Conference Paper

The Evolution Study Of 6-Cysteine Family Member Protein of *Plasmodium sp.* As a Potential Drug Candidate Against Malaria Infection

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Abstract

The increase of current antimalarial drug resistance was reported and lead to the greatest threats to malaria control; therefore, new methods should be applied to encounter this problem. Although the protein evolution study of Plasmodium may contain valuable information in finding a new antimalarial drug candidate, the cross-species antimalaria drug cannot be made because there is no sufficient information regarding the protein evolution between human-infecting and non-infectious *Plasmodium*. In this study, data mining from PlasmoDB discovers several proteins shared by Plasmodium where some of them include in a 6-Cysteine protein family. Previous studies revealed that 3 of 6-Cysteine family members (P41, P48/45, and P230) could be used as a vaccine candidate. From this information, the evolution properties and the characteristics of these proteins were further analyzed. Protein sequences of 6-Cysteine protein family members were retrieved from plasmoDB and the GenBank. Maximum likelihood phylogenetic tree and time trees were then constructed by using MEGAX, protein domain analysis was done by using InterPro, and all tertiary structures of these proteins were predicted by using PHYRE2. Phylogenetic tree and time tree analysis showed that the human-infecting and the non-infectious Plasmodium have a different cluster and evolutionary rates. Furthermore, several domains that can be used vaccine targets were found in P41, P48/45, and P230, such as transmembrane, signal peptide, and a coiled-coil domain. Tertiary structure prediction also revealed a different characteristic of these proteins. Thus, our findings provide valuable information to support the development of the cross-species antimalaria vaccine using 6-Cysteine protein family members.

Keywords: 6-Cysteine, drug target, protein domain, protein evolution, tertiary structure, *Plasmodium*

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1. Introduction

Malaria is one of the endemic diseases that occur in developing countries, including Indonesia. According to the world malaria report 2018, there are 219 million cases of malaria with 435 000 deaths worldwide [1]. On the other hand, the Indonesian

government estimated about 10 to 12 million people had suffered malaria, and 30 000 of them died each year [2]. From 34 province of Indonesia, Papua has the highest prevalence of malaria (12,07%) indicated that malaria infection still become one of the greatest threats towards human health in Indonesia [2].

A parasite called *plasmodium sp.* is responsible for the cause of malaria throughout many years. *Plasmodium spp.* have a complex life cycle alternating between female *Anopheles* mosquitoes and vertebrate hosts, which require the formation of unique zoite forms to invade different cell types [3]. When sporozoites enter the host, they will infect hepatocytes, followed by the asexual cycle in the blood [3]. Furthermore, sexual forms that develop during the blood-stage are ingested by a feeding mosquito and completing the cycle. Currently, there are five plasmodial species consider as health threats for humans, which are: P. *falciparum, vivax, ovale, knowlesi,* and *malariae* [3].

Artemisinin Combination Treatment (ACT) is one of the treatments for malaria that are commonly used in Indonesia [2]. The ACT is a combination of antimalaria drug artemisinin group with other antimalaria groups. Artemisinin act as a short-acting drug for rapid parasitemia reduction while the second antimalaria group serves as a longer-acting partner, which eliminates parasite that survives artemisinin [4]. Currently, there are three types of ACT that available in Indonesia, such as (i) Dihydroartemisinin+Piperaquine; (ii) Artesunate+Amodiaquine; (iii) and Artemether + Lumefantrine [2]. The duration of the ACT for each malaria patient depends on the cause of the malaria infection experienced by the patient [2]. However, the emergence and spread of ACT resistance lead to the most significant threats in malaria control [5].

Previous studies were demonstrated ACT resistance developed by plasmodial species. In 2018, Bakhiet et al. observed the evolution of known drug resistance markers in P*lasmodium falciparum* that lead to ACT failure [6]. In this study, Bakhiet et al. genotyped known drug resistance markers (Pfcrt, Pfmdr-1, Pfdhfr, Pfdhps, Pfk13 propeller) and their flanking microsatellite among *Plasmodium falciparum* which obtained between 2009 to 2016 in different regions in Sudan and data were compared with published findings pre-ACT [6]. The result showed a high prevalence of Pfcrt76T, Pfmdr-1-86Y, Pfdhfr51I, Pfdhfr108N, Pfdhps37G in all regions of Sudan, which indicate drug resistance genes in Sudan correlate with the drug deployment pattern [6]. Thus, a new method of treatment should be considered to encounter the spread of ACT resistance.

In this study, several proteins shared by *plasmodium* were found by data mining from PlasmoDB where some of them include in 6-Cysteine (6-Cys) protein family which expressed in different stages throughout the parasitic cycle of *plasmodium* [7]. Previous studies revealed that 3 of the 6-Cys family member could be used as vaccine target

candidate, which are: (i) P41 which has a signal sequence and located on the surface of the merozoite; (ii) P48/45 which involved in male or female gamete fusion in the mosquito midgut; (iii) and P230 that also associated with the gamete membrane by binding to P48/45 [7], [8].

Previous evolution study of each of these proteins contain valuable information regarding the characteristic of 6-Cys family member protein and support the development of malaria vaccine. The first study of polymorphism and natural selection of P41 was done by Ahmed et al. where they discover a low level of polymorphism in both s48/45 domains in P41 from P. *falciparum*, *vivax*, and *knowlesi* [9]. In 2018, Srisutham et al. also showed that P48/45 in P. *malariae* has low average pairwise nucleotide diversity and haplotype diversity compared to thrombospondin-related anonymous protein (TRAP) and apical membrane antigen 1 (AMA1) [10]. Another research conducted by MacDonald et al. discovers that recombinant P230 domain 1 in P. *falciparum* has a similar secondary structure after heating to denaturation levels and cooling which indicates P230 has suitable thermal stability for vaccine component [11]. All of these studies have revealed the characteristic of P41, P48/45, and P230 in P*lasmodium sp.* could be used as a vaccine target candidate in the future. However, the cross-species antimalaria vaccine cannot be made because there is no sufficient information regarding the protein evolution between human-infecting and non-infectious *Plasmodium*.

Vaccines are the most reliable alternative method for malaria control but their development has been limited by strain specificity in previously studied antigens which makes the proposed vaccine is ineffective against all five human-infecting *Plasmodium*. Furthermore, the specific characteristic of these studied antigens in human infecting *Plasmodium* has remained unknown. It is important to elucidate the differences between human infecting and non-human infecting *Plasmodium* of studied antigens to determine which antigens that effectively elicit immunity against malaria. Since the 6-Cys protein family members presence in all *Plasmodium* species, then the cross-species antimalaria vaccine could be made by using P41, P48/45, and P230 [7]. The objective of this study is to provide valuable information regarding the evolutionary relationship of P41, P48/45, and P230 by implementing the phylogenetic analysis. Furthermore, the characteristic differences of these proteins in each plasmodium were observed in 2D and 3D protein structure. Taken together, these results provide valuable information as well as support the development of the malaria vaccine using 6-Cys family member protein.

2. Methods

Eight protein sequences of each P41 and P230 were retrieved from PlasmoDB (https://plasmodb.org/plasmo/) while another eight protein sequences of P48/45 were taken from the GenBank (https://www.ncbi.nlm.nih.gov); detail is shown in table 1 [12]. From eight plasmodium, five of them could infect humans (*P. falciparum, P. vivax*, *P. knowlesi, P. ovale,* and *P. malariae*) and the other (*P. berghei, P. chabaudi,* and *P. reichenowi*) could not infect human but could infect other species. Multiple sequence alignment was done by using the MUSCLE algorithm [13]. Finding the best maximum likelihood model for each protein sample, phylogenetic tree analysis, and time tree analysis was conducted with MEGAX software [14]. All phylogenetic tree was constructed by using Jones, Taylor, and Thornton with gamma distribution and frequencies model (JTT+G+F) [15]. One thousand replication bootstraps were performed to test each of the tree robustness. The time tree was constructed by following Mello's protocol with the default parameter from MEGAX software [16]. Time tree calibration was retrieved from the time tree database (http://timetree.org/) [17]. Domain analysis and 3D structure prediction were done by using InterPro (https://www.ebi.ac.uk/interpro/beta/) and PHYRE2 fold recognition server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) [18], [19]. Each of the protein structures was visualized by using PyMol 2.3 [20].

Table 1: Protein sequence detail information.

P41		P48/45		P230	
Gene ID	**Species**	**Accession Number**	**Species**	**Gene ID**	**Species**
PF3D7_0404900	*P. falciparum* 3D7	SBS87718	*P. malariae*	PBANKA_0306100	*P. berghei* ANKA
PKNH_0303000	*P. knowlesi strain* H	SCM16869	*P. berghei*	PF3D7_0209000	*P. falciparum* 3D7
PVP01_0304300	*P. vivax* P01	SBS96099	*P. ovale curtisi*	PKNH_0412100	*P. knowlesi* strain H
PBANKA_1002600	*P. berghei* ANKA (putative)	SBT40052	*P. ovale wallikeri*	PmUG01_04023100	*P. malariae* UG01 (putative)
PmUG01_03015000	*P. malariae* UG01 (putative)	OTN66276	*P. knowlesi*	PocGH01_04021000	*P. ovale curtisi* GH01 (putative)
PocGH01_03012400	*P. ovale curtisi* GH01 (putative)	SCM18667	*P. berghei*	PVP01_0415800	*P. vivax* P01 (putative)
PCHAS_1003500	*P. chabaudi* (putative)	SCM13221	*P. chabaudi adami*	PCHAS_0308300	*P. chabaudi*
PRDC_0402500	*P. reichenowi* (putative)	CDO66251	*P.reichenowi*	PRCDC_0207900	*P. reichenowi*

3. Results and Discussions

3.1. P41

The phylogenetic analysis of P41 protein showed two different clades between human infecting and non-human infecting *plasmodium* (Figure 1). As shown in the phylogenetic tree (Figure 1) and time tree analysis (Figure 2), P41 protein of *P. vivax* and *P. knowlesi* have a close relationship with each other. This result is supported by the fact that both of these parasites is originated from the malarial parasite of non-human primates [21]. In 2007, Cornejo and Esalante proved this theory by analyzing the phylogeny of complete mitochondrial genomes data of *P. vivax* and non-human primates in Southeast Asia [21].

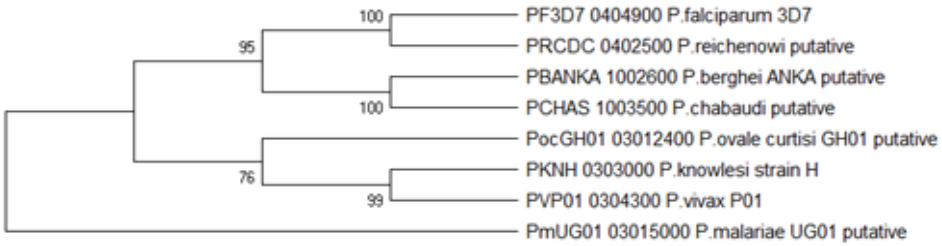

Figure 1: Phylogenetic analysis of P41 constructed by using the JTT+G+F model with 1000 bootstrap.

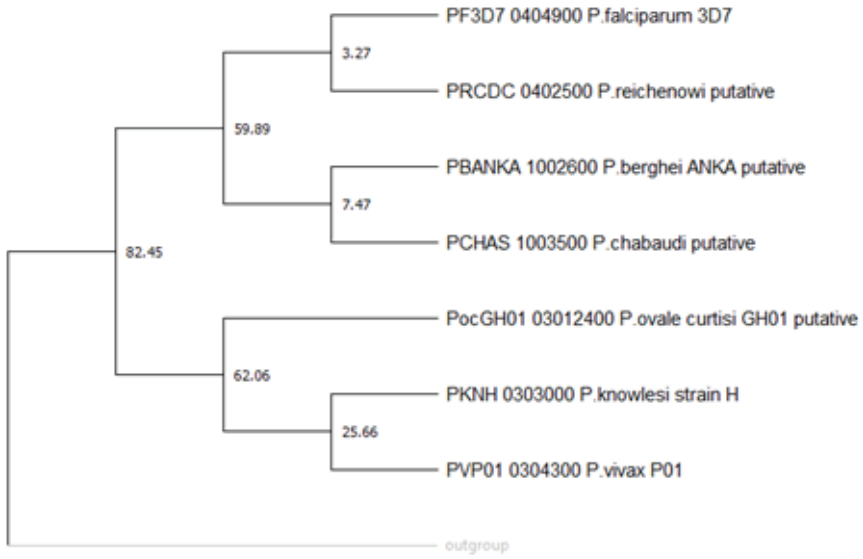

Figure 2: Time tree analysis of P41.

Another impressive result found in non-human infecting *Plasmodium* clade, where *P. falciparum* that initially known as human infecting *Plasmodium* was included in non-human infecting *Plasmodium* clade (Figure 1). Furthermore, *P. falciparum* has a close relationship with *P. reichenowi*, which only affects *chimpanzee*; as shown in the time

tree (Figure 2). This result may indicate that the P41 protein of *P. falciparum* has similar characteristics with non-human infecting *Plasmodium*. The previous study conducted by Prugnolle et al. found the same result by exploring the diversity of *Plasmodium* species infecting monkeys in Central Africa [22]. Their findings suggest that *P. falciparum* can switch host between non-human primates and humans [22].

From eight *Plasmodium* species, *P. malariae* has the farthest relationship (Figure 2) and does not include in either non-human infecting and human infecting *Plasmodium* clade (Figure 1). This may show that P41 protein from *P. malariae* has different characteristics compared to other human infecting *Plasmodium*. Therefore, MEGAX software was recognized *P. malariae* as the outgroup among P41 protein sequences [14].

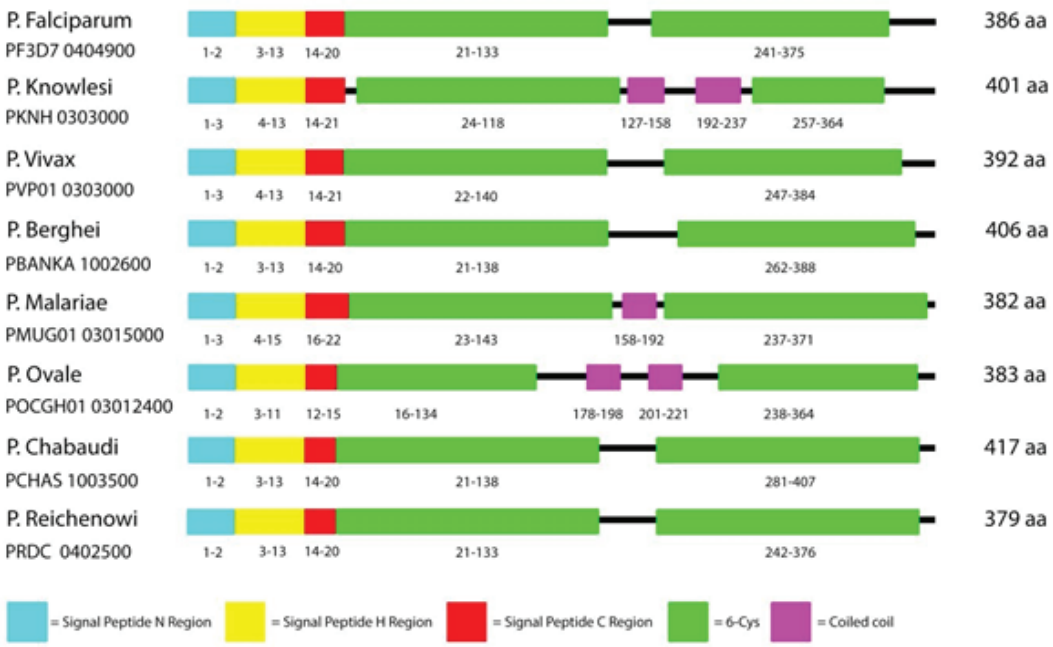

Figure 3: Domain prediction of P41protein by using InterPro.

Domain analysis detects the presence of signal peptide and coiled-coil region that could be used as a vaccine target [23], [24]. Signal peptide region present in all plasmodial species while the coiled-coil region only present in *P. knowlesi, P. malariae, P. ovale*. However, *P. malariae* has only one coiled-coil region compared to *P. knowlesi* and *P. ovale* that have two coiled-coil regions (Figure 3), which indicate the unique characteristic of P41 protein from *P. malariae*. The location of the signal peptide region and the coiled-coil region was described in Figure 3 where signal peptide present at the beginning of P41 protein sequences and coiled-coil present between two 6-Cys regions. *P. knowlesi* has a unique domain distribution where a small gap presents between signal peptide c-region and 6-Cys regions compared to other *Plasmodium* species.

Signal peptide consists of three main parts, which are: n-region, h-region, and c-region, which responsible for targeting proteins to the endomembrane system [23]. Targeting protein with a signal peptide region as a drug or vaccine candidate might influence protein targeting to various extracellular or subcellular compartments in the Plasmodial species [23]. On the other hand, the coiled-coil region known to induce the immune response and block critical functions in multiple pathogens, including *plasmodium* [24].

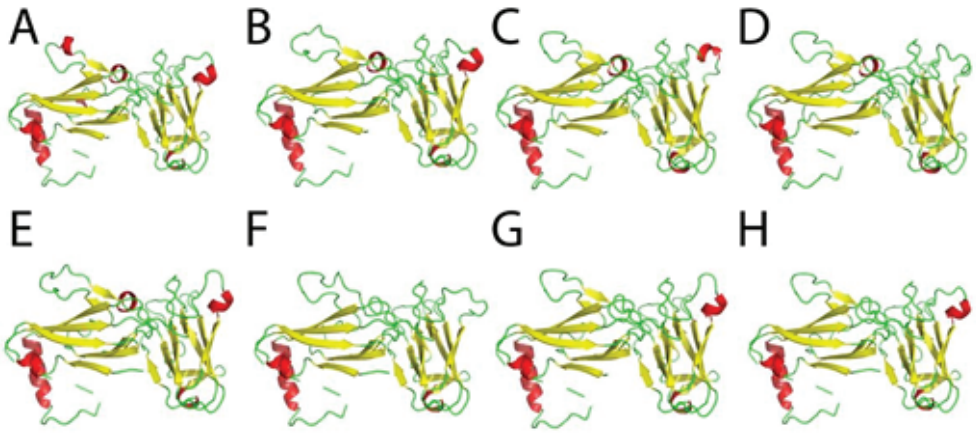

Figure 4: 3D protein structure of P41 protein predicted by using PHYRE2 and visualized by using PyMol version 2.3: (A) *P. berghei,* (B) *P. chabaudi* (C) *P. reichenowi*, (D) *P. falciparum*, (E) *P. vivax*, (F) *P. knowlesi*, (G) *P. ovale,* and (H) *P. malariae.*

In the 3D protein structure, several differences can be spotted between plasmodial species (Figure 4). For example, the present and the location of the small alpha-helix structure is different in each plasmodial species (Figure 4). However, there is no significant difference between human infecting, and non-human infecting *plasmodium* was spotted. Thus, P41 could be used as a cross-species vaccine for malaria infection.

3.2. P48/45

Phylogenetic analysis of P48/45 showed that *P. malariae* and *P. knowlesi* include in non-human infecting *Plasmodium* clade where *P. ovale* species stands as an outgroup. This result indicates P48/45 protein in *P. malariae* and *P. knowlesi* have a close relationship with non-human infecting *Plasmodium* (Figure 5). Furthermore, *P. malariae* and *P. knowlesi* have a homolog relationship with each other, which indicates the P48/45 in *P. malariae* and *P. knowlesi* have similar characteristics. These findings were supported by the fact that *P. malariae* and *P. knowlesi* are morphologically similar, which firstly mentioned by Coatney et al., back in 1971 [25].

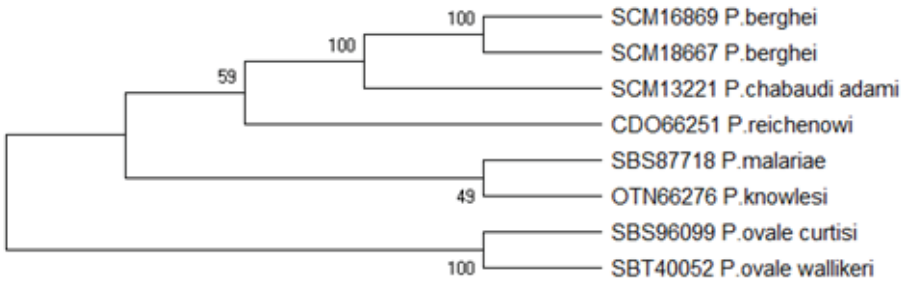

Figure 5: Phylogenetic analysis of P48/45 constructed by using the JTT+G+F model with 1000 bootstrap.

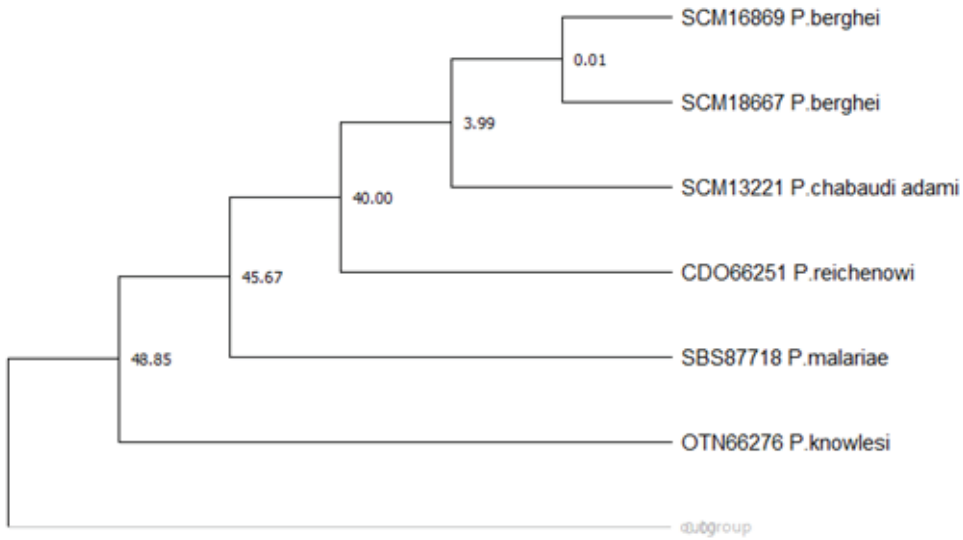

Figure 6: Time tree analysis of P48/45.

However, there is no homolog relationship between *Plasmodium* species found in the time tree analysis where human infecting *Plasmodium* have a distant relationship with non-human infecting *Plasmodium* (Figure 6). The result of the time tree analysis may suggest that each of the P48/45 protein in *Plasmodium* species have a unique characteristic. Time tree analysis also showed the farthest evolutionary rate of P48/45 protein from both *P. ovale* species compared to other *Plasmodium* species (Figure 6).

Compared to P41 domain distribution (Figure 3), the signal peptide region in P48/45 protein was only found in several *Plasmodium* species and with more extended size, such as *P. malariae, P. ovale curtisi, P. knowlesi, P. chabaudi* and, *P. reichenowi* (Figure 7). Furthermore, all of the P48/45 protein in all *Plasmodium* species contain a transmembrane region were mostly present at the end of P48/45 protein sequences which could be used as a vaccine target [26]. The different distribution of the transmembrane

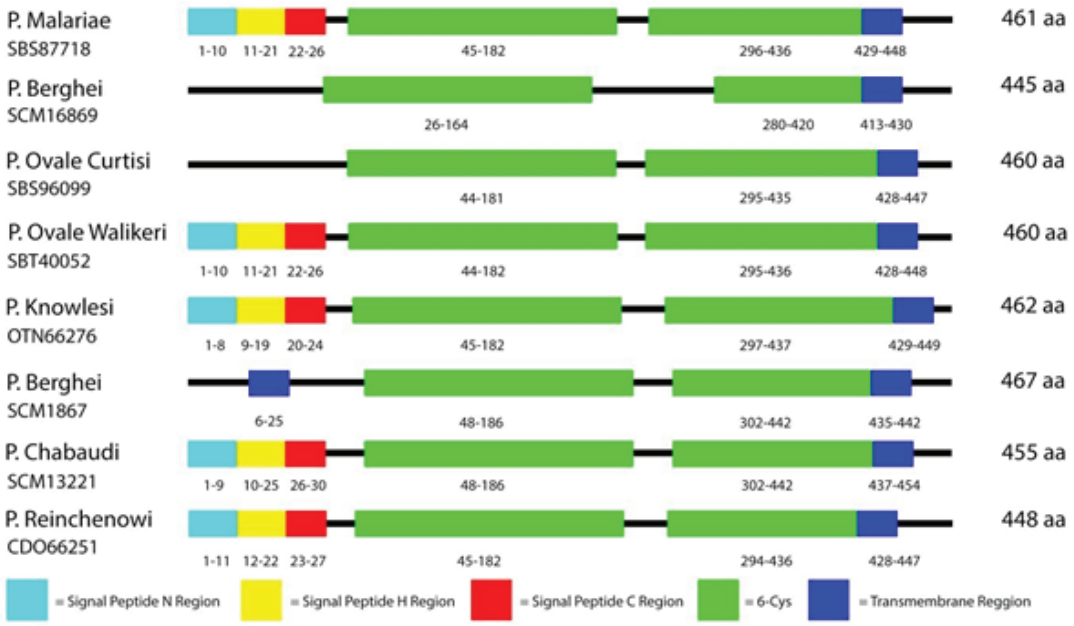

Figure 7: Domain prediction of P48/45 protein by using InterPro

region was observed in one of the P. *berghei* with accession number SCM 1687, where there are two transmembrane regions present in the sequences (Figure 7). Since the transmembrane region previously is known to have high CD8+ T cell epitope densities and broadly immunogenic, then targeting a transmembrane region in P48/45 protein in *Plasmodium* species can induce the maximal immune activation [26]. Therefore, these findings may suggest that P48/45 protein is suitable for a malaria vaccine.

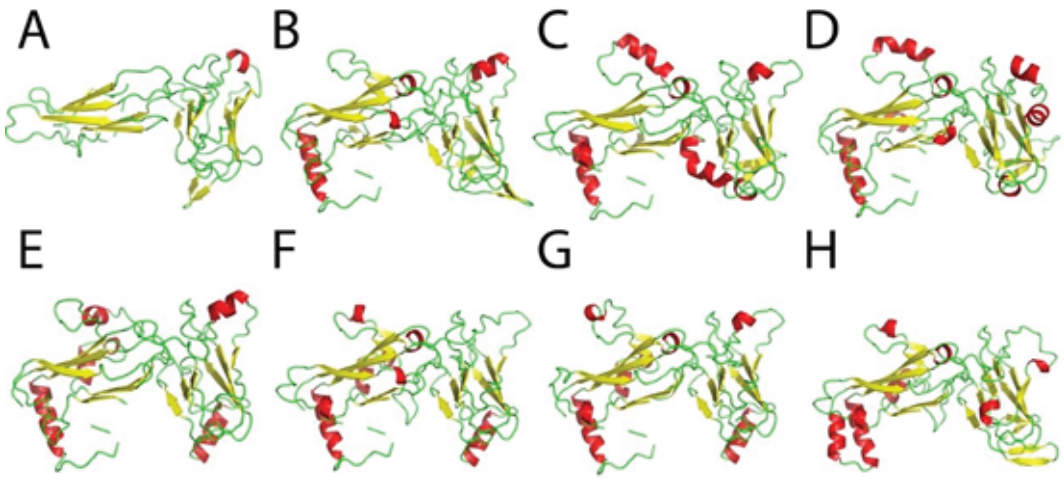

Figure 8: 3D protein structure of P48/45 protein predicted by using PHYRE2 and visualized by using PyMol version 2.3: (A) *P. berghei,* (B) *P. berghei*, (C) *P. chabaudi*, (D) *P. reichenowi*, (E) *P. knowlesi*, (F) *P. Ovale curtisi*, (G) *P. Ovale walikeri*, and (H) *P. malariae*.

Similar to P41 protein structure prediction (Figure 4), there are several differences observed in each *Plasmodium* species, such as the distribution of alpha-helix and beta-sheet (Figure 8). The unique protein structure was observed in *P. berghei* with accession number SCM 16869, where only one alpha-helix was found inside the protein structure (Figure 8A). However, there are no significant differences found between human infecting and non-human infecting *Plasmodium*. This result may indicate that P48/45 protein may be suitable for cross-species vaccine target similar to P41 protein.

3.3. P230

The phylogenetic analysis of P230 protein showed two different clades *Plasmodium* (Figure 9) where *P. knowlesi* and *P. vivax* were identified as the outgroup. *P. falciparum* and *P. reinchenowi* have a homolog relationship with a close evolutionary rate similar to P41 protein (Figure 9 and Figure 10). Not only that, *P. knowlesi* and *P. vivax* also have a homolog relationship with the distant evolutionary rate with other *Plasmodium* species (Figure 9 and Figure 10).

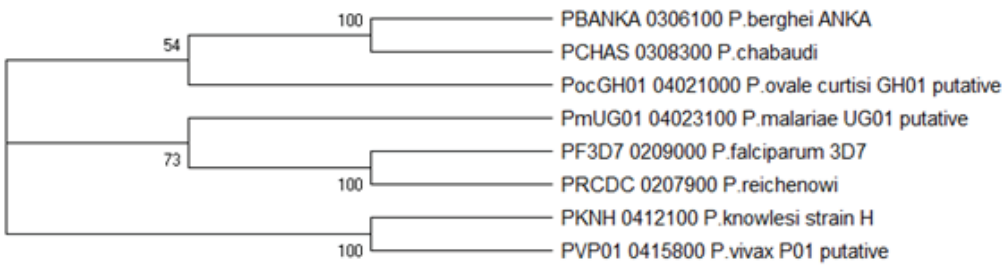

Figure 9: Phylogenetic analysis of P230 constructed by using the JTT+G+F model with 1000 bootstrap.

The unique relationship of *P. malariae* was observed in the time tree analysis where *P. malariae* stands alone in the time tree with the distant evolutionary rate as the outgroup (Figure 10). This finding may suggest there is a unique characteristic of P230 protein from *P. malariae* compared to other human infecting *Plasmodium*. The ortholog relationship was observed between *P. ovale* and rodent infecting *Plasmodium* (*P. berghei* and *P. chabaudi*).

The domain analysis showed that all *Plasmodium* species have a signal peptide region and some of them (*P. berghei, P. malariae,* and *P. vivax*) have a coiled-coil region. Both of these domains could be used as a vaccine target similar to P41 protein [23], [24]. However, the coiled-coil region that was detected in P230 protein is smaller compared to P41 protein. The bigger size of the coiled-coil region was only discovered

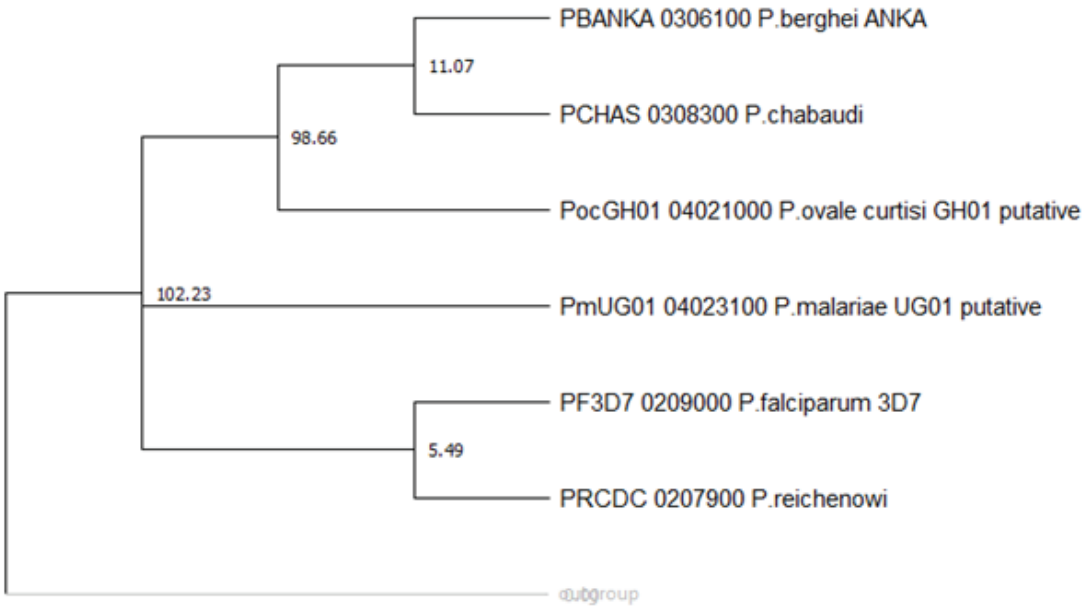

Figure 10: Time tree analysis of P230.

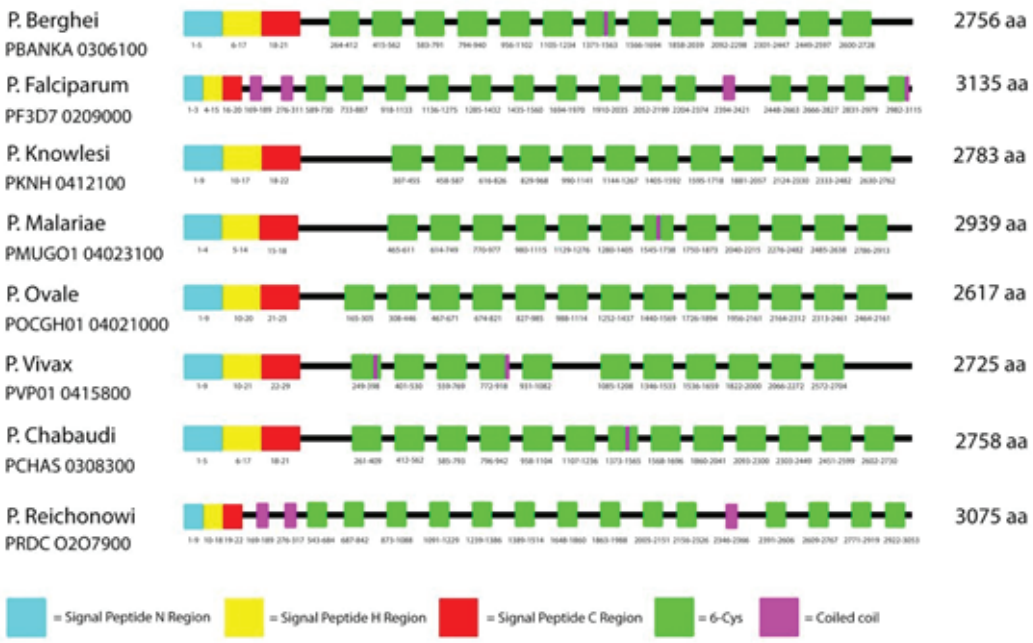

Figure 11: Domain prediction of P230 protein by using InterPro.

in *P. falciparum* and *P. reichenowi*. The distribution of coiled-coil regions in *P. falciparum* and *P. reichenowi* are similar where two coiled-coil regions are located after the signal peptide region and another one coiled-coil region present between 6-Cys region. Unlike P41 and P48/45 that have two 6-Cys domains, P230 has fourteen 6-Cys domains at most which mostly located after the signal peptide region.

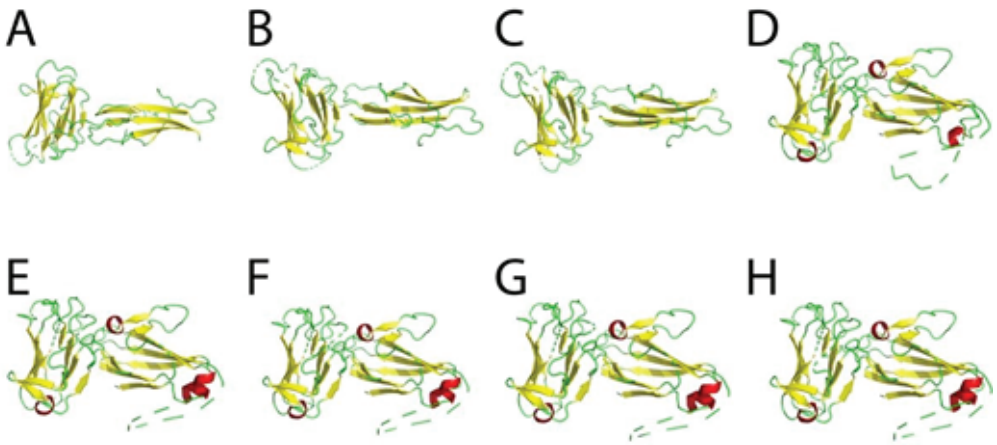

Figure 12: 3D protein structure of P230 protein predicted by using PHYRE2 and visualized by using PyMol version 2.3: (A) *P. berghei,* (B) *P. chabaudi*, (C) *P. reichenowi,* (D) *P. falciparum*, (E) *P. vivax,* (F) *P. knowlesi*, (G) *P. ovale,* and (H) *P. malariae*.

In 3D protein prediction, the significant differences in P230 protein structure were observed in human infecting and non-human infecting *Plasmodium*, where non-infectious *Plasmodium* does not have an alpha-helix structure compared to human infecting *Plasmodium*. The presence of alpha-helix structure in human-infecting *Plasmodium* may indicate that P230 protein in human infecting *Plasmodium* is more stable in a solution compared to non-human infecting *Plasmodium* [27].

4. Conclusion

Phylogenetic tree and time tree analysis revealed the unique evolutionary relationship in each of P41, P48/45, and P230 protein between human infecting and non-human infecting *Plasmodium*. Furthermore, the structural differences were observed in P230. Domain analysis also detected signal peptide, coiled-coil, and transmembrane region that could be used as a vaccine target. Thus, these results may support the development of a malaria vaccine using P41, P48/45, and P230 and may be useful for malaria control.

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Conflict of Interest

The authors declare no conflict of interest in this research.

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