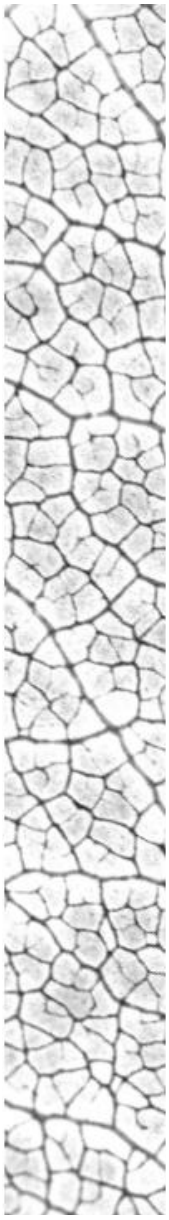

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The genetic relation of Indonesian *Calloselasma rhodostoma* based on ND4 gene and preliminary study of its venom storage condition

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Abstract
Calloselasma rhodostoma (Kuhl, 1824) is one species of Indonesian medically important snake that distributed in many Indonesia regions. With the recent snakebite cases of this snake and warrant of further improvement on anti-venin in Indonesia, the information on storage conditions for its venom is important for developing an anti-venin. The genetic identity that possibly holds a cryptic diversity that has not been resolved, could impact the future of anti-venin development of this species. We analyze the molecular data based on the ND4 gene to resolve the genetic relations of this snake from Java, Kangean, and Borneo population with the addition of the Thailand population by Bayesian Inference phylogenetic reconstruction. To evaluate the storage conditions, venom collection from six living specimens from the Java population was used to analyze the effect of svPLA2 activity under different storage conditions for 14 days long. The phylogenetic results show a polytomy tree, with a low p-distance value between populations. Only the storage at 37 ºC affects the performance of svPLA2 significantly. The phylogenetic indicating a single species even though divided by geographical barriers, more genes need to be compared to resolve the genetic relationship. More samples are needed to compare the venom properties throughout *C. rhodostoma* wide distribution, to enlight the anti-venin future development.

Keywords: *Calloselasma rhodostoma*, ND4, phylogenetic, svPLA$_2$, venom storage

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INTRODUCTION

Calloselasma rhodostoma (Kuhl, 1824) is distributed in South East Asia including Indonesia (Uetz and Hillermann, 2018). This species is naturally found in Java, Karimun Java, Kangean (Das, 2010), and in 2014 was reported to be found in Ketapang, West Borneo. The large distribution range, its habitat preference, and its venom content cause a high frequency of fatal snakebite envenomation. This place out *C. rhodostoma* as category I medically important snake (Warrel, 2010). Recent efficacy analysis of sole antivenom in Indonesia warranted for further improvement, especially on the species-specific basis (monovalent type) (Tan et al. 2016). Besides, the genetic identity that possibly holds a cryptic diversity hasn't been resolved, subsequently affecting the anti-venin development of this snake.

Calloselasma rhodostoma has been morphologically examined and were found variation in head scalation and morphometric characters among the populations from Java, Borneo, Karimun Java, and Kangean (Kadafi et al. 2018; Adeyeye, et al, 2016). But, several snakes species that are found to have different morphology characters yet are classified as one same species in the consideration of the molecular data (Wostl et al. 2016). However, the molecular data to support the finding of its morphological characters of *C. rhodostoma* in Indonesia has never been evaluated and in need to be examined.

The venom of *C. rhodostoma* contains various kinds of protein, both enzyme, and non-enzyme that have a role function in foraging activity and defense strategy (Vitt and Janalee, 2009). One major component inside the venom is Phospholipase A$_2$ (svPLA$_2$), which roles to hydrolyze glycerophospholipid into fatty acids and lysophospholipids (Kang et al. 2011). This enzyme damages phospholipid cellular membrane that leads to various pharmacological effects (Warrel 2010, Mackessy 2010, Tsai 2000). To consider this, some

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Table 1. The sequences used in this study. Abbreviations for deposited collection as follows: NK-Nia Kurniawan (Universitas Brawijaya, Indonesia); PT-Panupong Thammacoti (Chulalongkorn University, Thailand); AM-Anita Malbotra (Bangor University, United Kingdom); UMMZ-Museum Zoology, University of Michigan (United States).

No	Specimen Number	Taxon	Country	Accession Number	Locality	Source
1	Isolate K1	*C. rhodostoma*	Indonesia	MT722041	Kangean, East Java, Indonesia	This study
2	Isolate K3	*C. rhodostoma*	Indonesia	MT722043	Kangean, East Java, Indonesia	This study
3	Isolate K23	*C. rhodostoma*	Indonesia	MT722059	Kangean, East Java, Indonesia	This study
4	Isolate K2	*C. rhodostoma*	Indonesia	MT722042	Sapanjang (Kangean) East Java, Indonesia	This study
5	Isolate K5	*C. rhodostoma*	Indonesia	MT722044	Sapanjang (Kangean) East Java, Indonesia	This study
6	Isolate K9	*C. rhodostoma*	Indonesia	MT722047	Trenggalek, East Java, Indonesia	This study
7	Isolate K20	*C. rhodostoma*	Indonesia	MT722057	Majalengka, West Java, Indonesia	This study
8	Isolate K21	*C. rhodostoma*	Indonesia	MT722058	Majalengka, West Java, Indonesia	This study
9	NK 1270	*C. rhodostoma*	Indonesia	MT722045	Cilacap, Central Java, Indonesia	This study
10	NK 1271	*C. rhodostoma*	Indonesia	MT722046	Cilacap, Central Java, Indonesia	This study
11	Isolate K28	*C. rhodostoma*	Indonesia	MT722060	Cilacap, Central Java, Indonesia	This study
12	NK 1607	*C. rhodostoma*	Indonesia	MT722048	Ketapang, West Borneo, Indonesia	This study
13	NK 1608	*C. rhodostoma*	Indonesia	MT722049	Ketapang, West Borneo, Indonesia	This study
14	NK 1609	*C. rhodostoma*	Indonesia	MT722050	Ketapang, West Borneo, Indonesia	This study
15	NK 1610	*C. rhodostoma*	Indonesia	MT722051	Ketapang, West Borneo, Indonesia	This study
16	NK 1611	*C. rhodostoma*	Indonesia	MT72252	Ketapang, West Borneo, Indonesia	This study
17	NK 1612	*C. rhodostoma*	Indonesia	MT722053	Ketapang, West Borneo, Indonesia	This study
18	NK 1613	*C. rhodostoma*	Indonesia	MT722054	Ketapang, West Borneo, Indonesia	This study
19	NK 1614	*C. rhodostoma*	Indonesia	MT722055	Ketapang, West Borneo, Indonesia	This study
20	PT 297	*C. rhodostoma*	Thailand	MT722056	Nakon Si Thammarat, South Thailand	This study
21	UMMZ 184314	*C. rhodostoma*	Thailand	U41878	Thailand	Kraus and Brown 1998
22	Isolate RS-S	*Hypnale nepa*	Malaysia	KC347491	Sri Lanka	Pyron et al. 2013
23	Isolate RAP0552	*Hypnale zara*	India	KC347513	India	Pyron et al. 2013
24	Isolate A53-	*Hypnale hypnale*	India	AY352812	India	Malhotra and Thorpe 2004
25	AM B306	*Ovophis chaseni*	Malaysia	AY352825	Mt. Kinabalu, Sabah, Malaysia	Malhotra and Thorpe 2004

potential compounds against svPLA$_2$ activity is also have been studied (Xiao et al. 2017).

Despite the status as a major component, the abundance of svPLA$_2$ is relative to some factors such as kinds of snake and locality (Vija et al. 2009, Tan et al. 2015). Previous studies also pointed out the relation between snake venom toxicity and the prey preference of *Echis* sp. (Barlow et al. 2009). Thus, the consideration to use a fresh-milked venom solution is needed, especially with the fact that *C. rhodostoma* exists over a wide geographical range. As a consequence, the storage condition of *C. rhodostoma* is important to note. Previous studies carried by Munekiyo and Stephen (1998) evidence that svPLA$_2$ and other enzymes in the *Crotalus molossus molossus* venom have remained stable under -80–37 ºC for 7 days long. Nevertheless, the svPLA$_2$ and other enzymes need to be examined for this snake species.

Supported by the consideration of this snake's status in health matters, a relevant taxonomic status of this snake and the storage condition for the fresh-milked venom are important to be examined. Thus, our research purposes to analyze and construct a phylogenetic tree from the ND4 sequence from Java, Kangean, Borneo, and Thailand, also to evaluate the *C. rhodostoma* svPLA$_2$ activity as a major component of *C. rhodostoma* venom under various storage condition.

MATERIAL AND METHODS

Sample Collection

The tissue sample used was obtained from Java, Karimun Java, Kangean, Borneo Island, with addition to the Thailand population (**Table 1**). The dorsal muscle tissue was preserved in 95% ethanol in a small tube, and preserved specimens from the Indonesia population were deposited in the Laboratory of Ecology and Animal Biodiversity, Department of Biology, Brawijaya University.

Samples used for the evaluation of storage conditions in its influence in PLA$_2$ activity were taken from the Java population. A total of 6 snake individuals were maintained in an individual cage and were fed routinely. Venom milking was conducted after fasting, pooled together, and aliquoted into a smaller volume to be stored at the various condition. We performed three levels of storage time: 7, 9, and 14 days; and temperature: 37°C, 4ºC, and -80ºC.

Genetic Relationship Analysis

The DNA extraction procedure was performed by Qiagen (QIAamp DNA Mini Kit) company protocol with slight modification. Muscle tissue was crushed and dissolved in ATL Buffer, followed by vortex for 15 sec. The sample was incubated for 1–3 h at 56ºC. The homogenized sample was then added by 200 µl of AL buffer, followed by incubation at 70ºC for 10 min, and added by 200 µl of absolute ethanol. The whole solution was transferred into the QIAamp mini spin column, added by washing buffer AW1 and centrifuged at 8000 rpm (1 min). Pellet in the spin column was added by washing buffer AW2 and centrifuged at 14000 rpm (1 min). DNA precipitation in the spin column membrane was added by Elution buffer and was incubated at 15–25ºC for 1 min, followed by centrifugation at 8000 rpm for 1 min. DNA can be found in the supernatant part.

Amplification of collected DNA was performed by using ND4 forward primer F: 5' CAC CTA TGA CTA CCA

AAA GCT CAT GTA GAA GC 3'; and Leu reverse primer R: 5' CAT TAC TTT TAC TTG GAA TTT GCA CCA 3' (Arévalo 1994, Wostl et al. 2016). The amplification cycle comprises early denaturation (hot-start) which was performed at 94ºC (3 min), 30 times of denaturation at 94ºC (30 sec), annealing at 52ºC (45 sec), elongation at 72ºC (60 sec), and late elongation at 72ºC (7 min). Amplified results were qualitatively assayed through 2% gel electrophoresis and sequenced (FirstBase, Malaysia). The DNA sequences were contig and edited by sequencer 4.1.4 (Gene Codes, Ann Arbor, Michigan, USA) and ChromasPro 1.34 (Tomohiko Shimada, Kyoto University, Japan), and evaluated by eye for the quality. The sequences were deposited on GenBank (GenBank Accession No: MT722041-MT722060, **Table 1**).

As much as ~650 bp of alignment sequence were used for phylogenetic analysis and genetic distances. The similarity among DNA sequences and data in GenBank were analyzed through BLAST. Sequence alignment was conducted using MEGA7 (Kumar et al. 2016). The uncorrected p-distance values were grouped based on the locality of the sample. the ingroup including all of the *C. rhodostoma* from Indonesia and Thailand population, *Ovophis chaseni*, and *Hypnale* spp. are chosen as the outgroup (**Table 1**). The phylogenetic tree was reconstructed by Bayesian Inference model. The best evolution model was determined by Kakusan4 (Tanabe 2011). Bayesian inference was analyzed in MrBayes 3.04b (Huelsenbeck and Ronquist, 2001) using 5 million generations for the chain lengths of MCMC. The estimation was sampled every 1000 multiple generations with a topology consensus of 25%. The reconstruction result was considered valid as if the Bayesian posterior probability (BPP) reaches 0.95 or more (Huelsenbeck and Ronquist, 2001). and -80ºC.

Venom Storage Condition Analysis

The concentration of venom protein was measured as a prior step of the whole analysis series. 1 µl sample solution from each storage condition was measured the absorbance in the presence of 280 nm light wavelength through the NanoDrop instrument. The absorbance value was equalized into the protein content unit per solution sample volume (mg ml^{-1}). The data was used as the basis for further assays.

The crude venom solution that had been measured for the protein concentration was then dissolved by sterile equates and followed to be processed under reducing condition (Reducing Sample Buffer 1:1 v/v). The solution of the sample and RSB was heated at 100ºC for 15 min. A total of 23 µg protein content inside a 10 µg sample solution was then conducted into SDS-PAGE, with 3% and 15% gel concentration for stacking and separating gel respectively. Electrophoresis was performed in a constant voltage of 120 V. Jena Bioscience BlueEye Prestained Marker 10-245 kDa was used as a standard marker to measure the molecular weight of the separated protein sample. The CBB staining process was conducted to visualize the sample protein separation results.

Table 2. The intraspecies p-distance percentage value of *C. rhodostoma* from several populations with a range of minimum-maximum value

Population	Madura	Java	Borneo	Thailand
Madura	0			
Java	0.39 (0.3 – 0.6)	0		
West Borneo	0.39 (0.3 – 0.6)	0.35 (0.3 – 0.6)	0	
Thailand	0.28 (0.2 – 0.5)	0.28 (0.2 – 0.5)	0.27 (0.2 – 0.5)	0

The svPLA2 activity of *C. rhodostoma* was assayed by the acidimetric method based on Tan and Tan (1988). One part of egg yolk was mixed with 1 part of 18 mM $CaCl_2$ and 1 part of 8.1 mM sodium deoxycholate. The phospholipid substrate solution formed was mixed well and adjusted by 1 M NaOH to reach a pH of 8.0. The sample solution with a concentration of 50 µg/100 µl was poured into a 15 ml substrate solution. The pH was measured by meter using QIS Netherland Make Portable Meter in a 5–65 sec period, where the decrease of 1 pH unit was equal to the release of 133 µmol fatty acids as the products of phospholipids hydrolysis.

The statistical analysis was performed by SPSS 20.0. Normal and homogenous variant data were ANOVA tested, followed by univariate analysis and Tukey test.

RESULTS

Calloselasma rhodostoma ND4 Sequence Analysis

The p-distance value in this research does not show significant differences between each population. The highest p-distance can be found between the population of Madura and the populations of Java and Borneo (0.39 with a minimum-maximum value of 0.3–0.6) (**Table 2**). Meanwhile, the lowest p-distance value can be found between the population of Borneo and Thailand (0.27 with a minimum-maximum value of 0.2–0.5). The p-distance results are classified low, thus, the samples of *C. rhodostoma* tested were included in one same species even geographical barrier separate the populations. A supporting conclusion of this is only a little mutation occurs in the samples' genetic materials, or the sequences used are conserved.

Phylogenetic tree construction by Bayesian Inference forms a polytomy tree topology (**Fig. 1**). The topology arranged into 4 groups, where the grouping is referring to the locality of *C. rhodostoma*. Group I refer to the Java population of *C. rhodostoma* (BPP = 0.76). Group II refers to the Ketapang (West Borneo) population of *C. rhodostoma* (BPP = 0.5). Group III refers to the Thailand population of *C. rhodostoma* (BPP = 0.8). Group IV refers to *C. rhodostoma* from the

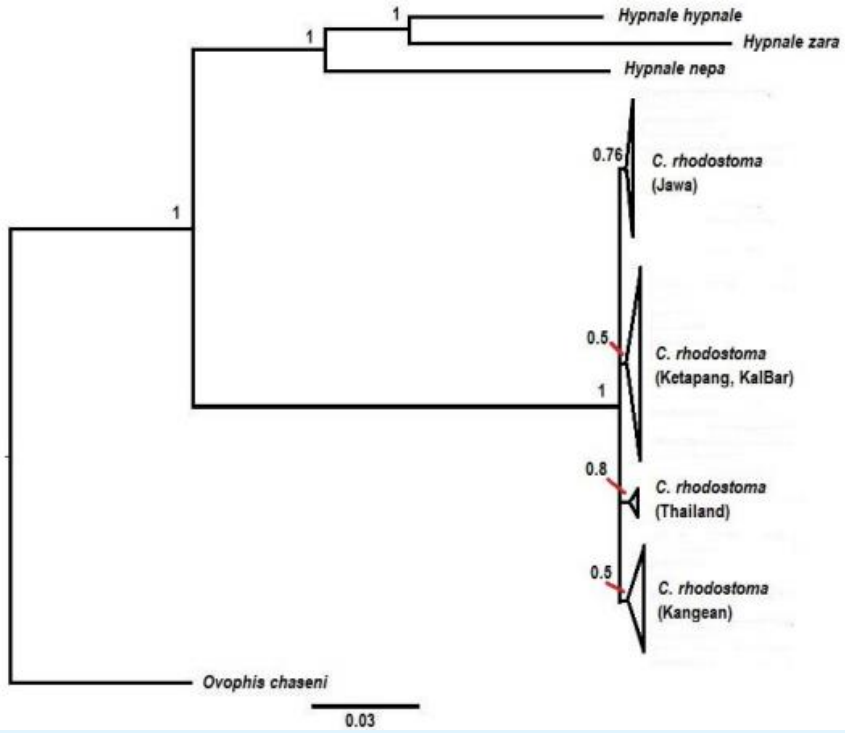

Fig. 1. Bayesian Inference (BI) phylogenetic tree of *C.rhodostoma* by ND4 gene. The number at each branch indicates bootstrap Bayesian posterior probability (BPP) value

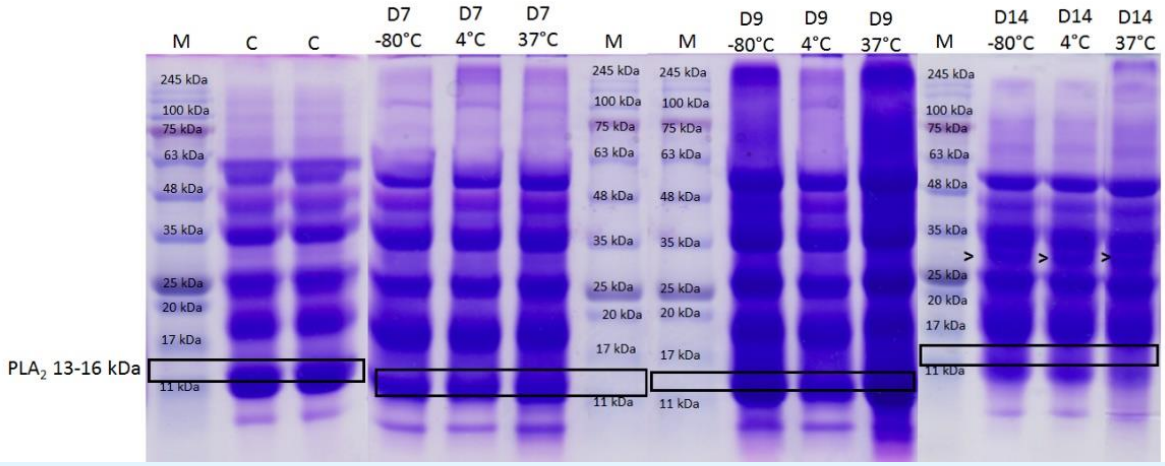

Fig. 2. Protein bands from *C. rhodostoma* venom through 15% SDS-PAGE Coomassie Brilliant Blue staining. M: Marker, C: Control (fresh-milked and separated immediately), D7: 7 separated after 7 days storage, D9: separated after 9 days storage, D14: separated after 14 days storage. The black arrow shows a new band appears only at 14 days stored venom solution

Kangean population *of C. rhodostoma* (BPP = 0.5) (**Fig. 1**).

Effect of Storage Condition on *C. rhodostoma* svPLA$_2$

Generally, the proteins inside *C. rhodostoma* crude venom solution are the proteins with a molecular weight ≤60 kDa. The electrophoretic result of the fresh and stored venom solution tends to be similar with a few different details. Some proteins with molecular weight 50-60 kDa in the stored sample show a thicker band compared to the control group. Also, the protein band in ~20–30 kDa appears only in the 14 days stored venom solutions (**Fig. 2**). The svPLA$_2$ of *C. rhodostoma* possibly presence inside the venom solutions. This can be observed from the 13–16 kDa proteins in both fresh and stored venom solutions. Observation of separation results shows that there is no significant change in 13–16 kDa proteins between the control group and other experimental groups, except that the bands seem less intense after 14 days of storage. However, the 13–16 kDa protein range does not refer to svPLA$_2$ only.

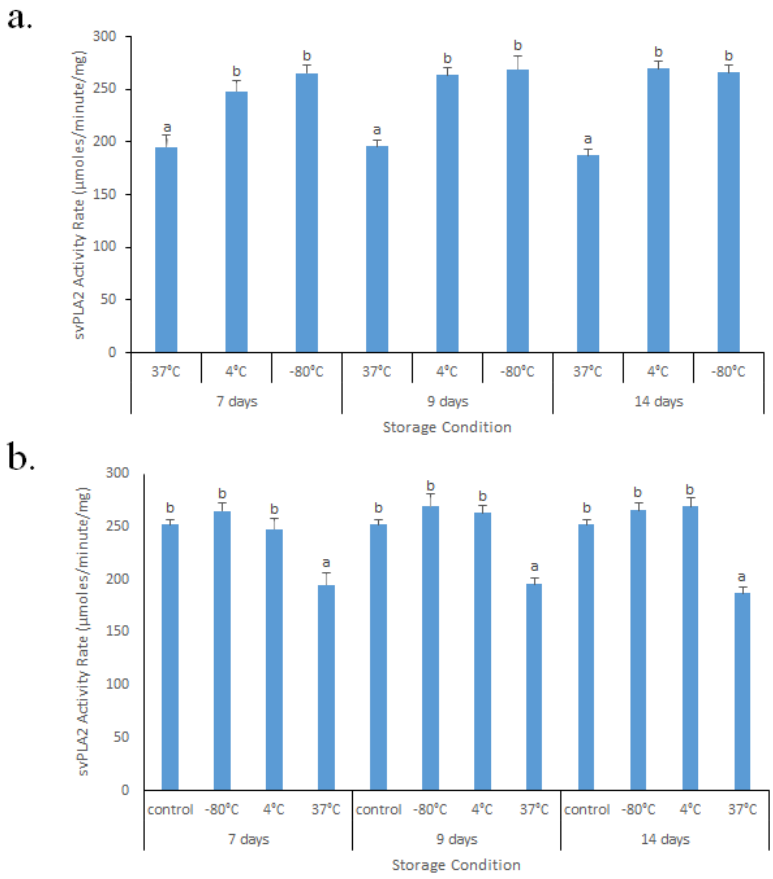

Fig. 3. *Calloselasma rhodostoma* svPLA$_2$ activity after various storage conditions, a) effect of storage temperature at the svPLA$_2$ activity rate, b) difference in svPLA$_2$ activity rate between storage and control (non-storage) condition, assayed based on the storage duration grouping. Letters a, b and c define notation by statistical analysis.

Our further examination of *C. rhodostoma* svPLA$_2$ through acidimetric assay shows that the storage temperature affects its performance significantly, thus, we examine the effect of storage temperature for each storage duration group (7, 9, and 14 days) (**Fig. 3a**). The *C. rhodostoma* svPLA2 activity of the venom stored for 7 days long at 37°C, 4°C and -80°C are 195.06 µmol minutes^{-1} mg^{-1}, 247.89 µmol minutes^{-1} mg^{-1}, and 265.11 µmol minutes^{-1} mg^{-1}, respectively; for 9 days long at 37°C, 4°C and -80°C are 195.95 µmol minutes^{-1} mg^{-1}, 263.34 µmol minutes^{-1} mg^{-1}, and 26.19 µmol minutes^{-1} mg^{-1}, respectively; for 14 days long at 37°C, 4°C and -80°C are 186.98 µmol minutes-^{1} mg^{-1}, 269.54 µmol minutes^{-1} mg^{-1} , and 266.00 µmol minutes^{-1} mg^{-1}. Here we can observe that the storage temperature at 37°C affects the *C. rhodostoma* svPLA$_2$ activity. This occurs in three different storage durations. Compared to the control (fresh-milked) group, the storage of venom solution at 37°C affects on the decreasing svPLA$_2$ activity. Meanwhile, storage at 4°C and -80°C does not give a similar effect on the svPLA$_2$ activity. There is no significant change in the svPLA$_2$ activity of the venom which has been stored at 4°C and -80°C compared to the control group. This condition also lasts for 14 days long (**Fig. 3b**).

DISCUSSION

The polytomy phylogenetic tree and the low p-distance value show a little difference in DNA sequences. This indicates that the samples used are categorized as one same species (Wostl et al. 2016). Allopatric speciation possibly happened in this species when Sundaland was separated in Paleocene age (60 million years ago). The previous study of Sumatran pit vipers, *Trimeresurus (Popeia) toba*, showed little differences in both morphology and genetic characters (p-distance) towards *Trimersurus (P.) barati*.Thus, *T. (P.) toba* and *T. (P.) barati* are concluded as one same species (Wostl et al. 2016). We show evidence that *C. rhodostoma* from various localities in Indonesia is grouped into one species, even though geographical barriers have existed. Our previous study about morphological character variation of *C. rhodostoma* from the various locality, thus, does not result similar to the molecular data. *C. rhodostoma* from various localities

may vary in morphological characters but remain in one same species due to the low variation in molecular data.

The absence and appearance of some protein bands in a venom solution sample has also been observed in a previous study (Munekiyo and Stephen 1998). The study pointed out that several high molecular weight bands in 37°C stored samples were found to have low intensity. Besides, a protein band with lower molecular weight was appearing. This may suggest autolysis in the venom solution under the applied storage condition. We find that a protein band with approximately 63 kDa in every stored sample appears with lower intensity compared to the control group, however, the protein band with approximately 52 kDa is more intense. We also find ~20-30 kDa protein band is appearing after 14 days storage, at -80°C, 4°C, and 37°C. The 13–16 kDa proteins, where svPLA$_{2}$' are estimated to be there, change are not observed in all experimental groups until 9 days of storage. In contrast, the 13–16 kDa bands are seemed to be less intense after 14 days of storage (**Fig. 2**). Several degradations possibly happen at the proteins after more than 9 days of storage.

The activity of svPLA$_2$ observed in this study show that 4°C and -80°C does not give a significant effect on the svPLA$_2$ activity. This also occurs in the 14 days stored samples, which have less intensity in protein bands compared to other groups (**Fig. 3a** and **3b**). If the degradation occurred on 13-16 kDa bands is related to the svPLA$_2$, it possibly happens in the non-functional residues of svPLA$_2$. Protein degradation is not accompanied by a decrease in toxicity also found in the previous study. The neurotoxicity of *Acanthophis praelongus* venom which has been stored since 1960 is found in a good condition when compared to the fresh venom, although some degradations are detected in the stored venom. Thus, the degradations are possibly restricted to the non-functional residues (Jesupret et al. 2014). The stability of venom components under various storage conditions is stated in previous studies. The SDS-PAGE patterns for crotalid venom are similar, since 0 days (fresh-milked) to 14 days storage under lyophilized, iced, evaporated, or desiccated condition. Additionally, the polyacrylamide electrophoretic profile and the svPLA$_2$ activity of crotalid venom are found stable during 7 days of storage under various conditions (Egen and Russel 1984, Munekiyo and Stephen 1998). The stability of svPLA$_2$ after several storage conditions is possibly an effect of svPLA$_2$ endogenous inhibitor inside the venom. A freezing cycle can also reduce the degradations process of the venom protein until several weeks or months (Francis et al. 1992, Carpenter et al. 2002)

Storage condition at 37°C affects the decreasing of svPLA$_2$ activity since the first 7 days of storage. The result of a similar study using the venom of *Crotalus molossus molossus* is not in accordance with our study. The activity of caseinolytic protease, thrombin-like protease, kallikrein-like protease, and svPLA$_2$ remain stable under 7 days of storage at 37°C (Munekiyo and Stephen 1998). However, in other studies, the stability of svPLA$_2$ at various temperature is concluded when the svPLA$_2$ are tested after incubation at various temperatures (included 37°C) for about 30 min (Shashidharamurthy and Kemparaju 2006, Avila et al. 2004). Thus, the stability of svPLA$_2$ at 37°C was not determined after longer storage at 37°C. In this study we observe the presence of precipitation in the 37°C stored venom solution, which is not observed in other sample groups. The presence of precipitation evidences a change inside a protein solution, which is initially started by aggregate formation from the protein molecules. Many factors can lead to aggregate formation: water and air exposure, change in pH, freeze-thawing cycles, and high-temperature exposure (Carpenter et al. 2002).

Snakebite envenomation does not always relate to a bacterial infection. In Central Taiwan (2005 – 2007), twenty-eight percent of snakebite victims undergo a secondary bacterial infection. *Morganella morganii* is found to be the most bacterial species which shows growth in the wound cultures (Huang et al. 2012). In other studies, seventy-five percent of snakebite victims who experience fasciitis are observed to have polymicrobial or monomicrobial infections. *Enterococcus faecalis* and *Morganella morganii* are two common bacteria found in snakebite victims' blood or wound in Taiwan. (Tsai et al. 2017). Recently a study of bacterial growth in the samples of venomous snake oral swab and venom solution pointed out that the venom gland is possibly a different ecological niche compared to the oral cavity. The bacterial in venom samples grow better compared to the bacterial from the oral cavity. Two new strains for *Enterococcus faecalis* are found in the study (Esmaeilishirazifard et al. 2018). *Morganella morganii* and *Enterococcus faecalis* can be found in the intestinal tract of humans and other warm-blooded animals. This is associated with the storage temperature we performed for the venom solution which undergoes a decrease in svPLA$_2$ activity (Lee et al. 2009, Dubin and Eric 2018), thus, bacterial growth is possibly another factor that leads to the decrease on svPLA$_2$ activity. This has to be investigated further to confirm the relation between svPLA$_2$ activity and the presence of bacteria.

The polytomy topologies and the low p-distance value from ND4 gene analysis indicate that *C. rhodostoma* populations in Indonesia and Thailand are grouped into one same species, regardless of the geographical barrier among the population and the differences in morphological characters. The storage condition of the C. rhodostoma venom at 4°C and -80°C do not affect the svPLA$_2$ activity. Contrastly, the storage at 37°C decrease significantly the svPLA$_2$ activity compared to the initial activity. Thus, the storage condition of *C. rhodostoma* venom better to be performed at 4°C and -80°C for up to 14 days long. More

samples are needed to compare the venom properties throughout *C. rhodostoma* wide distribution, to enlight the anti-venin future development.

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