A Tale of Two Parasites: A Glimpse into the clinical RNA Methylome of *P. falciparum* and *P. vivax* isolates

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ABSTRACT

Malaria continues to remain a global health burden. Understanding the molecular mechanisms governing these parasites' survival is crucial for developing effective interventions. Advances in RNA modification research have unveiled the significance of RNA methylation in gene regulation and pathogenesis. This is the first study to investigate the methylation pattern up to single-base resolution across RNA in clinical samples of *P. falciparum* and *P. vivax* with hepatic dysfunction complications using Oxford Nanopore Direct RNA Sequencing. Preliminary findings indicate distinct methylation profiles in *P. falciparum* and *P. vivax*, suggesting potential species-specific regulatory mechanisms. These methylation modifications are unevenly distributed across the mRNA's 3'UTR, CDS, and 5' UTR, which may influence mRNA export and translation initiation/repression of these primary transcripts. Furthermore, the data provides a glimpse into methylation events in Natural Antisense Transcripts (NATs). The correlation of these layers of regulation will facilitate a comprehensive understanding of the transcriptional environment of malaria parasites.

KEYWORDS: RNA methylation, *Plasmodium falciparum*, *Plasmodium vivax*, Oxford Nanopore

INTRODUCTION

Malaria remains the most prevalent mosquito-transmitted infectious ailment caused by the protozoan parasites of the *Plasmodium* species. The global disease burden, as per the World Malaria Report of WHO, accounts for 249 million cases in 2022 [1]. Despite efforts to stabilize deaths and disease fatality, they remain at an unacceptably high-rate post-pandemic. Clinical manifestations of malaria are non-specific and include fever, chills, headache, myalgias, and fatigue. However, if not treated promptly, it may also lead to a severe disease state manifesting as jaundice, seizures, renal failure, acute respiratory distress syndrome, and coma. Two of the five parasites – *Plasmodium falciparum* and *Plasmodium vivax*, are especially responsible for most of the disease burden. While the rapid progression of case morbidity in *P. falciparum* malaria has been well-documented, an increasing number

of reports of severe clinical complications caused by *P. vivax* infections have surfaced, with the recent bouts of *P. vivax* resurgence in the US [2], which had been certified malaria-free since the 1970s. We published the first detailed report of severe *P. vivax* infection from India validated by diagnostic PCR [3-5].

The life cycle of the *Plasmodium* parasite alternates between an exogenous sexual phase in the invertebrate host (female *Anopheline*) and an endogenous asexual phase in the vertebrate host – human. In the human host, the parasite undergoes schizogony by invading RBCs, host cell remodeling, genome replication, and RBC egress. Each step of this cascade involves a myriad of gene expressions to help the parasite survive in the host cell. While the parasite has been reported to be in a transcriptionally silent stage during RBC invasion [6], transcriptomic reports from field and laboratory strains have shown differential expression of various invasion genes in parasites to survive through schizogony. Several studies have shown that the involvement of long non-coding RNAs and natural antisense transcripts (NATs) are also crucial in mediating gene expression and influencing the development of parasites within human hosts [7-9]. In addition to mRNA abundance and differential gene expression, epitranscriptomic studies in recent years have highlighted certain parasite-specific post-transcriptional modifications in *P. falciparum* as an additional layer of gene regulation [10]. Cassandra C. et al. have further reviewed the role of chemical modifications in RNA in post-transcriptional gene regulation in *Plasmodium* sp. [11].

This study elaborates on the RNA-specific methylation signatures expressed in malaria manifesting as hepatic dysfunction from *Plasmodium vivax* and *Plasmodium falciparum* patient isolates. Since the genome of *P. falciparum* is extraordinarily abundant in adenine and thymine bases, with the average genome-wide AT content being 81% and often reaching 90–95% in intergenic regions and that of *Plasmodium vivax* is GC-rich to >50% [12-13], the study elaborates on the m5C and m6A chemical modifications of RNA in both sense and antisense transcripts, to single base resolution and presents a comparative between the two parasites.

METHODS

Sample Collection and processing

Venous blood samples (5 ml) from patients infected with malaria were collected from S.P. Medical College, Bikaner, after the informed consent of the patients. Malaria infection with *P. vivax* and *P. falciparum* was confirmed with RDTs (OptiMAL, Flow Inc., Portland, Oreg.) and microscopic investigation of peripheral blood films. The blood samples were separated on a Histopaque-gradient-based density separation (Histopaque 1077, Sigma Aldrich) to separate the erythrocytes from plasma and peripheral blood mononuclear cells (PBMCs). The erythrocytes were washed with sterile PBS, lysed with TRI Reagent (T9424, Sigma-Aldrich), and transported in a cold chain to BITS Pilani for further processing.

Total RNA was isolated from infected erythrocytes according to the manufacturer's protocol (Tri-Reagent, Sigma Aldrich) and resuspended in nuclease-free water. Molecular confirmation of infection with *P. vivax* or *P. falciparum* was done using 18S rRNA gene-based multiplex PCR and 28S rRNA gene-based nested PCR [14-15], and any possibility of

mixed infection was negated. Viral or other causes of hepatic dysfunction were ruled out at the point of collection. This study used one sample each of *P. vivax* and *P. falciparum*. The patient's diagnosis was confirmed as *hepatic dysfunction* as per WHO guidelines [16] and upon our team of clinicians' consultation. The clinical characteristics of the sample are tabulated in Table S1.

Library Preparation

The concentration and purity of the isolated total RNA were measured by the NanoDrop UV/Vis spectrophotometer (SimpliNano™ spectrophotometer, GEHealthcare) and Qubit 4 Fluorometer (Qubit RNA HS Assay kit (Q32855), Invitrogen). The quality of the isolated total RNA was analyzed by denaturing agarose gel. One µg of total RNA was used for library preparation as per the manufacturer's protocol (SQK-RNA002, Oxford Nanopore Technologies), including the optional reverse transcription step recommended by ONT. ~20 ng of reverse transcribed and adapter-ligated RNA was used for loading on Spot-ON Flow Cell (R9) and sequenced on MinION sequencer (FLO-MN106D, Oxford Nanopore Technologies). Each of the two samples was sequenced on individual flow cell, each with repeated loading of 20 ng of the final library until exhaustion of pores, which gave a run time >=24 hours.

Downstream bioinformatics analysis

Reads generated at the end of the sequencing procedure were basecalled using the base caller integrated into the MinKNOW software (version 23.07.15). After base calling, the reads categorized in the "pass" folder were concatenated and used for downstream analysis. ONT sequencing data was checked for quality using FastQCv0.12.1. The reads were indexed with Nanopolishv0.14.0 (https://github.com/jts/nanopolish) using preset commands. The reads were aligned with Minimap2v2.24 using the reference transcriptome *P. falciparum* 3D7 (PlasmoDBv64) and *P. vivax* Sal 1 (PlasmoDBv63) for the samples, PFC and PVC, respectively. For the alignment of antisense transcripts, the aforementioned reference transcriptomes were reverse complemented and used for read alignment. The mapped reads for sense and antisense transcripts were visualized in Integrative Genomics Viewer (IGV, https://www.igv.org/). Methylation calling in both sense and antisense transcripts was done by m6anet (https://github.com/GoekeLab/m6anet) for m6A modification calls and CHEUI: Methylation (CH3) Estimation Using Ionic current (https://github.com/comprna/CHEUI) for both m5C and m6A modification calls [17-18]. The concordant modification calls from the two tools were considered for further analysis. The analysis considered only the modified calls with a read_coverage of ≥20 and modified_probability >=0.9 to maintain data stringency. To increase the stringency of the analysis for the *P. falciparum* sample, the *modified probability* score was further increased to ≥0.999. The gene that satisfied the above two criteria for modified bases was used for GO enrichment analysis by DAVID (https://david.ncifcrf.gov/) with a *p-value* cut-off (EASE score) <0.05. Transcripts showing methylation of a base with the above-mentioned confidence were mapped to their position on the individual chromosome, and the modified base was mapped to genomic features using the annotation file of the reference genomes.

RESULTS

Total RNA from two enriched, PCR-validated severe malaria samples was extracted as detailed. (one each for *P. vivax* and *P. falciparum*). After a quality and integrity check, the RNA was used for library preparation for MinION sequencing. In the case of *P. vivax*, we obtained 672k reads, while for *P. falciparum*, we obtained 207k reads (Fig S1). Only the basecalled reads above Q>7 (as recommended by Guppy) was taken into further analysis. The total processed reads were 169464 for *P. falciparum* and 560535 for *P. vivax*, respectively. Consistently good-quality processed reads were observed in FASTQC analysis across the entire length of the reads, with a mean quality score of 15.7 for PFC and 15.8 for the PVC sample. A detailed summary of the read statistics is tabulated in Table S2.

Minimap2 was used to map PFC reads to the genome and the transcriptome of *P. falciparum 3D7* strain (PlasmoDBv64), and PVC reads to the genome and the transcriptome of *P. vivax Sal 1* strain (PlasmoDBv63), respectively. Mapping of the PFC reads with the *P. falciparum 3D7* genome gave a mean depth of 3.664x, while that of PVC reads with that of the *P. vivax Sal 1* genome was 0.9x. The longest mapped read length for PFC was ~5.2 kb, while that for PVC was ~3.5 kb. This exemplifies the potency of long-read sequencing technology, performed for the first time in malaria patient-derived RNA samples at the time of writing.

Methylation calling for mapped reads, using m6anet for m6A modified calls and CHEUI for both m6A and m5C modified calls revealed methylated RNA bases in both sense and antisense transcripts (NATs) to single base resolution. All the modification calls for m6A predicted by m6anet were covered in the modification calls predicted by CHEUI. Hence, we are reporting both tools' combined and concordant modification calls for m6A methylation. Only the transcripts with high-confidence methylation probability are considered for further analysis. The total number of transcripts predicted to be modified in both samples is summarized in Table S3.

Methylation modifications, both m5C and m6A, have been reported in mRNA, rRNA, ncRNA, and pseudogenic transcript types. While no transcript has m5C modification in both sense and antisense transcripts in both PFC and PVC samples, three transcripts show m6A modification in both their sense and their antisense complements in the PFC dataset (Table 1).

An overlap of the common transcripts considering both the m5C and m6A variables is depicted in Fig 1. Mapping of the genes showing RNA modifications to individual chromosomes of *P. falciparum* for PFC (Figure S2a-b) and *P. vivax* for PVC (Figure S2c-f) data has revealed no specific distribution of methylation modifications favoring any particular chromosome. In fact, some m6A and m5C modifications also lie in the sense transcripts encoded by the mitochondrial genome in both PFC and PVC and by the apicoplast genome in PFC. These transcripts are enlisted with their function in Table S4.

Further mapping of methylated bases in both sense and antisense transcripts to the genomic features revealed the positions of the modified base lying in 5’UTR, CDS, and 3’UTR

annotated regions of these transcripts. The methylated bases mapped to the exons for the genes with the modification lying in the rRNA transcripts, possibly due to a lack of annotated UTRs for these transcripts. Methylation points in antisense transcripts are hypothetically correlated with the sense mRNA regions, considering antisense to be a reverse complement of sense mRNA. Most of the methylation events of mRNA transcripts in the PVC dataset are mapped to CDS, and very few are in 3'UTR or 5'UTR, as depicted in Figure S3(a-d).

The total distribution of each of the methylated sense transcripts across 5'UTR, CDS, and 3'UTR regions of the mRNA is shown by the Venn diagram in Fig 2 and Fig 3. Only two transcripts in the PFC dataset, PF3D7_0202500 and PF3D7_1301200, have a m5C methylated base at more than one region of the mRNA, as depicted in Figure 2a-b. m5C methylation marks in the rest of all detected transcripts in PFC are distributed across only one annotated region of the mRNA. However, this is not true for the m6A methylation marks, which are highly abundant in comparison to m5C, owing to the parasite's A-T-rich genome (Fig 3a). In the PVC dataset, none of the m6A methylated transcripts are found to have methylation marks in more than one of the annotated mRNA regions (Fig 3c), whereas ten transcripts have shown distribution of m5C methylation marks in more than one annotated region of mRNA as shown in Figure 2c-d.

The Gene Ontology Enrichment analysis for the methylated genes for m5C and m6A modifications mapped to the following GO terms common between both datasets – structural molecule function, ribonucleoprotein, and structural constituent of ribosome. The GO terms for cellular components for m5C-modified genes are ribosome and cytosol. Some of the significant GO terms for the biological process for m5C modified genes are cellular protein metabolic process, translation, and organonitrogen compound biosynthetic process, detailed in Fig 4. Apart from this, some individual GO terms identified separately in PFC and PVC datasets are depicted in Figures S4, S5(a-b).

Further, in both the datasets, the enrichment of genes in the KEGG pathway revealed – 'Ribosome (pfa03010/pvx03010),' which was found with the genes associated with the biological process of translation in m5C methylated transcripts. Among the significant KEGG pathways identified in the PFC (m6A) dataset are - pfa00010:Glycolysis/Gluconeogenesis, pfa01230:Biosynthesis of amino acids, and pfa01200:Carbon metabolism associated with biological process terms like – hexose biosynthesis, synthesis of amino acid nucleotide phosphorylation which may suggest activation of pathways involved in cellular energy production and nucleotide metabolism. This suggests that m6A modifications in *P. falciparum* might alter the metabolic adaptations in the parasite in response to its' host cell environment.

The gene enrichment analysis of methylated natural antisense transcripts of the PFC dataset identified only one biological process - GO:0051383~kinetochore organization. This may suggest the involvement of these methylated genes in cellular proliferation dynamics, providing valuable insights into the molecular mechanisms underlying cell division in *P. falciparum*.

We further identified orthologs of methylated transcripts across *P. falciparum* and *P. vivax* data, revealing conserved epigenetic patterns. Two pairs of orthologous genes PVX_084190.1 and PF3D7_1202900.1, PVX_123845.1 and PF3D7_1224300.1 display m6A modifications in sense transcripts, encoding for High Mobility Group protein B1 & Polyadenylate-binding protein 1 respectively (Figure 5). This cross-species analysis hints at the potential evolutionary significance of methylated genes in *Plasmodium*, which may impact translational repression or activation, influencing the pathogenesis of severe malaria [19-20].

DISCUSSION

More than 170 chemical modifications of RNA are known to date. Amongst this, methyl-6-adenosine(m6A), methyl-5-cytosine(m5C), ribose-methylation(2'-O-Me), and pseudo-uridine (C) have been known for over 50 years [21]. m6A is the most abundant internal modification on mRNA, altering RNAs' splicing, translation, stability, and nuclear export. The m6A deposition levels in *P. falciparum* are comparatively greater than those of any other known eukaryotic species and are strictly regulated by methyltransferase writer complex (PfMT-A70) characterized by Baumgarten et. al. and identified by the YTH reader homolog [22-23]. Over 99% of m6A sequencing reads in these studies matched to regions that code for proteins. This is contradictory to what the current study in patient-derived RNA is reporting, with the almost equivalent distribution of m6A modification in the CDS and both 5' and 3' UTR regions of the mRNA. However, the negative correlation between translation efficiency and abundance of CDS m6A reported by Baumgarten et al. cannot be commented upon in this study.

Another interesting observation from this study is the occurrence of both the modifications multiple times over the annotated regions of the mRNA. This target specificity of m6A-methyltransferases and, consequently, m6A stoichiometry (that is, the fraction of a given site that is m6A-modified) might be altered by external determinants (m6A modulators), such as transcription factors and RNA-binding proteins that recruit the methyl transferase complex (MTC) to specific positions within certain transcripts [24]. It is conceivable to assume that m6A stoichiometry influences RNA destiny and, in turn, protein expression and cellular processes [25].

In the PFC dataset, the presence of m6A modification in the mRNA transcripts and its NAT (Table 3) reveals three key transcripts in *P. falciparum*. Of these, the Signal recognition particle subunit SRP68 (PF3D7_0621900.1) is shown to function as a transcriptional factor.[26] This is consistent with the enrichment of the GO term 'gene expression' in the gene ontology of m6A methylated transcripts for PFC (Fig S5b). The Early transcribed membrane protein 11.2 (PF3D7_1102800.1) is known to be widely expressed in the early asexual stages of the parasite at the host-cell interface [27]. Taken together, we hypothesize that this dual methylation pattern in the mRNA and antisense RNA may influence the fate of these two transcripts, thereby affecting the parasite's survival in the host.

Apart from m6A, m5C modification has been identified using bisulfite sequencing as a prevalent mRNA modification that may serve as an RNA epigenetic marker [28]. However, the investigation into the role of m5C in mRNA is not comprehensive. Some intriguing discoveries in gametocytes have linked m5C to transcript stabilization. This alternation is done by NOP2/Sun RNA methyltransferase 2(NSUN2). It has been observed that the gametocytogenesis of the rodent parasite *P. yoelii* is impaired when this protein is knocked out [29]. In the present study, we use Nanopore sequencing to report the first transcriptome-wide characterization of m6A and m5C modifications in clinical isolates of *P. vivax* and *P. falciparum*. We show that m6A and m5C are widespread modifications in sense transcripts, including mitochondria and apicoplast whereas the natural antisense transcripts have shown relatively fewer events for the same. However, the methylated antisense transcripts detected in the data may suggest an added layer of post-transcriptional regulation.

The methylation events in sense transcripts are primarily enriched in the coding regions, followed by 5'UTR and 3'UTR. However, in the case of antisense, the methylated transcripts in CDS, 5'UTR, and 3'UTR are sporadically dispersed. Additionally, we have seen that in *Plasmodium vivax*, there are twenty-three transcripts that contain both m6A and m5C in the sense transcripts and two transcripts that contain both these modifications amongst the NATs. In contrast, in the *Plasmodium* falciparum dataset, fifty-seven sense transcripts show both m6A and m5C modifications and two transcripts show both these modifications in the NATs.

Our investigations show that m6A and m5C are widely distributed and dynamically controlled base modifications in the mRNA of both parasites. Additionally, they pinpoint the mRNAs that are most likely to be impacted by metabolic pathways. Interestingly, ten transcripts are methylated in multiple mRNA regions by our profile of m5C in the *P. vivax* dataset. *The P. falciparum* dataset has two transcripts in m5C and thirty-four transcripts in m6A that showed distinct methylation patterns occurring at multiple annotated regions of mRNA. These may play a role in mRNA stability, translation dynamics, and subcellular localization.

Our study revealed a substantial enrichment in the translational dynamics by providing a thorough GO enrichment analysis of the m6A & m5C modified transcriptome of *P. vivax* and *P. falciparum*, indicating a significant impact of these modifications on the destiny of an mRNA transcript and the protein it encodes.

At the time of writing, this is the first report to comprehensively profile both the m5C and m6A modification distribution in both sense and natural antisense transcripts up to single-base resolution from the clinical transcriptome of *P. falciparum* and *P. vivax*. These modifications may potentially modify mRNA stability and/or repress/activate translation. This balance can serve to modify the result of protein synthesis. Further, we are the first group to identify methylated transcripts encoded from *Plasmodium*'s mitochondria and apicoplast genomes. Our comprehensive analysis elaborates on the post-transcriptional regulation of gene expression in *P. falciparum* and *P. vivax* clinical strains. This work opens wider avenues for the integrating RNA methylation data with proteomics to unlock key

insights into the intricate orchestration of gene expression, offering potential targets for therapeutic strategies against malaria.

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Ethics Approval - Sample collection was earlier approved by SP Medical College Hospital's Ethics Committee (No.F. (Acad)SPMC/2003/2395). Permission to use these samples for further studies was given through IERB approval No. F29(Acad)SPMC/2020/3151 dated 05.09.2020. We thank all the patients for their voluntary consent and participation.

AUTHOR CONTRIBUTIONS

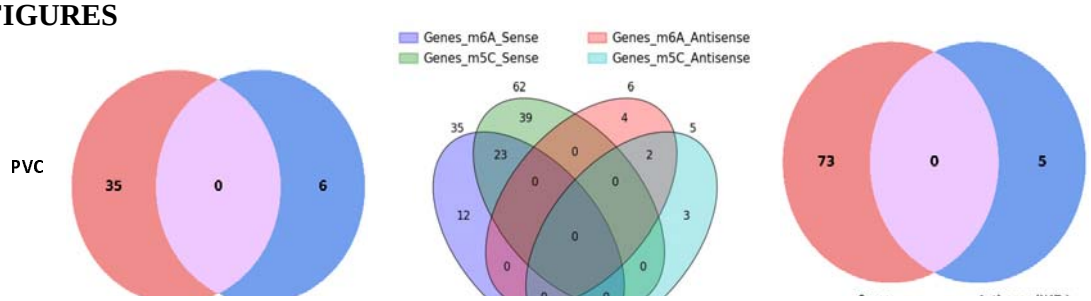
AD conceived the study, organized the material, and networked to facilitate the outcome. SG and PR were involved in performing the sequencing experimentation and subsequent analysis. AD, SG, and PR wrote the manuscript. TM supervised the application of various *in-silico* tools and assisted in troubleshooting when required. DG was involved in consultations on data analysis and related discussions. DKK and SKK supervised sample collection and clinical characterization. All authors read and approved the manuscript.

TABLES

Transcript ID	Gene Description	Region of the methylated base in the mRNA	Region of the methylated base in the NAT
PF3D7_1102800.1	Early transcribed membrane protein 11.2	3’UTR	3’UTR
PF3D7_0621900.1	Signal recognition particle subunit SRP68, putative	CDS	3’UTR
PF3D7_0904000.1	GTPase-activating protein, putative	5’UTR	3’UTR

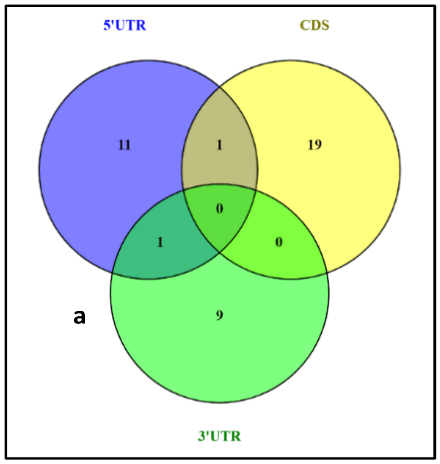
Table 1 Gene description with m6A modification in both sense and natural antisense transcripts in the PFC dataset.

FIGURES



(NATs)

Fig 1 Top - Representation of genes in PVC data (from left to right) - m6A modification in sense and antisense, both m6a and m5C modification in sense and antisense, m5C modification in sense and antisense;
Bottom - Representation of genes in PFC data (from left to right) – m6A modification in sense and antisense, both m6a and m5C modification in sense and antisense, m5C modification in sense and antisense;



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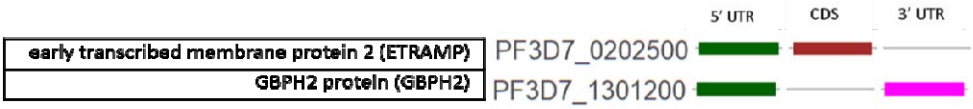

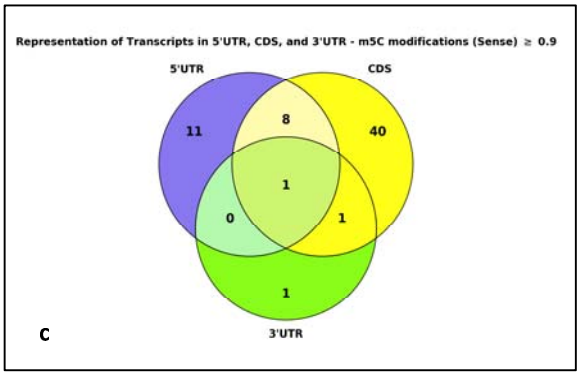

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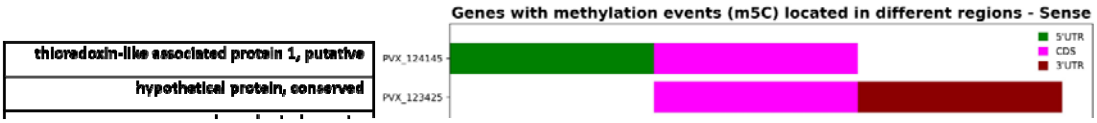

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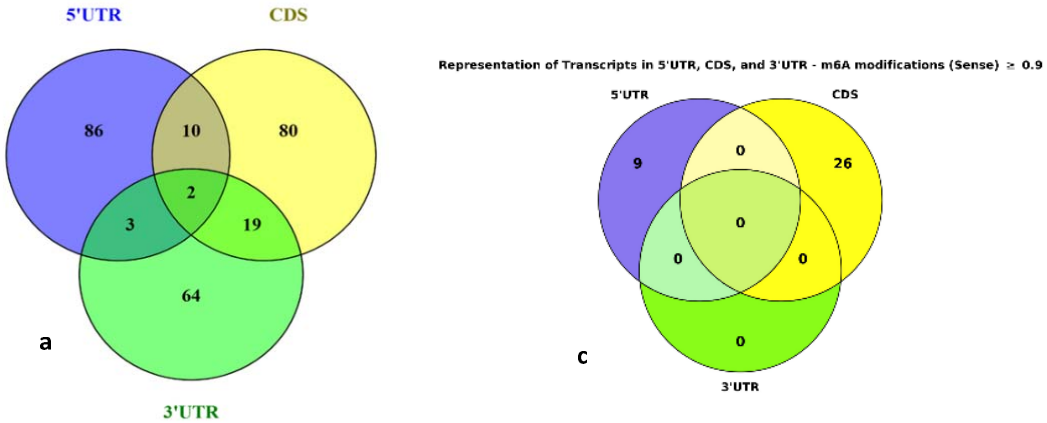

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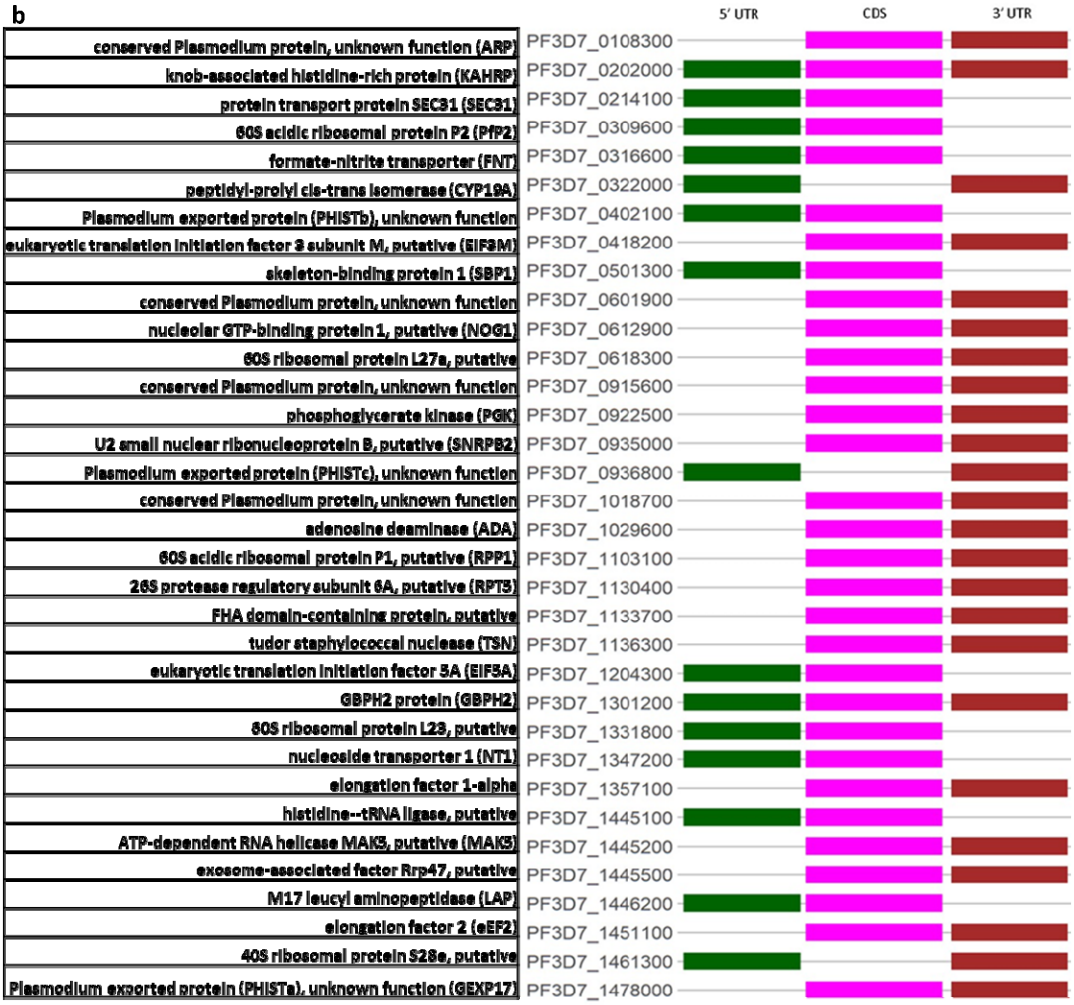

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Fig 2 a. Number of transcripts with methylation events (m6A) located in different annotated features of mRNA in PFC. Transcripts with

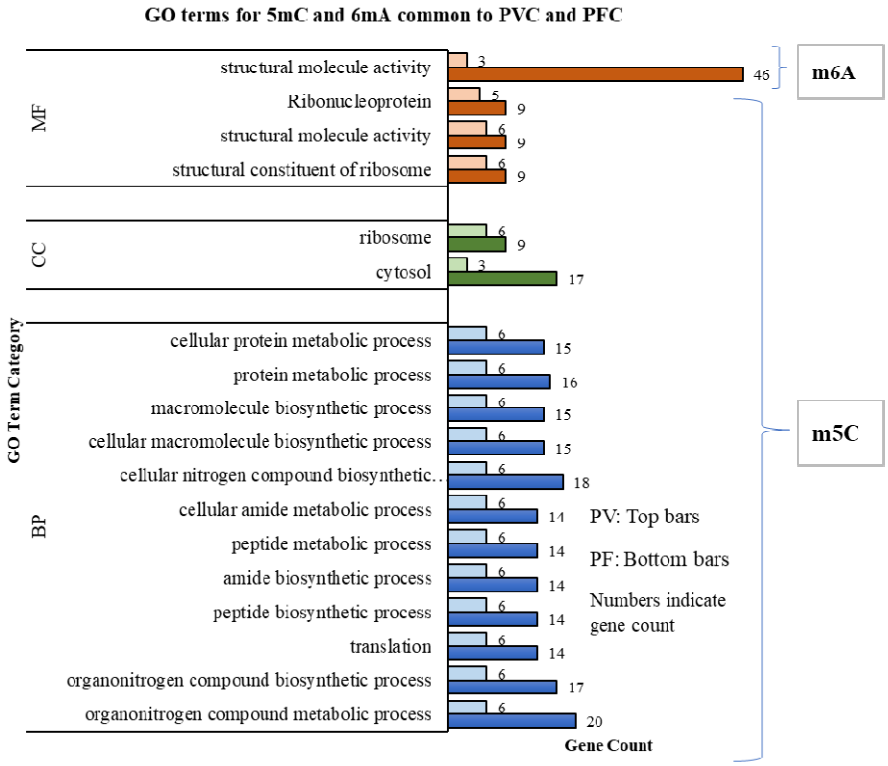

Fig 4 Common GO terms in both PFC and PVC datasets.
BP – Biological Process; CC – Cellular Component; MF – Molecular Function

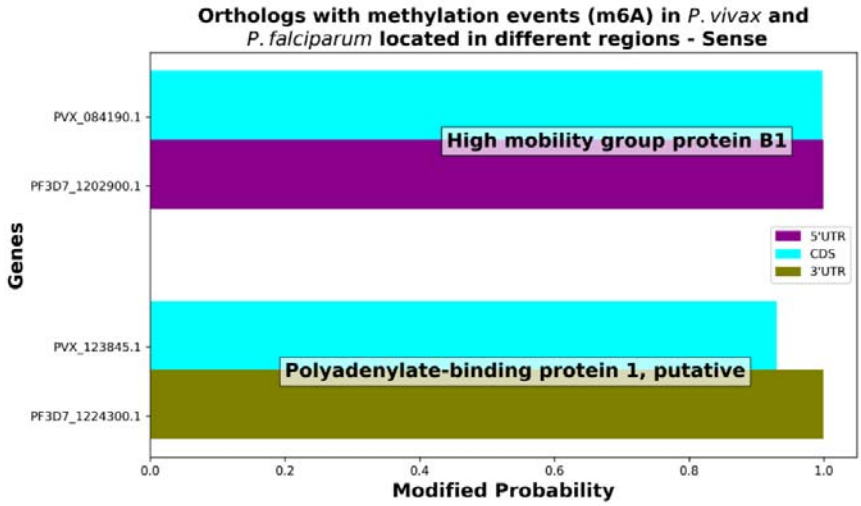

Fig 5 Ortholog details of methylated transcripts across PFC and PVC dataset.

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