Conference Paper

Investigating the Characteristics and Evolution of Apical Membrane Antigen 1 (AMA1) of *Plasmodium sp.* using Phylogenetic Approach in Searching for Drug Candidate

Rizky Nurdiansyah[1], Kevin Nathanael Ramanto[1], and Priscilla Jessica[2]

[1]Department of Bioinformatics, School of Life Sciences, Indonesia International Institute for Life Sciences, Jakarta, Indonesia
[2]Department of Biotechnology, School of Life Sciences, Indonesia International Institute for Life Sciences, Jakarta, Indonesia

Corresponding Author:
Rizky Nurdiansyah
rizky.nurdiansyah@i3l.ac.id

Abstract

Malaria is an endemic disease caused by *Plasmodium* parasite with female Anopheles mosquitoes as a vector. With the increase of drug resistance *Plasmodium* emerging around the world, a new method should be devised against the spread of *Plasmodium sp* lineage. Protein evolution of *Plasmodium sp.* can be an invaluable aid as an input for rational drug design and immune-informatics methods based on the novel virulent gene that exists in these protozoa. Previously, we did data mining in the PlasmoDB database and found several proteins shared by *Plasmodium*, one of them was the Apical Membrane Antigen 1 (AMA1). This protein was further analyzed for its characteristics and evolutionary properties. AMA1 protein sequences from human-infecting *Plasmodium* (*P. falciparum, vivax, knowlesi, ovale,* and *malariae*) and non-infectious (*P. berghei, coatneyi,* and *cynomolgi*) were retrieved from PlasmoDB. Maximum likelihood phylogenetic tree with molecular clock analysis was reconstructed from AMA1 sequences using MEGAX. Protein domain analysis was done using the INTERPRO server. Several domains that can induce protective immunity and vaccine target were found in AMA1 of those *plasmodium*, such as coiled-coil, disordered region, and signal peptide domain. Furthermore, molecular clock analysis showed a similar evolutionary rate between AMA1 protein in human-infecting and non-infectious *Plasmodium*. Interestingly, the phylogenetic tree showed a mixed cluster of human-infecting with non-infectious, indicating a unique evolutionary relationship between *Plasmodium* lineage. Thus, this information could be beneficial in developing the drug and vaccine for *Plasmodium*-related infection.

Keywords: AMA1, drug target, protein domain, protein evolution, *Plasmodium*.

1. Introduction

Malaria is a vector-borne disease caused by *Plasmodium* parasites that are commonly found in developing countries [1]. Even though the efforts to eradicate malaria and its

spread are numerous, starting from net, insect repellent, prophylaxis, early diagnostics, and combination therapies, the case of malarial infection is still very high. World Health Organization (WHO) estimated 219 million malaria cases with 435 thousand of mortality cases in 2017 [2]. Drug resistance poses as one of the most urgent issues in malaria eradication. Artemisinin combined therapy (ACT), the recommended first line of treatment for malaria, has hit a roadblock of partial resistance in Western Cambodia region [3]. The partial resistance itself is marked by the delayed blood-stage parasite [4]. This event causes delayed parasite clearance, allowing a small amount of *Plasmodium* parasite to survive over the course of treatment. This little amount of survived parasite would trigger a recrudescence, interpreted as the condition in which artemisinin-based therapy fails to cure the patient with resistant parasite phenotype.

Nowadays, WHO recommends treatment for uncomplicated malaria with the following ACTs: *artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine-pyrimethamine, and dihydroartemisinin-piperaquine* [5]. Firstly, one ACT will be used at a time in a country. When a high failure rate (>10%) is recorded, then another ACT will be deployed in place of the failing ACT. Despite the utilization of such ACT system deployment, malaria is still highly prevalent in South East Asia region. One of the reasons being is all ACT used artemisinin or its derivatives, despite the rise of partial artemisinin resistance in Greater Mekong Subregion: Cambodia, Laos, Myanmar, Thailand, and Vietnam [4]. The resistance is known to have spread from Cambodia, as the first country to report ACT treatment failure, to surrounding countries [3], [4].

These findings indicate that the artemisinin resistance is persistent and does not develop at random readily. The rise on resistance against ACT might eventually lead to the development of complete artemisinin resistance parasite and persistent infection from insufficient treatment. For this reason, there is a need to further study another drug candidate. Evolutionary relationship of the *plasmodium* genome may shed a new perspective in looking for new target candidate.

Interestingly, several proteins were found to be shared among *plasmodium* species after data mining in PlasmoDB database (https://plasmodb.org/), one of them is apical membrane antigen 1 protein (AMA-1). The mature form of this protein is found in the merozoite surface and it is well-established that AMA-1 is one of the essential proteins for the malarial parasite to infect the red blood cells (RBC) [6], [7]. Several studies had developed substantial research regarding the potency of AMA-1 to be a vaccine candidate. Drew et al (2012) reported the antigenic diversity of *P. falciparum* AMA-1 which could give some challenges in designing the vaccine [8]. The structural approach had been established to identify the binding site of AMA-1 and vaccine design [9],

[10]. The polymorphic feature of AMA-1 leads to a challenge in the process of vaccine design [8], [11]. The evolutionary relationship of the *plasmodium* species has been reported using several molecular markers [12]--[14], but the AMA-1 evolutionary relationship among the *plasmodium* species is not yet elucidated.

The objective of this study is to provide valuable information regarding the evolutionary relationship of AMA-1 protein by implementing the phylogenetic analysis from various species of Plasmodium. Furthermore, the characteristic differences of these proteins in each *plasmodium* were observed in 2D and 3D protein structure. The results obtained provided valuable information that can support the development of a malaria vaccine using AMA-1 protein.

2. Methods

2.1. Sequence retrieval and Domain analysis

PlasmoDB database (https://plasmodb.org/) was mined for the data in AMA-1 protein sequences for 8 species of *plasmodium*. Five of those could infect humans (*P. falciparum, vivax, knowlesi, ovale,* and *malariae*), while the rest, which could not infect human but could only infect other species (*P. berghei, coatneyi,* and *cynomolgi*), served as the comparison for the human infecting ones. A total of 24 protein sequences were retrieved from the database (Table 1). The retrieved sequences were analyzed for its domain and structure using the Interpro server (https://www.ebi.ac.uk/interpro/beta/search/sequence/).

2.2. Phylogenetic reconstruction

All of the phylogenetic reconstruction was based on Hall's protocols and performed using MEGA X program [15], [16]. Multiple sequence alignment was performed using the MUSCLE algorithm built in the MEGA X [17]. After that, the best maximum likelihood model was estimated and then used to create the ML tree. Jones, Taylor, and Thornton with gamma distribution (JTT+G) model was used to reconstruct the phylogenetic tree. Tree robustness test was performed using 1000 replication bootstraps. Validation test was performed by comparing the ML tree with other trees with different algorithms: neigbour-joining and minimum evolution; which also reconstructed using MEGA X.

Table 1: Retrieved AMA-1 protein sequences from PlasmoDB.

Sequence code	Accesion Number	Sequence code	Accesion Number
P. berghei ANKA	PBANKA_0915000	*P.falciparum_IT*	PfIT_110038000
P.knowlesi_strain_H	PKNH_0931500	*P.falciparum_KE01*	PfKE01_110038000
P.knowlesi_strain_Malayan _Strain_Pk1_A	PKNOH_S120150200	*P.falciparum_KH01*	PfKH01_110037800
P._vivax_P01	PVP01_0934200	*P.falciparum_KH02*	PfKH02_110038700
P.vivax_Sal-1	PVX_092275	*P.falciparum_ML01*	PfML01_110038300
P.falciparum_7G8	Pf7G8_110037300	*P.falciparum_SD01*	PfSD01_110036100
P.falciparum_CD01	PfCD01_110038900	*P.falciparum_SN01*	PfSN01_110036600
P.falciparum_Dd2	PfDd2_110036700	*P.falciparum_TG01*	PfTG01_110037900
P.falciparum_GA01	PfGA01_110037700	*P._malariae_UG01*	PmUG01_09042600
P.falciparum_GB4	PfGB4_110040000	*P.ovale_curtisi_GH01*	PocGH01_09039800
P.falciparum_GN01	PfGN01_110038000	*P.coatneyi_Hackeri*	PCOAH_00026700
P.falciparum_HB3	PfHB3_110036900	*P.cynomolgi_strain_M*	PcyM_0938200

2.3. Timetree reconstruction

Timetree was reconstructed based on Mello's protocol with the reconstructed ML tree as the base [18]. The default parameter from time tree tools of MEGA X was used. The time tree calibration for several *Plasmodium* species was retrieved from http://timetree.org/ [19].

3. Results and Discussion
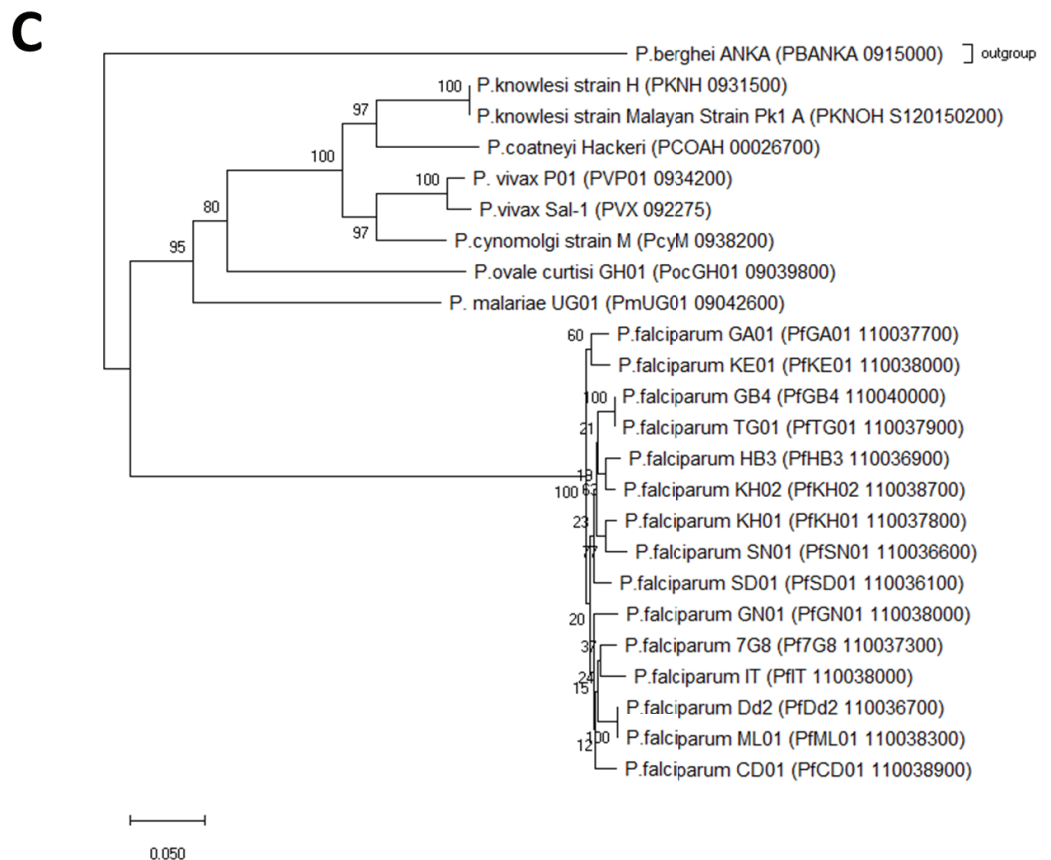
3.1. Phylogenetic tree reconstruction

The phylogenetic tree was reconstructed from 24 AMA-1 sequences, which came from various *plasmodium* species (Fig. 1). *P. berghei* was considered as the outgroup in the tree due to its closeness to the root judging from the tree topology. Surprisingly, the tree produced a similar topology using other algorithms, (Fig. 1b, 1c). This indicates the evolutionary hypothesis is similar within those algorithms. Several studies also reported the same topology as our tree although the molecular marker is different [12], [13]. The tight grouping, in terms of branch length, within the same species suggested that AMA-1 is conserved to a certain degree intraspecies. But an extensive study is needed to clarify the degree of AMA-1 protein sequence conservation.

The *P. falciparum* AMA-1 protein was clustered into one clade in all of the reconstructed trees, indicating the highly conserved sequence in *P. falciparum* AMA-1.

Although some reports stated the polymorphism of AMA-1 in this species is due to some evolutionary causes, the AMA-1 protein is still relatively conserved intraspecies [8], [11], [20]. Interestingly, the *P. falciparum* clade had a closer evolutionary relationship with *P. berghei*, which is not infectious to human (Fig. 1). *P. berghei* has been widely known to infect rodents and usually used as a proxy to study the malarial infection in rodents animal model [21], [22]. Several studies reported the positive results of the *P. berghei* ANKA expressed the gene from *P. falciparum,* like the p48⁄45 and PF83/AMA-1, indicating the similar expression system in both species [23], [24].

Another interesting result was observed in the clade of the *P. knowlesi* with *P. coatneyi* and *P. vivax* with *P. cynomolgi*. Both *P. coatneyi* and *P. cynomolgi* are infecting monkeys but the tree showed the close evolutionary relationship with human infecting *plasmodium*. Moreover, both clades shared the same ancestor with *P. malariae* and *P. ovale* (Fig. 1). These results suggest that the AMA-1 protein may not exclusively responsible for host species infection properties.

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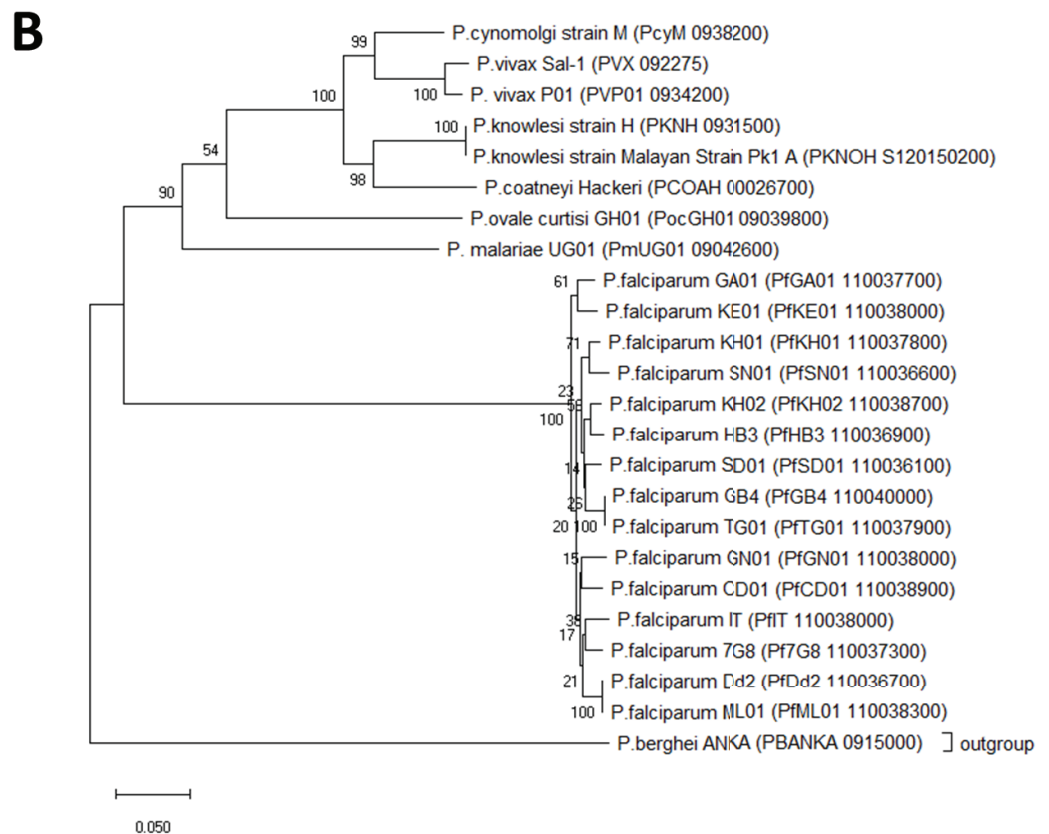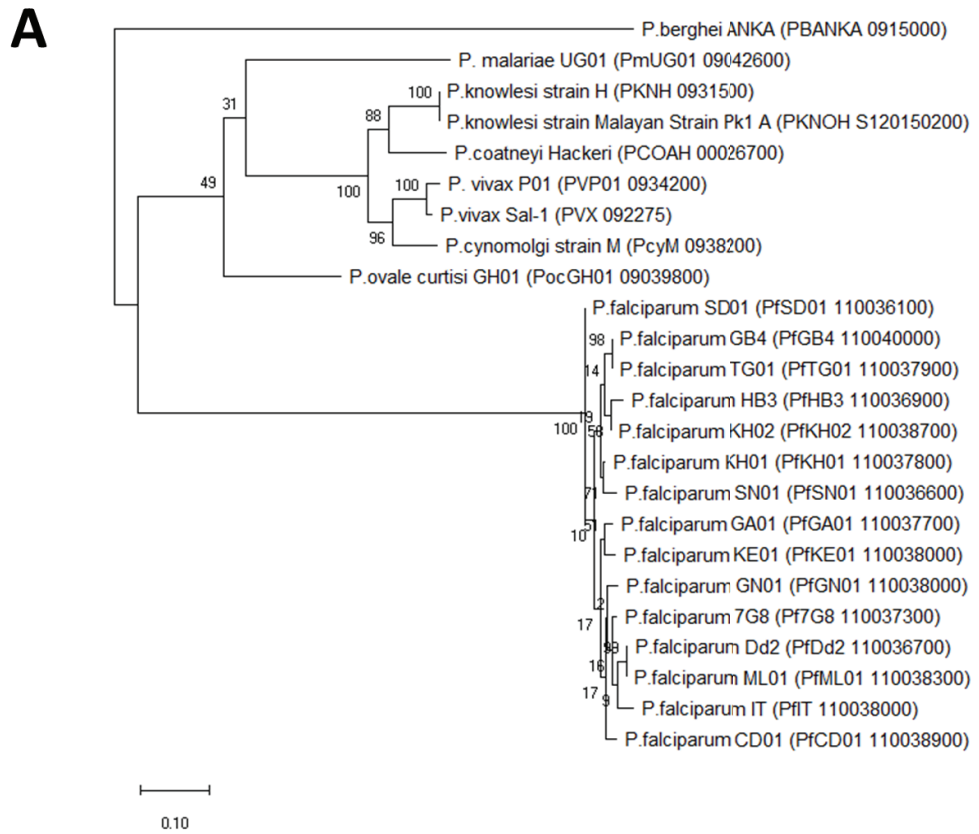
Figure 1: Reconstructed phylogenetic tree of AMA-1 protein from various *plasmodium* species. A. Maximum Likelihood tree with JTT + G model. B. Minimum evolution tree. C. Neighbor-joining tree. Every tree was reconstructed using 1000 bootstraps.

Both *P. coatneyi* and *P. cynomolgi* infect simians and show similar symptoms to the malarial infection to human. In details, *P. coatneyi* infection could induce severe anemia, coagulopathy, renal impairment, and metabolic dysfunction, similar to human cases of malaria [25]. An interesting study revealed that the genomes of *P. knowlesi, P. vivax, P. coatneyi* and *P. cynomolgi* were hypothesized coming from the similar ancestor and create the monkey clade in *plasmodium* phylogenetic tree [12], in accordance to our reported result in AMA-1 phylogenetic tree. The elucidation of the *P. cynomolgi* and *P. coatneyi* genomic sequences could serve a critical step in creating a model system to treat *P. vivax*, one of the major malaria agent outside Africa [12], [14]. It could also be true for AMA-1 protein vaccine design.

3.2. Time tree reconstruction

The time tree was reconstructed from the ML tree from the previous analysis. Based on the time tree, it was shown that some AMA-1 relative time divergence somehow supported the conserved hypothesis in the ML tree. Interspecies, the divergence is relatively narrow in the relative time divergence. The monkey clade of the *P. knowlesi* with *P. coatneyi* and *P. vivax* with *P. cynomolgi* was hypothesized to have the earliest divergence in 16.30 MYA. Our result is following the relative divergence of the genomics sequences [12]. Interestingly, the relative divergence time from *P. falciparum* with the monkey clades was shown to be a divergence in 77.85 MYA, indicating the AMA-1 protein sequence of *P. falciparum* is relatively different with the monkey clades and more closely related to the ancestor of AMA-1. Interestingly, *P. falciparum* and *P. vivax* AMA-1 structure have been reported to have similar structure even though their sequence is substantially different. Interestingly, the sequence could complement each other when combined, in terms of infecting host and avoiding the immune system, [26]. This report indicated the flexibility in retaining conserved structure in charge of infecting the host of AMA-1, which in turn could serve the ideal target for vaccine development.

A surprising result was observed in the placement of *P. berghei* AMA-1 in the time tree. Although previous trees showed that the *P. berghei* was on the outer clades and, hypothetically speaking, closer to the ancient AMA-1, the *P. berghei* was placed in the inner clades and closely related to *P. ovale* and diverged 25 MYA. This result is actually per another report using mitochondrial barcode sequence as the marker [13]. The report stated the *P. ovale* and *P. berghei* shared the same ancestry, while *P. falciparum* is the closest one to the outgroup.

3.3. Domain and structure analysis

To support the evolutionary relationship result, domain and structural analysis were done to elucidate the difference between the domain of AMA-1 of those *plasmodium* species. Due to the similarities of protein regions for each *plasmodium* strains within the same species, only the representative of each *Plasmodium* species were reported. Based on the result from InterPro, each of the protein belongs to the AMA-1 superfamily and has merozoitesa receptor proteins. Interesting result was found in the domain prediction of the AMA-1. Every AMA-1 protein had a similar pattern: starting from the signal peptide regions, followed by non-cytoplasmic domain, helix transmembrane region, and then the last part is the cytoplasmic domain of the protein. Surprisingly,
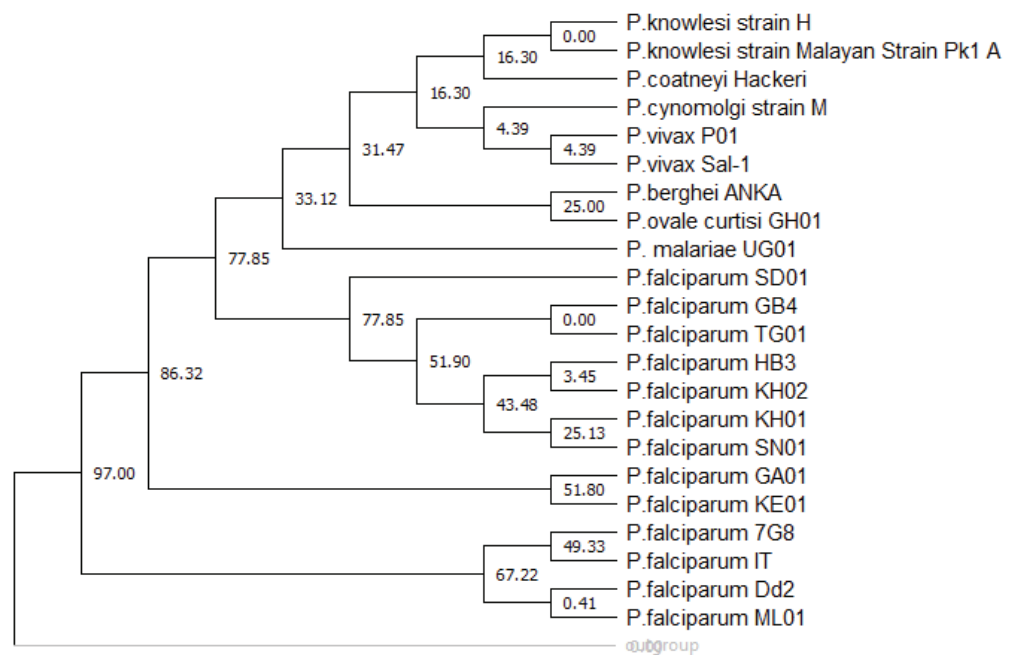
Figure 2: Time tree of AMA-1 protein divergence time. Numbers in each intersection indicates estimated divergence time in million years ago (MYA).

P. ovale and *P. vivax* were not predicted to have signal peptide regions, but it may be the prediction bias from the InterPro software (Fig. 3).

The previous report showed the flexibility in *P. falciparum* and *P. vivax* AMA-1 [26] yet the similar pattern in the regions of AMA-1 suggest some degree of structural conserveness in AMA-1 protein throughout the *Plasmodium* species. The similar pattern in the AMA-1 organization could serve as the ingredient for vaccine candidates. The signaling peptides have been studied as the candidate for a peptide-based vaccine to induce the immune system in targeting pathogen due to the similarity in structure and motifs [27].

Several unique patterns were observed as well, such as the coil region in the non-cytoplasmic region of *P. cynamolgi, P. knowlesi, P. ovale,* and *P. vivax*. Disordered regions were found in the cytoplasmic regions of *P. falciparum, P. vivax,* and *P. ovale*, which all of them are well-known for its human infecting properties. These results are very interesting when compared to the phylogenetic tree. The coil region was observed in the clade of *P. cynamolgi* and *P. vivax* but not in its sister clade member, *P. coatneyi*. The coil regions are mostly observed in the human-infecting *plasmodium*, except *P. falciparum*. The disordered region was also mostly found in human-infecting *plasmodium* except for *P. malariae* (Fig. 3).
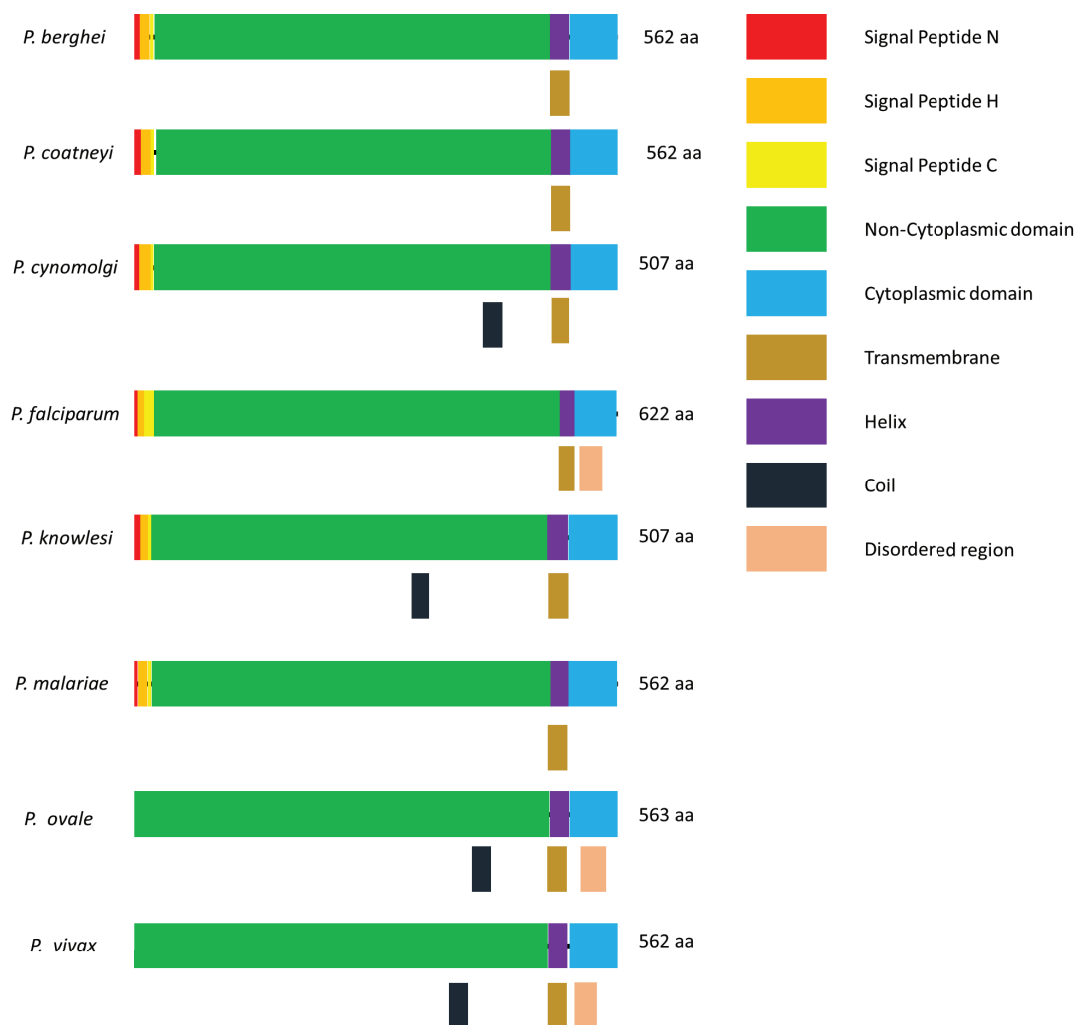
Figure 3: Interpro domain prediction analysis results from the representative sequences of each analyzed *Plasmodium* species.

These unique patterns could serve some pointers in the AMA-1 vaccine design. *P. vivax* and *P. cynomolgi* are clustered into one clade and both have the coil region in the non-cytoplasmic domain. The unique coil pattern in the protein organization may serve as the target for the same clade *plasmodium*. Several reports indicated the usage of coil regions in vaccine development in the *P. falciparum* [28], [29]. Surprisingly, the InterPro analysis didn't show any coil region in the *P. falciparum* but it had a disordered region in the cytoplasmic domain. The disordered region is the elusive part of the protein and it is well known for modulating the protein's function and enabling the protein to be involved in many cellular processes [30], [31]. The dynamic structure of this region is also contributing to its complexity [32] and even to the degree of oncogenicity [33]. The significance of the disordered region in protein is well understood which could serve as the target for drugs [30]. In addition to most of the human infecting *plasmodium* had a disordered region in their organization, this part could serve as another target. However,

due to the position of the region is in the cytoplasmic domain, some modification in the vaccine would be necessary to ensure the vaccine delivery.

4. Conclusion

This study elucidates some interesting aspects of AMA-1 protein characteristics and evolution in several *Plasmodium* species. The evolutionary relationship of AMA-1 is hypothesized to not following its infectious route to human, even though the protein itself is responsible for its infection to the host cell. The protein domain prediction found the coil region in the non-cytoplasmic region and the signal peptides and disordered region in the cytoplasmic domain could be a beneficial target for the vaccine in human-infecting *plasmodium*. However, substantial continuation studies are needed to elucidate the vaccine candidate potential for AMA-1. Nevertheless, this study could serve as the initial step for the fine-grained AMA-1 vaccine design for *plasmodium*.

Acknowledgments

Conflict of Interest

The authors declare no conflict of interest in this research.

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