

# Isolation and Characterization of Vaccine Candidate Genes Including *CSP* and *MSP1* in *Plasmodium yoelii*

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**Abstract:** Malaria is an infectious disease affecting humans, which is transmitted by the bite of *Anopheles* mosquitoes harboring sporozoites of parasitic protozoans belonging to the genus *Plasmodium*. Despite past achievements to control the protozoan disease, malaria still remains a significant health threat up to now. In this study, we cloned and characterized the full-unit *Plasmodium yoelii* genes encoding merozoite surface protein 1 (MSP1), circumsporozoite protein (CSP), and Duffy-binding protein (DBP), each of which can be applied for investigations to obtain potent protective vaccines in the rodent malaria model, due to their specific expression patterns during the parasite life cycle. Recombinant fragments corresponding to the middle and C-terminal regions of PyMSP1 and PyCSP, respectively, displayed strong reactivity against *P. yoelii*-infected mice sera. Specific native antigens invoking strong humoral immune response during the primary and secondary infections of *P. yoelii* were also abundantly detected in experimental ICR mice. The low or negligible parasitemia observed in the secondary infected mice was likely to result from the neutralizing action of the protective antibodies. Identification of these antigenic proteins might provide the necessary information and means to characterize additional vaccine candidate antigens, selected solely on their ability to produce the protective antibodies.

**Key words:** *Plasmodium yoelii*, MSP1, DBP, CSP, vaccine candidate, protective immunity, rodent malaria model

## INTRODUCTION

Malaria is an infectious disease affecting humans, which is transmitted by the bite of *Anopheles* mosquitoes harboring sporozoites of parasitic protozoans belonging to the genus *Plasmodium*. Five species, including *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*, infect humans in the respective endemic areas [1,2]. The parasitic disease remains a significant health threat with more than 3 billion people at risk of infection per year. However, substantial progress has been made in fighting malaria and thus, according to the latest estimates between 2000 and 2015, case incidence and mortality rate of the protozoan disease were reduced by 41% and 62%, respectively. During the period, the numbers of country, where malaria is endemic, was also decreased from 108 to 91 countries. In 2015, 212 million cases occurred globally leading to 429,000 deaths, most of which were children aged under 5

years in Africa [3].

Despite the obvious progress in antimalarial interventions, emergence and spread of drug-resistant parasites, as well as insecticide-resistant mosquitoes, are still existing risks that potentially threaten the recent gains in malaria control [4]. Approaches to develop molecules that effectively protect transmission of malaria from mosquito vector and/or generate defensive immune response in humans have been recognized as the greatest challenges in malaria elimination [5-7]. By virtues of these foresight activities, numerous *Plasmodium* proteins have been targeted for the development therapeutic vaccines by blocking any of the vector- or host-specific malarial stages. Some of the vaccine candidates have already entered diverse phases for clinical testing [3,7].

Of the 5 *Plasmodium* species infecting humans, *P. falciparum* is the most significant cause of malaria and thus has become the major concern in the relevant areas investigating molecular tools for malaria control. However, studies on human malaria are inherently restricted by difficulty either in using humans as an experimental system or in maintaining in vitro the complete life cycle of the protozoan parasites. Rodent malaria parasites, including *Plasmodium berghei*, *P. yoelii*, *P. chabaudi*, and *P. vinckei* can be a practicable choice to use as models of human

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malaria in the related experiments [8,9]. Small but significant biological difference among them in laboratory mice makes these rodent malaria particularly attractive models for the investigation of specialized aspects of human malaria. For example, *P. berghei* preferentially invades reticulocytes and induces severe pathology in mice [8]. *P. chabaudi* can be an appropriate model in studies associated with the drug resistance and immune evasion especially by antigenic variation [10]. In contrast, *P. yoelii* has been widely used in experiments concentrating on the liver-stage biology of malaria and on the host immune responses against that stage [9,11,12]. The last species is also highly applicable either in the investigations of receptors involved in erythrocyte binding [13] or in the evaluation of vaccine candidates [14-16].

In this study, we identified and cloned *P. yoelii* genes, including merozoite surface protein 1 (*MSP1*), circumsporozoite protein (*CSP*), and var 2 chondroitin sulphate A (*VAR2CSA*) genes, which can be applied in the investigation of immunization-induced protective immunity against *P. yoelii* malaria. The presence of antibodies specific to the recombinant fragments of *MSP1* and *CSP* proteins was also examined in mouse sera infected with *P. yoelii*.

## MATERIALS AND METHODS

### Experimental infections

Male ICR mice (4-week-old) were housed in the experimental animal center of Ewha Womans University School of Medicine, Seoul, Korea under the Institutional Animal Care and Usage Committee (IACUC) approved protocol for the animal care. Parasitemia of the non-lethal 17XNL strain of *P. yoelii* (courtesy of Prof. ET Han, Kangwon National University, Chuncheon, Korea) was monitored by microscopic examination of Diff-Quik stained blood smears from passage mice and expressed as the percentage of parasitized RBCs. Blood-stage infection was initiated by intraperitoneal injection of 150  $\mu$ l of the parasitized erythrocytes (5.5%). From day 2 post challenge, the resulting parasitemia was periodically monitored by enumerating parasitized erythrocytes in thin tail-blood smears stained with Giemsa, as previously described [17]. A total of 6 experimental mice that were recovered to normal levels 3 weeks after the primary injection were reinfected with the *P. yoelii* cells (5.5%) at 4 weeks after the first infection. Changes in the ear color and parasitemia were similarly checked in the experimental group.

### Parasite materials

Blood was collected through cardiac puncture from experimental mice ( $n=6$ ) at day 10 after the primary and secondary infections of *P. yoelii* 17XNL strain. Plasma samples prepared from the blood were used as the 1st and 2nd antisera, respectively, specific to *P. yoelii* antigens. Blood was also collected from uninfected normal mice with the same age ( $n=6$ ) to prepare the normal control sera. The sera were stored at  $-80^{\circ}\text{C}$  until use.

The trophozoite and schizont stage parasites in infected mice blood from the primary and secondary infections (parasitemia  $>35\%$ ) were highly enriched by the Optiprep (iodixanol; Axis-shield, Oslo, Norway) density gradient, as previously described [18]. Cellular fraction of normal mice blood was also prepared to use as control mouse proteins. Proteins were extracted by incubating the cellular fraction samples in a protein lysis buffer (155 mM  $\text{NH}_4\text{Cl}$ , 12 mM  $\text{NaHCO}_3$  and 0.1 mM EDTA) containing 1% SDS and protease inhibitors (Roche, Mannheim, Germany) for 1 hr on ice. The solutions were centrifuged at 12,000 rpm for 10 min at  $4^{\circ}\text{C}$  and the centrifugal supernatants were taken as protein samples. The total protein concentrations were determined by the Lowry method and stored at  $-80^{\circ}\text{C}$  until use.

Genomic DNA of *P. yoelii* 17XNL was extracted from whole blood collected from infected mice (parasitemia  $>35\%$ ) using the QIAamp DNA Blood Mini Kit according to the manufacturer's instruction (Qiagen, Hilden, Germany). The concentration and quality of the prepared genomic DNA was determined by a spectrophotometer by measuring absorbance at 260 and 280 nm. The DNA sample was stored at  $4^{\circ}\text{C}$  until use.

### Identification of *P. yoelii* genes

As vaccine candidates applicable in the examination of protective immunity, 4 malaria proteins (*MSP1*, *CSP*, *VAR2CSA*, and liver stage antigen 1 [*LSA1*]) were selected in this study. *P. yoelii* genes homologous to the selected molecules were searched in the genomic database of the rodent malaria in PlasmoDB (<http://plasmodb.org/plasmo/>) using BLAST algorithms. Protein and mRNA sequences used as queries in the homology searches were retrieved from the GenBank (<https://www.ncbi.nlm.nih.gov/>) database of *Plasmodium* spp. (no. AAA66185.1 for *MSP1*, partial sequence of *P. yoelii* protein; no. AAY34563.1 for *CSP*, *P. yoelii*; no. ALB01043.1 for *VAR2CSA*, *P. falciparum*; and no. CD064792.1 for *LSA1*, *P. reichenowi*). The genomic nucleotide sequences were compared to the respec-

tive mRNA sequences and then, their genomic structures were determined by referencing the general GT-AG rule [19].

#### Amplification of full-unit genes

Forward and reverse primers were designed to match the 5'- and 3'-ends of *P. yoelii* genes. The primer sequences were as followings: 5'-ATGAAGAAGTGTACCATTTTATTGTTAGC-GTC-3' and 5'-TTAATTAAGAATACTAATACTAATAATATTAC-3' for CSP gene; 5'-ATGAAGGTGATTGGACTTTTATTTTCTTTC G-3' and 5'-TTAAAATATATTAATAACAATTAATGTGATAA-3' for MSP1 gene; 5'- ATGAACAAAATAATAACTTATTTTTCTTT-TATAC-3' and 5'- TTATTTTAAAAATATGACTATGCATACCATC-3' for VAR2CSA gene. The primers were used in long-range PCR to amplify each of the full-length genes from the genomic DNA of *P. yoelii*. The typical 2-step thermal cycling PCR was performed for the amplification using the LA *Taq* polymerase (Takara, Shiga, Japan) (initial denaturation at 95°C for 5 min, 35 cycles of 45 s at 94°C and 5 min at 64°C, and a final extension at 70°C for 10 min). After verifying their correct sizes and purity on 0.8-1%-agarose gels, the products were cloned into a pGEM-T Easy vector (Promega, Madison, Wisconsin, USA). The nucleotide sequences were automatically determined using a BigDye Terminators Cycle Sequencing Core Kit (Ver. 3.0; Perkin Elmer, Foster City, California, USA) and an automated ABI PRISM 377A DNA Sequencer (Applied Biosystems, Foster City, California, USA).

#### Sequence analysis

Open reading frame (ORF) encoded in each of the cloned genes was predicted using ORF Finder (<http://www.ncbi.nlm.nih.gov/>) and their similarity profiles were further verified using BLASTp with the theoretically translated amino acid (aa) sequences. The aa sequences of orthologous proteins identified in the PlasmoDB and GenBank databases were aligned with ClustalX and used in phylogenetic analysis with MEGA 6.0 (neighbor-joining algorithm, Jones-Thornton-Taylor model for aa substitution, rate heterogeneity with 4 gamma category, pairwise-deletion of gapped region sequences). The statistical reliability of branching nodes was assessed by the standard bootstrapping test of 1,000 replicates. The aa sequences were also applied in the prediction of functional protein domains using the SMART program (<http://smart.embl.de/>).

#### Generation of recombinant MSP1 and CSP proteins

The coding DNA sequences of MSP1 and CSP genes were

segmentally amplified from the genomic DNA of *P. yoelii* with specific primers containing restriction sites for endonucleases (Supplementary Table 1). After digestion with the corresponding enzymes, the amplicons were cloned into pET-28a or pGEX-6p-1 plasmid and transformed into competent *Escherichia coli* BL21 (DE3) cells. Expression of these recombinant proteins were induced by incubating the cells in Luria-Bertani medium supplemented with 0.5 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) for 4 hr at 37°C. The proteins were purified by nickel-nitrilotriacetic acid (Qiagen) or glutathione sepharose (GE Healthcare, Little Chalfont, UK) chromatography according to the manufacturers' instructions.

#### Western blot analysis

The cellular proteins (each 20  $\mu$ g) extracted from the bloods of normal and infected mice were resolved through 10% SDS-PAGE, together with a pooled normal mice serum (n=5). The protein bands were either visualized by staining with Coomassie brilliant blue R-250 or transferred electrophoretically onto nitrocellulose membranes. The membranes were reacted with the specific mouse antisera prepared during the primary and secondary infections of *P. yoelii* 17XNL, as well as normal mouse serum (1:1,000 dilution). Antigen-specific antibodies were detected with the goat anti-mouse IgG antibody conjugated with IR-Dye 800cw (1:20,000 dilution). The positive reaction was scanned on an Odyssey CLx imager (800 channel; Lincoln, Nebraska, USA). Images were acquired on the CLx imager at 169  $\mu$ m resolution. The recombinant MSP1 and CSP fragments (each 200 ng) were similarly applied in the western blot analysis.

## RESULTS

#### *P. yoelii* MSP1 gene (*PyMSP1*)

A partial protein sequence (459 aa, 2104291A) corresponded to the N-terminal portion of *PyMSP1* was identified in the GenBank database [20]. The sequence was used as a query during the tBLASTn analysis of *P. yoelii* transcripts in PlasmoDB to isolate the full *PyMSP1* gene sequence. A total of two 5,319-bp transcripts (sequence identity=100%) were detected in the similarity analysis (PY05748 of *P. yoelii* yoelii 17XNL and PYYM\_0834100 of *P. yoelii* yoelii YM). By using the sequence as a query in BLASTn search, the full-length sequence of a single exonic *PyMSP1* gene was identified in the genomic databases of *P. yoelii* 17XNL/17X and *P. yoelii* YM strains (se-

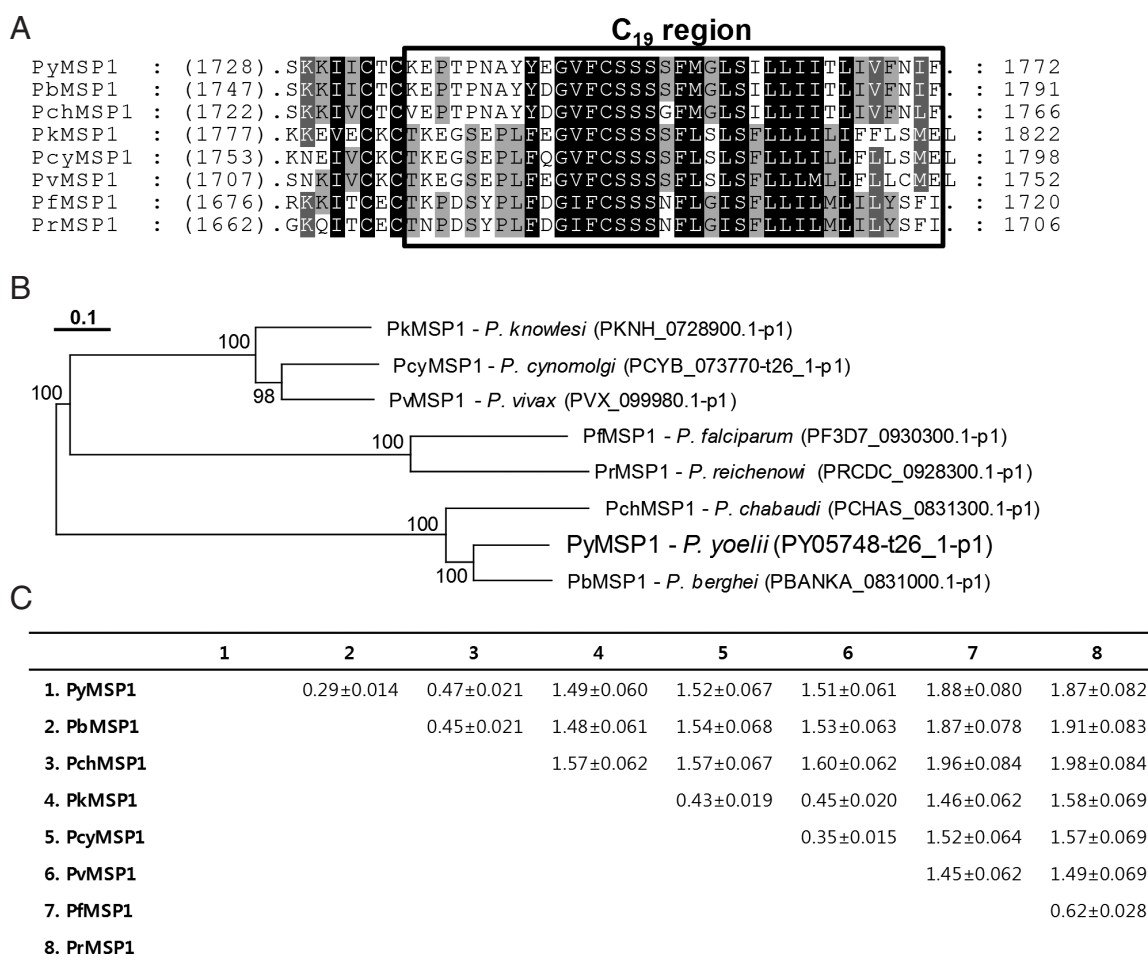
quence identity= 100%). The full gene sequence was amplified by a long-range PCR from the genomic DNA of *P. yoelii* 17XNL (Supplementary Fig. 1).

The 1772-aa PyMSP1 protein detected orthologous proteins in almost all of the *Plasmodium* databases in PlasmoDB, except for that of an avian malaria *P. gallinaceum*, and exhibited identity values ranging from 32% (*P. falciparum* MSP1) to 74% (*P. berghei* MSP1) with its plasmodial orthologs. As previously shown during characterization of *P. berghei* MSP1 [21], PyMSP1 also contained the structural/functional domains conserved in the plasmodial MSP1 proteins (data not shown). In a phylogenetic analysis, plasmodial MSP1 proteins showed a

clustering pattern identical to that of their donor organisms [22]. The distance values were similarly estimated as  $0.403 \pm 0.1028$  and  $0.408 \pm 0.5110$  among MSP1 proteins identified in rodent and primate malaria, respectively. However, the value was greatly increased to  $1.535 \pm 0.0398$  between rodent and primate malaria proteins (Fig. 1).

*P. yoelii* CSP gene (PyCSP)

The genomic sequence of PyCSP gene was searched in PlasmoDB using BLAST programs with the mRNA (1284 bp, DQ012939.1) and aa (427 aa, AAY34563.1) sequences of *P. yoelii* CSP, which had been isolated from GenBank. The gene



**Fig. 1.** Comparison of primary structures among merozoite surface protein 1 (MSP1) identified in various *Plasmodium* spp. (A) The full amino acid sequence of *P. yoelii* MSP1 (PyMSP1) was aligned with those of orthologous proteins identified in other *Plasmodium* spp. Dots in the alignment were introduced to increase identity values among them. Only the region corresponding to the B cell-activating epitopes (C19 region) is presented in this panel. (B) Phylogenetic relationships among the plasmodial MSP1 proteins were analyzed with the neighbor-joining algorithm of MEGA. All the sequences used in the analysis were retrieved from PlasmoDB (their respective entry numbers are presented in the parentheses). Numerals at branch nodes indicate their percentage of appearances in 1,000 bootstrap replicates. (C) The pairwise distance among the plasmodial MSP1 proteins was calculated with the MEGA program.

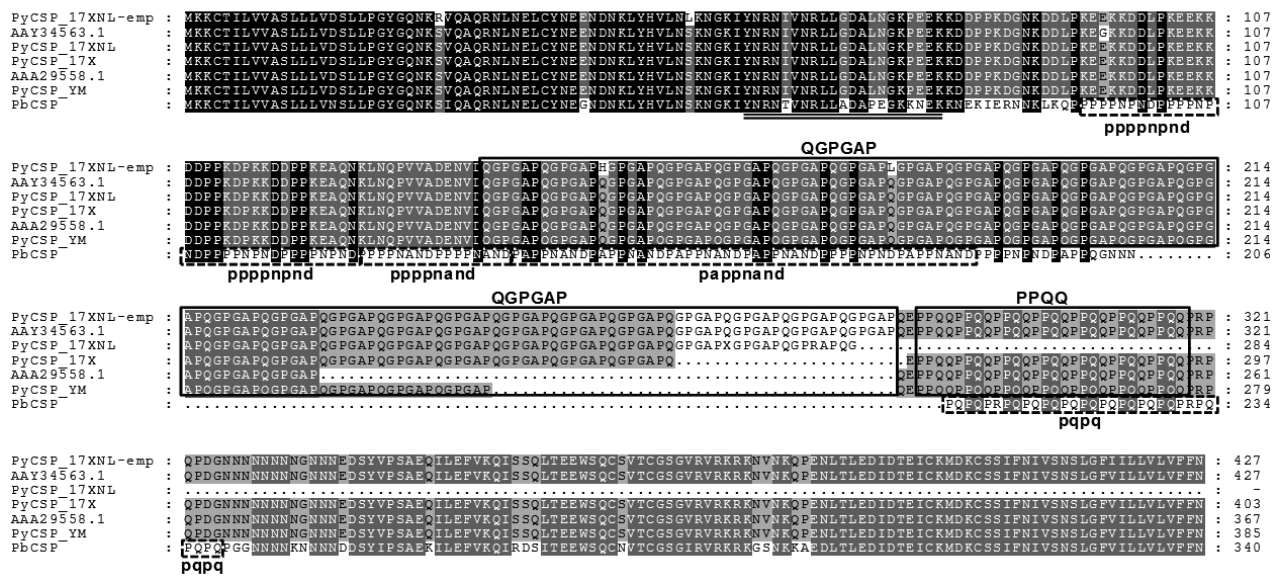
was also found to be single exonic. However, in contrast to *MSP1*, there were several gaps with various lengths in the resulting sequence alignment between the mRNA query and matched genomic sequences. Gap lengths in the alignment were slightly different among genomic sequences of *P. yoelii* strains, mainly due to the nucleotide length polymorphism in the repetitive region detected in the middle region of rodent malaria CSP genes [23]. The full-unit *PyCSP* gene empirically determined (*PyCSP\_17XNL-emp*; Supplementary Fig. 1), had a size identical to that of DQ012939.1, although 7 nucleobase mismatches were observed between them (99.5% identity). Of the 7 mismatched bases, 5 bases changed the decoding amino acids (see *PyCSP\_17XNL-emp* and AAY34563.1 in Fig. 2).

To verify the length polymorphism among the *PyCSP* proteins of different *P. yoelii* strains, orthologous sequences were isolated from PlasmoDB and GenBank. Two unique sequences were identified in 17XNL (PY07368-t26\_1-p1/XP\_728216.1 and AAY34563.1) and 17X (PY17X\_0405400.1-p1/CDZ 10959.1 and AAA29558.1) strains, whereas only a single unique sequence was detected in the proteomic database of YM strain (PYYM\_0405600.1-p1/CDU16435.1). Sequence alignment of

these *PyCSP* proteins together with that of *P. berghei* (PbCSP) demonstrated that the length polymorphism was induced by shrinkage/expansion of the repeat unit 'QGPGAP' found in the middle region of *PyCSP* (Fig. 2). It was also apparent that the repeat-region sequences, but not the 5'- and 3'-flanking regions, has been specifically evolved in each of the rodent malaria species. CSP proteins identified in primate malaria also exhibited repeat sequences specific to the lineages (data not shown). Amino acid sequence acting as the cytotoxic T lymphocyte epitope was tightly conserved in the *PyCSP* proteins with different strain origins (double underlined in Fig. 2) [24].

*VAR2CSA* and *LSA1* genes in *Plasmodium* spp.

The *VAR2CSA* gene was screened in the genome of various *P. yoelii* stains by tBLASTn program using the aa sequence of *P. falciparum* *VAR2CSA* (partial, ALB01043.1) as a query. The sequence retrieved an mRNA transcript putatively encoding Duffy-binding protein (DBP; PY04764 of 17XNL strain, PY17X\_1337400 of 17X strain and PYYM\_1334400 of YM strain; *E*-value=7e-13). The 5'- and 3'-region sequences of PY04764 (2361 bp) differed from those of PY17X\_1337400



**Fig. 2.** Polymorphic repeat sequences of circumsporozoite proteins (*PyCSP*) encoded in the different *Plasmodium yoelii* strains. The amino acid sequence of *PyCSP* protein was empirically determined from the non-lethal 17XNL (*PyCSP\_17XNL-emp*) strain in this study and compared with those identified in the PlasmoDB and GenBank databases of 17XNL (*PyCSP\_17XNL* and AAY34563.1), 17X (*PyCSP\_17X* and AAA29558.1), and YM (*PyCSP\_YM*) strains. Sequence of an orthologous protein in *P. berghei* (PbCSP) was also included in the comparison. Dots in the alignment were introduced to increase identity values among them. Boxes with solid lines indicate repeat units conserved in the central region of CSPs of the multiple *P. yoelii* strains, while those with dotted lines mark the repeats detected in the *P. berghei* CSP protein. The amino acid stretches that are involved in the activation of cytotoxic T cells are double underlined.

and PYYM\_1334400 (2559 bp; data not shown). The nucleotide sequence of homologous gene, which was empirically determined from the genome of 17XNL strain (Supplementary Fig. 1), was found to be identical to those of PY17X\_1337400 and PYYM\_1334400. Therefore, there might have been some fault during determination of PY04764 sequence. The genomic version of the *P. yoelii* DBP gene (*PyDBP*, 3,228 bp) was comprised of 5 exons and 4 intervening introns.

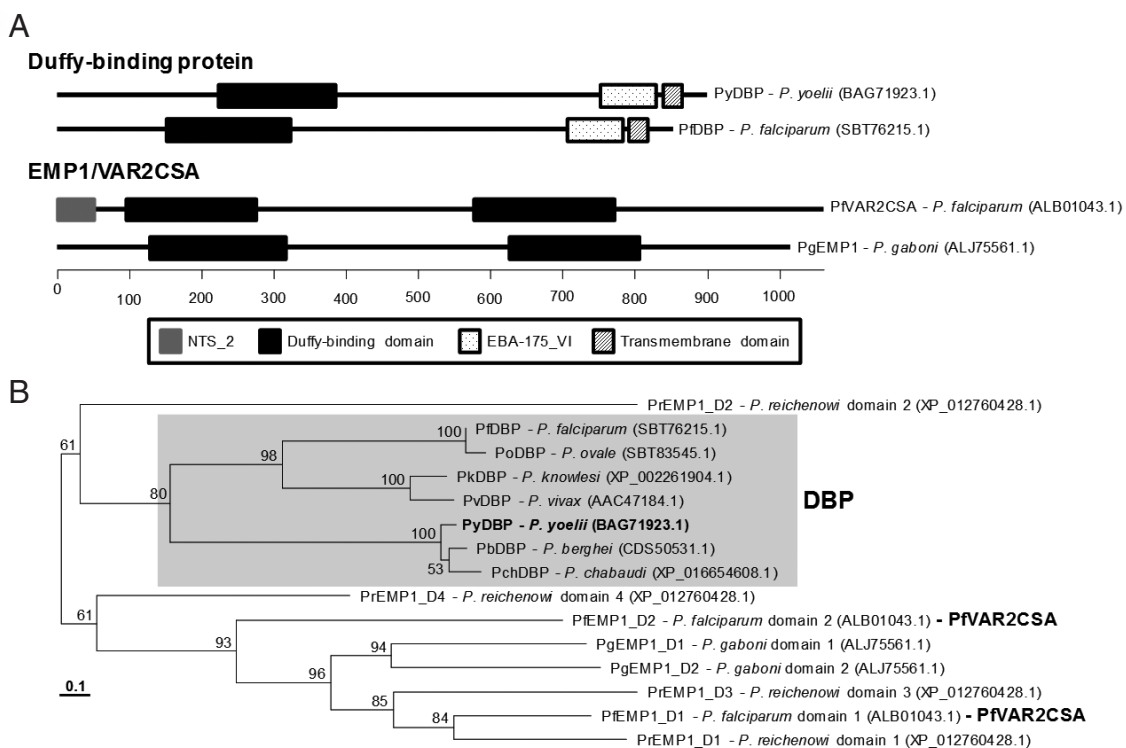
BLASTp search of the GenBank plasmidial databases using the *PyDBP* sequence retrieved numerous DBPs from the rodent and primate malaria databases including *P. falciparum* DBP (SBT76215.1) (identity > 33%, *E*-value < 2e-43). However, the orthologs of *P. falciparum* VAR2CSA, named erythrocyte membrane protein 1 (EMP1), were detected only in the proteome databases of *P. reichenowi* and *P. gaboni* (identity > 35%, *E*-value < 6e-23). The DBP and EMP1/VAR2CSA protein groups possessed the well conserved Duffy-binding domain, although the domain was duplicated in the EMP1/VAR2CSA (Fig. 3A). *P. reichenowi* EMP1 (XP\_012760428.1) had 4 different Duffy-

binding domains. Other group-specific domains such as EBA-175\_VI and NTS-2 were further found in the proteins of each group. In a phylogenetic tree, the Duffy-binding domains comprising DBP formed a tight clade, which were clearly separated from those consisting of EMP1/VAR2CSA (Fig.3B).

The plasmidial orthologs of LSA1 were screened with the amino acid sequence of *P. reichenowi* LSA1 (CD064792.1) against *Plasmodium* databases in PlasmoDB. However, matches with statistical significance were detected only in *P. falciparum* (PF3D7\_1036400.1-p1 of 3D7 strain and PFIT\_1035600.1-p1 of IT strain; *E*-value < 7e-155). Therefore, *LSA1* gene appeared to have evolved specifically in the genomes of *P. falciparum* and *P. reichenowi*.

### Protective immune responses in *P. yoelii* 17XNL-challenged mice

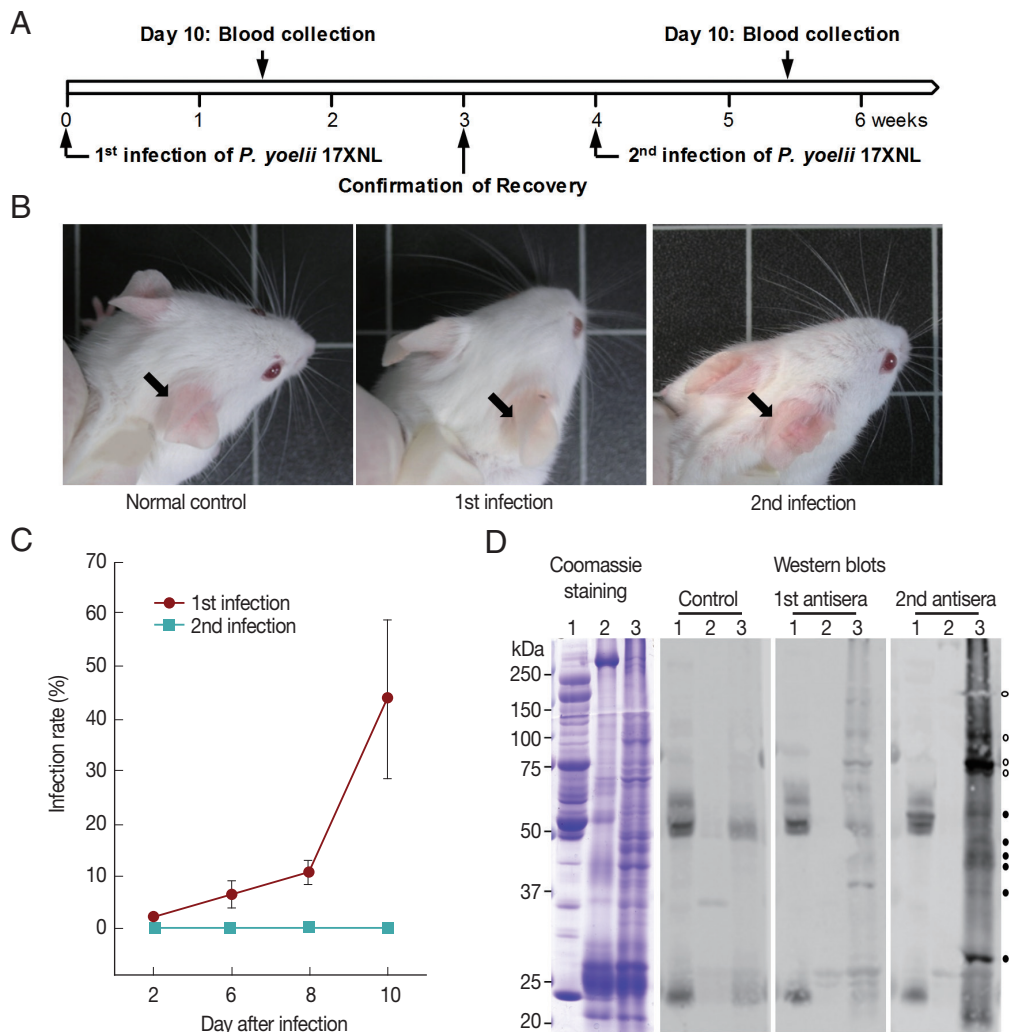
The ICR mice (n = 6) were challenged with the non-lethal *P. yoelii* 17XNL and monitored over the course of infection for parasitemia (Fig. 4). The percent parasitemia in the peripheral



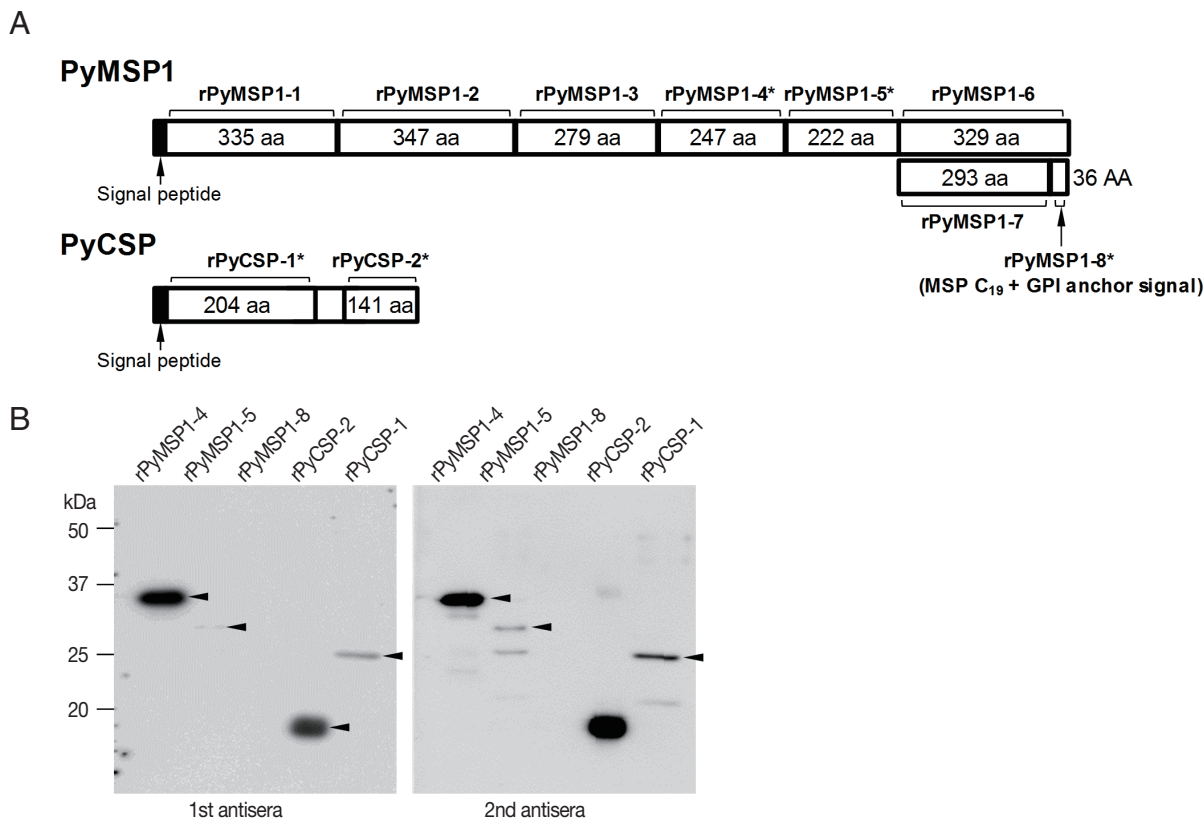
**Fig. 3.** Structural comparison between Duffy-binding proteins (DBP) and erythrocyte membrane protein 1 (EMP1)/VAR2CSA. (A) Functional domains conserved in the representative DBP and EMP1/VAR2CSA were predicted using the SMART program. (B) A neighbor-joining tree was constructed with MEGA using the Duffy-binding domain sequences conserved in DBP and EMP1/VAR2CSA proteins of *Plasmodium* spp. Multiple Duffy-binding domains encoded in EMP1/VAR2CSA were marked as D1, D2, and so on. Numerals at branch nodes indicate their percentage of appearances in 1,000 bootstrap replicates. Proteins annotated as DBP are highlighted in grey box.

blood was increased from  $2.03\% \pm 0.403$  at 2 days to  $43.82\% \pm 15.146$  at day 10 post-infection. The peak parasitemia at day 10 was gradually decreased to a background level and finally, complete convalescence from malaria was confirmed at week 3 after the primary challenge. The mice ears, which had turned to be pale during the infection with high parasitemia, recovered their original color (Fig. 4B). Interestingly, the parasite was rarely detected in the peripheral blood,

when the recovered mice were secondary challenged with an identical dose of *P. yoelii* 17XNL ( $n=6$ ; Fig. 4A-C). The mice ears also maintained the normal pinkish color (Fig. 4B). Western blot analysis of *P. yoelii* proteins with a pooled serum collected from the primary and secondary infected mice ( $n=6$ ) demonstrated that multiple proteins invoked humoral immune response to generate specific antibodies (Fig. 4D). Some of the specific antigen-antibody reactions were uniquely de-



**Fig. 4.** Infection of the non-lethal *Plasmodium yoelii* 17XNL strain to experimental ICR mice. (A) Schematic presentation of time schedule for the primary and secondary injection of the parasite into the mice and for blood collection to prepare specific antisera and parasitic materials. (B) Observation of ear color during the primary and secondary infections (black arrows). The images were taken from the experimental- (1st and 2nd infections) and control-group mice at 10 days post-infection. (C) Monitoring of parasitemia by enumerating parasitized RBCs in thin tail-blood smears stained with Giemsa. Mean  $\pm$  SD. (D) Detection of *P. yoelii* proteins-specific antibodies in the mice antisera by Western blot analysis. The sera were collected from the primary- (1st antisera) and secondary (2nd antisera)-infected mice as well as normal mice (control). Lane 1, plasma proteins in normal mice blood; lane 2, proteins extracted from cellular portion of normal mice blood; lane 3, proteins extracted from cellular portion of the primary-infected mice blood at 10 days post-infection. Closed circles mark protein bands reacted only with the 2nd antisera, while open circles indicate protein bands, of which positive signal was stronger with the 2nd antisera than that with the 1st antisera.



**Fig. 5.** Antigenicity of recombinant fragments of *Plasmodium yoelii* MSP1 and CSP proteins. (A) Schematic representation of cloned sites for the generation of each recombinant fragment. Asterisks mark proteins, which had been successfully induced in this study. (B) Detection of the recombinant protein-specific antibodies in mouse antisera collected from the first and second infections. Arrowheads indicate the positions of positive signals.

tected in the secondary infected serum (closed circles), while the others were found to be intensified probably due to the repeated challenge of identical antigens in the re-infected mice (open circles).

#### Antigenicity of recombinant MSP1 and CSP proteins

The coding DNA sequences of *P. yoelii* MSP1 and CSP genes were segmentally cloned to generate recombinant fragments of these proteins (Fig. 5A and Supplementary table 1). Of the 10 gene segments transformed into *E. coli* cells, 5 gene segments were successfully induced by IPTG (3 rPyMSP1 and 2 rPyCSP segments; recombinant fragments marked with asterisks). The recombinant proteins were purified and reacted with the *P. yoelii*-specific mouse antisera. As shown in Fig. 5B, the rPyMSP1-4 and rPyCSP-2 fragments exhibited strong positive signal, while rPyMSP1-5 and rPyCSP-1 reacted weakly with the mouse sera. All the signal intensity was slightly increased in reactions with sera from the secondary infection.

However, no significant reaction was observable between the mouse antisera and rPyMSP1-8 containing C<sub>19</sub> fragment, which had been known to act as a potent B-cell epitope in *P. falciparum* MSP1.

#### DISCUSSION

Plasmodial proteins that are specifically expressed during erythrocyte stage have been targeted for the development of protective vaccines [7,25,26]. Up to date, several proteins expressed on the surface of the merozoites and infected red blood cells such as merozoite surface proteins 1/2/3 (MSP1/2/3), serine-repeat antigen, erythrocyte binding antigen, glutamate-rich protein (GLURP) and apical membrane antigen 1 (AMA1) have been developed as potent vaccines [7]. MSP1 engaging in the initial attachment of merozoites to erythrocytes is a leading erythrocyte-stage vaccine candidate, which has been most extensively studied in the relevant fields [27].



Both of the N-terminal and C-terminal fragments of MSP1 have been found to be effective for the generation of immune response and protection against malaria [28,29], although the C-terminal fragment appeared to exhibit larger effect than the N-terminal counterpart [26]. The protective immunity has also been examined with rodent model using the C-terminal fragment of MSP1 protein expressed in *P. yoelii* [16,30]. However, it was unclear whether the C-terminal fragment of native PyMSP1 invoke generation of specific antibody during *P. yoelii* infection, since no significant immune reaction was detected against the C-terminal C<sub>19</sub>-containing fragment of PyMSP1 (rPyMSP1-8; Fig. 5). In addition, the middle fragment of PyMSP1 A (rPyMSP1-4) also exhibited strong reactivity against mouse sera infected with the parasite.

CSP is a sporozoite stage-specific protein that expresses a unique immunodominant epitope recognized by immunized or repeatedly infected hosts [24,31,32]. The plasmodial protein contains N- and C-terminal regions of relatively low immunogenicity, which flank a central region with tandemly repeated aa units. The central repeats differ from species to species and have been targeted as vaccine candidates for protective immunity against malarial infection due to their highly immunogenic properties [23,33,34]. The central repeats of CSP encoded in the *P. yoelii* genome were 'QGPGAP' and 'PPQQ', which were separated by a di-amino acid sequence 'QE'. The repeat units were well-conserved in the CSP orthologs identified in different *P. yoelii* strains (Fig. 2). The significant length polymorphism observed among these orthologs were mainly resulted from the shrinkage/expansion of the former 'QGP-GAP' repeat. Meanwhile, the repeats were 'PPPPNPND' and 'PQPQ' in CSP protein of *P. berghei*, while the sequence of former unit was slightly deviated to 'PPPPNAND' and 'PAPP-NAND' in the downstream regions. More recent investigations demonstrated that the N-terminal region of CSP proteins (double underlined sequence in Fig. 2) can also be applicable for the development of protective vaccines, even though its protective potential was relatively weak compared to that of the central repeat [24,34]. In *P. yoelii* system, the specific mouse antisera reacted strongly with the C-terminal fragment of CSP (rPyCSP-2), but not with the N-terminal fragment containing the 'QGPGAP' repeat (rPyCSP-1) (Fig. 5B). Taken together, these facts may reflect the collaborated actions between multiple regions of CSP proteins, which invoke specific immune response(s) against the parasite infection, are uniquely evolved among *Plasmodium* species.

The adhesion of infected erythrocytes to vascular endothelium or placenta is the key event in the pathogenesis of severe malaria induced by *P. falciparum* infection. By binding to receptors in the vascular bed, the parasite avoids being filtered through spleen, where they are removed from the circulation [35,36]. The plasmodial EMP1 proteins are adhesion molecules mediating the interaction between infected erythrocytes and the host endothelium. Of the multiple paralogous EMP1 proteins, VAR2CSA is a unique variant of EMP1 that is expressed in pregnant women and is responsible for the adhesive binding in the placenta. The VAR2CSA protein interacts with a low-sulfated form of chondroitin sulfate A attached to proteoglycans in the intervillous spaces of the placenta [37-40]. Protective immunity against the pregnancy-related placental malaria is acquired over multiple pregnancies and thus, VAR2CSA can be a major target for the development of vaccine against placental malaria [39,41]. In this study, VAR2CSA homologs are detected only in *P. reichenowi* and *P. gaboni*, but not in other primate and rodent malaria (Fig. 3). Currently, it is uncertain whether the EMP1 protein plays the adhesive role, similar to the VAR2CSA protein of *P. falciparum*, in rodent malaria. Nevertheless, the functional analog(s) of VAR2CSA is likely to be expressed in rodent malaria, considering many features that are commonly observed in the murine and human placental malaria [42]. Screening of the functional analog(s) in murine system is necessary to use the rodent malaria as an effective experimental model of human placental malaria.

To date, numerous recombinant proteins and crude blood-stage antigens of plasmodial species have been applied in the experimental investigations of protective immunity against malaria using model animals [14,26,43]. The target proteins mixed with various adjuvants were injected into experimental animals in a large amount to invoke potent immune response in these studies [14]. However, the ability to stimulate host immune system for the generation of specific protective antibodies appears to be quite different among vaccine candidates in practical patients [26]. Therefore, there have been more recent efforts to overcome/compensate the clinical limitation of previously established vaccine candidates by selecting immunogenic antigens against naturally acquired antibodies [43-45]. Several protein bands expressed in the blood-stage *P. yoelii* 17XNL were highly reactive with specific antibodies in experimental mice sera recovered from the primary challenge of the parasite. The low or negligible parasitemia observed in these

mice might result from the neutralizing action of the protective antibodies (Fig. 4). In addition to the generation of recombinant proteins characterized in this study for protective test, experiments for identification and cloning of the immunogenic antigens shown in Fig. 4D via mass spectrometry and following Mascot analyses are under preparations. Vaccination effects of the 17XNL proteins will also be further examined in mice challenged with the lethal *P. yoelii* 17X strain. The results obtained by the near-future studies might provide the necessary information and means to characterize additional vaccine candidate antigens, selected solely on their ability to produce the protective antibodies.

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### CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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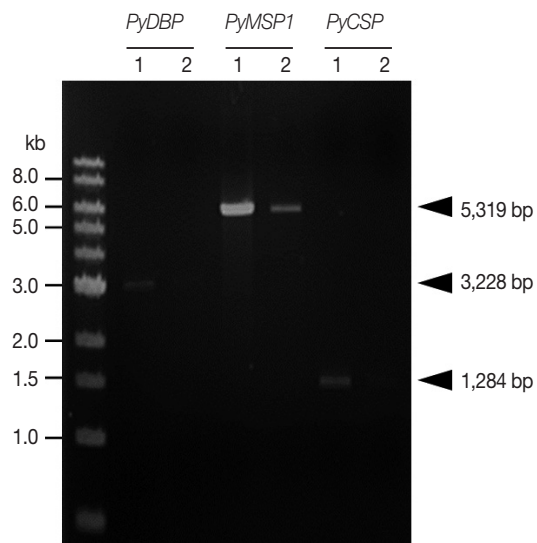
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**Supplementary Table 1.** Primers used in the amplification of *Plasmodium yoelii* MSP1 and CSP gene segments for the generation of recombinant proteins

Protein <sup>a</sup>	Oligo name	Sequence (5'→3') <sup>b</sup>	Amplified base positions	Recombinant protein
rPyMSP1-1	PyMSP1Nt1-F	<u>CCGGATCC</u> 'ACAATTGAAGTTTATAATG	61 - 1065	
	PyMSP1Nt1-R	GCGCGGCCCGC <sup>2</sup> GTTAAGTTGGAACGGGTTGC		
rPyMSP1-2	PyMSP1Nt2-F	<u>CCGGATCC</u> 'CTTACATTAGAAGAAAAACAG	1066 - 2103	
	PyMSP1Nt2-R	GGGCGGCCCGC <sup>2</sup> GTTATTTCTTTCGATACTTTC		
rPyMSP1-3	PyMSP1Mt1-F	<u>CAGGATCC</u> 'ATTGCTAAAGAGAAG	2104 - 2490	
	PyMSP1Mt1-R	GCGCGGCCCGC <sup>2</sup> GTTATGATGCTTCTGTTGAG		
rPyMSP1-4	PyMSP1Mt2-F	<u>CAGGATCC</u> 'TCATCAGCACCTGGCGAAGG	2941 - 3678	Yes
	PyMSP1Mt2-R	GGGCGGCCCGC <sup>2</sup> GTTAAGAAGTAAAATATTTAG		
rPyMSP1-5	PyMSP1Ct1-F	<u>CAGGATCC</u> 'ATGGAAGCTGTTCTTTAAAAAC	3679 - 4344	Yes
	PyMSP1Ct1-R	GCGCGGCCCGC <sup>2</sup> GTTATAATCCATTTGTCATG		
rPyMSP1-6	PyMSP1Ct2-F	<u>CGGGATCC</u> 'AATAATAAATCACAAAAAAG	4345 - 5331	
	PyMSP1Ct2-R	GGGCGGCCCGC <sup>2</sup> GTTAAAATATATTAATAC		
rPyMSP1-7	PyMSP1Ct2-C19-F	<u>CGGGATCC</u> 'ATGGAAGCTGTTCTTTAAAAAC	4345 - 5223	
	PyMSP1Ct2-C19-R	GCGCGGCCCGC <sup>2</sup> GTTATTTACATGTACATATAATT		
rPyMSP1-8	PyMSP1C19-F	<u>CCGGATCC</u> 'CCGAACCAACCOCTAATGC	5224 - 5331	Yes
	PyMSP1C19-R	GGGCGGCCCGC <sup>2</sup> GTTAAAATATATTAATAC		
rPyCSP-1	PyCSPNterm-F	<u>CGGGATCC</u> 'AATAAAAGTGCCAAGCCC	73 - 684	Yes
	PyCSPNterm-R	GCGCGGCCCGC <sup>2</sup> GTTATGGTGCTCCTGGTCCTTG		
rPyCSP-2	PyCSPCterm-F	<u>CGGGATCC</u> 'CCACAAGAACCACCCCAAC	862 - 1284	Yes
	PyCSPCterm-R	GCCTCGAG <sup>3</sup> GTTAATTAAGAATACTAATAC		

<sup>a</sup>Amplified gene segments were cloned into pET-28a plasmid except for Clone 8, which was inserted into pGEX-6p-1.

<sup>b</sup>Underlined sequences are recognition sites for *Bam*HI (1), *Not*I (2) and *Xho*I (3), respectively.



**Supplementary Fig. 1.** Amplification of *Plasmodium yoelii* genes. The *P. yoelii* genes encoding Duffy-binding protein (PyDBP), merozoite surface protein 1 (PyMSP1), and circumsporozoite protein (PyCSP) were amplified from genomic DNA of the parasite by a long-range PCR using respective gene-specific primers. The PCR products were electrophoresed on a 0.8%-agarose gel and visualized by ethidium bromide staining. Lane 1, products of secondary PCR (cycling numbers=25); lane 2, products of first PCR (cycling numbers=35).