

Replication and Review of Githinji & Bull 2017

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The malaria-causing parasite *Plasmodium falciparum*'s virulence factor (*P. falciparum* erythrocyte membrane protein 1, PfEMP1) adheres to the infected surface of human erythrocytes. Natural malaria immunity is facilitated by the immune system's response to PfEMP1, which could also be the target of malaria vaccines. However, this protein is encoded by a diverse family of about 60 *var* genes. This diversity gives rise to antigenically diverse binding properties of the protein and therefore varying severity of malaria. There have been extensive analyses of PfEMP1 sequences from the currently available seven parasite genomes. Due to the limited number of full-length *var* gene sequences available, many studies have also classified a specific conserved subdomain ("tag") of PfEMP1 called Duffy binding-like alpha (DBL α) to draw information about cytoadhesion properties of the parasite's virulence factor and severity of malaria. Githinji & Bull 2017 compared DBL α tag classifications with sequence features of full-length *var* genes to show that the tags may provide insight into the functional specializations of *var* genes. In this review, we attempted to reproduce the results presented in Githinji & Bull 2017 and found that they were almost completely reproducible. As part of this replication, we provide open Python code, allowing the authors and others to see in detail how we used the datasets and implemented the methods in Python. This project was a 2-month rotation project in the Interdisciplinary Quantitative Biology program at the University of Colorado Boulder.

I. INTRODUCTION

The *Plasmodium falciparum* parasite is the most lethal of the five *Plasmodium* parasites responsible for malaria in humans. Once transmitted by the *Anopheles* mosquito to humans, the parasite exports to the surface of an infected red blood cell (RBC) a virulence factor called *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) (Figure 1), which is the target of the host immune system in malaria infections [4]. The type of PfEMP1 present on the infected RBC plays a key role in the clinical severity of the infection. However, the parasite can produce antigenically different proteins by switching on and off about 60 different PfEMP1-encoding genes, called *var* [5]. The hyper-variant types of PfEMP1 have different binding properties to human endothelial receptors, and cause the immune system's antibodies to not always recognize the protein to kill the infected cell [5]. Thus, dissecting PfEMP1 diversity is a problem with possible clinical significance.

PfEMP1 molecules are made up of two to nine domains: N-terminal segment (NTS), Duffy binding-like (DBL), cysteine-rich inter-domain region (CIDR), transmembrane (TM), and acidic terminal segment (ATS) domains [10] (See Figure 1 for an overview diagram of the PfEMP1 structure). The head structure of the protein contains DBL and CIDR domains that are known to mediate important binding properties of the parasite. Based on sequence similarity, the DBL and CIDR domains have been divided into subclasses ($\alpha, \beta, \gamma, \delta, \epsilon, \zeta$ and $\alpha, \beta, \gamma, \delta$ respectively) [10]. CIDR α domains encode PfEMP1 binding to the host receptors called

cluster determinant 36 (CD36) and endothelial protein C receptor (EPCR), which in turn are linked to particular clinical symptoms such as cerebral malaria [7]. A subset of DBL α domains are linked to rosetting, a process that causes infected RBCs to bind to uninfected RBCs and has been clinically linked to respiratory distress [8]. Understanding the structure and composition of PfEMP1 proteins by analyzing the diverse makeup of the *var* genes that encode them is therefore critical to understanding malaria's abilities to evade the immune system and cause severe disease.

Many different approaches have been taken to categorize *var* genes, which are characterized by their modular domain structure and diversity, in an effort to understand how *var* categories might represent functional or evolutionarily important groups. Based on full-length sequences from seven *P. falciparum* parasites, *var* genes have been classified by multiple structural characteristics. The upstream promoter sequence (UPS) classification divides the sequences into groups A-E [10, 12]. Domain alignment of full-length sequences yields 23 *var* "domain cassettes" (DCs), some of which are linked to clearly defined functions, such as DC8 *var* gene proteins binding to brain endothelial cells.

Past studies have also explored the classification of short PCR-derived sequences from the DBL domain, called "tags." The first approach involves grouping the tag sequences based on the number of cysteines and the mutually exclusive motifs MFK and REY [3]. These groups are called cys/positions of limited variability (Cys/PoLV) groups. The second approach uses network analysis to group together the sequences that share blocks of sequence (position-specific polymorphic blocks, or PSPBs) with each other, with the two prominent groups being block-sharing groups 1 and 2 (BS1 and BS2) [2]. [DM: Figure 1 shows an example PfEMP1 structure with its DBL α sequence with labeled PSPBs, cysteines, and a PoLV motif, MFK].

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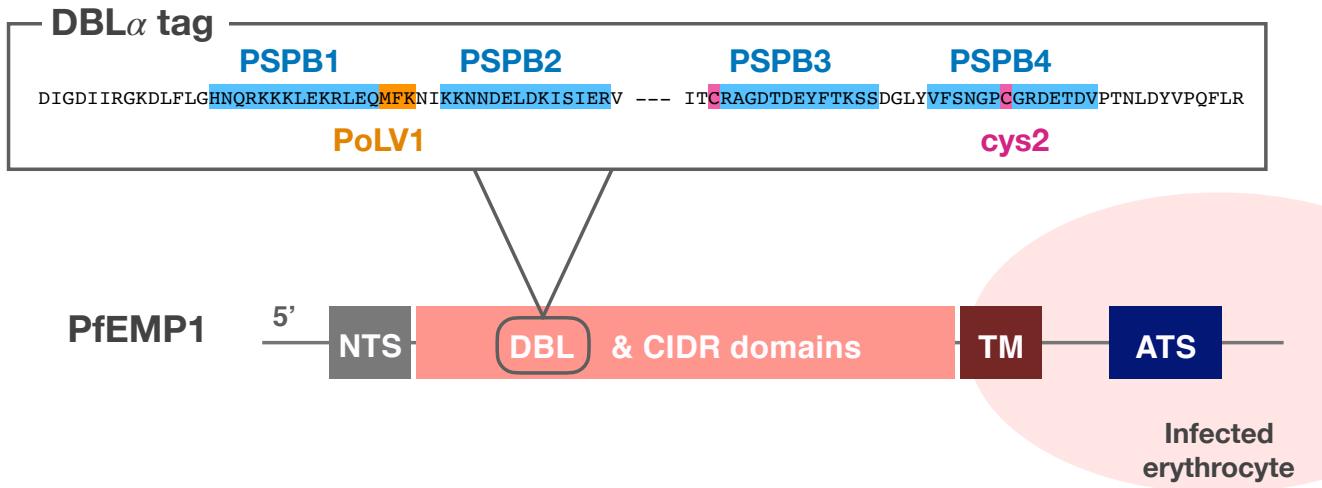


FIG. 1. Overview of PfEMP1, the *Plasmodium falciparum* virulence factor encoded by *var* genes. PfEMP1 molecules are usually made up of two to nine domains: N-terminal segment (NTS), Duffy binding-like (DBL), cysteine-rich inter-domain region (CIDR), transmembrane (TM), and the intracellular acidic terminal segment (ATS) domains. The DBL α subdomain, called “tag,” can be classified by two methods: 1) the Cys/PoLV groups based on number of cysteines and the presence of either (or neither) motif MFK or REY, 2) block-sharing groups based on sequences sharing position-specific polymorphic blocks (PSPBs). This diagram shows an example DBL α tag sequence, with the PSPBs highlighted in blue, the two cysteines in pink, and the motif MFK belonging to PoLV group 1 in orange.

In total, there are four common classification schemes for *var* genes, and two additional schemes for DBL α tags. In an effort to map the similarities and differences among these various classifications, Githinji & Bull 2017 [6] assessed the relationships between DBL α tag classifications and the features of full-length *var* gene sequences. They showed in detail that tag features and full-length *var* features are mutually related in various ways. Here, we aim to reproduce the results of this paper, provide open Python code for the two approaches the authors have used to classify the DBL α tags (Cys/PoLV and block-sharing group classifications), and reproduce all figures presented in the paper. We also refer to [2, 3] and [10] for more details on methods for DBL α tag and full sequence classifications used in Githinji & Bull [6].

This replication effort is the result of a two-month rotation project for the Interdisciplinary Quantitative Biology program at the University of Colorado Boulder. Replication code is written in Python 3.6.2 and network visualizations were done in webweb (See Code Repository).

II. METHODS & RESULTS

A. DBL α tag classifications

We first explored the two different approaches that the authors have used to classify DBL α tags in previous papers, referred to as Cys/PoLV [3] and block-sharing groups [2]. For both approaches, we obtained the 1548 DBL α sequences from the file “1548_tags.fa” from the authors’ Open Science

Framework (OSF) storage [?] under “datasets.”

1. Cys/PoLV classification

[DM: Bull et al. 2007 observed that different tags have different number of cysteines (with 2 and 4 being the most frequent counts), indicating that tags may be classified into separately recombining groups. Further investigation showed that there were two mutually-exclusive motifs (MFK and REY) that may be present or absent in DBL α sequences, reinforcing the idea that the tags may be divided into intra-mixing but not inter-mixing groups. [3]]

Thus, the Cys/PoLV approach, described in detail in [3], involves extracting two features from each tag: 1) the number of cysteines and 2) motifs located at positions of limited variability (PoLV) – in particular, the presence or absence of mutually exclusive motifs MFK at PoLV1 and REY at PoLV2. As seen in Figure 2A, cys2 and cys4 groups have the most DBL α sequences, explaining the rationale behind the use of the two cys groups as the main groups for the Cys/PoLV classifications. The sequences are further grouped into six Cys/PoLV groups based on the [3]’s definitions (an asterisk denotes any amino acid):

- Group 1: cys2, MFK* motif present at PoLV1
- Group 2: cys2, *REY motif present at PoLV2
- Group 3: cys2, not in groups 1, 2
- Group 4: cys4, not group 5
- Group 5: cys4, *REY motif present at PoLV2

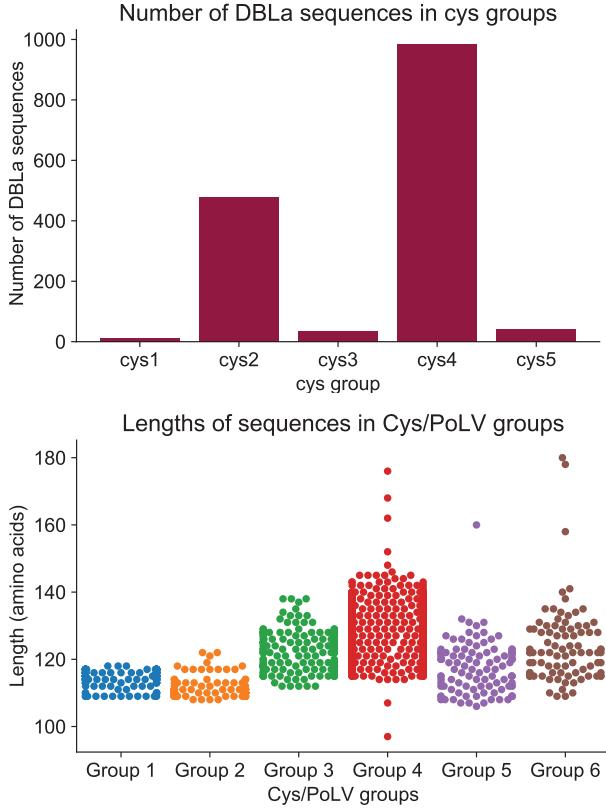


FIG. 2. Analysis of the Cys/PoLV classification of DBL α tags.
(A) Number of DBL α sequences in each Cys group. **(B)** Comparisons of sequence lengths (based on number of amino acids) of DBL α sequences in different Cys/PoLV groups.

Group 6: cys1, 3, 5, or >5

Bull et al. 2007 [3] hypothesized that groups of genetically isolated sequences that do not recombine with other groups maintain distinct distributions in sequence length. If the Cys/PoLV grouping based on some sequence similarities is accurate, the sequences in each group should have similar lengths. As expected, we confirm this finding that the lengths of the sequences are similar within groups, and that groups follow a similar distribution of lengths (Figure 2B).

2. Block-sharing network& classification

While Cys/PoLV groupings classify sequences based on features of individual sequences, the BS network approach classifies sequences based on their relationships [2]. For each sequence, we identify four polymorphic blocks at fixed locations based on three conserved anchor points which are annotated in Figure 3 (similar to [2] Figure 1B): D at the beginning, WW (or W followed by another amino acid) in the middle, and R at the end of the sequence. Each 10-amino acid (aa) block is a “position-specific polymorphic block” (PSPB).

These PSPBs are then used to construct a “block-sharing network” structure, in which each node represents a sequence, and two nodes are linked if their corresponding sequences match at one or more PSPBs. Edges are not weighted in regard to number of shared PSPBs. The network structure is shown in Figure 4, in which we can observe that the network has two prominent lobes: a large one in the center and a smaller one on the right of large lobe [2] [6]. When only links of 14-aa or more are considered, the network of DBL α tags from Kenyan children [2] fragments into two large components, which have been annotated as block-sharing group 1 (BS1) and 2 (BS2), shown in Figure ??.

From the perspective of reproducibility, we note that partitioning the sequences into BS1 and BS2 was challenging. Figure 4C from Bull et al. 2008 [2] shows the network components obtained by using 14-aa long PSPBs, giving seven block-sharing groups, of which the two most prominent ones (BS1 and BS2) are used for sequence classifications in Githinji & Bull. We could not find more details on how to identify the seven BS groups, so we followed the Perl script (“mmi0068-1519-SD3.pl”) from [2] to assign sequences to BS1, BS2, or neither. In this way, the block-sharing groups are hard-coded within the Perl script, but cannot be reproduced *de novo*.

B. Full-length *var* gene sequence classifications

In Githinji & Bull [6], the authors obtained full-length *var* genes classifications from the literature, notably [10]. These classifications include: 33 DBL α subdomains (DBL α 0.1-0.24, DBL α 1.1-1.8 and DBL α 2), 5 UPS groups (A-E), 628 homology blocks (HB), and 23 domain cassettes (DC). Some of these classes have been associated with severe malaria and are further discussed below.

C. Figures: Relationships between DBL α tags and full-length *var* sequences

Below is our reproduction of the figures in Githinji & Bull in the same order as the paper. Because we’ve confirmed above that the Cys/PoLV and block-sharing classifications were successfully reproduced, for the visualizations below, we use the data from Githinji & Bull file “curated_data_set.csv” (also on OSF Storage under “datasets”) because it also includes full-length *var* gene classifications from [10] and other sources that we otherwise do not have access to.

I. Bar graphs

The bar graphs provide a straightforward visualization of the relationship between different *var* gene classifications (upstream promoter sequence (UPS), Cys/PoLV, BS, and HB

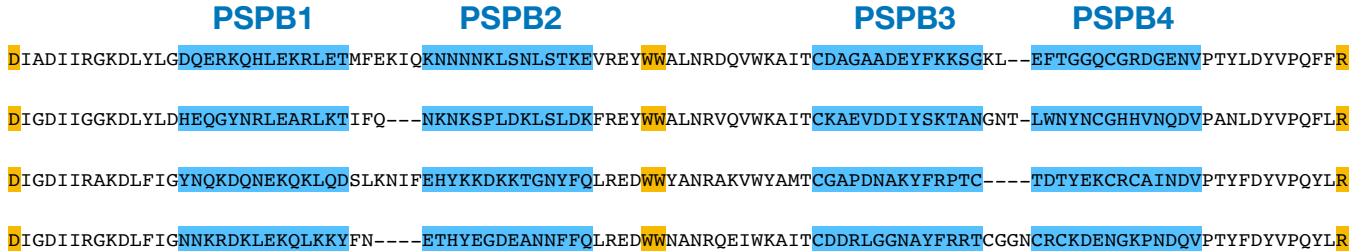


FIG. 3. **Position specific polymorphic blocks (PSPBs).** Four polymorphic blocks (purple) for four example tag sequences at fixed locations based on three conserved anchor points shaded in orange.

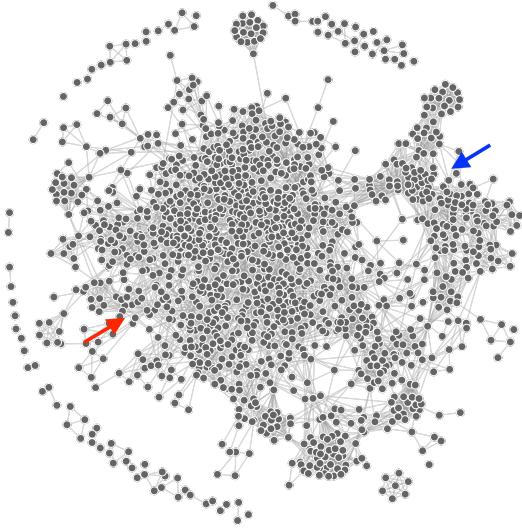


FIG. 4. **Block-sharing network.** The network is constructed based on DBL α tag sequences matching at one or more 10 amino acid PSPBs. Each sequence is represented by a node. Nodes that share PSPBs are linked by edges and are in the same region of the network. Two main lobes are observed: a large one in the center (pointed to by red arrow) and a smaller one right of large lobe (blue arrow). Nodes with few or no connections are placed at the perimeter of the network.

and the specific DBL α domains, CIDR1 domains, and domain cassettes (DCs) contained in the sequences. We use the same color scheme and arrangement of information (in decreasing UPSA order) as the authors did, for easy comparison. Overall, the bar graphs below (Figures 5, 6, and 7) are identical to those in Githinji & Bull Figure 1-3. As seen across the 3 figures, BS1 sequences are closely associated with UPSA, while BS2 sequences with UPSB and UPSC. Most cys2 sequences (CP groups 1-3) are found in UPSA sequences, but some are also found in UPSB and UPSC. Furthermore, DC8 cassettes, which are associated with severe malaria [9, 10], tend to contain CP groups 2, 3, and 4 as well as most of the BS2 tags. This is consistent with the clinical finding of DC8-like sequences in two severe cases of malaria in Kenya [1]. Although this is based on limited information, as Githinji & Bull suggests, these findings may imply that

var genes sampled from Africa may commonly share BS2 sequences.

2. Network visualizations

Built on the analysis in Bull et al. 2008 [2], the network visualizations in Githinji & Bull Figures 4 and 5 provide information on how specific subsets of full-length *var* sequences are mapped onto the network based on the sharing of PSPBs by the DBL α tags. In Figure ??, we show our network analyses of several classifications: Cys/PoLV, block-sharing groups, UPS, DC (4,5,8,13), predicted EPCR binding, and CD36-binding. The clustering tendencies of the tag sequences in our networks are similar to those in Githinji & Bull Figure 4. Consistent with the bar graph analysis, DC8 sequences occupy the same region of the network as UPSB and UPSC sequences.

Figure 8 shows the analysis of the DBL α tags from *var* genes with DC8 cassettes. We are able to reproduce the largest connected component (star-shaped), the three groups outside of this largest component, and the isolated nodes/sequences shown in Githinji & Bull Figure 5. We also show the same results for the block-sharing group classification of each sequence, color-coded in the figure.

3. Receiver operator characteristic curves

When evaluating the quality of a parameterized prediction scheme, a common approach is to plot the relationship between sensitivity (false positives) and specificity (true positives). These curves, called receiver operator curves (or ROC curves [sic]), were used in Githinji & Bull Figure 6 to illustrate how three DBL α tag classifications (cys2, cys2bs1, cys2bs_Cp1) predict four *var* gene features (UPSA [13], DC8 [9] [10], DC13 [13], CIDR α 1 [11]) which have been associated with malaria severity in previous papers. Our ROC curves in Figure ??A, C, D are similar to those in Githinji & Bull Figure 6A, C, D. As the authors noted, CIDR α 1 domains, which are associated with severe malaria due to binding to EPCR [11], are associated with “group A-like sequences” (cys2bs1). Previous reports have also shown as-

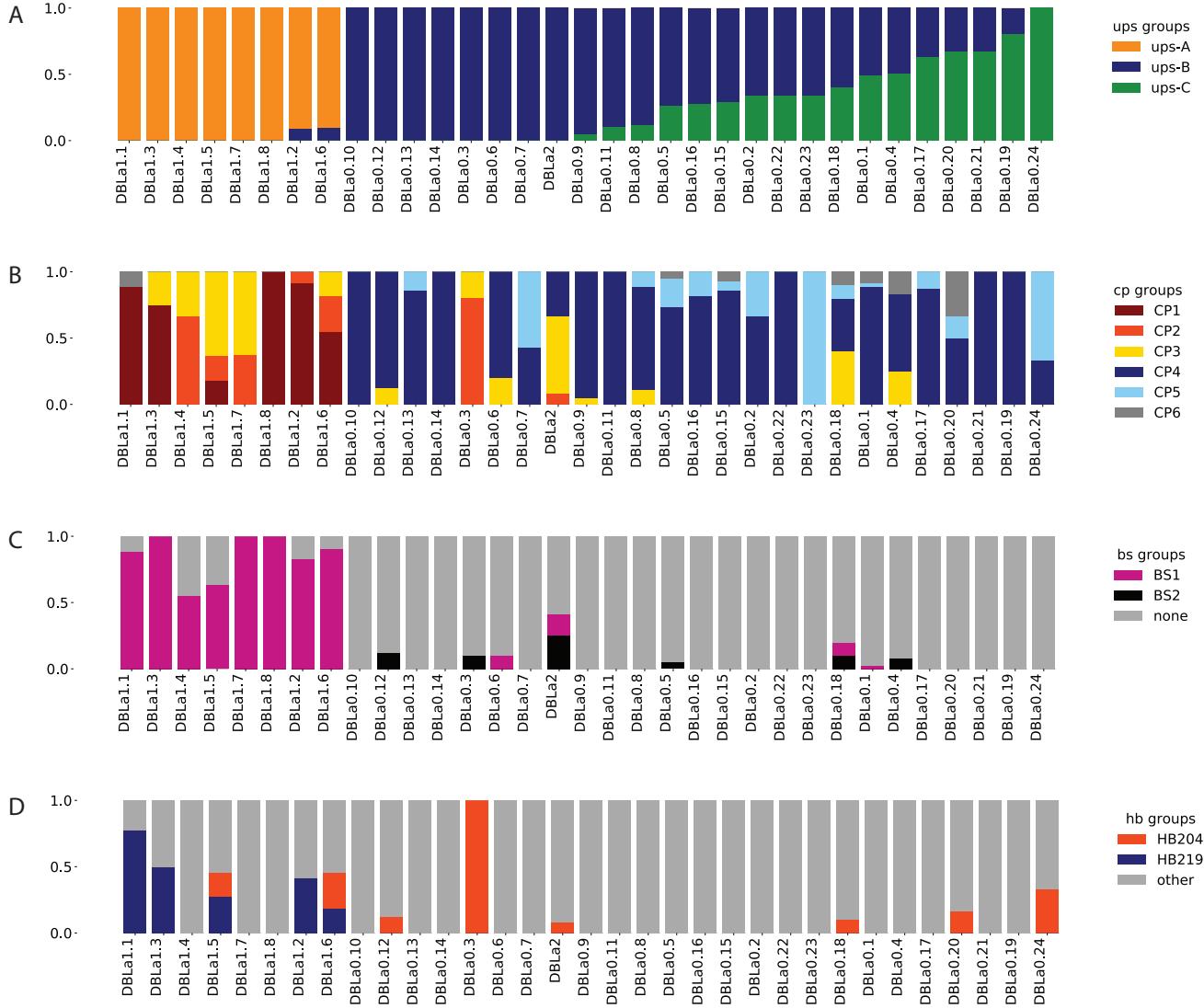


FIG. 5. Correspondence between *var* sequence classifications and presence of specific DBL α domains. *var* sequences are classified based on DBL α domains (horizontal axis) they contain. The proportion of the genes carrying other sequence features (UPS, Cys/PoLV, block-sharing groups, select homology blocks) is shown on the vertical axis. Like in Githinji & Bull, the DBL α domains are, from left to right, in order of decreasing UPSA sequences.

sociations between subsets of cys2 sequence tags and DC8 and DC13 *var* genes with severe disease phenotypes [13]. We reproduce this relationship between cys2 and DC13 in Figure ??C. However, our ROC curves show higher sensitivity for predicting DC8 from cys2, and both lower sensitivity and lower specificity for predicting DC8 from cys2bs1 and cys2bs1_CP1 such that these two curves are below the diagonal of the ROC space. (Githinji & Bull Figure ??B shows the ROC curves for the prediction of DC8 from cys2bs1 and cys2bs1_CP1 as roughly lying on the 45° diagonal.) Together with our results, it seems that these two tag classifications are not highly accurate in providing prediction of the DC8 cassettes of *var* genes.

III. CONCLUSION

In summary, we have studied and reproduced the methods and results in Githinji & Bull, which brings together previous papers to present an analysis of the correspondence between the biologically complex full-length *var* genes' features and one of their domains, the DBL α tags. This analysis shows that despite their diversity, DBL α tag classification can partially predict the features of the full-length *var* genes. Being able to predict the features that are associated with severe malaria is clinically valuable, especially when sequencing the hyper-variable *var* genes is challenging but DBL α tags are more accessible.

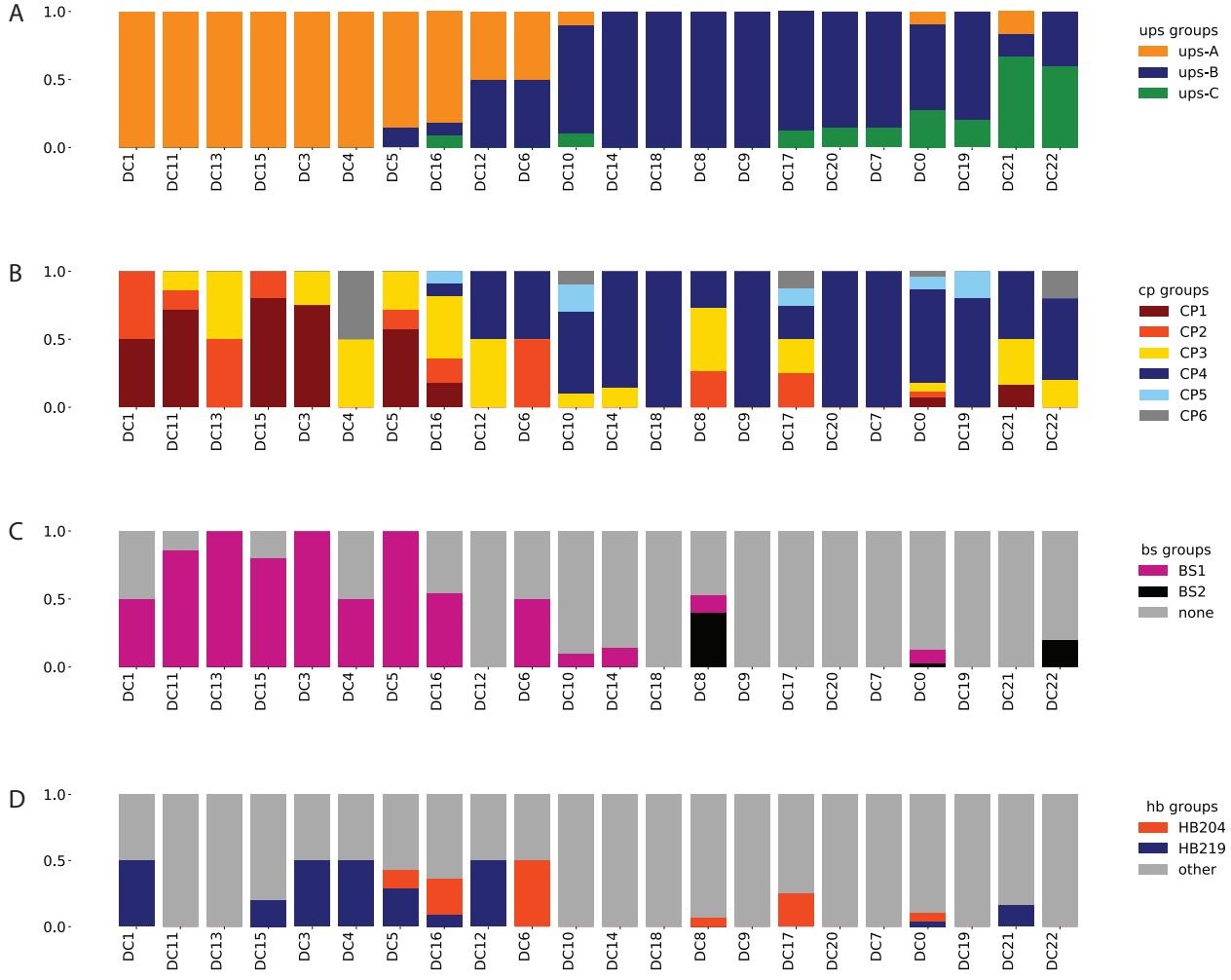


FIG. 6. Correspondence between *var* sequence classifications and presence of specific domain cassettes (DCs). *var* sequences are classified based on DCs (horizontal axis) they contain. The proportion of the genes carrying other sequence features (UPS, Cys/PoLV, block-sharing groups, select homology blocks) is shown on the vertical axis. Like in Githinji & Bull, the DCs are, from left to right, in order of decreasing UPSA sequences.

The figures and methods described in Githinji & Bull are clear and easily understood, making the paper almost completely reproducible, except for a minor difference in the ROC curves discussed in section C above. The open datasets and authors' code provide us a convenient way to access and use the same datasets in our replication and to compare our results. Reproducing this work has been a productive experience to learn the biology of malaria as well as the analysis methods and findings this community of researchers. This paper also opens future directions for continuous exploration of the DBL α tags as a predictor of functional features of full-

length *var* gene sequences, especially with the Sanger Institute releasing more *P. falciparum* whole genomes in the near future.

Code Repository:

All Python code and relevant datasets are available at: https://github.com/dieumynguyen/githinji_vargenes. Webweb network creator is available at: <http://danlarremore.com/webweb/>

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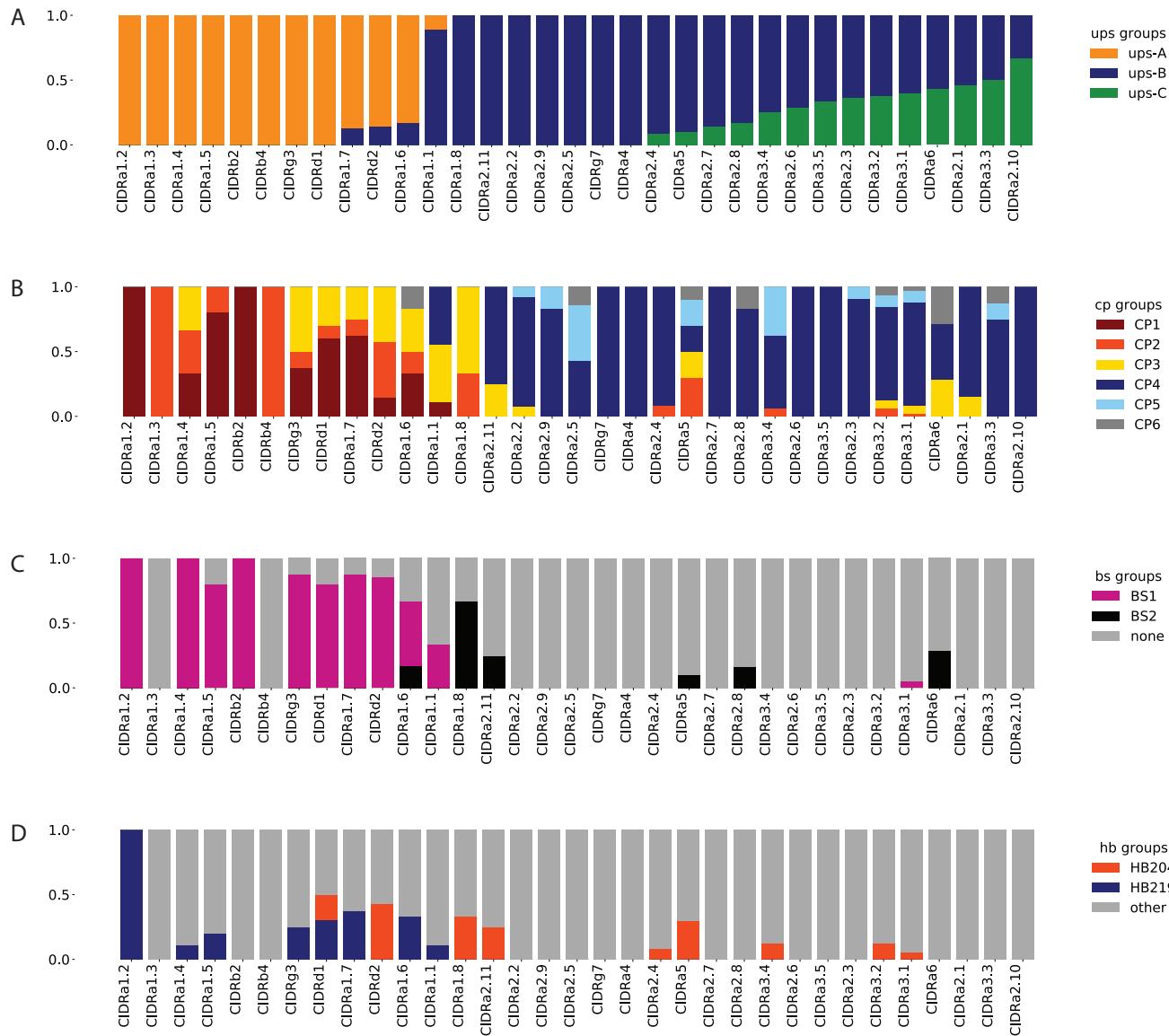


FIG. 7. Correspondence between var sequence classifications and presence of specific CIDR1 domains. var sequences are classified based on CIDR1 domains (horizontal axis) they contain. The proportion of the genes carrying other sequence features (UPS, Cys/PoLV, block-sharing groups, select homology blocks) is shown on the vertical axis. Like in Githinji & Bull, the CIDR1 domains are, from left to right, in order of decreasing UPSA sequences.

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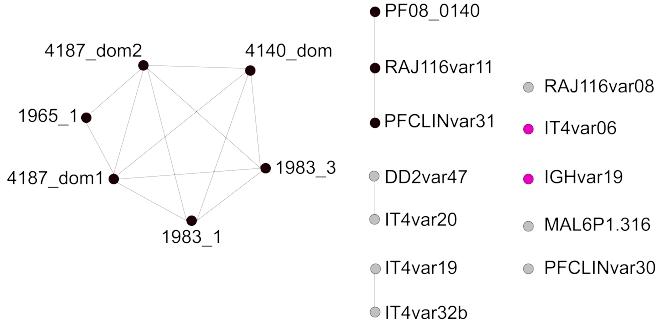


FIG. 8. Network analysis of DBL α tag sequences from known DC8 var genes. Visualization created with Gephi 0.9.2. Node colors: grey = not in a BS group; pink = BS1; black = BS2.

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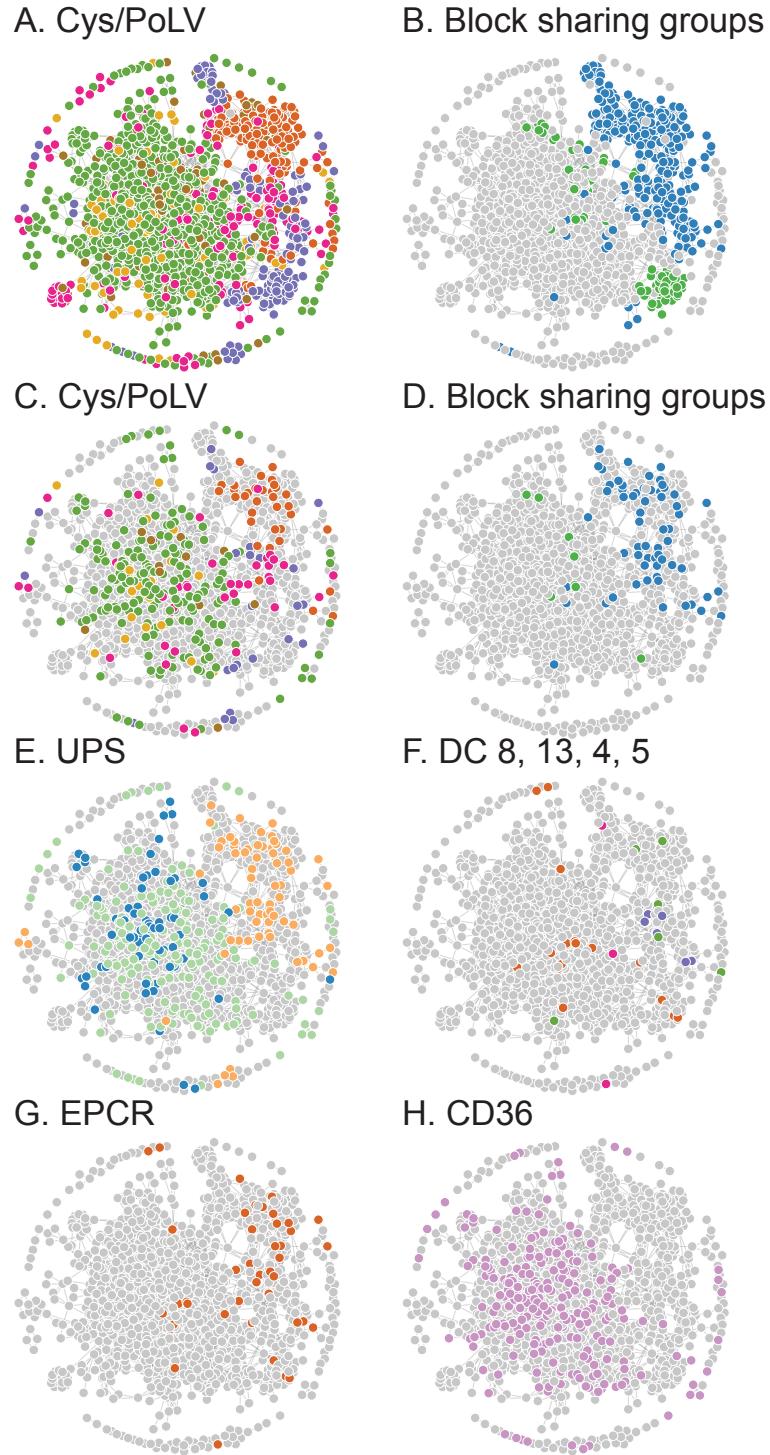


FIG. 9. Various DBL α tag classifications mapped onto the block-sharing network. (A) Cys/PoLV analysis for all sequences; (B) BS analysis for all sequences; (C) UPS grouping; (D) Cys/PoLV analysis for full length *var* gene sequences from 6 laboratory isolates; (E) BS analysis for full length *var* gene sequences from 6 laboratory isolates; (F) domain cassette (DC) classification for DC4, DC5, DC8 and DC13; (G) predicted EPCR-binding phenotype due to CIDR α 1.1, CIDR α 1.4, CIDR α 1.5, CIDR α 1.6, CIDR α 1.7 or CIDR α 1.8 (Lau et al., 2015) for sequences with CIDR α information available; (H) predicted CD36-binding phenotype due to CIDR α 2, CIDR α 3, CIDR α 4, CIDR α 5 (Robinson et al., 2003) for sequences with CIDR α information available. Node colors: For all, unclassified = 0. (A&D) red = CP1, purple = CP2, pink = CP3, green = CP4, yellow = CP5, brown = CP6. (B& E) blue = BS1, green = BS2; (C) UPSA = orange, green = UPSB, blue = UPSC; (F) pink = DC8, purple = DC5, green = DC13, orange = DC4; (G) orange = predicted EPCR binding; H) purple = predicted CD36 binding.

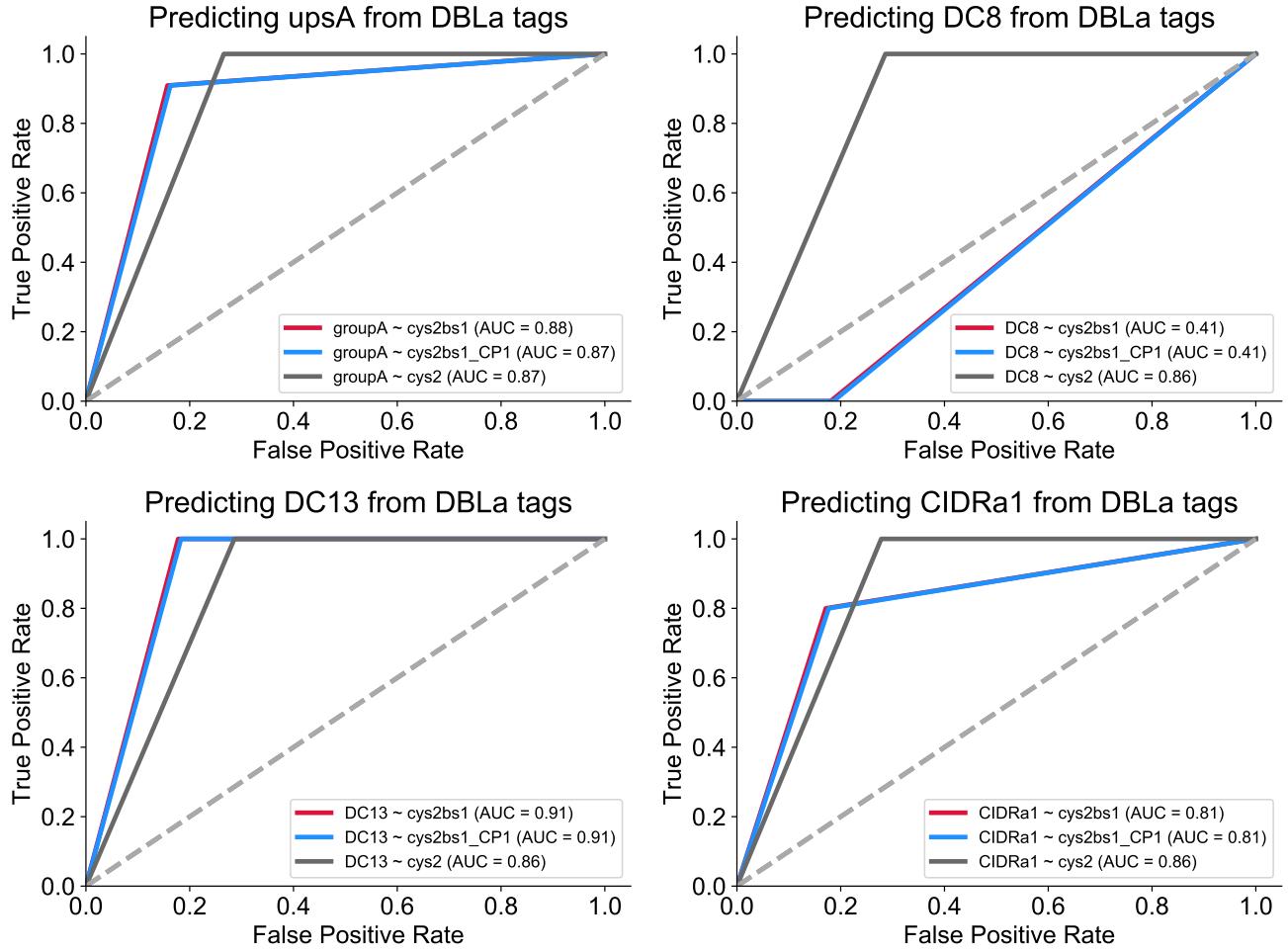


FIG. 10. Receiver operator curves showing the sensitivity (true positive rate) and specificity (false positive rate) of three DBL α tag classifications (cys2, cys2bs1, cys2bs1_CP1) in predicting four *var* gene features associated with malaria severity: UPSA, DC8, DC13, CIDR α 1. Sequences from the genomes 3D7 and IT4 were excluded because they were used in developing the BS classification. cys2 = two cysteines within the tag region; cys2bs1 = tag sequences in block-sharing group1 AND have two cysteines, defined as “group A-like”; cys2bs1_CP1 = cys2bs1 OR in Cys/PoLV group 1.