Identification and expression of *maebl*, an erythrocyte-binding gene, in *Plasmodium gallinaceum*

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Abstract

Avian malaria is of significant ecological importance and serves as a model system to study broad patterns of host switching and host-specificity. The erythrocyte invasion mechanism of the malaria parasite *Plasmodium* is mediated, in large part, by proteins of the erythrocyte binding-like (*ebl*) family of genes. However, little is known about how these genes are conserved across different species of *Plasmodium*, especially those that infect birds. Using bioinformatical methods in conjunction with PCR and genetic sequencing, we identified and annotated one member of the *ebl* family, *maebl* (merozoite apical erythrocyte binding ligand) from the chicken parasite *Plasmodium gallinaceum.* We then detected the expression of *maebl* in *P. gallinaceum* by PCR analysis of cDNA isolated from the blood of infected chickens. We found that *maebl* is a conserved orthologous gene in avian, mammalian, and rodent *Plasmodium* species. The duplicate extracellular binding domains of MAEBL, responsible for erythrocyte binding, are the most conserved regions. Our combined data corroborate the conservation of *maebl* throughout the *Plasmodium* genus, and may help elucidate the mechanisms of erythrocyte invasion in *P. gallinaceum* and the host specificity of *Plasmodium* parasites.

Keywords

Plasmodium gallinaceum; MAEBL; EBL; EBA-175; Avian Malaria

1. Introduction

Malaria is caused by the parasite genus *Plasmodium* and undergoes three major life stages: a sexual stage within the mosquito's midgut, the generally asymptomatic exoerythrocytic stage, and the symptomatic blood stage in the vertebrate host, which is characterized by merozoite invasion of erythrocytes. This process is mediated by receptor/ligand interactions that can vary depending on the species of *Plasmodium* or host polymorphisms in genes encoding the receptors (Chitnis and Blackman 2000; Ghai et al. 2002). For example, *Plasmodium vivax* requires the Duffy Binding Protein (DBP) to recognize Duffy blood group antigens on the surface of human erythrocytes (Wertheimer and Barnwell 1989). In contrast, *Plasmodium falciparum* has several proteins that recognize receptors on human erythrocytes (Brown and Higgins 2010). A major group of these receptors are part of the family of erythrocyte binding-like (EBL) proteins that includes EBA-175, a *P. falciparum*

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Supplementary data associated with this article

protein that binds to glycophorin A on human erythrocytes and plays an important role in host specificity and the junction formation involved in erythrocyte invasion (Orlandi et al. 1992; Tolia et al. 2005). Other members of the EBL family in *Plasmodium falciparum* include JESEBL, PEBL, EBL-1, BAEBL and MAEBL (Adams et al. 1992)

MAEBL (Merozoite Apical Erythrocyte Binding-ligand) has been described in *Plasmodium* species that infect mammals and rodents (Blair et al. 2002; (Michon et al. 2002; Verra et al. 2006b). MAEBL is a protein of the EBL family with characteristics that distinguish it from other family members (Blair et al. 2002). All *ebl* genes are single copy and share similar gene structures; conserved exon-intron boundaries, and conserved amino and carboxyl cysteine-rich domains (Adams et al. 2001). In addition, most *ebl* genes encode duplicate cysteine-rich DBL (Duffy Binding-like) ligand domains; however, MAEBL does not. Instead, MAEBL has duplicate amino cysteine-rich regions (M1 and M2) with similarity to domains I and II of Apical Membrane Antigen-1 (AMA-1) (Kappe et al. 1998). The MAEBL M1 and M2 domains are responsible for the recognition of a presumed specific receptor (Ghai et al. 2002). Both domains are conserved but only the M2 MAEBL domain appears to be essential for binding its receptor on erythrocytes (Ghai et al. 2002). The unknown MAEBL receptor is not a sialylated protein but is affected by both papain and trypsin digestion (Ghai et al. 2002). Unlike its EBP counterparts, which are expressed in micronemes, MAEBL is expressed in rhoptries; apical organelles that secrete proteins during merozoite invasion. Furthermore, MAEBL is detected in late trophozoite and early schizont stages prior to erythrocyte rupture and Duffy Binding Protein expression in the rodent parasite *Plasmodium yoelli* (Noe and Adams 1998).

MAEBL has been promoted as a target for vaccine production because of its expression in both merozoite and sporozoite stages within the host (Ghai et al. 2002; Preiser et al. 2004). *Maebl* is alternatively spliced, producing different transcripts at the sporozoite and merozoite stages (Singh et al. 2004). It has also been shown that the *maebl* transcript codes for a bicistronic message, containing the mitochondrial ATP synthase subunit gene upstream of the transcript (Balu et al. 2009). For these reasons *maebl* serves as a model for post-transcriptional mRNA processing of Apicomplexans. Additionally, MAEBL plays an important role in many developmental stages, both in the host and mosquito vector (Florens et al. 2002). The protein has been detected in zygote, ookinete, merozoites, midgut and salivary sporozoites, indicating that it is present at various developmental stages and remains important throughout the life of the parasite (Blair et al. 2002; Ghai et al. 2002; Patra et al. 2008; Preiser et al. 2004; Saenz et al. 2008; Singh et al. 2004).

The *maebl* gene has been identified in *P. falciparum* isolates and has high conservation with orthologs of rodent malaria parasites (Blair et al. 2002). Among both laboratory clones and field isolates, *maebl* shows conservation with few polymorphisms in the M1 and M2 extracellular binding domains (Verra et al. 2006a). As of yet, *maebl* has not been characterized in an avian malaria parasite. Avian *Plasmodium spp.* are numerous, are evolutionary basal to mammalian *Plasmodium spp.* and provide a natural model system to study the ecology and evolution of malaria parasites (Martinsen et al. 2008; Ricklefs and Outlaw 2010). The gene is of ancient origin and appears to have evolved as a single locus prior to *Plasmodium* speciation (Michon et al. 2002). The isolation of a functional *maebl* gene in an avian *Plasmodium* species may aid in deciphering the evolution of erythrocyte invasion processes of the *Plasmodium* genus. Thus, pending identification of *maebl* in an avian species, we hypothesized that there would be a close phylogenetic relationship of the avian malaria *maebl* to its mammalian orthologs. Here, we sought to identify the sequence and expression of *maebl* in the chicken parasite, *Plasmodium gallinaceum*, the only avian malaria parasite with a published genomic sequence (Wellcome Trust Sanger Institute) (Silva et al. 2011). Our statistical and molecular data show that the sequence and gene

structure of *P. gallinaceum maebl* is orthologous to other species *Plasmodium* infecting mammals. Results confirm a high level of amino acid conservation (based on synonymous and non-synonymous changes) among *P. gallinaceum maebl* and human *Plasmodium* species, lending evidence to the biological importance of the gene. In addition, we confirm the expression of *maebl* in *P. gallinaceum*, and use DNA sequencing to annotate the gene.

2. Materials and Methods

2.1 Identification and sequence analysis of the full length P. gallinaceum maebl gene

BLAST searches of the *P. gallinaceum* genome sequence project (accession number PRJNA12649) carried out with the full-length *P. falciparum* EBL genes *eba-175, jesebl, pebl, baebl and ebl-1* (Adams et al. 2001) yielded no significant hits. Only one BLAST search of the *P. gallinaceum* genome sequence project (accession number PRJNA12649) carried out with the full-length *P. falciparum* MAEBL sequence (accession number XM_001348117) as query (Altschul et al. 1990) was significant. We also performed local BLAST analysis using the same *P. gallinaceum* FTP genome database (PRJNA12649; Parasite Genomics Group at the Wellcome Trust Sanger Institute) that yielded nearly identical results.

All BLAST searches, using *Plasmodium falciparum* as query, identified gal28as.d000006237.Contig1 (a super phusion contig) to contain the *P. gallinaceum maebl* ortholog sequence. The downloaded super phusion contig was originally 10,549 base pairs and contained an unknown sequence, which was labeled N_{10} by the Parasite Genomics Group. Primers flanking the N_{10} sequence were designed in order to obtain the missing sequence (Fig. S1), and confirm the downloaded sequence. The unknown N_{10} sequence was 235 base pairs, making the super phusion contig 10,774 base pairs long. The complete super phusion contig (gal28as.d000006237.Contig1) was recorded and translated to identify open reading frames (ORFs) using Sequencher 4.8 (GeneCodes, Ann Arbor, MI) and Apollo (Lewis et al. 2002).

2.2 Annotations

Multiple sequences obtained from GenBank, included EST and proteomic sequences, were uploaded onto the automated genome annotation pipeline MAKER (Cantarel et al. 2008) to annotate and create a model of the *P. gallinaceum* MAEBL gene region. Annotations were viewed in Apollo (Lewis et al. 2002) and were manually adjusted so that they were consistent with *maebl* orthologs of various *Plasmodium* species.

2.3 Phylogenetic analysis

We used Bayesian analysis to construct a phylogenetic tree using the M2 domain sequences (of seven *Plasmodium spp*: P. falciparum, P. gallinaceum, P. yoelli, P. vivax, P. knowlesi, P. chabaudi and *P. berghei* (GenBank accession numbers XM_001348117, JQ780838, XM_724578, AY042083, XM_002259441, and plasmoDB.org identifiers PCHAS_070250 and PBANKA_090130 respectively). All individual sequences were grouped into a consensus that was 1,231 bp long, with *P. falciparum* (XM_001348117) as the outgroup. Data was generated by MrBayes version 3.1.2 (Huelsenbeck et al. 2001), using the model of sequence evolution obtained from MrModeltest: Generalized Time Reversible, Gamma and Proportion Invariant (GTR + I + G). Two Markov chains were run simultaneously for ten million generations and sampled every 200 generations, producing 50,000 trees. 25% of the trees were discarded, and the remaining 37,500 trees were used to construct a majority consensus tree.

2.4 Gene/protein alignments and divergence levels

Gene and protein sequences were aligned using ClustalX (Larkin et al. 2007) and rates of synonymous and non-synonymous changes ((K_a/K_s ratios), were measured with the PAL2NAL package (Suyama et al. 2006).

2.5 DNA/RNA extractions, PCR, and Sequencing

Genomic DNA from White-Leghorn chicken blood infected with *Plasmodium gallinaceum* strain A8 was used for all molecular work (Frevert et al. 2008) The blood was provided by Dr. Ute Frevert. Parasite DNA was extracted from blood following the animal tissue protocol of the Wizard® SV Genomic DNA Purification Kit (Promega Corporation, Madison, WI). All polymerase chain reactions (PCR) involving genomic DNA were carried out in a 25µl reaction mixture containing 10-100 ng of genomic DNA (1µl of template DNA), 0.4mM of each dNTP, 0.4 mM of each primer, 2.5µl of 5X CL Buffer (Qiagen, Valencia, CA), 0.625 units Taq (Qiagen, Valencia, CA) and 0.4mg/ml Bovine Serum Albumin (BSA). Several primer combinations were used to amplify smaller fragments of *maebl* (Table S1). Positive PCR products were purified using ExoSap (following the manufacturer's instructions, USB Corporation, Cleveland, Ohio), then sent out for Bi-directional sequencing to Elim Biopharmaceuticals Inc., Hayward, CA. All PCR reactions were tested in duplicates, to verify all sequence data. The GenBank accession number for the *Plasmodium gallinaceum* 6866 base pair *maebl* gene is JQ780838. These data have also been uploaded to PlasmoDB.org.

To obtain total RNA, we infected chickens with *Plasmodium gallinaceum*. Six White Leghorn chickens were hatched and raised at UC Davis. After 6 days, 5 of the chickens were infected by intraperitoneal injection of infected brain emulsions, again provided by Dr. Frevert (Frevert et al., 2008). One chicken was left uninfected to serve as a negative control. Infected blood was drawn 7 days after infection and stored in TRIzol® LS Reagent (Invitrogen, Grand Island, New York) and processed at San Francisco State University. Blood was drawn a second time at day 15 and third time at day 21 post infection. Infection status was confirmed visually both by microscopy, after staining blood smears with Giemsa, and using a standard PCR protocol to amplify the Cytochrome *b* gene (Valki nas et al. 2009). RNA was extracted from the second bleed using the TRIzol® LS Reagent (Invitrogen, Grand Island, New York) protocol with slight modifications. Heavy phase-lock gel tubes were used to separate nucleic acids into an aqueous phase. RNA was precipitated using isopropyl alcohol and a 250 µl of a high-salt solution (0.8M sodium citrate, 0.2M NaCl in DEPC treated water). The RNA was resuspended and treated with Ambion® TurboDNase™. RNA was re-extracted with phenol-chloroform isoamyl and precipitated in 100% ethanol. The RNA pellet was resuspended in DEPC treated water. cDNA was synthesized via Reverse Transcriptase PCR using 2 µg of pure RNA from infected chickens using an iScript™ cDNA synthesis kit (BIORAD Laboratories).

All PCR reactions using cDNA as template were carried out using *AccuPower*® HotStart PCR PreMix (Bioneer, Alameda, California). Primers were mixed with sterile water and added to the PCR tubes to make a total volume of 20 µl including the cDNA template. The final concentration of each primer was 0.75 µM.

To confirm cDNA synthesis chicken α-globin genes were amplified with primer sets AlphaA (αA) and AlphaD (αD) (Rincon-Arano et al. 2009). PCR was conducted using thermal cycling profiles described by the authors. Sequencing was carried out in the same manner as for genomic DNA.

Maebl expression was detected by using a nested PCR protocol. For the first PCR we amplified a 736 bp fragment using primers A12_F5: 5'-

GAAGATCGACTAAATGATCCAACAA-3' and M2A_R3: 5'-CTAAGCAAGGTCCTACACAGTATG-3'. Initial denaturation was 94° C for 3 min, followed by 20 cycles of 94° C for 30 s, 58° C for 30 s, 72° C for 1 min, and a final extension at 72° C for 10 min. The second PCR amplified a 199 bp fragment using primers A13_F5: 5'-GATTGCCATGCATCATGGTG-3' and A12_R3: 5'-ACCTAATCTCCCACCTAAACC-3'. Initial denaturation was 94° C for 3 min, followed by 35 cycles of 94° C for 30 s, 60° C for 30 s, 72° C for 30 s, and a final extension at 72° C for 7 min. Expression was visualized on an agarose gel.

To confirm splice sites we performed a nested PCR. The first PCR used 3 µl of cDNA and primers complimentary to regions in predicted exons 2 and 5: A10_F5: 5'-GCTTCTGGAAAACCAACACC-3' and E4A_R3: 5'-AAGTTTACACATTAGCAATGATATG -3' respectively. Initial denaturation was 94° C for 3 min, followed by 35 cycles of 94° C for 30 s, 58° C for 30 s, 72° C for 1 min, and a final extension at 72° C for 10 min. The second PCR used 3 µl of the first PCR product and primers complimentary to regions in predicted exons 3 and 4: E2B_F5: 5'-TGTTCTAATGAAGAAAGAGAACAT-3' and A9_R3: 5'-TGGAGCAGGAATAGCACT-GAT-3 respectively. The thermal cycling profile was the same as the first PCR. The final 189 base pair product was sequenced and further enhanced our *maebl* gene structure model (Fig. S2).

3. Results

3.1 Identification and Conservation of maebl in Plasmodium gallinaceum

We identified an ortholog of *maebl* in the avian parasite, *P. gallinaceum* in the Wellcome Trust Sanger Institute database, and confirmed the sequence using PCR and genomic sequencing. The resulting sequence is approximately 6,866 base pairs (including introns) (GenBank accession number JQ780838). Although we performed DNA sequencing to confirm the majority of the gene, we were unable to sequence a 1,491 base pair region due to high repeat density (positions 7278-8771), which codes for the repetitive region of MAEBL (Fig. 1c). Other groups studying mammalian *Plasmodium* species have also had difficulty obtaining sequence data for this region (Michon et al. 2002).

DNA sequences were translated and the resulting amino acid sequences showed 45% amino acid identity to *P. falciparum* MAEBL and 63% DNA identity (Fig. S3, S4). Like all the MAEBL proteins characterized so far, the most conserved regions are the cysteine-rich domains M1 and M2 (Michon et al. 2002). We found that *P. gallinaceum maebl* M1 and M2 domains have 16 cysteine residues that are conserved when compared to 14 other *Plasmodium* species (Fig. 2). Additionally, *P. gallinaceum* MAEBLs M1/M2 domains also share similarity to AMA-1 domains I and II with only 10 of the 16 cysteines conserved (Ghai et al. 2002). The remainder of the *P. gallinaceum maebl* amino acid sequence still had a high degree of similarity to other MAEBL proteins.

3.2 Gene structure of P. gallinaceum maebl

The *P. gallinaceum maebl* gene contains an amino signal peptide, extracellular binding domains (M1/M2), a transmembrane domain and a cytoplasmic tail (Fig. 1c). Using annotation evidence from MAKER, we were able to predict the exon-intron boundaries from *P. gallinaceum maebl* and show five exons by using expression data (ESTs) from *Plasmodium spp*. We also performed a nested PCR to determine the intron-exon boundary between predicted exons 3 and 4. The sequence obtained was sequenced and enhanced our *maebl* gene structure model (Fig. 1b). The M1 and M2 domains are coded at the end of exon 1 and continue through the first half of exon two. The rest of exon two codes for the tandem

repeat region of MAEBL, containing mainly lysine, alanine, glutamic acid and arginine residues. The carboxyl-cysteine (C-Cys) domain homologous to region VI of the *ebl* family (Adams et al. 1992), the transmembrane domain, and the cytoplasmic tail are all shifted relative to the known *maebl* genes, and are instead coded in exons three, four, and five (Fig. 1a). The total lengths of the exons differ between *P. gallinaceum* and *P. falciparum* (1809 bp and 2055 bp, respectively), and result from different lengths of the repeat region.

3.3 Phylogenetic analysis of M2 domain

Based on the identified sequence of *P. gallinaceum* MAEBL, using the M2 domains of seven *Plasmodium* species, we constructed a maximum-likelihood tree (Fig. 3). The *maebl* gene from *P. gallinaceum* is significantly divergent from that of the mammalian parasites *P. knowlesi, P. vivax, P. berghei, P. yoelli* and *P. chabaudi*. However, the sequence data did not provide enough resolution to establish an informative relationship with *P. falciparum.*

3.4 Molecular variation of the M2 domains of Plasmodium species compared to P. gallinaceum

Sequence variation of *P. gallinaceum* M2 *maebl* domain was calculated by counting non-synonymous and synonymous substitutions compared to other *Plasmodium* M2 MAEBL domains (Table 1). *Maebl* sequences were aligned and the K_a/K_s ratios were calculated. The rate of non-synonymous (K_a) changes were all less than 0.1023, and the rate of synonymous (K_s) changes were high, suggesting that purifying selection is acting on the M2 domain.

3.5 maebl is expressed by P. gallinaceum

After identifying *maebl* within the genome of *P. gallinaceum*, we verified its expression. Following the synthesis of cDNA from the blood of an infected chicken, we used PCR with *maebl* specific primers within the M2 domain to detect the expression of the gene. Results showed that a 199 bp fragment from *maebl* was present and that *P. gallinaceum* indeed expresses *maebl* in the blood stages (Fig. 4).

4. Discussion

Malaria parasites have evolved to infect a wide variety of hosts including mammals, reptiles, and birds. It is known that within each host, proteins expressed by the parasite mediate erthyrocyte invasion. In this study, we investigated the *ebl* gene known as *maebl* in *Plasmodium gallinaceum.* Using bioinformatics, we were able to identify *maebl*, annotate it, and show its conservation across different species of *Plasmodium.* In addition, we verified that *P. gallinaceum* expresses *maebl.*

The 16 cysteine residues in each M1 and M2 domain of the *P. gallinaceum maebl* are conserved, which suggests that the residues may be essential in the formation of the protein's tertiary structure, specifically in the extracellular binding domains. Comparison of the AMA-1 crystal structure to its paralogue MAEBL reveals that the 10 conserved cysteines form similar disulfide bridges, but not enough information was present to predict the structure of the six remaining cysteine residues and their possible functions (Chesne-Seck et al. 2005). Thus, determining MAEBL's crystal structure will confirm the importance and function of the conserved cysteine residues.

All five exons and exon-intron boundaries are conserved among P. falciparum, P. knowlesi, P. chaubadi, P. yoelli, P.vivax and *P. berghei* (Fig. 1a), but the last three 3' end exons have different exon-intron boundaries in *P. gallinaceum* (Fig. 1c). The conserved carboxyl-cysteine rich region is coded in exon 3 of *P. gallinaceum* MAEBL instead of exon 2, further validating the distant relationship of *P. gallinaceum* compared to other mammalian

Plasmodium species. Although the repetitive region cannot be confirmed, the fact that our cDNA primers (in a nested PCR reaction) were able to amplify a cDNA region downstream suggests that the predicted exons 2 and 5 exist in the cDNA.

The constructed M2 MAEBL phylogenetic tree is comparable to the known phylogenetic relationships of *Plasmodium* species, and reveals the divergence of the M2 domain between *P. gallinaceum* and mammalian *Plasmodium spp.* (Martinsen et al. 2008; (Outlaw and Ricklefs 2011). We focused on the M2 domain because studies show it is essential for erythrocyte binding when compared to the M1 domain (Ghai et al. 2002). The M2 domain of *P. gallinaceum* MAEBL showed no evidence of diversifying or positive selection based on the high rate of synonymous changes compared to the rate of non-synonymous substitutions. A ratio below 0.25 is indicative of purifying selection, and the quantification of evolution upon the *P. gallinaceum* M2 MAEBL domain shows K_a/K_s ratios below 0.1023 (Table 1). These calculated ratios strongly suggest that the M2 domain is functionally constrained and important as previously described (Verra et al. 2006). Our analysis of substitution rates in *P. gallinaceum* M2 domain adds to Verra's study and confirms the functional constraints on the M2 domain. Thus, although we find that the M2 domain differs at the nucleotide level from the same region of mammalian parasites (Fig. 3), we find that the domain is highly functionally conserved in all tested malaria parasites. The importance of the M2 domain in avian *Plasmodium* species is unknown, but it would be interesting to further study the M2 domain's role in erythrocyte invasion in birds, and also in sporogony in mosquitoes.

Studies have found that MAEBL is alternatively spliced in different *Plasmodium* stages (Ghai et al. 2002; Preiser et al. 2004; Singh et al. 2004). The MAEBL isoform containing the transmembrane domain is important in the invasion of the mosquito salivary glands and for erythrocyte binding in merozoites (Noe and Adams 1998; Saenz et al. 2008). MAEBL exemplifies a complex gene structure in *Plasmodium* because of its multiple isoforms and possible bicistronic transcript. These post-transcriptional processes appear to be conserved in the chicken parasite, and with the identification of *maebl* in *P. gallinaceum*, future studies can ascertain whether higher-level transcript regulation is occurring in avian *Plasmodium spp.*

The complex structure of MAEBL suggests that different receptors may be present in the host and vector. The high conservation observed in MAEBL may indicate the essential function of its receptor within the host. Thus, identification of the MAEBL receptor and further study of avian nucleated RBCs will lead to understanding the purpose and mechanisms of MAEBL. In a study by Fu et al. (2005), it was shown that *P. falciparum* survives without the protein, but the loss of MAEBL in merozoites alters the parasite invasion pathway to a novel alternative pathway, which is sialic acid-dependent and trypsin-insensitive. We can only speculate that *P. gallinaceum* may use this or a similar invasion pathway. The receptors of EBL proteins in mammalian *Plasmodium* species are mostly sialylated proteins such as glycophorins (Iyer et al. 2007; Mayer et al. 2009). Glycophorins from nucleated chicken erythrocytes have been isolated and characterized. It was found that they are highly divergent from their human counterparts, and therefore the *ebl* genes of different *Plasmodium spp.* may differ as well (Duk et al. 2000). Although MAEBL is highly conserved, MAEBL from rodent *Plasmodium spp.* cannot bind to human RBCs and vice versa (Kappe et al. 1998). Crosnier et al. 2012 was able to identify the human erythrocyte receptor of PfRh5 (an essential merozoite erythrocyte-binding protein of *P. falciparum*) using a human erythrocyte protein library for an AVEXIS assay (avidity-based extracellular interaction screen). A similar approach could potentially lead to the identification of the MAEBL receptor(s) in different hosts.

The identification of *Plasmodium* proteins involved in erythrocyte invasion is important in understanding the mechanism by which *Plasmodium* infects its host. For example, infected avian erythrocytes with *P. gallinaceum* develop furrow-like structures instead of knob-like structures like most *Plasmodium spp.* (Nagao et al. 2008). Avian parasites encounter different immunological pressures from their hosts, which result in different methods of erythrocyte invasion. In addition, erythrocyte binding proteins are involved in the junction formation that commits the parasite to invade red blood cells. Thus, these proteins are being added to the list of possible vaccine targets against malaria because of their importance in the invasion mechanism (Drummond and Peterson 2005).

We focused on *P. gallinaceum,* because it is the only avian *Plasmodium* to have had its genome sequenced. Our analysis using annotated *Plasmodium ebl* sequences, as well as using EBL Duffy Binding domains, did not detect any other *ebl* members. One possible reason may be that other *ebl* genes in *P. gallinaceum* are too divergent from mammalian parasites or that other *ebl* genes found in *P. falciparum* are not present in *P. gallinaceum*. For example, *Plasmodium vivax* has only one *ebl* gene, the Duffy Binding Protein (Wertheimer and Barnwell 1989). The complete annotation of *P. gallinaceum*'s genome will reveal whether other *ebl* members or proteins involved in erythrocyte invasion are present and functional. To obtain this information, high throughput sequencing of the parasite's transcriptome would confirm or refute the expression of other *ebl* genes in the parasite.

Avian malaria provides an ideal model system to test whether EBL proteins are involved in the host specificity of *Plasmodium* species. The extinction of several avian populations in the Hawaiian Islands was in part due to a newly introduced Plasmodium strain (*P. relictum*) that was capable of infecting multiple naïve bird species (a generalist parasite) (Beadell et al. 2009; Loiseau et al. 2010; Vanriper et al. 1986). Unlike generalist parasites, host-specific parasites (specialists) can infect only one species, and it is evident that certain malaria parasites are specialists to certain bird species (Iezhova et al. 2005; Loiseau et al. 2012). Moreover, previous work has shown that specialists may become generalists in host switching events, with increased virulence (Garamszegi 2006). Although the molecular basis of host specificity in avian *Plasmodium* parasites is largely unstudied, it is known that EBA-175 is a protein potentially involved in the host-specificity of *P. falciparum* and *P. reichenowi* of humans and chimpanzees respectively (Chattopadhyay et al. 2006; Martin et al. 2005; Tolia et al. 2005). EBA-175 is highly conserved between *P. falciparum* and *P. reichenowi*, but neither can infect each other's host. Chattopadhyay (2006) found that by comparing the 3D structure of EBA-175 of both species, the only significant difference was the distribution of protein surface charge in the channel containing the duplicated Duffy binding domains. Here we have shown that *maebl*, another member of the *ebl* family is also conserved and expressed across various species of *Plasmodium.* Thus, the identification and functional characterization of *ebl* genes in avian parasites will aid in the prediction of potential emerging diseases in avian populations, and help researchers to better understand the emergence of novel strains of malaria in both birds and other animals across the globe.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

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MAEBL	merozoite apical erythrocyte binding ligand
EBA-175	erythrocyte binding antigen-175
DBP	duffy binding protein
EBL	erythrocyte binding-like
PCR	polymerase chain reaction
RBC	red blood cells
EST	expressed sequence tags

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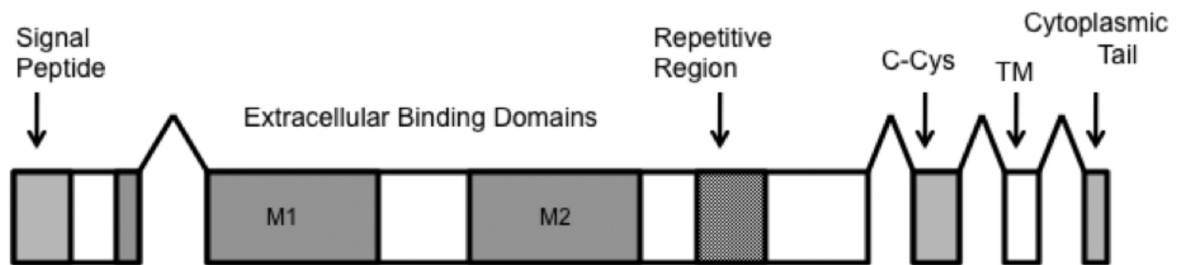
a

Plasmodium maebl conserved gene structure
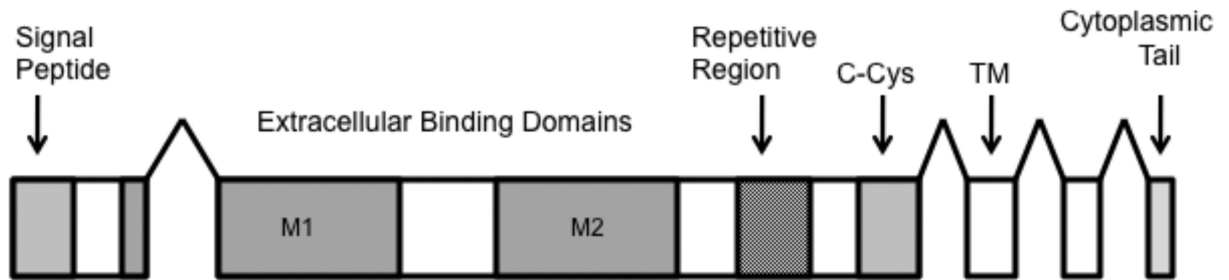
b

c

Plasmodium gallinaceum maebl conserved gene structure

Fig. 1.
Schematic Gene Structure of *Plasmodium* MAEBL. *a*, The conserved gene structure of *maebl* (above) in known *Plasmodium* species consists of five exons, coding 1) signal peptide, 2) ligands domains M1 and M2, repetitive region, and carboxyl-cysteine rich domain, 3) transmembrane domain, and a 4/5) cytoplasmic domain. *b*, Apollo model of *Plasmodium gallinaceum maebl* based on EST, BLAST, Protein2protein evidence (bars in black box). *P. gallinaceum maebl* gene structure appears conserved with five exons. *P. gallinaceum* transcript *maebl* model is indicated in the light gray box. *c,* The gene structure of *P. gallinaceum maebl* as predicted by the Apollo and Maker, B. The carboxyl-cysteine

rich domain and transmembrane domain are located in different exons than the conserved *Plasmodium* species gene structure, A.
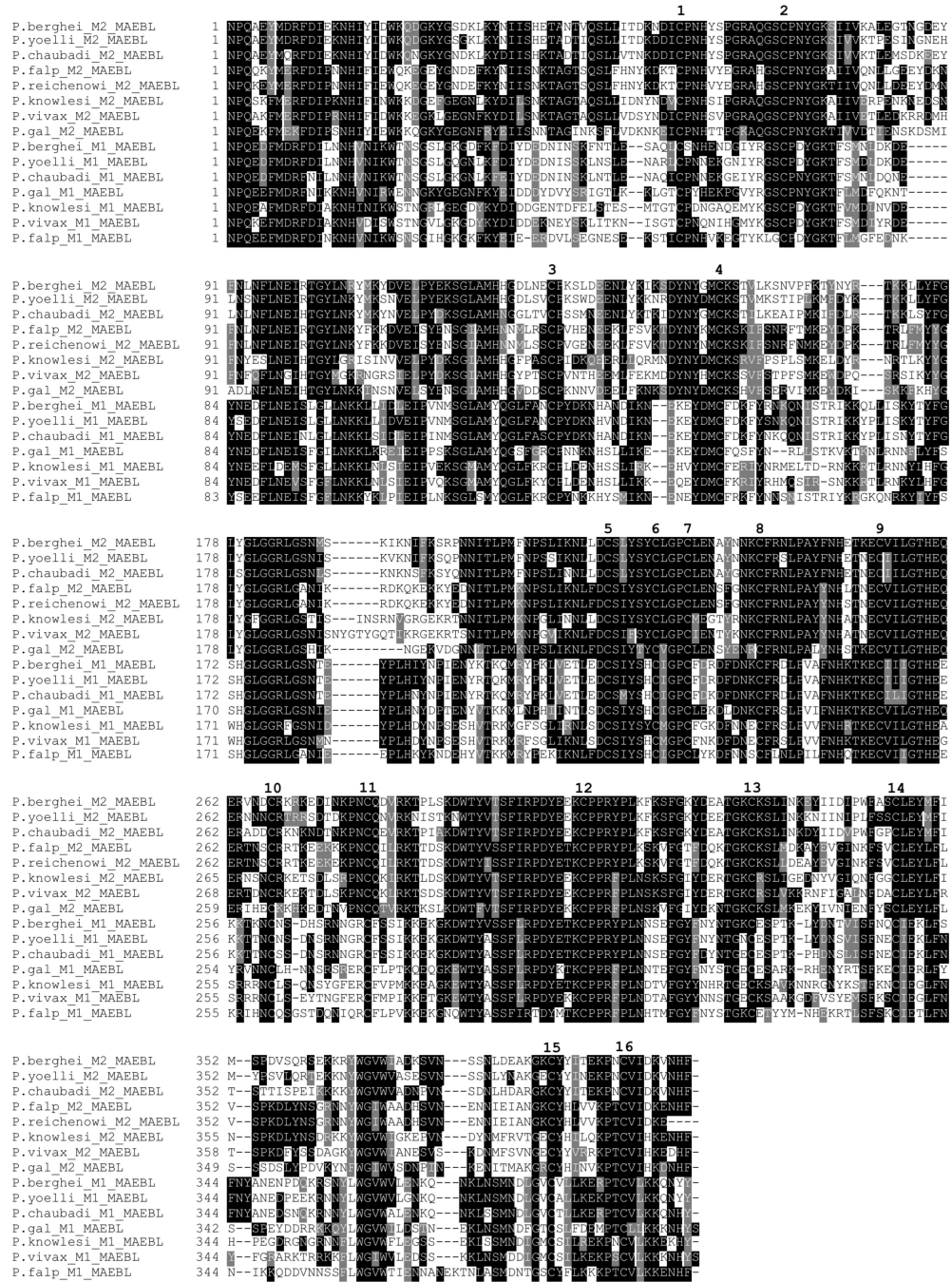
Fig. 2.

Plasmodium gallinaceum M1 and M2 domains have conserved cysteine residues.
CLUSTAL alignment of the *P. gallinaceum* MAEBL M1 and M2 domains. The 16
conserved cysteines are numbered. Black highlighted regions represent conserved/identical
amino acids. Gray regions represent similar amino acids. Spaces have been introduced to
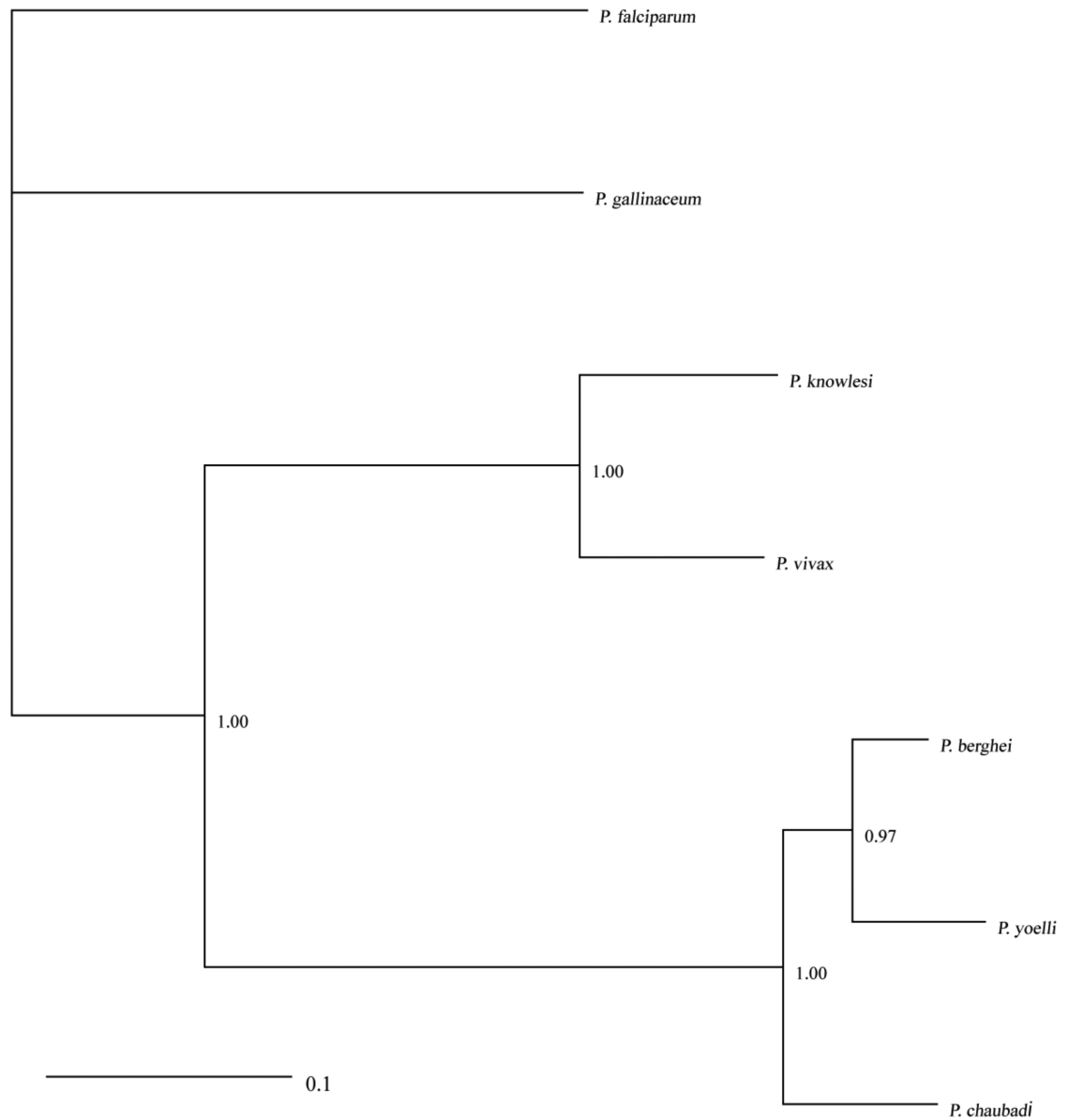maximize alignment.

Fig. 3.
Plasmodium gallinaceum MAEBL M2 domain diverges from rodent and mammalian *Plasmodium spp.* Phylogenetic relationships among the M2 domains of *maebl* from *P. gallinaceum* and mammalian *Plasmodium* species. Numbers along branches correspond to node support from Bayesian Analysis.
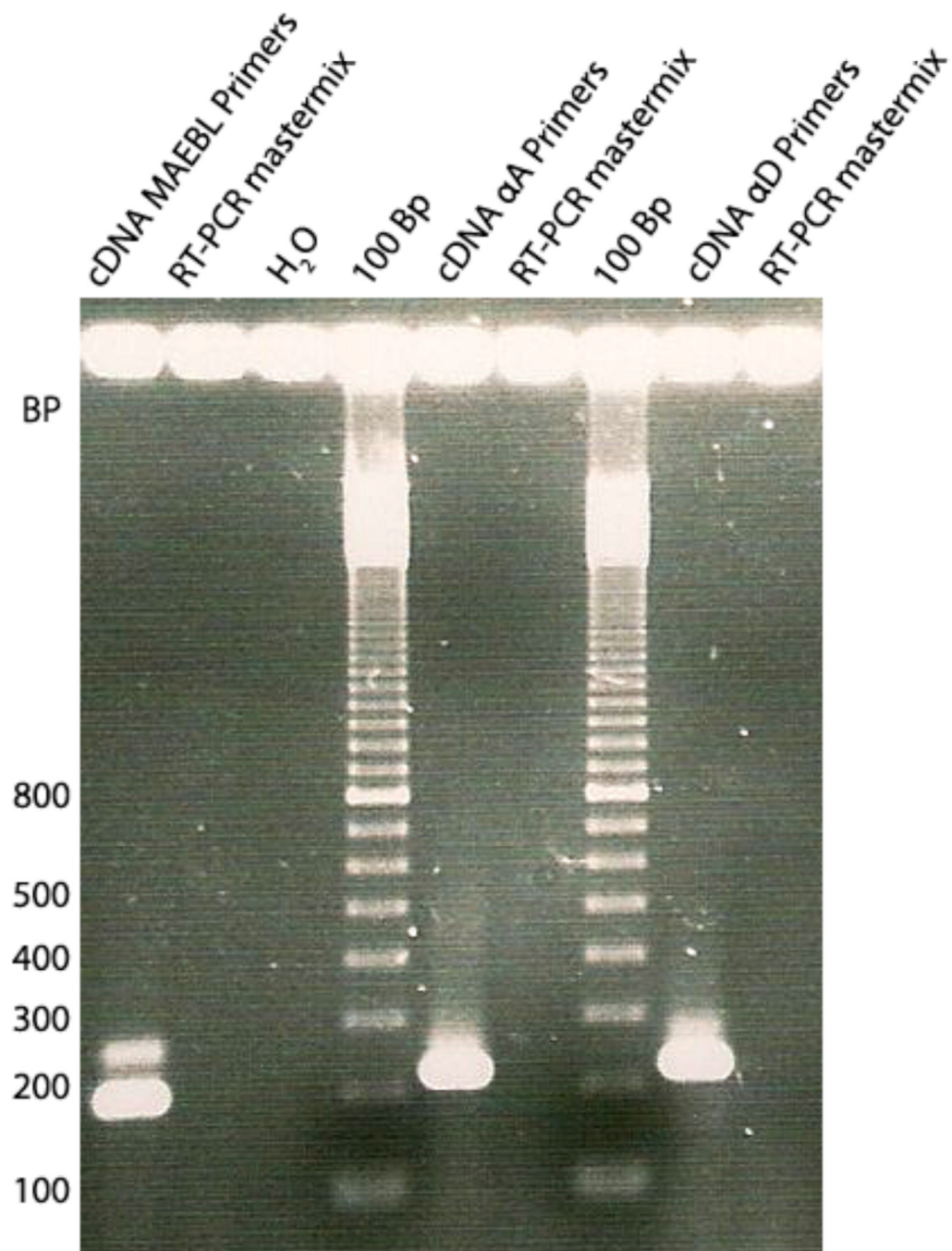
Fig. 4.
Expression of *maebl* by *P. gallinaceum*. Lane 1 (left) shows amplification of a 199 bp fragment amplified from cDNA using a nested PCR with *maebl* primers. Reverse Transcriptase PCR mastermix without RNA and water were used as the template for the negative control lanes. αA primers and αD primers were used to amplify expressed chicken alpha-globin indicating the presence of cDNA.

Table 1

Plasmodium gallinaceum **MAEBL M2 domain is under purifying selection**

Molecular variation: Values of non-synonymous and synonymous substitutions among *maebl Plasmodium* sequences as compared to *maebl* of *Plasmodium gallinaceum.*

P. gallinaceum	P. falciparum	P. vivax	P. knowlesi	P. berghei	P. yoelli	P. chaubadi
Number of Synonymous Sites	169.7	217.2	199.4	160.1	151.3	153.2
Number of non-synonymous sites	1033.3	985.8	1003.6	1042.9	1051.7	1049.8
Synonymous substitution rate (Ks)	4.8209	3.1401	2.9886	4.3808	4.712	4.7267
Non-synonymous substitution rate (Ka)	0.2621	0.2991	0.3057	0.3046	0.3124	0.3039
Ka/Ks Ratio	0.0544	0.0953	0.1023	0.0695	0.0663	0.0643