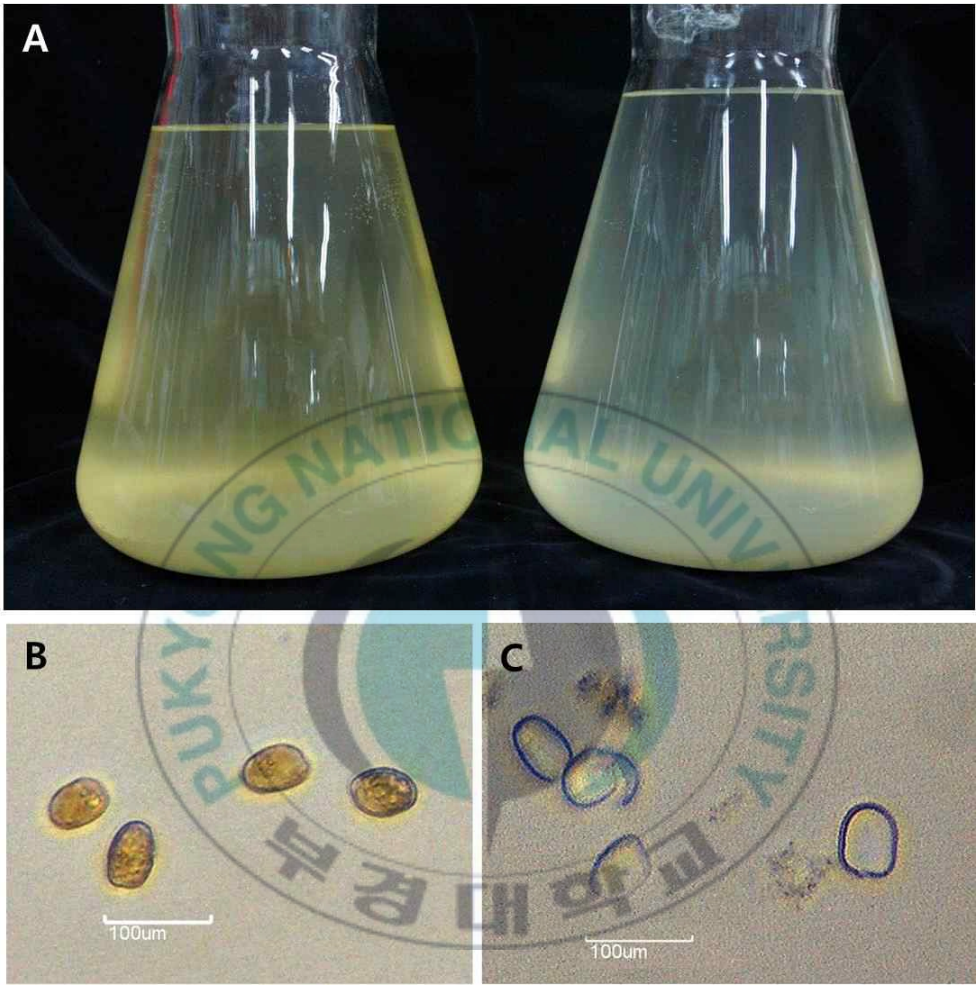

Figure 2. *Prorocentrum minimum* NF-F-PMI-1d.

(A) Picture of healthy host culture (Left) and completely lysed PminDNAV01 inoculated host culture at 120 hpi (Right). (B) Optical microscopic observation of intact cells. (C) Optical microscopic observation of PminDNAV01-infected cells at 160 hpi.

2. Next generation sequencing

Two contigs, PMV53 and PMV93 of 52,425bp and 46,021bp, respectively were assembly from NGS.

By using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), ORFs of 2 contigs were identified. By using BLAST, the upstream and downstream ORFs, 149 coding sequences of contig-53 and 91 cording sequences of contig-93, were considered, respectively.

19 ORFs sites of PMV53 genome information (52,425bp) showed similarity with known algal viruses (Fig. 3, Table 6).

Ribonucleodside-diphosphate reductase small subunit of *Emiliania huxleyi* virus were matched with PMV53-001 (E value=2e-90).

ORFs related to RNA polymerase subunit1 of African swine fever virus were found such as PMV53-002 (E value=4e-62) and PMV53-003 (E value=7e-122). Additionally, in PMV53-002, DNA-directed RNA polymerase subunit of *Heterocapsa circularisquama* DNA virus 01 in (E value=7e-58) and DNA-directed RNA polymerase subunit of *Acanthamoeba polyphaga lentillevirus* in (E value=1e-42) showed matched.

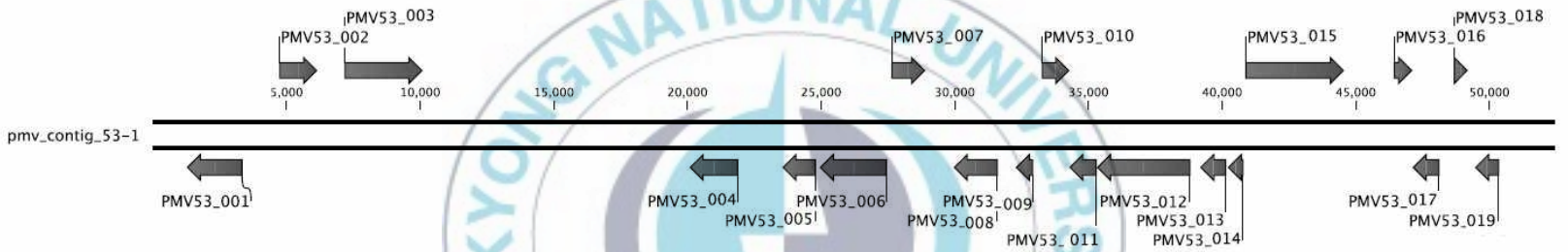

Figure 3. Linear map of the PminDNAV01 contig-53 indicating the location of ORFs

Table 6. ORFs information of the PminDNAV01 contig-53

ORF	Start	End	Direction	Length(N)	Length(A)
PMV53-001	3358	1271	reverse	2088	695
PMV53-002	4718	6145	forward	1428	475
PMV53-003	7165	10086	forward	2922	973
PMV53-004	21899	20079	reverse	1821	606
PMV53-005	24804	23554	reverse	1251	416
PMV53-006	27466	24950	reverse	2517	838
PMV53-007	27621	28892	forward	1272	423
PMV53-008	31613	29961	reverse	1653	550
PMV53-009	32929	32282	reverse	648	215
PMV53-010	33252	34286	forward	1035	344
PMV53-011	35311	34289	reverse	1023	340
PMV53-012	38815	35318	reverse	3498	1165
PMV53-013	40160	39180	reverse	981	326
PMV53-014	40799	40215	reverse	585	194
PMV53-015	40874	44557	forward	3684	1227
PMV53-016	46416	47111	forward	696	231
PMV53-017	48127	47129	reverse	999	332
PMV53-018	48663	49187	forward	525	174
PMV53-019	49462	50370	reverse	909	302

PMV53-004 showed ring-finger-containing E3 ubiquitin ligase of *Acanthamoeba castellanii* mamavirus (E value=5e-65); putative helicase of Megavirus courdo 7 (E value=2e-90), Megavirus chilinsis (E value=8e-90), Moumouvirus Monve (E value=4e-89), *Acanthamoeba polyphaga* mimivirus (E value=9e-65), Lausannevirus (E value=2e-54); zinc-finger protein of Maseillevirus (E value=1e-53), putative superfamily II helicase of *Cafeteria roenbergensis* virus BV-PW1 (E value=1e-25), putative ATPase/DNA helicase of *Amscta moorei* entomopoxvirus 'L' (E value=1e-15).

PMV53-006 showed best match with major capsid protein: African swine fever virus (E value=1e-65), Tiger frog virus (E value=4e-08), Epizootic haematopoietic necrosis virus (E value=5e-08), King grouper iridovirus (E value=6e-08), Bohle iridovirus (E value=7e-08), *Rana catesbeiana* virus JP (E value=7e-08), Chinese giant salamander virus (E value=7e-08), Pike-peach iridovirus (E value=7e-08), European catfish virus (E value=7e-08), *Rana esculenta* virus (E value=7e-08), Common midwife toad ranavirus (E value=7e-08) and Soft-shelled turtle iridovirus (E value=8e-08). Phylogenetic tree (Fig. 4) showed major capsid protein was more related with *Asfarviridae* (African swine fever virus) than *Iridoviridae* (King grouper iridovirus, Bohle iridovirus, Tiger frog virus, Soft-shelled turtle iridovirus, common midwife toad ranavirus, Pike-perch iridovirus, *Rana esculnta* virus, Chinese giant salamander virus, European catfish virus, and Epizootic haematopoirtic necrosis virus).

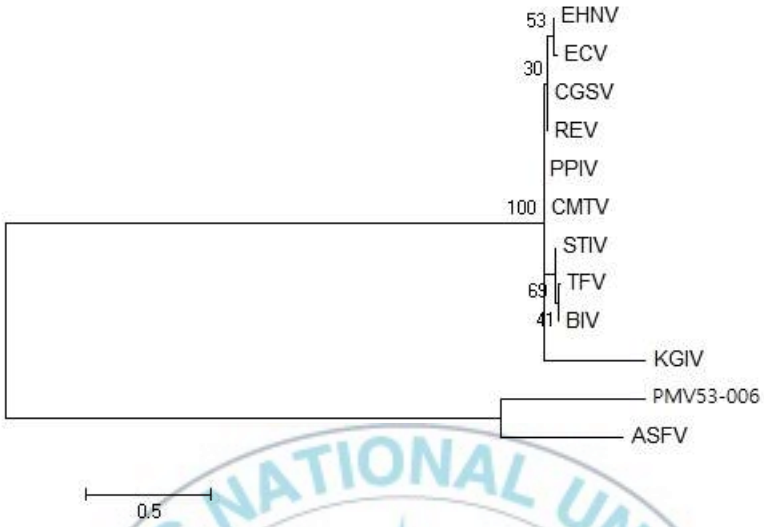

Figure 4. Phylogenetic tree of major capsid protein of ORFs PMV53-006; ASFV, African swine fever virus (Accession no. Q8V9S6.1); KGIV, King grouper iridovirus (AEI85924.1); BIV, Bohle iridovirus (ACO90022.1); TFV, Tiger frog virus (AAL77814.1); STIV, Soft-shelled turtle iridovirus (ACF42314.1); CMTV, common midwife toad ranavirus (AFA44920.1); PPIV, Pike-perch iridovirus (ACO90019.1); REV, Rana esculnta virus (ACO90020.1); CGSV, Chinese giant salamander virus (AET51835.1); ECV, European catfish virus(YP006347613.1); EHNV, Epizootic haematopoirtic necrosis virus (AAO32315.1). The scale bar indicates a distance of 0.5 fixed mutations per amino acid position.

PMV53-007 best matched with HNH endonuclease of *Heterocapsa circularisquama* DNA virus 01 (E value=6e-16), *Acanthamoeba castellanii* mamavirus (E value=2e-12), *Acanthamoeba polypage* lentillevirus (E value=0.068), *Acanthamoeba polypage* mimivirus (E value=0.54), Marseillevirus (E value=4e-10); HNH homing endonuclease of Lausannevirus (E value=2e-12).

PMV53-008 matched with glutamine-dependent asparagine synthetase of Monumouvirus Monve (E value=4e-103), Megavirus courdo7 (E value=2e-96), *Acanthamoeba polypage* virus (E value=6e-95) ; asparagine synthetase of *Acanthamoeba castellanii* mamavirus (E value=6e-95), *Ostreococcus lucimarinus* virus OIV4 (E value=3e-85), *Ostreococcus lucimarinus* virus OIV3 (E value=3e-84), *Ostreococcus lucimarinus* virus OIV6 (E value=7e-84), *Ostreococcus tauri* virus 2 (E value=7e-84).

PMV53-011 matched with HNH endonuclease of *Heterocapsa circularisquama* DNA virus 01 (E value=3e-38), *Acanthamoeba castellanii* mamavirus (E value=4e-13); HNH homing endonuclease of Lausannevirus (E value=1e-10).

PMV53-012 showed similarity with early transcription factor large subunit, VETF-L of Fowlpox virus (E value=1e-04).

PMV53-13 matched with HNH endonuclease of *Heterocapsa circularisquama* DNA virus 01 (E value=5e-145), Marseillevirus (E value=8e-17), *Acanthamoeba castellanii* mamavirus(E value=2e-08); HNH homing endonuclease of Lausannevirus (E value=2e-12); HNH endonuclease family protein of *Enterococcus* phage EFRM31

(E value=1e-08).

Also, PMV53-014 showed similarity with HNH endonuclease of *Heterocapsa circularisquama* DNA virus 01 (E value=3e-126).

PMV53-015 with type B DNA polymerase of *Heterocapsa circularisquama* DNA virus 01 (E value=0.0); DNA polymerase of African swine fever virus (E value=1e-81) and putative B family DNA polymerase of *Pyramimonas orientalis* virus (E value=3e-26) were matched. PMV53-015 showed highest homology with Heterocapsa circularisquama DNA virus 01 with 97% similarity (Fig. 5). A phylogenetic tree of PMV53-015 with viruses that showed high similarity was drawn. PMV53-015 showed closest relationship with *Heterocapsa circularisquama* DNA virus 01 than African swine fever virus (Fig. 6).

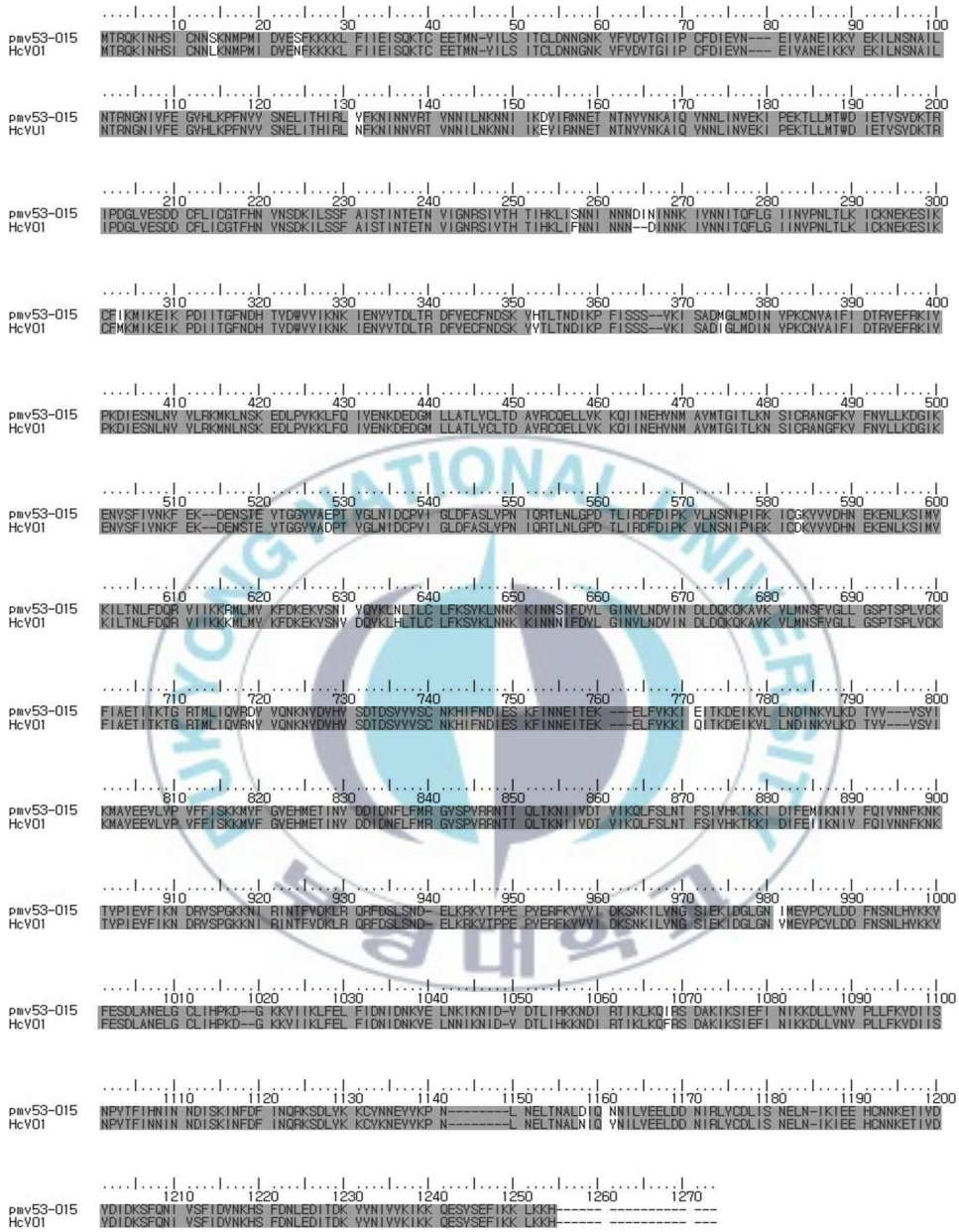

Figure 5. Alignment of DNA polymerase sequence of PMV53-015 with *Heterocapsa circularisquama* DNA virus 01 (Accession no. BAI48198.1). Grey box referred to matched sequences between the two proteins.

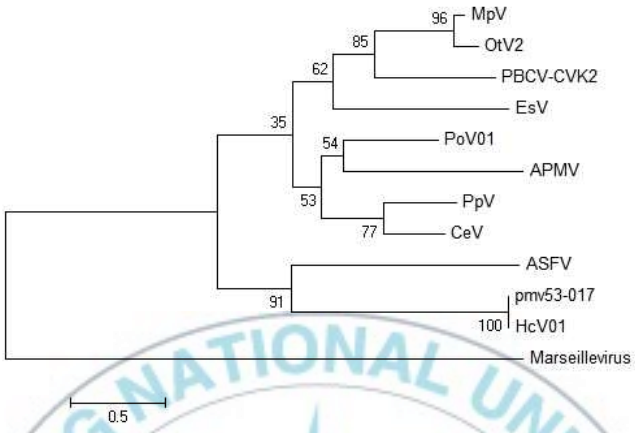

Figure 6. Phylogenetic tree of DNA polymerase sequence of ORFs. PMV53-015; *Micromons pusilla* virus PL1 (Accession no. AET43521); *Ostreococcus tauri* virus 2 (YP_004063640); *Paramecium busaria Chlorella* virus CVK2 (AB011500.1); *Ecotocarpus siliculosus* virus 1 (AF204951.2); *Pyramimonas orientalis* virus (ABU23717.1); *Acanthamoeba polyphaga* mimivirus (AAV50591); *Phaeocystis pouchetii* virus (A7U6F3.1); *Chrysochromulina ericina* virus (A7U6F1.1); ASFV, African swine fever virus (NP042783.1); HcV01; *Heterocapsa circularisquama* DNA virus 01 (BAI48198.1); Marseillevirus (AFM52350). The scale bar indicates a distance of 0.5 fixed mutations per amino acid position.

PMV53-018 showed homology with viral transcription factor 2 of Megavirus bus (E value=4e-06), Megavirus terra 1 (E value=1e-05), Moumouvirus ochan (E value=0.002), Mimivirus pointerouge 1 (E value=0.026), Moumouvirus moumou (E value=5.6) ; VLTF2-like transcription factor of Lymphocystis disease virus 1 (E value=0.21). However when the phylogenetic tree was observed, PMV53-018 is not in the same cluster with the *Mimiviridae* (Fig 7).

PMV53-019 showed match with TATA-box binding protein of *Acanthamoeba polyphaga* mimivirus (E value=0.10); TATA-box-binding protein-like protein of *Acanthamoeba polyphaga* mimivirus (E value=0.41), Megavirus courdo 7 (E value=2.5).

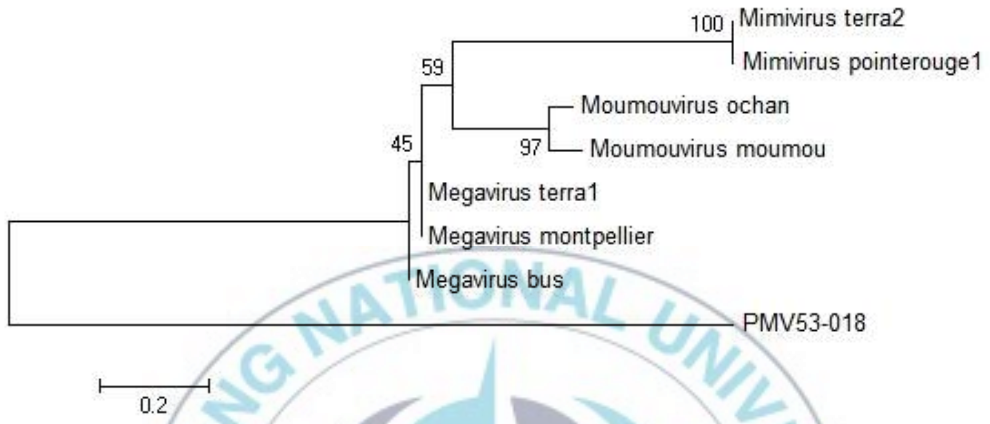

Figure 7. Phylogenetic tree of viral transcription factor 2 of ORFs. PMV53-018; Megavirus bus (Accession no. AEY9928401); Megavirus montpellier (AEY99282.1); Megavirus terra1 (AEY99281.1); Moumouvirus moumou (AEY9278.1); Moumouvirus ochan (AEY99279.1); Mimivirus pointerouge1 (AEY99269.1). The scale bar indicates a distance of 0.2 fixed mutations per amino acid position.

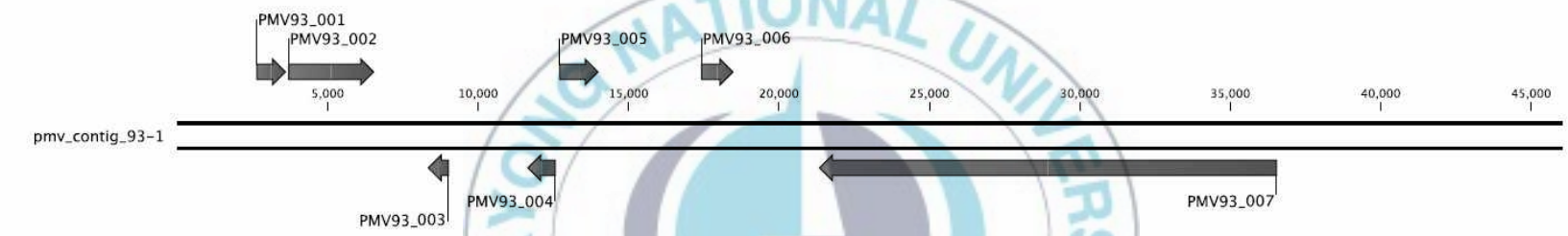

Figure 8. Linear map of the PminDNAV01 contig-93 indicating the location of ORFs.

Table 7. ORFs information of the PminDNAV01 contig-93

ORF	Start	End	Direction	Length(N)	Length(A)
PMV93-001	2632	3615	forward	984	327
PMV93-002	3686	6547	forward	2862	953
PMV93-003	9033	8323	reverse	711	236
PMV93-004	12582	11638	reverse	945	314
PMV93-005	12691	14007	forward	1317	438
PMV93-006	17408	18487	forward	1080	359

7 ORFs sites of PMV93 (46,021bp) matched with known algal viruses (NCBI database) (Fig. 8, Table 7).

PMV93-001 showed similarity with HNH endonuclease. HNH endonuclease of *Heterocapsa circularisquama* DNA virus 01 (E value=3e-54), *Acanthamoeba castellanii* mamavirus (E value=1e-11), Marseillevirus (E value=1e-19); HNH homing endonuclease of Lausannevirus (E value = 7e-15).

PMV93-002 showed homology with DNA mismatch repair protein of *Heterocapsa circularisquama* DNA virus 01 (E value=0.0), MutS protein of *Pyramimonas orientalis* virus (E value=3e-118), *Chrysochromulina ericina* virus (E value=3e-112), *Phaeocystis pouchetii* virus (E value=9e-110), DNA mismatch repair ATPase MutS of *Acanthamoeba castellanii* mamavirus (E value=1e-113) and *Acanthamoeba polyphaga* mimivirus (E value=1e-103).

PMV93-003 showed homology with putative Fe2OG dioxygenase of MoumouVirus Monve (E value=1.5), Megavirus chiliensis (E value=3.1), Megavirus courdo 7 (E value=3.2); alkylated DNA repair of Acanthamoeba polyphaga mimivirus (E value=6.4).

PMV93-004 showed homology with proliferating cell nuclear antigen-like protein of African swine fever virus (E value=2e-2).

Table 8. PminDNAV01 ORFs grouped by their functions

Nucleotid transport and metabolism	
PMV53-001	ribonucleodside - diphosphate reductase small subunit
PMV53-008	glutamine-dependent asparagine synthetase
PMV53-008	asparagine synthetase
Transciption	
PMV53-002	DNA-directed RNA polymerasesubunit
PMV53-003	RNA polymerase subunit1
PMV53-004	RING-finger-containing E3 ubiquitin ligase
PMV53-004	zinc-finger protein
PMV53-012	early transcription factor large subunit, VETF-L
PMV53-018	viral transcription factor 2
PMV53-018	VLTF2-like transcription factor
PMV53-019	TATA-box binding protein
PMV53-019	TATA-box-binding-like protein
DNA and RNA Replication, Recombination, and Repair	
PMV53-004	putative superfamily II helicase
PMV53-004	putative helicase
PMV53-004	putative ATPase/DNA helicase
PMV53-015	type B DNA polymerase
PMV53-015	DNA-directed DNA polymerase
PMV53-015	DNA-dependent DNA polymerase
PMV93-002	DNA mismatch repair protein
PMV93-002	MutS protein
PMV93-002	DNA mismatch repair ATPase MutS
PMV93-003	putative Fe2OG dioxygenase
PMV93-003	alkylated DNA repair
Structural Protein	
PMV53-006	Major capsid protein
Integration and Transposition	
PMV53-007	HNH endonuclease
PMV53-011	HNH endonuclease
PMV53-013	HNH endonuclease
PMV53-014	HNH endonuclease
PMV93-001	HNH endonuclease
PMV53-007	HNH homing endonuclease
PMV53-011	HNH homing endonuclease
PMV53-013	HNH homing endonuclease
PMV53-014	HNH homing endonuclease

Table 9. NCLDVs that have close relationship with PminDNA01

Poxviridae	**Mimiviridae**
Amscta moorei entomopoxvirus 'L'	Acanthamoeba polyphaga lentillevirus
Fowlpox virus	Acanthamoeba polyphaga mimivirus
	Acanthamoeba castellanii mamavirus
Asfarviridae	Megavirus courdo 7
African swine fever virus (ASFV)	Megavirus chiliensis
	Moumouvirus Monve
Iridoviridae	Megavirus bus
Epizootic haematopoietic necrosis virus	Megavirus terra1
Tiger frog virus	Megavirus montpellier
Chinese giant salamander virus	Moumouvirus ochan
Soft-shelled turtle iridovirus	Mimivirus pointerouge1
Pike-peach iridovirus	Moumouvirus moumou
King grouper iridovirus	
Bohle iridovirus	**Unclassified**
Common midwife toad ranavirus	Heterocapsa circularisquama DNA virus 0 1 (HcV01)
Chinese giant salamander virus	Cafeteria roenbergensis virus BV-PW1
European catfish virus	Ostreococcus lucimarinus virus OlV4
	Ostreococcus lucimarinus virus OlV3
Phycodnaviridae	Ostreococcus lucimarinus virus OlV6
Emiliania huxleyi virus	**Maseillevirus**
Ostreococcus tauri virus 2	**Lausannevirus**
Pyramimonas orientalis virus (PoV01)	
Chrysochromulina ericina virus	
Phaeocystis pouchetii virus	

Table 8 showed the 26 PminDNAV01 ORFs that have homolog in the GenBank data base and are grouped by their functions. Nucleo-cytoplasmin large DNA viruses (NCLDVs) that have similarity with PminDNAV01 (contig-53, contig-93) are summarized in Table 9.

Additionally, PMV53-005, 009, 010, 016, 017 and PMV93-005, 006 showed similarity with hypothetical protein of NCLDVs (Data not shown).

3. Virus preparation and DNA extraction

The result of electrophoresis showed that the genome size of nucleic acids extracted from PminDNAV01 was over 10kb (Fig. 9).

3.1. PCR of major capsid protein gene and DNA polymerase gene

PCR amplification of the virus with the primer set of mcp Fwd.-Rev. produced DNA fragment of 450bp (Fig. 10). In DNA polymerase primer sets of AVS1-AVS2, no amplification was observed. However, in AVS1-AVS3 produced multiple bands of 1,000bp, 750bp and 500bp (Fig. 11). Sequence analysis result of the 750bp band showed high similarity with *Heterocapsa circularisquama* DNA virus 01 (data not shown) and this confirmed the earlier shown result of NGS analysis (Fig. 5).

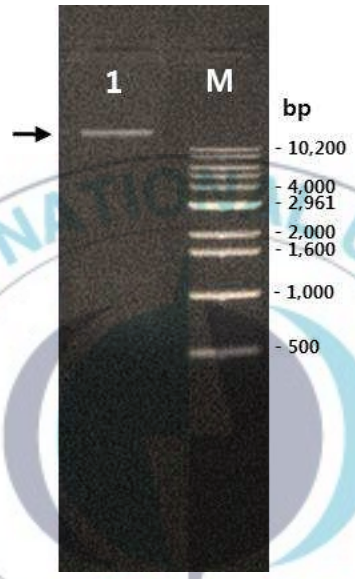

Figure 9. Electrophoresis of total viral genome of PminDNAV01

Lane 1: Nucleic acids extracted from PminDNAV01

Lane M: 1kb DNA ladder

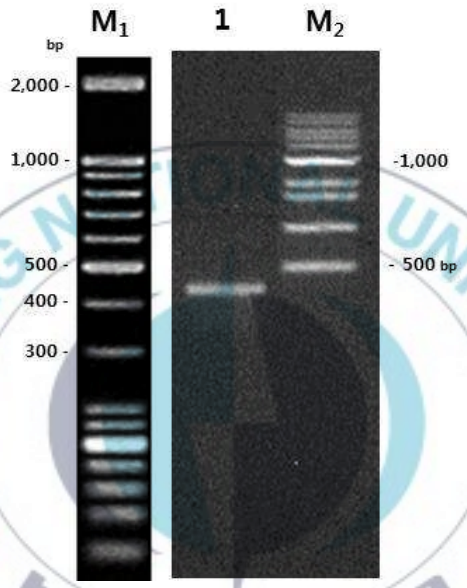

Fig. 10. Analysis of major capsid protein of PminDNAV01

Lane 1: PCR product of PminDNAV01 with mcp primer set

Lane M_1: 100bp DNA ladder

Lane M_2: 1kb DNA ladder

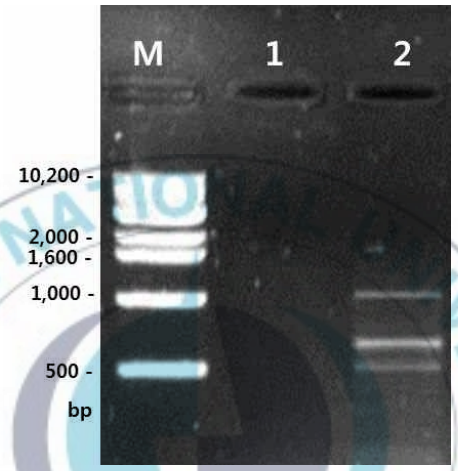

Figure 11. Analysis of viral DNA polymerase gene primer .

Lane 1: PCR product of PminDNAV01 with primer set of AVS 1-AVS 2

Lane 2: PCR product of PminDNAV01 with primer set of AVS 1-AVS 3

Lane M: 1kb DNA ladder

3.2. SDS-PAGE

The size and number of structural proteins were estimated using SDS-polyacrylamide gel electrophoresis. PminDNAV01 contained at least 3 proteins of 120, 37.5 and 14 kDa in molecular weight (Fig. 12)

3.3. PCR of 16S rRNA for confirmation of bacteria contamination

PCR using 16s rRNA primer sets, 1492RF-27RF, EUB338RF-Bac514RF and Bac514RF-Bac785RF, were carried out. No amplification was observed and thus confirmed that the extracted viral DNA was cleared from bacteria contamination (Fig. 13).

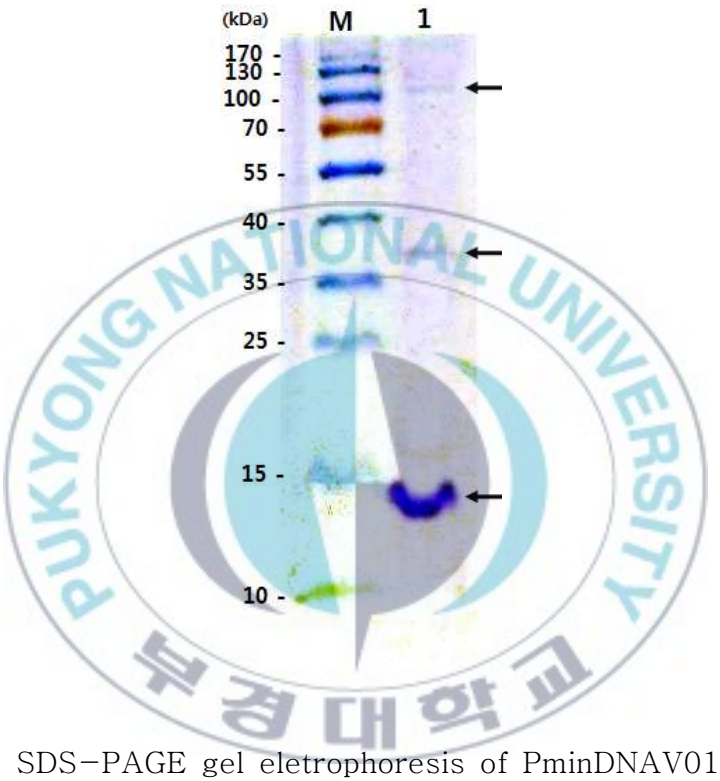

Figure 12. SDS-PAGE gel eletrophoresis of PminDNAV01 protein

Lane 1: PminDNAV01 protein

Lane M: 10 to 170 kDa protein ladder

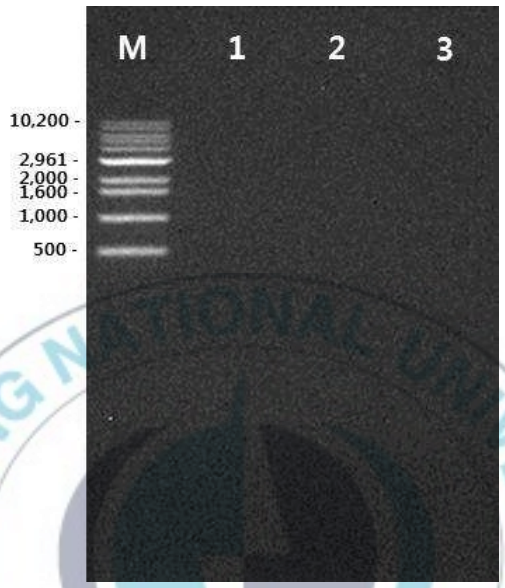

Figure 13. Analysis of 16S rRNA for confirmation of bacteria contamination

Lane 1: PCR product of PminDNAV01 with primer set of 1492RF-27RF

Lane 2: PCR product of PminDNAV01 with primer set of EUB338RF-Bac514RF

Lane 3: PCR product of PminDNAV01 with primer set of Bac514RF-Bac785RF.

Lane M: 1kbp DNA ladder

DISCUSSION

Sequencing the whole genome of PminDNAV01 was a great challenge. In traditional virus genome sequencing methods, the virus has to be extracted from the infected host. In this study, many problems occurred especially at the purification step of the virus. Therefore, various purification and concentration methods have been tried to overcome the problems. Hence, next generation sequencing was carried out because it allows whole genome sequencing of PminDNAV01 without virus purification. However, whole genome sequence of the virus could not been obtained but partial genome of the virus has been successfully sequenced.

Two major problems in purifying virus were overcome in this study. The first problem was high concentration of viral DNA was needed for genetic analysis. However, PminDNAV01 virus titer was low. In order to obtain sufficient concentration of viral DNA, large scale culture of the host and virus was carried out. Capsule filters and ultra-filtrator were used to minimize the time of filtering large volume of host inoculated with virus.

The other problem was elimination of contaminants like cell debris, bacteria and polyethylene glycol 6000 (PEG #6000). PEG #6000 was needed for virus concentration during ultracentrifugation, but process of removing the chemical was difficult. Hence, according

to recent researches, a chloroform treatment was added to the virus purification process (Shirai *et al.*, 2008; Toyota *et al.* 2011). Chloroform treatment was helpful in removing bacteria, cell debris and PEG #6000 in the viral suspension. Additionally, after chloroform treatment, the viral suspension was treated with DNaseI and RNaseA to make sure all bacteria have been removed (Fig. 13).

Next generation sequencing has successfully sequenced two contigs of PminDNAV01. The two contigs are 52,425bp long PMV53 and 46,021bp long PMV93. PMV53 contains 149 ORFs and 19 of the ORFs showed homology with proteins in GenBank database. PMV93 contains 91 ORFs and 7 of the ORFs have homologs in GenBank database. The rest of the ORFs in PMV53 and PMV93 showed homology with putative or hypothetical proteins.

The 26 ORFs of PminDNAV01 showed high homology with nucleo-cytoplasmic large DNA Virus (NCLDVs) proteins (Table 9). NCLDVs are viruses from the family of *Poxviridae, Asfarviridae, Iridoviridae, Ascoviridae, Phycodnaviridae, Mimiviridae, Megaviridae* and *Marseilleviridae* (Iyer *et al.* 2001). The NCLDVs have common ORFs like the major capsid protein, family B DNA polymerase, transcription factor, primase-helicase and DNA packaging ATPase (Van Etten, 2011). Typically, the NCLDVs do not exhibit much dependence on the host replication or transcription systems for completing their replication because, even in viruses like *Paramecium bursaria* Chlorella virus (PBCV), which initiate replication in the nucleus, disruption of a functional host nucleus by irradiation does not

abrogate replication (Van Etten *et al.*, 1986). This relative independence of the NCLDVs from the host cells is consistent with the fact that all these viruses encode several conserved proteins performing most key life-cycle processes, such as DNA polymerases, helicases, and DNA clamps for DNA replication and topoisomerases for genome manipulation, transcription factors involved in transcription and elongation, ATPase pumps for DNA packaging, and chaperones involved in the capsid assembly (Iyer *et al.*, 2001).

PminDNAV01 has 5 ORFs that showed homology to the NCLDVs common ORFs. 4 ORFs from contig PMV53 showed homology with Asfaviridae major capsid protein (PMV53-006; E value=1e-65), Asfaviridae DNA polymerase (PMV53-015; E value=1e-81), Miniviridae transcription factor 2, A2L (PMV53-018; E value=0.21) and Mimiviridae helicase (PMV53-004; E value=1e-25). PMV53-004 also showed homology with Mimiviridae ATPase (E value=1e-15). PMV93-004, the only ORF from PMV93, showed homology with mimiviridae ATPase (E value=1e-103).

The virions of different NCLDVs have dramatically different structures. The major capsid proteins of iridoviruses and phycodnaviruses, both of which have icosahedral capsids surrounding an inner lipid membrane, showed a high level of sequence conservation. A more limited, but statistically significant sequence similarity was observed between these proteins and the

major capsid protein (p72) of ASFV, which also has an icosahedral capsid. It was surprising, however, to find that all of these proteins shared a conserved domain with the poxvirus protein D13L, which is an integral virion component thought to form a scaffold for the formation of viral crescents and immature virion (Iyer *et al.*, 2001; Sodeik *et al.*, 1994).

All NCLDVs share a DNA polymerase of the B family, which, like the principal replicative polymerases of archaea and eukaryotes, contains the polymerase catalytic domain fused to N-terminal 3'→5' exonuclease domain (Leipe *et al.*, 1999). Another notable ancestral protein family of NCLDVs consists of homologs of proliferating cell nuclear antigen (PCNA), a protein that is ubiquitous in cellular life forms and functions as the sliding clamp during DNA replication (Bruck *et al.*, 2001; Iyer *et al.*, 2001). Like NCLDVs, PMV93-004 showed homology with Asfaviridae PCNA with an E value of 2e-2.

The D5R-like ATPase, typified by the eponymous vaccinia protein essential for DNA replication, appears to be the replicative helicase that is conserved in all NCLDVs. The D5R family belongs to the helicase Superfamily III within the AAA+ ATPase class, which includes the primary replicative helicases of many other DNA and RNA viruses (Gorbalenya *et al.*, 1990; Iyer *et al.*, 2004).

The NCLDVs clade is defined by a unique set of three transcription factors (typified by Vaccinia proteins A1L, A2L, and A7L), of which A1L and A2L are conserved in all NCLDVs. These

transcription factors might form a NCLDVs specific complex required for the recognition of viral promoters. The ancestral NCLDVs also encoded a TFIIS-like Zn-finger protein that functions in transcription elongation. (Iyer *et al.*, 2006). Same with NCLDVs, PminDNAV01 also showed homology with Maseilleviridae Zn-finger protein with an E value of 1e-53.

Many of PminDNAV01 ORFs showed homology to hypothetical proteins. There is so far no classification of hypothetical proteins (HPs) and working terms are replacing definitions of hypothetical proteins. In the strict sense, HPs are predicted proteins, proteins predicted from nucleic acid sequences and that have not been shown to exist by experimental protein chemical evidence. Moreover, these proteins are characterised by low identity to known, annotated proteins. In an attempt to define HPs Galperin (2001) and Galperin and Koonin (2004) defined "conserved hypothetical proteins" as a large fraction of genes in sequenced genomes encoding those that are found in organisms from several phylogenetic lineages but have not been functionally characterised and described at the protein chemical level. Shmuely *et al.* (2004) mention a representative fraction, however, consisting of HPs lacking any significant sequence similarity to other ORFs in the databases and are termed orphan ORFs or "poorly conserved ORFs" (Siew and Fischer, 2003a, b). And it is these HP that form a myriad of structures that match no other sequence in the databases. Another possibility for HP classification would be based

upon the presence or absence of a known gene name for the HP (Lubec *et al.*, 2005).

About half the proteins in most genomes are candidates for HPs (Minion *et al.*, 2004). This group is of utmost importance to complete genomic and proteomic information. Detection of new HPs not only offers presentation of new structures but also new functions. There will be new structures with so far unknown conformations and new domains and motifs will be arising. A series of additional protein pathways and cascades will be revealed, completing our fragmentary knowledge on the mosaic of proteins per se. The network of protein-protein interactions will be increasing logarithmically. New HPs may be serving as markers and harmacological targets. Last but not least, detection of HP would be of benefit to genomics enabling the discovery of so far unknown or even predicted genes (Lubec *et al.*, 2005).

Although many algal viruses have been isolated, many genome of the isolated viruses have not been completely sequenced. Many algal virus whole genome have not been sequenced as many problems were faced by scientists trying to obtain the complete sequence of algal viruses. This research conducted molecular biological approaches on microalgal virus (PminDNAV01). However, many problems have arised when the research was carried out. Therefore, improvement in the experimental stages have been carried out. The new virus purification process constructed in this study proved to be

effective in producing sufficient pure viral DNA for whole genome sequencing. Hopefully this new method could be used in future research on algal virus genome. Although only two contigs were found in this research and most of the proteins encoded by the ORFs are hypothetical proteins, this research can be a stepping stone to future research on sequencing of whole genome of algal viruses.

해양 미세조류 *Prorocentrum minimum*에 감염하는 신종 바이러스의 순수분리를 통한 유전자 분석

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요 약

해양 미세조류 바이러스 연구에서 가장 큰 어려움은 바이러스의 순수 분리 과정이다. 대부분의 조류의 경우 인공 배양액에서 10^3-10^4 particle/cell 정도에 이르기 때문에 분석에 필요한 바이러스의 대량확보가 쉽지않다.

지금까지 바이러스를 농축하여 충분한 양의 viral DNA를 확보함에 큰 문제는 작은 ultracentrifugation 튜브를 사용하여 여러 번 반복하여 ultracentrifugation를 해야 하는 것이 단순한 번거로움의 문제가 아니라 시간상으로 많은 시간이 필요한 단계이기 때문에 그 과정에서 바이러스의 손실이 생기게 된다는 지적을 받았고, 그 과정을 통해 필요한 충분한 양의 바이러스 확보하고 유전자 분석을 하는 과정에 어려움이 따랐다. 이러한 문제점을 해결하기 위해 capsule형 filter를 이용한 pre-filtration 및 mini-size hollow fiber ultracentrifugation 법을 이용하여 농축시간을 단축시켰다. 실험방법에 있어서의 문제를 해결하기 위한 다양한 방법의 접

근을 하였고, 그 하나의 방법으로 PminDNAV01에 감염된 숙주의 전체 DNA를 Next generation sequencing (NGS)법으로 분석하였다. 분석 결과 52kb의 contig_53 (PMV53)과 46kb의 contig_93 (PMV93)을 확보하였으며, 이 2개의 contig에 포함된 ORFs에 대한 분석 결과 총 250개의 ORFs 중 26개의 ORFs는 기존에 알려진 조류 감염 바이러스, *Heterocapsa circulaarisquama* DNA virus 01, *African sqine fever* virus, Acanthamoeba polyphaga mimivirus, *Pyramimonas orientalis* virus, *Chrysochromulina ericina* virus, *Phaeocystis pouchetii* virus, *Paramecium bursaria* Chlorella virus 01 등의 바이러스가 속하는 nucleo-cytoplasmic large DNA Virus (NCLDVs)과의 연관성을 가지는 신규 바이러스임을 확인할 수 있었다.

하지만 이번 분석을 통해 알게 된 것은 PminDNAV01 바이러스 전체 게놈의 분석의 시작에 불과하며 두 개의 contig를 통해 알게 된 ORFs 중 상당수는 확인되지 않은 해양미세조류 바이러스의 hypothetical protien이라는 결과가 나왔다. 이는 전체 해양 미세조류 바이러스 분야에 대한 연구부족에 기인하는 것이며 이번 연구를 통해 좀 더 적극적인 연구가 필요하다.

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