

Genetic variability of Indonesian *Oryctes rhinoceros nudivirus* (OrNV) as genus of *Alphanudivirus*

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Manuscript received: 8 January 2020. Revision accepted: 16 April 2020.

Abstract. *Rahayuwati S, Kusumah YM, Prawirosukarto S, Dadang, Santoso T. 2020. Genetic variability of Indonesian Oryctes rhinoceros nudivirus (OrNV) as genus of Alphanudivirus. Biodiversitas 21: 2047-2055. Oryctes rhinoceros nudivirus (OrNV) is known as a successful classical biological control agent. Recent reports have revealed differences in pathogenicity among OrNV isolates, with correlation suspected between virus virulence and genetic variability. The aim of the research is to study the genetic variability of OrNV isolates based on the DNA polymerase (DNA pol) gene and their phylogenetic relationship with Genus Alphanudivirus. Oryctes rhinoceros (Coleoptera: Scarabaeidae) as an OrNV host were collected from Sumatra, Belitung, Java, Kalimantan, and Sulawesi. Primers were designed to amplify complete gene of DNA pol. Sequencing, phylogenetic, and investigate genetic diversity scores were used to determine variability at nucleotide level. Five primers successfully amplified the entire DNA pol gene. Genetic variability of OrNV was high, from nine isolates found eight genetically different. Isolates could be divided into two groups of genetic diversity: high and low. High genetic diversity of OrNV supposed as an old population that occupied Sundaland and Wallacean paleogeography at first appearance in Miocene Era until early Pliocene 5 Mya. New population of OrNV had low genetic diversity were travel cross-island that supposed to correlate with oil palm plantation development and the genetic drift. The benefit information of the OrNV genetic variability is as preliminary guideline for virulence test. OrNV is a member of Nudiviridae family, Genus Alphanudivirus, and at recent moment they consist of eight species. Alphanudivirus infect Coleoptera, Diptera, Hemiptera and Orthoptera order that soil as immature niche may explain their closely related taxa.*

Keywords: DNA polymerase gene, genetic variability, Nudiviridae family, *Oryctes rhinoceros nudivirus*, OrNV

INTRODUCTION

Indonesia has a biological wealth of virus, *Oryctes rhinoceros nudivirus* (OrNV), from the Nudiviridae family. OrNV was discovered by Huger (1966) as part of an exploration of *Oryctes rhinoceros* (Coleoptera; Scarabaeidae) natural enemies in south-east Asia as endemic areas. OrNV is known as successful classical biological control that has been used to manage *O. rhinoceros* in the Pacific (Huger 2005; Huger 1966). Unfortunately, *O. rhinoceros* is important pest in Indonesian and Malaysian. These beetles attacking young oil palms less than four years during replanting (Bedford 2014). Adults are the most troublesome during the establishment of young palm, and the damage has increased with current extensive replanting schemes (Ramle et al. 2005). Minister Agriculture of RI regulation number 18/permentan/KB.330/5/2016 about oil palm replanting program, entail to chipped oil palm trunks with thickness 5-20 cm of 45-60° angle direction. Prohibited of land clearing by burning also stated in UU RI no 32-year 2009 about environmental protection and management. Chipped trunk with zero burnings was good regulation although still as optimum habitat for the breeding immature stage of *O. rhinoceros* in 9 until 18 months after felling and chipping (Abidin et al. 2014). *O. rhinoceros* behavior in

coconut slightly different than adult attacks the tree throughout the year. The adults population fluctuates influenced by breeding sites such as coconut trunks, cow dung, sawdust, husks, piles of organic matter (Salim and Hosang 2013).

Nudiviridae is a nonoccluded Baculovirus that has been reported to infect a wide range of insect orders (Huger and Krieg 1991). The lack of an occlusion body as reported by Wang et al. (2007a) justifies its classification as a naked virus that has been termed Nudivirus. Nudivirus has a genome size of 95-228 kb, rod-shaped virions, circular dsDNA, and it replicates in the host nucleus (Wang et al. 2007b). There was a proposal in 2013 to make Nudivirus into a new family, Nudiviridae, with two genus members *Alphanudivirus* and *Betanudivirus* (Jehle et al. 2013). By 2019, the genera had grown to become four-plus *Gammanudivirus*, *Deltanudivirus* with the members of this family infecting not only insects but also crustaceans (Holt et al. 2019; Bézier et al. 2015). *Gammanudivirus* is suggested to be a genus that specializes in infecting diverse aquatic crustacean taxa (Holt et al. 2019). *Alphanudivirus* is the most growing genus of Nudivirus as a result of viral metagenomics study. Viral metagenomics is the study of viral genetic material recovered directly from environmental samples. As sequencing technology continues to develop causing an increasingly large

database. These databases provide important information that can be used for study of viral taxonomy, evolutionary relationship, genome organization, finding organism, finding genes and its pathways (Segata et al. 2011; Simmonds et al. 2017). By using these metagenomic databases, four species of *Alphanudivirus* has been found infected *Drosophila melanogaster* (Diptera: Drosophilidae): *Mauternbach nudivirus*, *Kallithea nudivirus*, *Tomelloso nudivirus*, *Esparto nudivirus* (Webster et al. 2015).

OrNV has not been optimally used as an *O. rhinoceros* biological control. It is hoped that success in the use of OrNV in the Pacific will indicate similar success for rhinoceros management in Indonesia. OrNV has different geographical pathogenicity (Moslim et al. 2011) that could be favorable in virus development. The pathogenicity of viruses can be used to control the insect host population (Federici and Maddox 1996); a relationship is also suspected between virus virulence and its genetic variability. The genetic variability of OrNV in Indonesia has not yet been reported.

Nucleotide sequences provide much in the way of phylogenetic information. Systematic studies have shown that some genes are visibly better than others for reconstructing evolutionary relationships at particular levels of divergence (Simon et al. 1994). The DNA changes exhibit a regular pattern that could be formulated as a tree using a mathematical model. Using this model, it is possible to formulate the DNA change and compare it with distantly related taxa (Sutrisno 2006). DNA pol is one of the genes used to construct phylogenetic of virus (Federici and Bigot 2003). All known types of virus DNA that infect animal cells encode their own DNA pol (Braithwaite and Ito 1993). DNA pol was likely present in the earliest virus ancestors. This suggestion is supported by the observation that phylogenies constructed by viral polymerase match with viral taxonomic classifications (Shackelton and Holmes 2004).

The aim of this research is, therefore, to look for the genetic variability of Indonesian OrNV based on DNA pol gene and then mine the relationship information between other Genus *Alphanudivirus* that have been reported by National Center for Biotechnology Information (NCBI).

MATERIALS AND METHODS

Sampling

Oryctes rhinoceros were taken from single point in predetermined provinces (Table 1). The sampling and midgut collection was carried out between January and April 2018. Individual midguts were placed in a sterilized Eppendorf tube for storage at -20°C at the Insect Pathology Laboratory of IPB University, Bogor, Indonesia.

Primer design

Primers were designed to amplify the complete OrNV DNA pol gene with isolate Ma07 from Malaysia with accession number EU747721.1 as a primer reference (Wang et al. 2008). DNA pol gene nucleotides were

submitted to the online primer design www.idtdna.com. Each primer was manipulated up to its end with C or G nucleotide and then ordered to First Base Asia Sdn Bhd.

DNA isolation and PCR (Polymerase Chain Reaction)

The Quick-DNA miniprep plus kit from Zymo Research was used for DNA extraction (Jackson et al. 2010). The PCR thermal cycle was based on the Dream Taq Green PCR master mix protocol from Thermo Scientific. The PCR master mix was prepared in 50 µL containing 25 µL Dream Taq Green PCR master mix, 1µM forward and reverse primer, 10 pg-1µg templates, and up to 50 µL of nuclease-free water.

The primer annealing temperature was determined using the TM calculator by Thermo Fisher Scientific. Annealing temperature was optimized by increased 4°C and decreased 2°C from temperature suggestion. Temperature yielded the thickest DNA band selected as annealing temperature for PCR reaction.

Amplified polymerase sequences were visualized with 1.5% agarose. DNA was stained with 2 µL peqGreen DNA and RNA dye from peqlab, a VWR company. Electrophoresis was conducted with TBE 1x buffer at 100 volts for 30 minutes. The DNA bands were observed with UV light and documented (Jackson et al. 2010).

Sequencing and data analysis to investigate the nucleotide diversity and its phylogeny

The amplified PCR products with size approximately 700 bp of primer OrNV 200F/R and product with size approximately 1500 bp of Primer OrNV1 F/R, OrNV2 F/R, OrNV3 F/R, OrNV4 F/R were sent to First Base Asia Sdn Bhd to be sequenced. Batch assembly of multiple DNA sequences was automatically undertaken by DNA Sequence Assembler V5.15.0 (32 bit) (2013). The sequences were visually checked for correction. DNA pol gene was identified using the BLAST program in NCBI, and the start and stop codons of the DNA pol gene were determined using the NCBI ORF finder: <https://www.ncbi.nlm.nih.gov/orffinder>. DNA pol gene was deposited to NCBI for accession number requesting.

A phylogenetic tree was constructed using NCBI's recent deposit of complete gene DNA pol of *Alphanudivirus* and an outgroup was *Heliothis zea Nudivirus* 1 (HzNV1), Genus *Betanudivirus* (Table 2). The DNA pol sequences were aligned with clustal W multiple alignments and find the sequence identity matrix with BioEdit V 5.0.7 (Hall 1999). The alignment was trimmed in order to minimize end gaps. Second align use Clustal X for fasta format output (Thompson et al. 1997) and then processed for maximum likelihood phylogeny tree construction by MEGA 7 (Kumar et al 2016). Best DNA model was Tamura 3 parameter (Nei and Kumar 2000) and the tree branch integrity was tested using Bootstrap (Pellock et al. 1996) with 1000 replicates. The DnaSP program (Rozas et al. 2017) was used to dig information about genetic variability of OrNV DNA pol gene like number of haplotypes, variable sites (S), number mutation (Eta), nucleotide diversity (Pi), and haplotype (gene) diversity (Hd).

Table 1. Sampling site including location, type of sample, commodity, counterpart, and GPS coordinate

Location	Individual of <i>Oryctes rhinoceros</i>	Commodity and counterpart	GPS coordinate
Marihat Baris, Siantar Sub-district, Simalungun District, North Sumatra	Adult = 57 Larvae = 50	Oil palm; Indonesian Oil Palm Research Institute (IOPRI)	2.9223, 99.0894
Kalianta Estate, Rokan Hulu District, Riau	Adult = 61 Larvae = 50	Oil palm; IOPRI	0.465870, 100.820694
Sijuk Village, Bangka-Belitung Islands	Adult = 50 Larvae = 50	Coconut; undergraduate student of IPB Univesity	-2.55847, 107.74773
Parungkuda Sub-district, Sukabumi District, West Java	Adult = 50 Larvae = 50	Coconut; Indonesian Industrial Beverage Crops Research Institute (IIBCRI)	-6.844, 106.7526
Bulumanis Lor, Margoyoso Sub-district, Pati District, Central Java	Adult = 60 Larvae = 50	Coconut; <i>Dinas Kehutanan dan Perkebunan</i> , Central Java Province	-6.608368, 111.068438
Rubung Buyung, Cempaga, East Kotawaringin District, Central Kalimantan	Adult = 74	Oil palm; PT. Bumitama Gunajaya Agro (BGA)	-2.19719, 113.06786
Pundu, Cempaga Hulu, East Kotawaringin District, Central Kalimantan	Larvae = 50		-1.97657, 113.10685
Babulu Darat, Babulu Sub-district, Penajam Paser Utara District, East Kalimantan	Larvae = 50	Oil palm; farmer	-1.495287, 116.406840
Kumasari, Sarudu Sub-district, Mamuju Utara District, West Sulawesi	Adult = 54	Oil palm; PT Surya Lestari 1, Astra Agro Group	-1.701959, 119.367979
Tammarunang, Duripoku Sub-district, Mamuju Utara District, West Sulawesi	Larvae = 50	Oil palm; IOPRI outlet Mamuju	-1.648731, 119.411832
Beteleme Village, Lembo Sub-district, Morowali District, Central Sulawesi	Adult = 3 Larvae = 17	Oil palm; IOPRI outlet Morowali	-2.1396, 121.2839

Table 2. National Center for Biotechnology Information (NCBI) accession number of DNA polymerase gene as source of phylogenetic tree. The isolates with bold words were samples in this research. As out group of the tree was *Heliothis zea nudivir* 1 (HzNV1)

Isolate's name	NCBI accession number
<i>Oryctes rhinoceros nudivir</i> (OrNV) Riau01	MK241543.1
OrNV Philippines PV505	AH015832.2
OrNV Sulteng01	MK241541.1
OrNV Malaysia Ma07	EU747721.1
OrNV Sumut01	MK241540.1
OrNV Kaltim01	MK241544.1
OrNV Jabar01	MK241547.1
OrNV Belitung01	MK241548.1
OrNV Kalteng01	MK241545.1
OrNV Jateng01	MK241546.1
OrNV Sulbar01	MK241542.1
Tomelloso Nudivir Dros EU28 (Tomelloso NV)	KY457233.1
<i>Drosophila innubila nudivir</i> (DiNV)	MF966379.1
Mauternbach Nudivir (Mauternbach NV)	MG969167.1
Kallithea Nudivir (Kallithea NV)	KP714105.1
Esparto Nudivir (Esparto NV)	KY608910.1
<i>Nilaparvata lugens endogenous nudivir</i> (NiNV)	KJ566528.1
<i>Gryllus bimaculatus nudivir</i> (GbNV)	NC_009240.1
<i>Heliothis zea nudivir</i> 1 (HzNV1)	AF451898.1

RESULTS AND DISCUSSION

Preparation for phylogenetic construction

OrNV infect both larvae and adult of *O. rhinoceros*. OrNV replicate in nucleus of midgut and fat body both of

larvae and adults (Huger 1966, Huger 2005). OrNV infection in adult is simply detected by dissection and examining their midgut. Midgut of infected adult is swollen, white color, approximately 4 mm diameter, and have milky contents. Midgut uninfected adult contains brown, narrow (1 mm diameter) with clear contents (Crawford and Zelazny 1990). Larvae with severe OrNV infection also have prominent appearance: shiny, beige, waxen, increased turgidity, and their rectum was completely prolapsed (Huger 2005).

OrNV DNA was taken from individual adults of *O. rhinoceros* which belong infected feature as mentioned above. The substantial DNA extraction from single host was to ensure purely of the OrNV isolate. It could be any mixed infection of different isolates in one spot of *O. rhinoceros* habitat, although mixed infections themselves were rare (Crawford and Zelazny 1990). Investigate signature adaptation gene of Nudivir by Hill and Unckless (2018) was also using single individual of *Drosophila innubila* (Diptera: Drosophilidae).

The Quick-DNA miniprep plus kit from Zymo Research yielded a reasonably good quality of total DNA from midgut with obvious symptoms of OrNV infection. The DNA showing a relatively high concentration and purity. Quick-DNA miniprep is a DNA purification method that uses a matrix column made of silica, glass, or diatoms. Nucleic acid is absorbed by the column during the purification process. Pure DNA is then elucidated using buffer elution (Tan and Yiap 2009). DNA concentration from infected midgut was rise significantly by crushing, enzyme digestion at 55°C for 3 hours, and pre-warmed buffer elution at 65°C.

A total of five primers were designed to amplify the complete gene sequence of OrNV DNA pol (Table 3). The amplicons have different sizes of 650 bp and 1500 bp. The primer amplicons overlap with each other. The primer order from the start to the stop codon of DNA pol gene was primer 200F/200R, OrNV4F/OrNV4R, OrNV3F/OrNV3R, OrNV2F/OrNV2R, and OrNV1F/OrNV1R. The five primers designed had a high ability to detect OrNV infection in both adults and larvae of *O. rhinoceros*. The primers are quite sensitive to detect an early infection of the OrNV by extraction of midgut tissue. Thick PCR band was produced from extraction of white swollen midgut diameter of 4 mm while thin PCR band was resulted from not swollen midgut diameter 1 mm. These positive PCR results were strongly correlated with visual disease symptoms in *O. rhinoceros* adult also reported by Richard et al. (1999). Sequencing requires sufficient DNA concentration. Sequencing for thin PCR band sample, comprised two PCR steps aimed at increasing the DNA pol gene amplicon. The first solution carried out for PCR processes was then applied as a DNA template for the second PCR.

Oryctes rhinoceros nudivirus (OrNV) variability

Genetic variability that is caused by spontaneous mutations makes the evolution of OrNV possible (Hall et al. 1984). Spontaneous mutations occur by errors in DNA replication, but the polymerase gene can control production of point mutation by selected correct polymerization or removed of incorrect bases using exonuclease (Hall et al. 1984). DNA pol genes of virus are usually essential genes that they are evolved closely with the virus and thus reflect their evolution (Pellock et al. 1996). The mutations that propagate are the ones that increase survival. Investigation of the genetic variability basically looks at tiny changes in microevolution.

The main task of an OrNV DNA pol gene is to ensure complete copy of its genome. Since OrNV replicates in the nucleus of infected host cells (Wang and Jehle 2009), OrNV does not need to encode their own DNA pol. Genome replication and mRNA synthesis of OrNV depends on host cell machinery (Choi 2012). OrNV has double-stranded (dsDNA) (Wang and Jehle 2009) that replicate their genomes using DNA-dependent DNA polymerases (or called DNA polymerases) and transcribe

mRNA using DNA-dependent RNA polymerases (or called RNA polymerases) (Choi 2012).

The evolution can be presented as a phylogenetic tree whose leaves correspond to extant species and the nodes to extinct ancestors. Phylogenetic analysis is usually carried out with DNA sequences to interpret closely related species rather than protein (Wolf et al. 2002). In the study of OrNV evolution, the phylogenetic tree uses its nucleotides. The phylogenetic tree tells exactly how OrNV from Indonesia related to others, and how recently or distantly with other particular *Alphanudivirus* genus (Figure1).

Kobayashi and Somowiyarjo (1995) mentioned that there are only slight differences in the pattern of restriction for EcoRI and HindIII fragments from Indonesian isolates. They suggested that there are many of OrNV isolates from Indonesia. DnaSP program (Rozas et al. 2017) could show how high levels of variabilities of Indonesian OrNV (Table 4).

Number for haplotype of Indonesian OrNV were eight: haplotype 1 (isolate Sumut01), haplotype 2 (Sulteng01 and Riau01), haplotype 3 (Sulbar01), haplotype 4 (Kaltim01), haplotype 5 (Kalteng01), haplotype 6 (Jateng01), haplotype 7 (Jabar01), and haplotype 8 (Belitung01). Haplotype refers to a group of genes within an organism that was inherited together from a single parent (Nature 2005). It was supposed that Sulteng01 and Riau01 isolate of OrNV have the same parental origin although both were far apart for 2205 km.

Genetic variability within population mainly estimated using score of haplotype diversity (Hd) and nucleotide diversity (Pi) (de Jong et al. 2011). The meaning of haplotype diversity or gene diversity is probability that two randomly sampled alleles are different. While nucleotide diversity is the average number of nucleotide differences per site in pairwise comparisons among DNA sequences. Based on the definition by de Jong et al. (2011) show that randomly sampled alleles of Indonesian OrNV were different one with another, except OrNV isolate Sulteng01 and Riau01 (Table 4). Four groups of tests showed a maximum Hd score (1.00) which means these maximum scores for gene diversity. One group test (Sulteng01 and Riau01) was without Hd score because two isolates were no gene diversity. As mentioned above, Sulteng01 and Riau01 supposed from single parental.

Table 3. Primers for amplifying complete gene of *Oryctes rhinoceros nudivirus* (OrNV) DNA polymerase

No	Name	Sequence	GC content (%)	The amplicon length (bp)
1	OrNV1 F	5'-GGA GAG GAT GTG TGT GAC TTT-3'	47.6	1 504
	OrNV1 R	5'-CGC GCA ACA TTC GGT TAT AC-3'	50.0	
2	OrNV2 F	5'-GTT TCA CTG ACC GAC AAG CTA-3'	47.6	1 509
	OrNV2 R	5'-GGC CGA AAG GTC TCC ATT AT-3'	50.0	
3	OrNV3 F	5'-GAA GAA CTC CGC TAG ATA CGT TAA-3'	41.7	1 516
	OrNV3 R	5'-GAC CCT CCC ATA TCA CGA TTA C-3'	50.0	
4	OrNV4 F	5'-ACT ACC AGT CGA ACG AAG TAT TG-3'	43.5	1 498
	OrNV4 R	5'-TAG CTT GTC GGT CAG TGA AAC-3'	47.6	
5	OrNV200 F	5'-CGC ATA CTG CAA TAC GTA CAC-3'	47.6	666
	OrNV200 R	5'-CTA AAT TCC CAG GTC TGC AAA C-3'	45.5	

Table 4. Five running tests between isolate using DnaSP program to investigate OrNV variability. Isolate groups were determined based on phylogenetic tree results (Figure 1)

OrNV isolate	Variable sites (s)	Number of mutation (Eta)	Nucleotide diversity (Pi)	Haplotype (gene) diversity (Hd)	Fu and Li's D test	Fu and Li's F test
Sulbar01, Jateng01, Kalteng01	86	88	0.01553	1.00	0.00 (statistical significance)	0.00 (statistical significance)
Belitung01, Jabar01	13	13	0.00347	1.00	0.00	0.00
Sumut01, Sulteng01	3	3	0.00080	1.00	0.00	0.00
Sulteng01, Riau01	0	0	0.00	-	0.00	0.00
Riau01, Kaltim01	2	2	0.00053	1.00	0.00	0.00

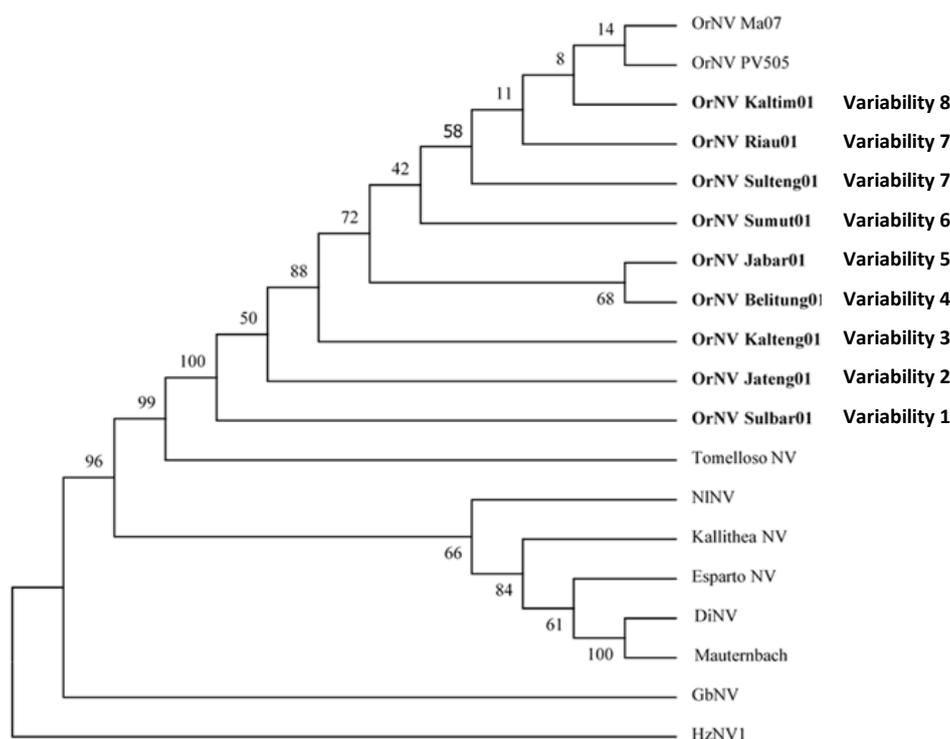


Figure 1. Maximum likelihood phylogenetic based on DNA polymerase gene of Indonesian *Oryctes rhinoceros Nudivirus* (OrNV) (write in bold words) and recent National Center for Biotechnology Information (NCBI) deposited of Genus *Alphanudivirus*. Percent bootstrap values with 1000 replications were listed for each node.

Fu and Li test both D and F are calculating the distribution of alleles or haplotypes under the assumption of neutrality of mutation (Fu and Li 1993). The result for Fu and Li's D and F tests in Table 4 were all statistical significance. The Fu and Li test score was not negative which indicated that the OrNV population in neutral evolution (de Jong et al 2011). The rate of sequence DNA changes almost like living fossils whose morphological characters hardly to change. Sequences of DNA is genetic information transmitted from generation to generation (Ohta 1992). In neutral theory of evolution, evolution is a consequence of two processes genetic variability generated by mutation and changes of alleles frequency within-population over time. These mutations are not deleterious but its rapidly removed by natural selection. A neutral

mutation is one that does not affect an organism's ability to survive and reproduce. In other words, the mutation is not deleterious but in neutral rather than beneficial. Allelic frequencies may change simply as random gamete sampling in reproduction process. This process is called genetic drift. Genetic drift could lead to fixation of some alleles or loss of the others. Gene duplication may get fixed in population by random genetic drift because they are not deleterious. The neutral theory suggests that a mutant gene can arise within a population and reach fixation by chance, rather by selective advantage. Most evolution changes at molecular level are due to selectively neutral (Duret 2008). Although Sulteng01 and Riau01 were supposed low level of nucleotide diversity, both isolates faced neutral evolution based on Fu % Li's D and F test.

OrNV epizootiology

Insect epizootiology is science of causes and form of mass phenomena of diseases at all levels of intensity in an animal host population (Vega and Kaya 1993). Definition that explains the epizootiology term is epizootic and enzootic. Epizootic refers to unusual large number of diseases cases in host population, while enzootic is low prevalence and is constantly present of the diseases in the host population. OrNV spread in its host population, the *O. rhinoceros*, are influenced by their pathogenicity and transmissibility. The transmission pathways by the OrNV must ensure their survival in its host. They infect host but are only a non-lethal infection. This OrNV transmitted by horizontal normal route from host to host, and vertical transmission from parent to offspring (Vega and Kaya 1993). Non-lethal transmitted OrNV disease may be dispersed in various *O. rhinoceros* populations in Indonesia.

Nucleotide diversity (Pi) score of isolate Sulbar01, Jateng01, Kalteng01 were 0.01553 and Pi score of isolate Belitung01 and Jabar01 was 0.00347. These two Pi values were classified as high score. Pi score of isolate Sumut01 and Sulteng01 were 0.0080, Sulteng01 and Riau01 were 0.00000, while isolate Riau01 and Kaltim01 were 0.00053 (Table 4). These two Pi values were classified as low scores (de Jong et al 2011). High score of Pi have correlated with value of variable sites (S), number of mutation (Eta) that also showed high points. High score of nucleotide diversity, variable sites, and number of mutations are typically considered a function of population history and the initial origin of the OrNV. Long period of

OrNV existence, means a long time for mutation accumulation and hence increased level of nucleotide diversity. The occurrence of a bottleneck in population size, accompanying with dispersion of OrNV to other locations would further increase genetic drift, which reduced genetic variation (Relethford 2015).

Initial origin of the OrNV comes from West Sulawesi (isolate Sulbar01), non-lethal infected host *O. rhinoceros* than headed to Central Java (isolate Jateng01). Some non-lethal infected of host then fly from Central Java to Central Kalimantan (isolate Kalteng01), to Belitung (isolate Belitung01) and to West Java (isolate Jabar01) (Figure 2). These dispersion routes were not at long distances. Nudivirus ancestor supposed to appear at 310 Mya, in Paleozoic era in the Carboniferous period, suggesting that they already infected the first insects which appeared during Devonian and Carboniferous period (Theze et al. 2011). The emergence of Kalimantan, Java, and Sulawesi island was relatively new compared to the emergence of Nudivirus ancestral. These islands had been formed in Miocene Era 20 Mya until early Pliocene 5 Mya. Central Kalimantan, the north part of Central Java, almost all region of West Java was part of land in Sundaland paleogeography, while West Sulawesi was first appeared land in Wallacean paleogeography (Hall 2013). It had been estimated that OrNV ancestral arrived on those first appeared land and continues to regenerate until now. The mutation site in DNA polymerase gene accumulated which amounts to 88 in West Sulawesi, Central Java and Central Kalimantan OrNV populations, and 13 mutations site of OrNV populations from Belitung and West Java.

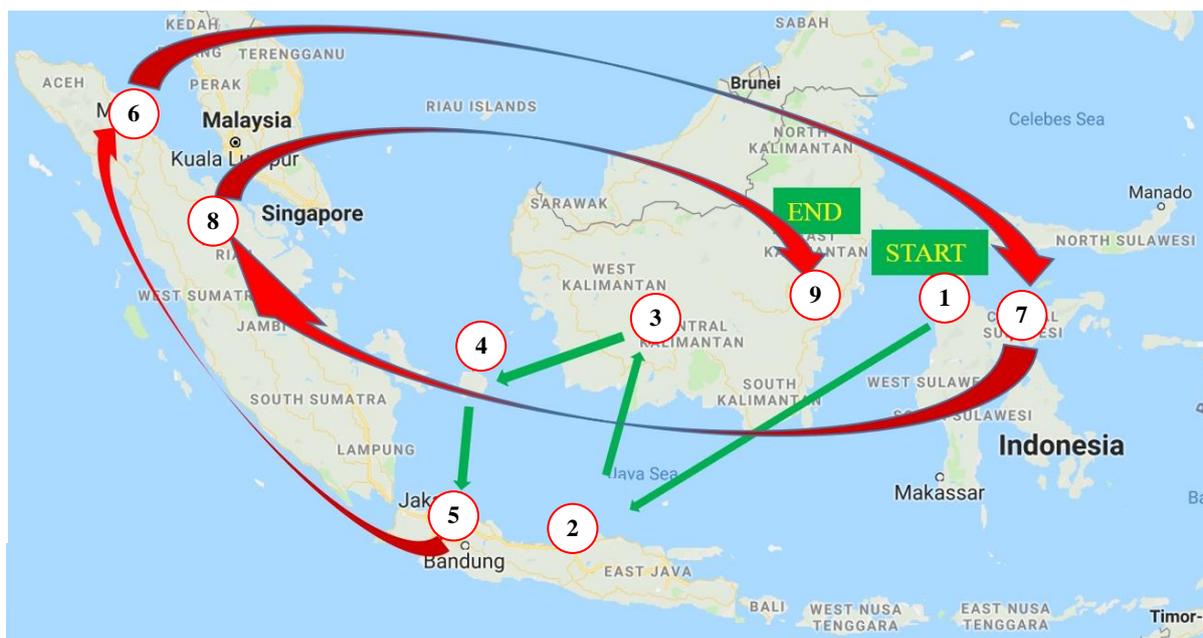


Figure 2. The epizootiology dispersion pattern of Indonesian OrNV starts from (1) West Sulawesi which spread to (2) Central Java, then to (3) Central Kalimantan, to (4) Belitung and to (5) West Java. These consecutive four isolates have high score of nucleotide diversity. Route of the epizootiology of OrNV began to spread across the island from (5) West Java to (6) North Sumatra, then to (7) Central Sulawesi to (8) Riau province and ended in (9) East Kalimantan. OrNV which experiences cross-island travel has low Nucleotide diversity as shown in Table 3.

OrNV with non-lethal infection to its host from old ancestry then disperse across the island with relatively long distance. The OrNV from West Java (isolate Jabar01) disperses to North Sumatra (isolate Sumut01), then to Central Sulawesi (isolate Sulteng01), to Riau province (isolate Riau01) and to East Kalimantan (isolate Kaltim01). This long-distance of OrNV dispersion was probably as a result of oil palm plantation development in Indonesia. Oil palms have been cultivated since 1911 in Sumatra, and 1981 in Kalimantan. Sumatra and Kalimantan become production center of Indonesian oil palm (Pamin 1998). Oil palm plantation began to be expanded to Central Sulawesi and West Sulawesi in 2004 (Fatmasari et al. 2018). Sulawesi island is not a major oil palm plantation as Sumatra and Kalimantan islands, areas under oil-palm cultivation are only 354,000 ha. Java island is also not intensively cultivated with oil palms, the area occupied by oil palm as only 36,163 ha in Banten province (Gapki, 2015; Directorate General of Estate Crop RI 2016).

The advantage of OrNV epizootiology dispersion information related to the next OrNV's virulence test, that could be explored between high and low nucleotide diversity of isolates. Isolates with high genetic variability were Sulbar01, Jateng01, Kalteng01, Belitung01, Jabar01 and isolate with low genetic variability were Sumut01, Sulteng01, Riau01, and Kaltim01. We could choose two strategies to manage *O. rhinoceros* by selecting high virulence or low virulence of the isolate. High virulence is needed to control *O. rhinoceros* population as quickly as possible. Once an OrNV application is applied, the *O. rhinoceros* population will be reduced immediately. This condition is requested if *O. rhinoceros* population on highly conditioning as devastating pest. Current status of *O. rhinoceros* is main pest of coconut and oil palm (Bedford 2014). Mild virulence of OrNV isolate is requested to manage *O. rhinoceros* population under economic threshold. By this research, it is supposed that OrNV from Java and Belitung island consists of only high nucleotide diversity. Sumatra island supposed consists only OrNV with low nucleotide diversity, while Kalimantan and Sulawesi island consist mixture of low and high nucleotide diversity. Crawford and Zelazny (1990) detected three types of genomic changes of OrNV isolate that had been released in Maldives Island after four years. It was supposed after 109 years of oil palm plantation in Sumatra island (Pamin et al. 2018) and the genetic drift changed the old population of OrNV with young populations that have low nucleotide diversity. Oil palm development in Kalimantan and Sulawesi island is relatively new (39 years and 16 years) so that it still found both young and old OrNV populations.

Relationship of OrNV with other *Alphanudivirus*

Since developed into a new family (Jehle et al. 2013), the discovery of *Nudiviridae* members has been increasing. Jehle et al. (2013) reported GbNV (Orthoptera order), OrNV (Coleoptera order) as a member of Genus *Alphanudivirus* and HzNV 1, HzNV 2 (Lepidoptera order) as a member of genus *Betanudivirus*. The following year,

Bézier et al. (2015) added PmNV (Decapoda order) and ToNV (Diptera order) as members of the new genus *Gammanudivirus*. The discovery of *Homarus gammarus nudivirus* (HgNV) by Holt et al. (2019) therefore suggested ToNV as a separate genus, *Deltanudivirus*, while *Gammanudivirus* had HgNV and PmNV as members. The *Gammanudivirus* genus was proposed as a specialized member group of *Nudiviridae* that infects aquatic crustaceans (Holt et al. 2019).

Some genes are visibly better than others to build evolutionary relationship among taxa at particular level of divergence. DNA sequences data could better understand the way genes and species evolved (Simon et al 1994). Viruses lack universal genes that all viruses could be placed in unified phylogeny (Simmonds et al 2017). Even though, DNA polymerase has been shown distinct phylogenetic of DNA viruses of Genus *Alphanudivirus* as presented in Figure 1. Phylogenetic tree constructed by Hill and Unckless (2018) using Nudiviral 20 conserved ORF resulted in similar phylogenetic pattern with Figure 1.

The *Alphanudivirus* has grown and now have eight species: (i) *Oryctes rhinoceros nudivirus* (OrNV); (ii) Tomelloso nudivirus; (iii) *Drosophila innubila nudivirus* (DiNV); (iv) Mauternbach Nudivirus; (v) *Kallithea nudivirus*; (vi) Esparto nudivirus; (vii) *Nilaparvata lugens endogenous nudivirus* (NINV); (viii) *Gryllus bimaculatus nudivirus* (GbNV). The host of these genera: Orthoptera (GbNV); Hemiptera (NINV); Diptera (DiNV, Tomelloso NV, Mauternbach NV, *Kallithea* NV, Esparto NV); and Coleoptera (OrNV). OrNV closely related to Tomelloso Nudivirus. Mauternbach Nudivirus, DiNV, Esparto Nudivirus, and *Kallithea* Nudivirus clustered together have the same host, *Drosophila* sp. (Diptera: Drosophilidae) (Webster et al. 2015). These clustered taxa were a sister group of the OrNV and Tomelloso Nudivirus. Tomelloso Nudivirus also has *Drosophila* sp. as its host but is closely related to OrNV. GbNV has been described as taxa from the earliest branch of *Alphanudivirus* phylogenetic tree (Wang et al. 2007c) and this matches our study (Figure 1).

Palmer et al. (2019) also reported the phylogenetic of 8 species of Genus *Alphanudivirus* using maximum likelihood method of DNA polymerase protein. This tree pattern was similar to Figure 1 as presented above, except the position of the species NINV out of the cluster: Mauternbach Nudivirus, DiNV, Esparto Nudivirus, *Kallithea* Nudivirus. The NINV displacement in phylogenetic tree supposed because other species of *Alphanudivirus* have not yet been found. With more members of a cluster founded, the phylogenetic tree pattern became stable (Pellock 1996). Investigated using Fu and Li test both D and F represent negative scores for cluster with NINV or without NINV. Negative score of Fu and Li score indicate that this cluster stands on non-neutrality evolution. The evolution of organisms supposed to be in neutral condition as presented with statistical test significant (Fu and Li 1993).

Nudivirus has been infected with diverse arthropods hosts suggesting their ancient origin and complex evolutionary history. Nudivirus infect early

hemimetabolous Orthoptera to the most recent emerged holometabolous Lepidoptera. Nudivirus also present in marine crustacean hosts (Wang and Jehle 2009). Order infected by this *Alphanudivirus* i.e. Orthoptera, Coleoptera, Hemiptera, Diptera. Although phylogenetic patterns of its host different from *Alphanudivirus* pattern but they have resemblance of immature life cycle niche. All members correlated with soil as a habitat during immature. This shared ecological niche may explain their closely related taxa. Phylogenetic incongruence between virus and their insect hosts suggests that host ecology rather than host phylogeny was the driving force of virus evolution (Wang et al. 2007c).

Insect and crustacean lineages associated with dsDNA viruses appeared in middle of Paleozoic Era at 310 Mya (Theze et al. 2011). Nudivirus ancestor with its sister families diverged at late Carboniferous as well as the insect was diversifying into new order. Unfortunately, their evolution way was independent, lack of co-phylogeny. It was supposed that viruses may have colonized new insect and crustacean hosts many times. Nudivirus ancestor lack of occlusion bodies as environment protectant. It has been proposed that evolution association with their host involving viral persistence or latency. Ancestral insect becomes main resources for ancestral Nudivirus survival. When new insect species evolved, virus individual adapted to that new ancestral host. The ecological speciation mechanism at some point produced new virus species as the *Alphanudivirus* genus have the same immature soil niche.

In conclusion, nine of OrNV isolates collected from the research were found eight haplotypes with two kinds of high and low genetic diversity. These genetic diversities supposed to correlate with development of oil palm plantation and genetic drift. OrNV epizootiology of old population with high genetic diversity starts from West Sulawesi to Central Java, to Central Kalimantan, to Belitung Island, and to West Java. Epizootiology then continued from West Java to North Sumatra, to Central Sulawesi, to Riau, to East Kalimantan with its properties were low genetic diversity. Java and Belitung were sources sites of old OrNV population, otherwise Sumatra as sources site of new OrNV population. Kalimantan and Sulawesi were found of mixed populations both old and new OrNV. This OrNV belongs to Genus *Alphanudivirus* where the closeness of each member was based on immature ecological niche.

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