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by Mursyidin

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Phylogenetic Relationship of *Cymbidium Mosaic Virus* (CymMV) from the Native Orchids of South Kalimantan, Indonesia

Dindin Hidayatul Mursyidin* **and Ahmad Winarto Saputra**

Laboratory of Genetics and Molecular Biology, Faculty of Mathematics and Natural Sciences, University of Lambung Mangkurat, South Kalimantan, 70714, Indonesia

*Corresponding author: dindinhm@gmail.com

Abstract

Information of viral genomics, particularly their phylogenetic relationships, is valuable in controlling the viral infection and screening for the development of virus-resistant cultivars in the future. The objectives of our study were to characterize and analyze the partial genome (RdRp region) of *Cymbidium mosaic virus* (CymMV), one of the most pathogenic viruses in the world, from the native orchids of South Kalimantan, Indonesia, by the RT-PCR method. Also, to reconstruct their phylogenetic relationship by ML and MP methods. Following RT-PCR analysis, from 10 samples of native orchids used, only one was positively infected by CymMV. In early detection, the RdRp region of CymMV has approximately 530 bp in size. After being sequenced and aligned with other isolates, this region has 121 polymorphic or mutation sites, a GC content of 45.21%, a transition/transversion bias value of 3.52, and nucleotide diversity (0.0415). The phylogenetic analysis was revealed that CymMV from South Kalimantan, Indonesia, has closest related to similar isolates from Korea Type 2 (AF016914.1), Niigata, Japan (AB197937.1), Hawaii (EF125180.1), and Taiwan M2 (EU314803.1), with the coefficient divergence of 0.025. But, it has very distantly related to Hawaii 18-1 (EF125178.1) with a coefficient of 0.142. Our results provide urgent information in supporting the native orchid's conservation and breeding efforts, locally and globally,

including mitigating or controlling the viral infection and screening for the development of virus-free or resistant cultivars in the future.

Keywords: Breeding and conservation, mosaic virus, orchids, plant protection, RT-PCR.

Abbreviations: ELISA = Enzyme-Linked Immunosorbent Assay; ML = Maximum Likelihood; MP = Maximum Parsimony; ORFs = Open Reading Frames; PCA = Principal Component Analysis; RdRp = RNA-dependent RNA polymerase; RT-PCR = Reverse Transcriptase-Polymerase Chain Reaction.

1. Introduction

The native orchids are valuable germplasm for conservation and breeding purposes, particularly as a parental or broodstock, because they serve many beneficial genes with essential traits. According to Yusop *et al*. (2022), this germplasm is spread globally in the diverse region of the world, particularly in the tropics. However, they are narrowly distributed in specific habitats and are extremely susceptible to habitat disturbance comparing to other plants (Zhang *et al*., 2015). The Meratus Mountains of South Kalimantan, Indonesia, is one of the habitations of many native orchids. Muslimah *et al*. (2011) reported that over 115 native orchids were present in this region with uniques characteristics, such as *Phalaenopsis*, *Dendrobium*, *Paphiopedilum*, and *Vanda*. Most of those orchids have a high economic value. For instance, *Phalaenopsis amabilis* var. 'Pelaihari' is the most popular and high-value of moth orchid in the world because they have a beautiful spot in their flower labellum. Besides, this orchid has a blossom that reaches 50 units at its stalk and has a long-lasting flowering period until six months (Mursyidin *et al*., 2021).

However, due to many human impacts, like illegal logging and trading, including natural disasters and climate change, some orchid species are very hard to find in the wild habitat, even among them are being threatened (Zahara and Win, 2019; Liu *et al*., 2021). The Commission of International Trade of Endangered Species (CITES) even included some of them as endangered species, like *Phalaenopsis* (Zhang *et al*., 2018). Consequently, the conservation and breeding efforts of the orchids are very urgent to employ. Factually, although some native orchids have been incorporated into breeding and conservation programs, they are constrained by many factors, one of which is a viral infection.

Cymbidium mosaic virus (CymMV), which belongs to the genus Potexvirus, is the most pathogenic problem that causes the loss of orchid cultivation worldwide (Park *et al*., 2016; Yusop *et al*., 2022). Genetically, this virus is characterized as a positive-sense single-stranded RNA with approximately 6.3 kb in length. The viral genome contains five open reading frames (ORFs) and potentially encodes RNA-dependent-RNA polymerase (RdRp) for genome replication (Lee *et al*., 2021). Phenotypically, the orchid plants infected by CymMV show the mosaic, necrotic, and chlorotic symptoms, and imperfection of flower growth (Liu *et al*., 2009). However, this virus attack was difficult to distinguish by this view and may be confused with other disease problems, particularly by fungal infections, like *Fusarium* (Srivastava *et al*., 2018; Wang *et al*., 2018). Thus to ascertain whether a virus attack causes the disease, we require an accurate technique such as a molecular approach.

The virus infection in orchid plants can be detected by the Enzyme-Linked Immunosorbent Assay or ELISA (Pradhan *et al*., 2016). However, this method is time-consuming and have other limitation (Seoh *et al*., 1998). Reverse Transcriptase/RT-PCR is a molecular-based method commonly used to detect and characterize virus infection in plants (Sudha and Rani, 2016). This method has more advantages than others, like ELISA, such as

being more effective and efficient (faster, accurate, and sensitive) in detecting orchid plants' virus infection (Lai *et al*., 2013).

The objectives of this study were to characterize and analyze the partial genome of CymMV, namely RdRp (RNA-dependent RNA polymerase) region, from the native orchids of South Kalimantan, Indonesia, following the RT-PCR method. This study was also employed to evaluate and determine the phylogenetic relationship of this virus compared to others from several countries. This information is valuable as an essential reference locally on the cultivation and preservation of orchids germplasms in South Kalimantan, Indonesia, and globally in controlling the virus infection for orchids breeding purposes, particularly screening for virus-resistant cultivars in the future.

2. Materials and Methods

2.1 Plant Materials

A total of 10 samples of native orchids of the Meratus Mountains of South Kalimantan, Indonesia, which are symptomatically infected by CymMV, were collected randomly from some private collectors, particularly at Banjarmasin, Banjarbaru, and Tanah Laut regencies (Figure 1, Table 1). All samples were then brought to the Laboratory of Genetics and Molecular Biology, Faculty of Mathematics and Natural Sciences, Universitas Lambung Mangkurat for further (molecular) analysis.

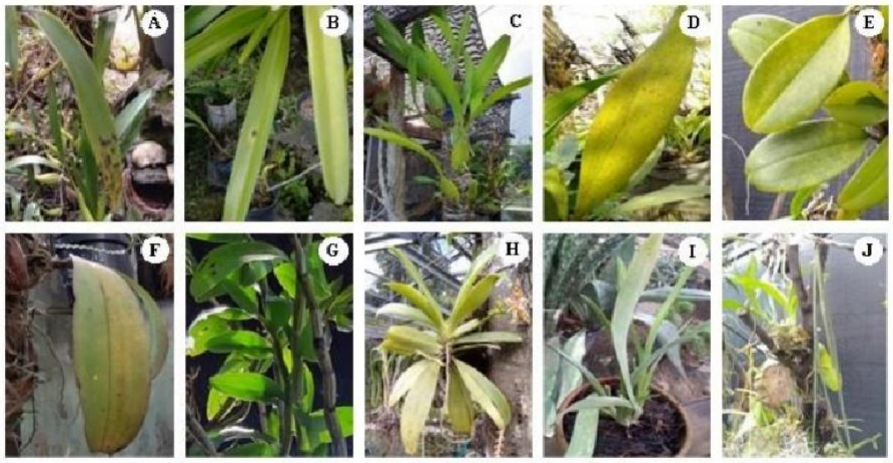

Figure 1. Orchid samples used in this study, show a viral infection symptom, e.g., a chlorotic, necrotic, and mosaic. The name of each sample is listed in Table 1.

Table 1. List of symptomatic orchid samples used in the study.

Name of Species	Code	Origin	Symptom
Dendrobium spurium	A	Tanah Laut	Necrotic
Cymbidium bicolor	B	Tanah Laut	Necrotic; Mosaic
Coelogyne pandurata	C	Banjarmasin	Necrotic; Chlorotic
Paphiopedilum lowii	D	Tanah Laut	Chlorotic; Necrotic
Bulbophyllum macranthum	E	Banjarmasin	Chlorotic; Mosaic
Phalaenopsis amabilis	F	Banjarbaru	Chlorotic; Mosaic
Phalaenopsis cornu-cervi	G	Tanah Laut	Necrotic
Vanda dearei	H	Tanah Laut	Chlorotic; Mosaic
Oncidium sp.	I	Banjarmasin	Necrotic; Mosaic
Paraphalaenopsis serpentilingua	J	Banjarmasin	Chlorotic; Necrotic

2.2 RNA Preparation and Molecular Analysis

The RNAs were isolated and purified from symptomatic orchid leaves following the viral RNA kit (Invitrogen, USA) and quantified using a UV-Vis spectrophotometer (NanoVue, GE Healthcare, UK). The RNAs were then amplified directly using the One-Step RT-PCR kit

(SuperScript® III, Invitrogen, USA) and a pair of specific primers, namely CymMV-F: 5'-GGGATCTTCGCACACCCAA-3' and CymMV-R: 5'- ACGATCATATT CATCGCATGG'3' (Park *et al*., 2016). The PCR reaction was employed, using a thermal PCR system (BioRad, MyCycler, USA) in a total volume of 25 μL. PCR reaction was done with a cycling condition: initial denaturation 94°C for 2 min, denaturation 94°C for 30 sec, annealing 55°C for 30 sec, extension 68°C for 1 min, as well as final extension 68°C for 5 min. The PCR products were separated by 1.5% agarose gel electrophoresis and documented using a digital camera. The target cDNA fragment of the virus (RdRp region), which was positively detected, was then purified and sequenced directly using the Sanger method bi-directionally by 1st Base Ltd., Malaysia. The RdRp sequence was deposited into the GenBank database with an accession number of MN150525.

2.3 Data Analysis

Two (forward and reverse) sequences of the RdRp region of CymMV were combined and manually edited using the MEGA-X software to obtain a consensus (Kumar *et al*., 2018). The sequence then was traced with the BLAST method in GenBank or NCBI database (https://www.ncbi.nlm.nih.gov/). Several RdRp regions of CymMV found in this database, including the target region, were aligned using Clustal X software (Larkin *et al*., 2007). In this analysis, indels (insertions or deletions) were introduced into the alignment coded in the following ways. Shared indels were treated as single characters. Indels of uniform length were coded as absence (1) or presence (0) characters independent of the indel length. The gapped 4 of 10 regions in the alignment were excluded from subsequent analysis unless some positions included nucleotide diversity (Petersen and Seberg, 2002). The phylogenetic relationship was performed using the maximum likelihood (ML) and maximum parsimony (MP) methods. The phylogram's topological robustness was assessed by bootstrap analysis with 1,000 replicates

(Loog, 2018). The principal component analysis (PCA) and Pearson correlation were also applied to confirm this relationship, following the criteria of Taylor (1 990).

3. Results

3.1 RT-P C"R Anol‹sis

Following RT-PCR analysis, only one of 10 samples of native orchids from the Meratus Mountains of South Kalimantan, Indonesia, was positively infected by C yrnMV (Figure 2, Table 2). Based on Figure 2, the RdRp region of C yrnM V has approximatel y 530 bp in size.

Figure 2. A positive infection of CymM V to one native orchid sample of South Kalirnantan, namely Dnr return sp. (lane 9), showed the RdRp virus with approximatel y 530 bp in size. Note: M = DN A mar ker (I kb); lanes 1- 10 = the orchid earn ples, see Table 2 for details.

Table 2. List of orchid samples with the viral symptoms collected from three regions of South Kalirnantan, 1ndonesia

Species	Origin	Syrnptom	RT-PCR
Dendrobium spurium	Tanah Laut	Necmtic	
Cymbidium bicolor	Tanah Laut	Neurotic; Mosuic	
Coelogyne pandurata	Banjarmasin	Neurotic; Chlomtic	
Paphiopedilum lowii	Tanah Laut	Chlomtic; Necmtic	
Bulbophyllum macranthum	Banjarmasin	Chlomtic; Mosaic	
Phalaenopsis amabilis	Banjarbaru	Chlomtic; Mosaic	

Phalaenopsis cornu-cervi	Tanah Laut	Necrotic	-
Vanda dearei	Tanah Laut	Chlorotic; Mosaic	-
Oncidium sp.	Banjarmasin	Necrotic; Mosaic	+
Paraphalaenopsis serpentilingua	Banjarmasin	Chlorotic; Necrotic	-

3.2 Genetic Diversity and Polymorphism

The RdRp region of CymMV from native orchids has been sequenced by the Sanger method bi-directionally. It was recorded with 525 bp in length (Table 3). Following Table 3, the partial RdRp region of CymMV has 121 polymorphic or mutation sites, with the GC content (45.21%) and transition/transversion bias value of 3.52. Besides, this region has a nucleotide diversity of 0.0415. Figure 3 shows multiple alignments, where many mutational events on the RdRp region of CymMV were present. According to Figure 3, most mutations are substitutions, i.e., transition and transversion. Furthermore, one deletion only was found in this region.

Table 3. The genetic information of a partial RdRp region of CymMV*

Parameter	Value
Sequence length (bp)	525
Number of variable sites	121
Number of Parsimony informative sites	73
Number of singleton sites	48
Bayesian information criterion (BIC)	3708.652
Akaike information criterion (AICc)	3431.876
Maximum likelihood value (*ln*L)	-1677.801
GC content (%)	45.21
Transition/transversion bias value (*R*)	3.52
Nucleotide diversity (π)	0.0415

* following Kimura 2-Parameter

Figure 3. Multiple aligriments, showing many mutational events on the RdRp region of CymMV, both substitutions (black rectarigle) and deletion (green rectangle).

3.3 Phylogenetic Relationship

The phylogenetic **analyses,** both ML arid MP, were revealed **that CymMV** from South Kalimantan, Indonesia, and other countries have unique relationships. Generally, this virus was grouped into nine clades, both for ML (Figure 4) arid MP (Figure 5). In this case, a CymMV isolate from South Kalimantan, Indonesia, was grouped into a similar clade with isolates from Korea Type 2 (AF0169I4.1), Nllgata, lapan (AB197937.1), Hawaii (EFI25180.1), and Taiwan M2 (EU314803.1). Hence, it has closely related to these isolates

mentioned with the coefficient divergence of 0.025 (Table 4). In conoast, the CymMV of this region has very distantly related to Hawaii 18-1 (EF125178.1) with a coefficient of divergence of 0.142 (Table 4).

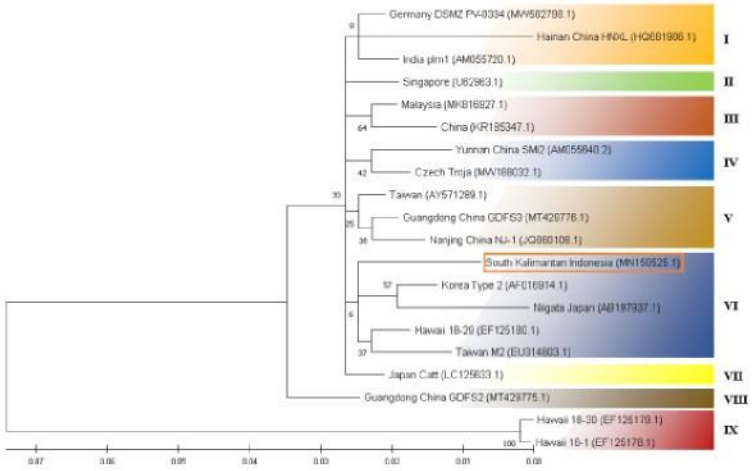

Figure 4. Phylogenetic relationship of CymM V from a native orchid of South Kalirnantan, Indonesia, compared to others, revealed by ML and bootstrap 1,000 replicates.

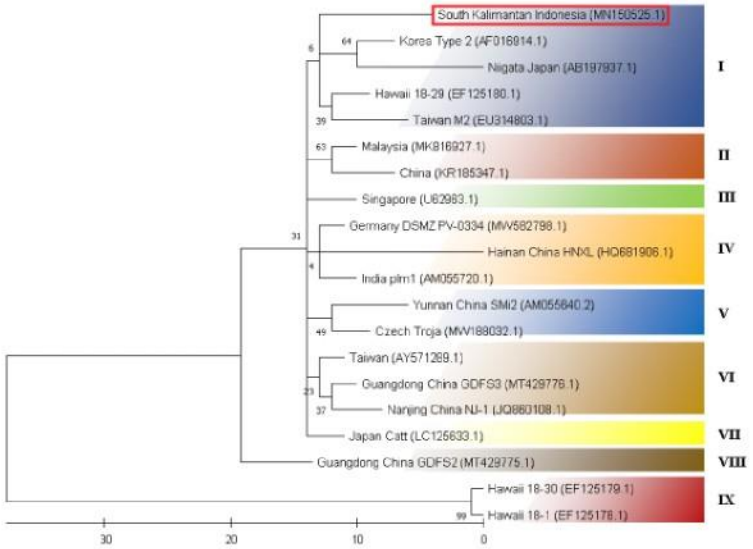

Figure S. Phylogenetic relationship of CymM V from a native orchid of South Kalirnantan, Indonesia, compared to others, revealed by M P and bootstfap 1,000 replicates.

Following Table 4, the CymMV with closest related was shown by two Hawaii isolates (EF125l79.1 and EF125178.1, respectively) with a coefficient of 0.004, whereas the farthest by Niigata, Japan (AB197937.1) with two Hawaiian as well. The Pearson correlation analysis was confirmed that most CymM V isolates have a strong relationship, except for Japan (LC125633.1) and Hainan, China (HQ681006.1) isolates, and from Guangdong, China (MT429775.1) with all isolates observed (Figure 6). The PCA was also corresponding to the ML and MP results, that two Hawaiian (EFl25l79.1 and EF125178.1) and Guangdong, China (MT429775. 1) isolates are far separated from other ones (Figure 7).

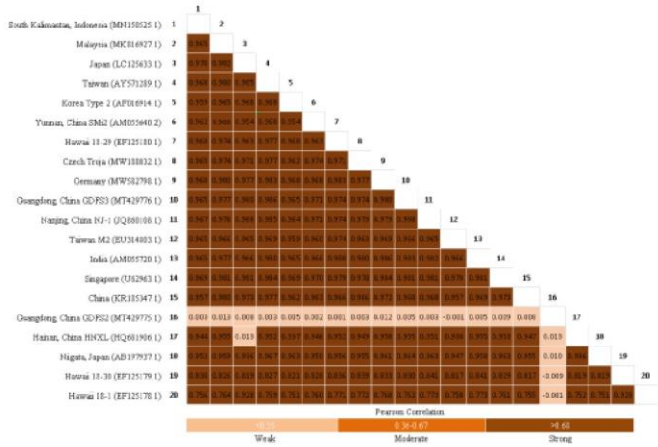

Figure 6. Pearson correlation between CymMV isolates from a native orchid of South Kalimantan, Indonesia, compared to others, based on RdRp region.

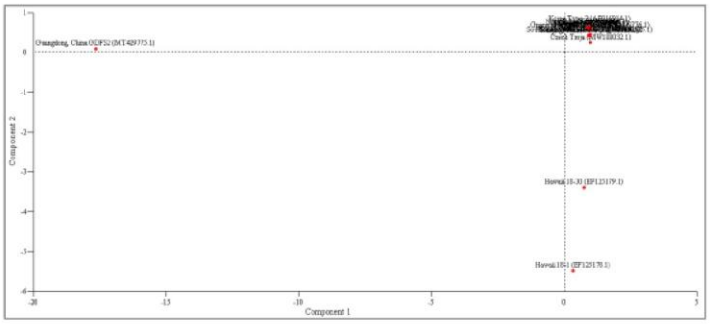

Figure 7. Grouping of CyrnMV isolates from South Kalimantan, Indonesia, and others based on RdRp region and PCA analysis.

Table 4. Genetic divergence of CymMV between South Kalimantan, Indonesia isolate and others.

OTUs	Csde	0	2	3	4	5	6	7	41	9	00	0¥	OI	¥3	¥4	¥5	!6	17	18	19	20
South Kalimantan, Indonesia_(MN150525.1)	1																				
M Haysia(M K 8ld427.t)	2																				
Japan (LC125633.1)		0.025	0.014																		
Taiwan (AY57I289.1)	4	0.025	0.0I4	0.012																	
Korea Type 2 (AF016914.1)	S	0.029	0.021	0.019	0.019																
Yunnan, China (AM055640.2)	6	0.029	0.023	0.A2t	0.A2l	0.029															
Hawaii 18-29 (EF125180.1)	7		0.017	0.015	0.016	0.019	0.025														
Czech (MW 188032.1)	8	0.027	0.017	0.015	0.016	0.023	0.017	0.019													
Germany (MW582798. I)	9		0.014	0.012	0.0I2	0.019	0.021	0.012	0.016												
Guangdong, China GDFS3 (MT429T76. I)	10	**0.027**	0.016	0.014	0.010	0.02 I	0.019	0.017	0.017	0.014											
Nwjny,ChirmNJ1NQ8dN08.I	11	**0.027**	0.017	0.015	0.012	0.023	0.021	0.019	0.015	0.015	0 B10										
Taiwan M2 (EU3 I4803.I)	12	0.027	0.023	0.021	0.021	0.025	0.027	0.017	0.025	0.021	0.023	0 B25									
India pIm I (AM055720.1)	13	0.027	0.016	0.014	0.014	0.02 I	0.023	0.014	0.014	0.010	0.012	0.014	0.023								
Singapore (U62963.1)	14	0.027	0.015	0.013	0.014	0.02 I	0.023	0.017	0.017	0.014	0 B15	0 B17	0.023	0.015							
Chinu (KR 185347. I)	15	0.033	0.014	0.019	0.0]6	0.023	0.025	0.023	0.023	0.019	01121	0 B23	0.029	0.021	0.021						
Guangdong, China GDFS2 (MT429775. I j	16	0.039	0.027	0.025	0.025	0.029	0.033	0.029	0.029	0.025	0.027	0.029	0.036	0.027	0.027	0B29					
Hainan, China Ht'4XL (HQ68I%6.1)	17	0.039	0.031	0.033	0.033	0.041	0.037	0.033	0.035	0.029	0.031	0.035	0.044	0.031	0.035	0fi37	0048				
Niigata, Japan (AB197937.1)	18	0.039	0.031	0.029	0.025	0.025	0.038	0.033	0.033	0.029	0 B27	0 B29	0.040	0.031	0.031	0D33	0D39	0.048			
Hawaii 18-30 (EF125179.1)	19	0.139	0.135	0.132	0.133	0.135	0.133	0.125	0.123	0.128	0.130	0.123	0.142	0.121	0.135	0.142	0.128	0.139	0.145		
Hawaii 18-31 (EF125178.1)	20	0.142	0.135	0.133	0.133	0.137	0.133	0.123	0.123	0.128	0.130	0.123	0.140	0.121	0.135	0.142	0.128	0.139	0.145	0.004	

Green highlight = closest related, Red highlight = fartest related

4. Discussion

The RNA-dependent RNA polymerase (RdRp) is the core of virus replication and transcription complex (Jiang *et al*., 2021). According to Jia and Gong (2019), this region was first identified in the 1950s in the Mengovirus and Poliovirus (PV) and has responsibility for the viral genome replication and transcription processes. In the 1970s and 1980s, the RdRp was studied extensively and shown to be induced by virus infection in several plant species (Carr *et al*., 2010). In this study, the partial RdRp region of CymMV has 121 polymorphic or mutation sites, with the GC content (45.21%) and transition/transversion bias value of 3.52. Following Figure 3, the most prevalent mutations are substitutions, i.e., transition and transversion, and very lack in indels (only one found).

According to Venkataraman *et al*. (2018), RdRp has a high mutation rate due to the error during the copying (≈10−4) process of a proofreading exonuclease activity (Venkataraman *et al*., 2018). In the progeny of the viral population, the increased mutation rates allow some variants to be selected under the pressures imposed by the host defense mechanisms and other environmental factors. Furthermore, changing of RdRp strand during replication allows for recombination, which allows for gene reorganization or the introduction of new genes from other viruses or hosts (Venkataraman *et al*., 2018).

Further analysis shows that the RdRp region of CymMV has a nucleotide diversity of 0.0415 (Table 3). According to Venkataraman *et al*. (2018), the molecular phylogeny of RdRp demonstrates diversity in hosts, capsid morphologies, and genomic features originating from the loss of ancestral genes, gene exchange between distantly viruses, and transfer of viruses between hosts. Shu and Gong (2016) reported that viral RdRP is very diverse in size and structural organization, from the ~50-kDa to the ~260-kDa, and forms a unique enclosed right-hand structure with palm, fingers, and thumb protein domains.

Apart from their mutation and diversity, RdRp is the most conserved gene in RNA viruses that is ideally suited to understand their evolutionary patterns (Venkataraman *et al*., 2018). Then, this gene is an attractive system for understanding the fundamentals of nucleic acid synthesis and for developing antiviral strategies (Jia and Gong, 2019). Carr *et al*. (2010) explained that cellular RdRPs have crucial roles in plant RNA-silencing pathways, providing amplification of silencing through the generation of siRNA-primed dsRNA synthesis and initiation of antiviral silencing through *de novo* synthesis of dsRNA. Thus, this region is necessary for basal resistance maintenance to several RNA viruses, for example, TMV and PVY (Carr *et al*., 2010).

The phylogenetic analysis revealed that CymMV from South Kalimantan, Indonesia, has closely related with isolates from Korea Type 2 (AF016914.1), Niigata, Japan (AB197937.1), Hawaii (EF125180.1), and Taiwan M2 (EU314803.1) but very distantly related to Hawaii 18-1 (EF125178.1) with a coefficient of divergence of 0.142 (Table 4). According to Domingo (1997), mutations, homologous and non-homologous recombinations, and changes in viral RNA segments can contribute to genetic variation and the relationship of these viruses (Domingo, 1997). Conceptually, a virus's natural ability to adapt to its environment is a factor that causes mutations and the two factors mentioned, namely the recombination and changes in viral RNA segments (Domingo, 1997).

In general, RNA viruses have a mutation substitution rate in the range of 10-3 to 10-5 substitutions/copies of nucleotides (s/nt) (Domingo, 1997). Acosta-Leal *et al*. (2011) reported that the RNA viruses of the family Potyviridae, Tobamoviridae, and Sobemovirus have an evolutionary rate exceeding 10–5 s/nt/year. Meanwhile, Geminiviridae and Nanoviridae ssDNA viruses evolved faster by 10–3 s/nt/year (Acosta-Leal *et al*., 2011). Acosta-Leal *et al*. (2011) added that plant viruses show higher mutation rates and different evolutionary dynamics than bacterial and fungal phytopathogens.

In brief, an understanding of the dynamics of virus evolution, including other aspects of biology, such as reproductive strategies, transmission (virulence), and ecology, is most beneficial in mitigating or controlling the virus in the future (Elena *et al*., 2014). In other words, the management of virus control is necessary to employ.

5. Conclusion

The CymMV isolate from a native orchid (*Oncidium* sp.) of South Kalimantan, Indonesia, has closest related to similar isolates from Korea Type 2 (AF016914.1), Niigata, Japan (AB197937.1), Hawaii (EF125180.1), and Taiwan M2 (EU314803.1), with the coefficient divergence of 0.025. But, it has very distantly related to Hawaii 18-1 (EF125178.1) with a coefficient of 0.142. This finding is urgent in supporting the native orchid's conservation and breeding efforts, locally and globally, including mitigating or controlling the viral infection and screening for the development of virus-free or resistant cultivars in the future.

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