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# Prevalence of *Plasmodium falciparum* delayed clearance associated polymorphisms in adaptor protein complex 2 mu subunit (*pfap2mu*) and ubiquitin specific protease 1 (*pfubp1*) genes in Ghanaian isolates

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## Abstract

**Background:** *Plasmodium falciparum* delayed clearance with the use of artemisinin-based combination therapy (ACTs) has been reported in some African countries. Single nucleotide polymorphisms (SNPs) in two genes, *P. falciparum* adaptor protein complex 2 mu subunit (*pfap2mu*) and ubiquitin specific protease 1 (*pfubp1*), have been linked to delayed clearance with ACT use in Kenya and recurrent imported malaria in Britain. With over 12 years of ACT use in Ghana, this study investigated the prevalence of SNPs in the *pfap2mu* and *pfubp1* in Ghanaian clinical *P. falciparum* isolates to provide baseline data for antimalarial drug resistance surveillance in the country.

**Methods:** Filter paper blood blots collected in 2015–2016 from children aged below 9 years presenting with uncomplicated malaria at hospitals in three sentinel sites Begoro, Cape Coast and Navrongo were used. Parasite DNA was extracted from 120 samples followed by nested polymerase chain reaction (nPCR). Sanger sequencing was performed to detect and identify SNPs in *pfap2mu* and *pfubp1* genes.

**Results:** In all, 11.1% (9/81) of the isolates carried the wildtype genotypes for both genes. A total of 164 *pfap2mu* mutations were detected in 67 isolates whilst 271 *pfubp1* mutations were observed in 72 isolates. The majority of the mutations were non-synonymous (NS): 78% (128/164) for *pfap2mu* and 92.3% (250/271) for *pfubp1*. Five unique samples had a total of 215 *pfap2mu* SNPs, ranging between 15 and 63 SNPs per sample. Genotypes reportedly associated with ART resistance detected in this study included *pfap2mu* S160N (7.4%, 6/81) and *pfubp1* E1528D (7.4%, 6/81) as well as D1525E (4.9%, 4/81). There was no significant difference in the prevalence of the SNPs between the three ecologically distinct study sites (*pfap2mu*:  $\chi^2 = 6.905$ ,  $df = 2$ ,  $P = 0.546$ ; *pfubp1*:  $\chi^2 = 4.883$ ,  $df = 2$ ,  $P = 0.769$ ).

**Conclusions:** The detection of *pfap2mu* and *pfubp1* genotypes associated with ACT delayed parasite clearance is evidence of gradual nascent emergence of resistance in Ghana. The results will serve as baseline data for surveillance and the selection of the genotypes with drug pressure over time. The *pfap2mu* S160N, *pfubp1* E1528D and D1525E must be monitored in Ghanaian isolates in ACT susceptibility studies, especially when cure rates of ACTs, particularly AL, is less than 100%.

**Keywords:** *Plasmodium falciparum*, Antimalarial drug resistance, Artemisinin, ACT, *pfubp1*, *pfap2mu*, Mutations, Ghana

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## Background

Malaria is still a debilitating disease, especially in sub-Saharan Africa (sSA) where there were 212 million cases and 429,000 malaria-related deaths in 2015 [1]. There has been a 21 and 29% reduction in morbidity and mortality, respectively, since 2010, probably as a result of the implementation of integrated control strategies [1, 2]. The control efforts employed included the use of insecticide-treated mosquito nets (ITNs), indoor residual spraying (IRS), chemoprevention in pregnant women and children as well as chemotherapy with artemisinin-based combination therapy (ACT). As such, the development of *Plasmodium falciparum* resistance to artemisinin (ART) derivatives as reported from Southeast Asia (SEA) is quite worrying [1]. Chemotherapy, which is one of the core control strategies for the disease, has been hindered over the years by the emergence and spread of parasites resistant to the commonly used antimalarial drugs [1]. Currently, the World Health Organisation (WHO) has initiated the containment of drug resistance in the SEA region with the deployment of a multi-sector strategy [1]. Although this initiative is commendable, the need for country-level monitoring of the genome of parasite populations for possible evolution and selection due to drug pressure is also crucial for the early detection of emerging drug resistance.

For over a decade, molecular markers of antimalarial drug resistance have been used to monitor the emergence and spread of drug resistance in malaria endemic areas. These genetic markers are mainly single nucleotide polymorphisms in genes encoding drug target proteins in essential biochemical pathways of the parasite. The levels of drug susceptibility in the parasites have been linked to SNPs or haplotypes of the genes, and these markers are relevant in antimalarial drug efficacy studies. The recent observation of parasite resistance to ART in SEA set into motion the need to discover a molecular marker for surveillance of drug susceptibility. Ariey et al. [3] discovered the SNPs in the kelch propeller domain on chromosome 13 of the *P. falciparum* genome known as k13 in drug resistance isolates *in vitro*. Three of the k13 polymorphisms, C580Y, R538T and Y493H were also present in slow clearing clinical isolates with ART use. The presence of the SNPs showed varying parasite clearance half-life in patients; however, the C580Y mutation was linked to longest parasite clearance half-life compared to the other SNPs. So far these SNPs have not been detected in African isolates [4–6] and the quest for novel markers for ART resistance is ongoing. Henriques et al. [7] linked SNPs in two genes, the *P. falciparum* adaptor protein complex 2 mu subunit (*pfap2mu*) and ubiquitin-specific protease 1 (*pfubp1*) to delayed clearance of parasites. The *pfap2mu* gene mutation was at codon 160 resulting in the amino acid change from serine to either asparagine or threonine (S160N/T). The *pfubp1*

gene mutations were at codon 1525, a change from aspartic acid to glutamic acid (D1525E) and codon 1528 from glutamic acid to aspartic acid (E1528D) in the Kenyan isolates. In addition, Sutherland et al. [8] reported four UK residents with imported malaria who showed recurrent malaria after AL treatment [8]. These mutations, *pfap2mu* S160N and *pfubp1* E1525D/Q were observed in the recurrent parasites but none of the known K-13 gene mutations were observed [8]. Although the role played by both genes in artemisinin action is not clearly understood, the *ap2mu* gene is known to encode the  $\mu$ -subunit of the adaptor protein 2 complex (AP2) involved in clathrin-mediated endocytosis into the parasite vacuole [9]; *ubp1* encodes a deubiquitinating (DUB) enzyme that functions by cleaving ubiquitin from any protein or peptide to which it is joined [10]. The polymorphic homologues of these two *P. falciparum* genes were first identified in the rodent malaria parasite, *P. chabaudi* (*pcubp1* encodes ubiquitin carboxy-terminal hydrolase 1) and *pcap2mu* encodes clathrin vesicle-associated adaptor 2 mu subunit), as being associated with ART resistance [11]. More studies are therefore needed to validate these polymorphisms and their role in antimalarial drug resistance.

The use of ACTs in Ghana began in 2005 and since then the cure rate of the drugs in use, artesunate-amodiaquine (AS-AQ) and artemether-lumefantrine (AL), has been 100 and 97.6%, respectively, as of 2014 [12]. Surveillance studies using the *P. falciparum* multidrug resistance gene (*pfmdr1*) SNPs (haplotype N86-F184-D1246) linked to reduced parasite susceptibility to AL showed an increasing trend over the years in Ghana from 2005 to 2010 [13]. In addition, increased *pfmdr1* gene copy number linked to parasite reduced susceptibility to artesunate (AS), mefloquine (MQ), halofantrine and AL [14–16] were also detected in Ghanaian isolates [13]. The findings from the reported studies above are indicative of a subtle emergence of parasite resistance to ART and to ACTs especially AL in Ghana. Therefore, the monitoring of newly discovered molecular markers is essential as an early warning signal to the emergence of resistance in Ghana. This study determined the prevalence of known and novel SNPs in the *pfubp1* and *pfap2mu* in Ghanaian isolates collected from three ecologically distinct areas for monitoring antimalarial drug efficacy in Ghana to serve as baseline data for antimalarial drug resistance surveillance in Ghana.

## Methods

### Study sites

The Noguchi Memorial Institute for Medical Research (NMIMR) in collaboration with the National Malaria Control Programme (NMCP) have set up ten sentinel sites in the ten regions of Ghana for monitoring antimalarial drug efficacy. These sites lie in the three distinct ecological zones in the country. Of the ten sites, samples

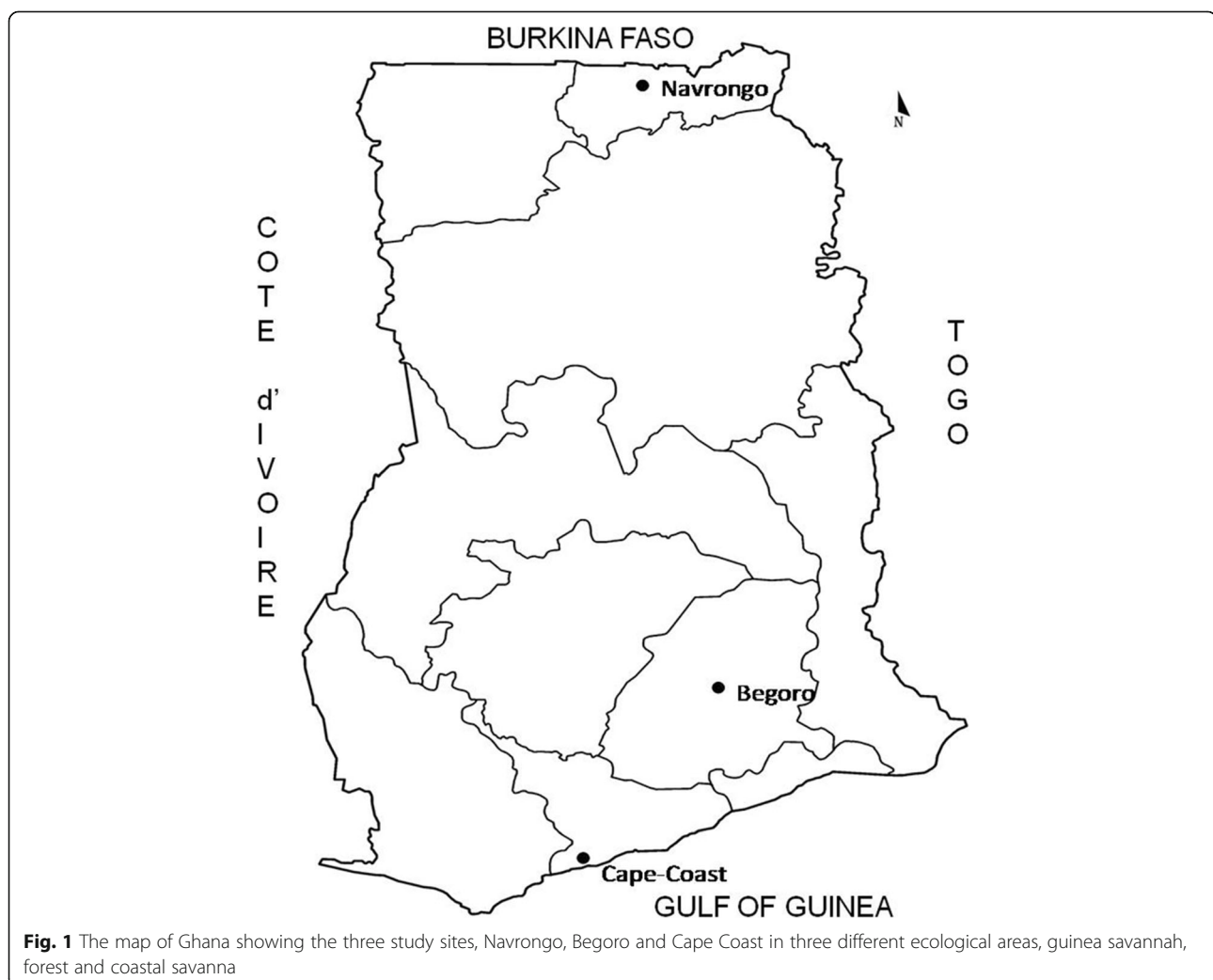
from three sites were used for this study. The sites include Navrongo (10°53'44.05"N, 1°05'31.56"W) located in the Kassena Nankana District in the Upper East Region and lies in the guinea savannah zone; Begoro (6°23'29.76"N, 0°22'46.20"W) located in the Fanteakwa District of the Eastern Region lies in the forest zone; Cape Coast (5°06'00"N, 1°15'00"W), the capital town of Central Region lies in the coastal savannah zone (Fig. 1). The forest and coastal savannah zones experience perennial malarial transmission whilst the guinea savannah zone experiences a seasonal malaria transmission pattern with almost all cases occurring during rainy months between May-June and October-November of each year.

### Study samples

Filter paper blood blots collected in 2015–2016 from children aged below 9 years with uncomplicated malaria reporting at designated health care facilities in Navrongo, Begoro and Cape Coast were used for the study. In total 120 samples (40 from each site) were used for this investigation.

### Detection of *pfubp1* and *pfap2mu* gene polymorphisms

Parasite DNA was extracted from 120 pre-treatment blood blot samples on filter paper (Whatman™ 3 Little Chalfont, United Kingdom) using the QIAmp DNA mini kit (Qiagen GmbH, Hilden, Germany) as per the manufacturer's protocol. This was followed by amplification of the *pfap2mu* and *pfubp1* genes using nested polymerase chain reaction (nPCR) following a previously published protocol [7] with minor modifications. The PCR was performed in a total volume of 25 µl with the following reaction mixture: 0.2 µM of each primer (Table 1), 4.0 mM MgCl<sub>2</sub>, 0.4 µM deoxynucleotides triphosphate (dNTPs), 1 U One-Taq polymerase (New England Biolabs, Massachusetts, USA), 1× PCR buffer, nuclease free water and 2 µl of the extracted parasite DNA. One microlitre of the first round product was used as a template in a 50 µl inner PCR reaction. A DNA sample extracted from the 3D7 parasite strain was used as a positive control. The PCR thermal conditions were the same for both genes but different annealing temperatures as shown in Table 1. The



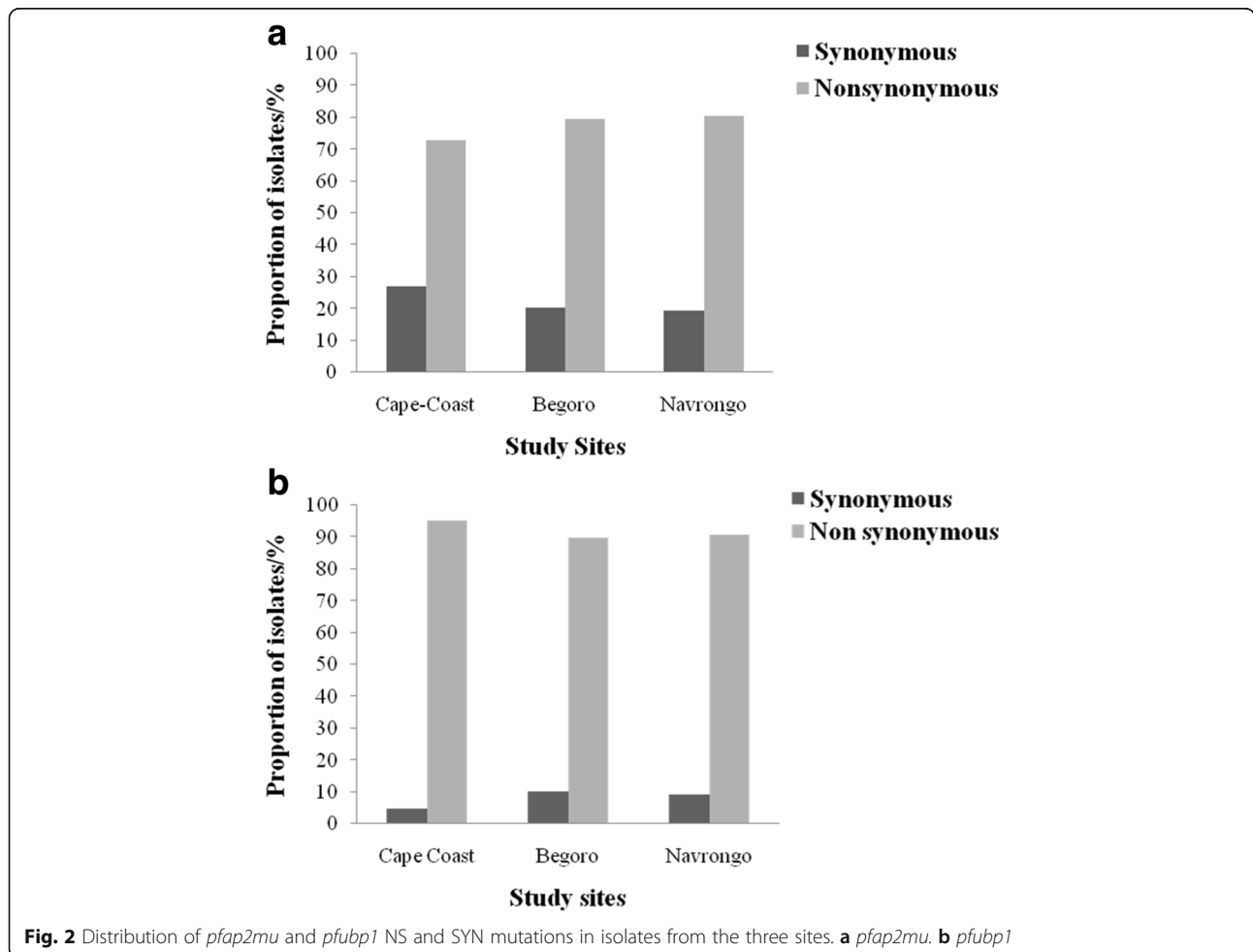
**Fig. 1** The map of Ghana showing the three study sites, Navrongo, Begoro and Cape Coast in three different ecological areas, guinea savannah, forest and coastal savanna

**Table 1** Primer sequences, sizes of PCR amplicons and annealing temperature of the amplification of *pfap2mu* and *pfubp1* genes

Gene	Primers (5'-3')	Size of PCR amplicon (bp)	Annealing temperature (°C)
<i>pfap2mu</i>	Primary amplification	2247	50
	Forward: AAGACTGTCAAATGTAAAAGACCC		
	Reverse: CTCATGTAAAACAAAAAGTGAGG		
	Secondary amplification	841	52
	Forward: GATATCCACAAACATTAGAAGTG		
	Reverse: CCATCTGGTGGTGTGAAGG		
<i>pfubp1</i>	Primary amplification	484	52
	Forward: CGCCCGTACTATGAAGAAGATC		
	Reverse: GGCTTTTACCTGAACTGTTTCAGG		
	Secondary amplification	304	57
	Forward: CGTAAACAGAATATTCAGGATTGC		
	Reverse: CTAGCCCTTTATTATCATTATCG		

thermal cycle programme for each 1st amplification was 94 °C for 3 min, and 30 cycles of 94 °C for 30 s, annealing temperature for 30 s and 68 °C for 1 min with a final extension of 68 °C for 15 min. The second round of PCR consisted of initial denaturation at 94 °C for 3 min,

followed by 40 cycles of 94 °C for 30 s, annealing temperature for 30 s and 68 °C for 45 s with a final extension of 68 °C for 10 min. The PCR amplicons for the fragments of the two genes were sequenced using Sanger sequencing.



**Data analysis**

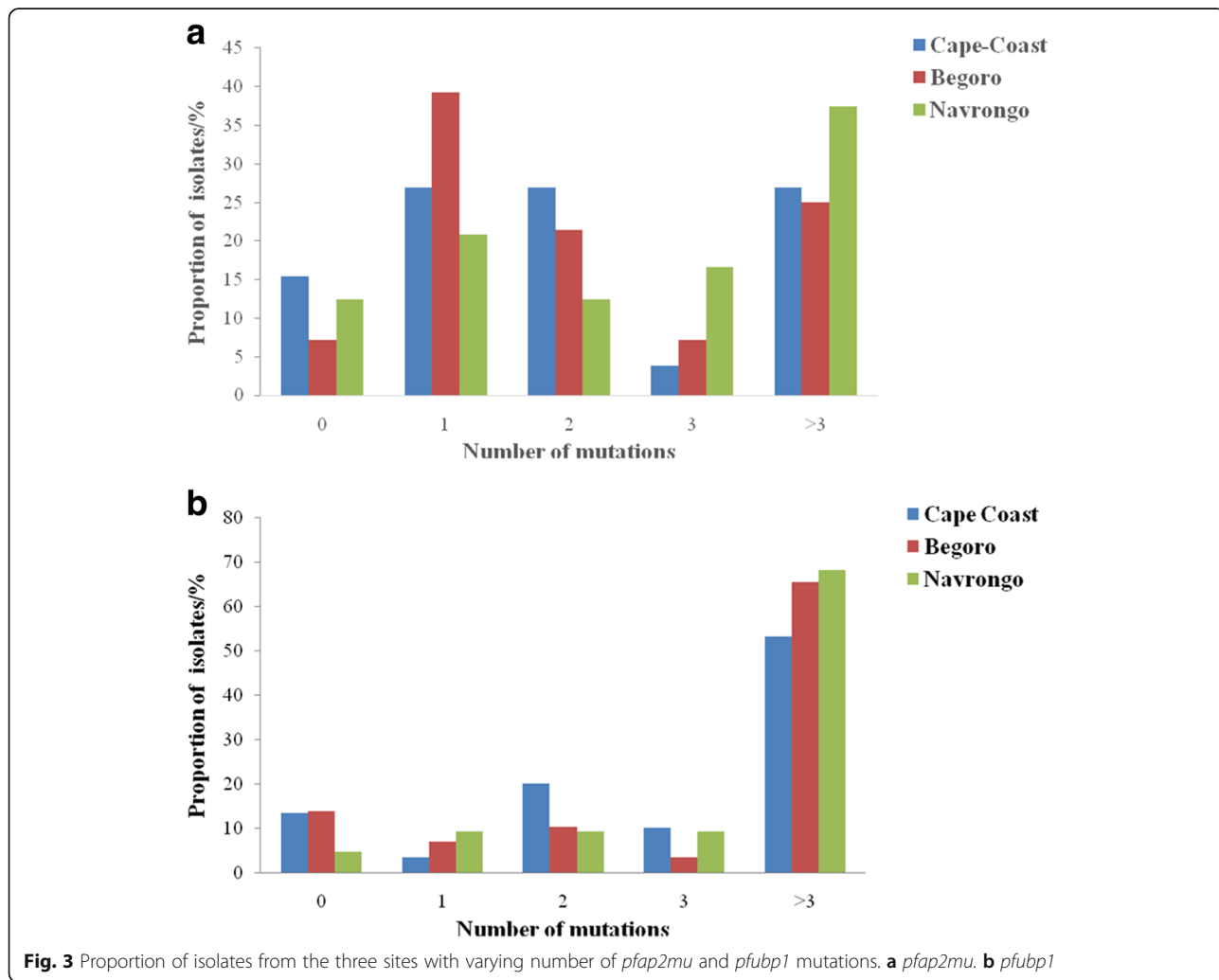
The sequence data of the isolates were analysed using the CLC Genomics Workbench 10.01 software (Qiagen, Aarhus, Denmark) and Benchling.com (California, CA, USA). PF3D7\_1218300 and PF3D7\_0104300 (PlasmoDB) were used as reference sequences to detect SNPs in the *pfap2mu* and *pfubp1* respectively. Poor quality sequences of isolates after three sequencing trials were not analysed. The prevalence of individual SNPs was determined for each site. Chi-square tests were used to compare the proportions of mutations occurring in the three sites and to determine any significant differences in the prevalence of the mutations among the three sites using the GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA, USA). Statistical significance was defined as a *P*-value ≤ 0.05.

**Results**

**Polymorphisms in *pfap2mu* and *pfubp1***

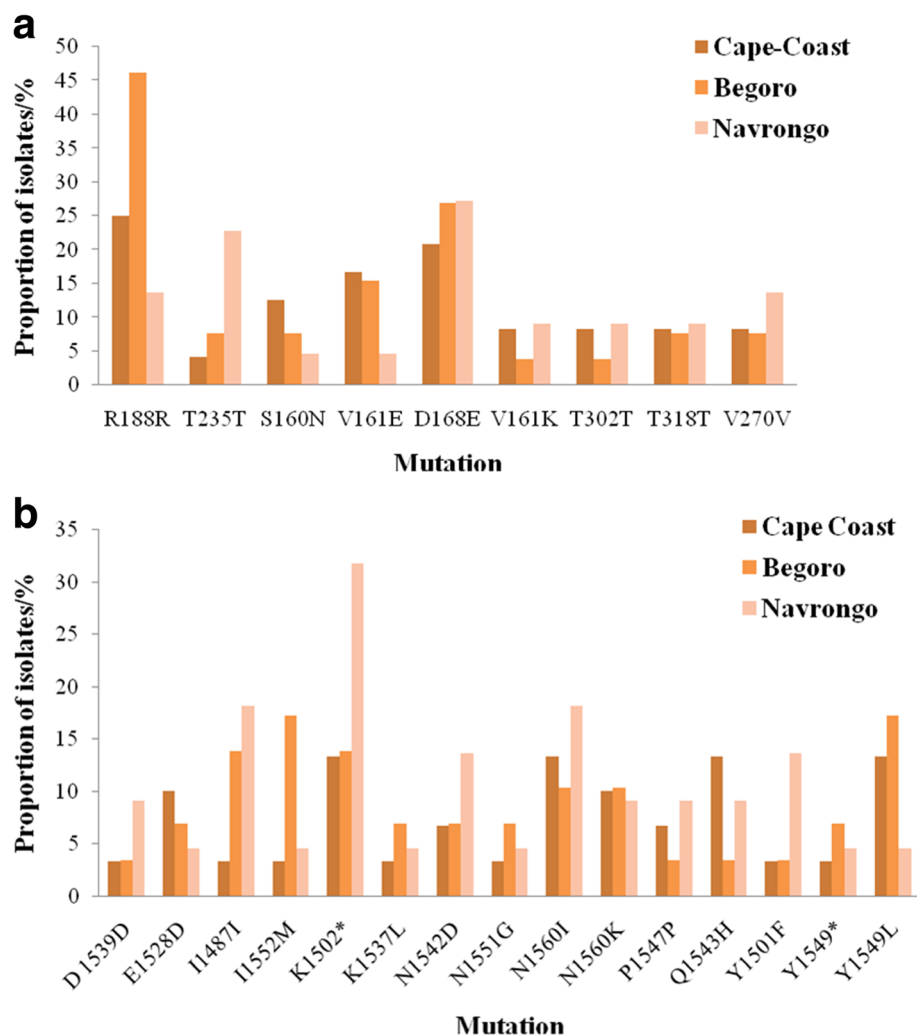
For the *pfap2mu* gene, 96 samples were sequenced and 15 were of low quality as determined by quality

assurance analysis. Of the 81 good sequences, 35% (28/81), 35% (28/81) and 31% (25/81) were from Cape-Coast, Begoro and Navrongo, respectively. The proportion of isolates with no mutations in the *pfap2mu* gene, that is wildtype sequence as the 3D7 strain, was 11.1% (9/81). The sequence analysis revealed several SNPs and the total number observed in 67 samples (of the 72 with mutations) was 164 SNPs with ≤ 5 mutations per sample. Of the 164 SNPs, 78.0% (128/164) were non-synonymous (NS) and 22.0% (36/164) were synonymous (SYN) mutations. The distribution of *pfap2mu* NS and SYN mutations in isolates from the three sites is shown in Fig. 2a. There was no significant difference in the type of mutation (SYN or NS) present between the three sites ( $\chi^2 = 1.960$ , *df* = 2, *P* = 0.360). The distribution of the single or multiple mutations for each site is shown in Fig. 3a. About 68.6% (46/67) of the isolates with mutations had more than one mutation and 36% (9/25) of the isolates from Navrongo had more than three mutations per isolate. In addition, there were insertions and



deletions in some of the isolates from the three sites. In all 22 common SNPs were detected in either two of the three sites or all three sites. These include Q149R, S160N, V161K, V161E, D168E, R188R, D203F, D203Y, E206\*, T235T, N240Y, N240F, K256\*, D263V, V270V, I272I, G284G, K285E, T302T, N317S, T318T and T325T. Nine of these SNPs were shared in all three sites and the proportion of isolates from the study sites is shown in Fig. 4a. Of these SNPs, three NS mutations were present in isolates from the three sites: S160N, D168E and V161K. The nucleotide changes for the SNPs are shown in Table 2. The amino acid sequences alignment for 21 isolates are shown in Fig. 5. There were 5 other isolates (of the 72 with mutations) with a total of 215 *pfap2mu* SNPs (G020, 63 SNPs; G022, 60 SNPs; G025, 47 SNPs; G029, 30 SNPs; G034, 15 SNPs) and were all from Begoro. The amino acid sequences for these 5 isolates are shown in Fig. 6.

For the *pfubp1* gene, 81 quality sequence data were analysed comprising 37% (30/81), 35.8% (29/81) and 27.2% (22/81) from Cape Coast, Begoro and Navrongo, respectively. About 11.1% (9/81) of the isolates had no mutations in their *pfubp1* gene. A total of 271 SNPs were observed in the 72 sequences of which 92.3% (250/271) were NS and 7.7% (21/271) were SYN mutations. The proportion of isolates with either *pfubp1* NS or SYN mutations for each site is shown in Fig. 2b. The proportion of isolates with varying number of mutations is also shown in Fig. 3b for the three study sites. Overall, 93.1% (67/72) of the isolates with mutations had more than one *pfubp1* SNP. The majority of isolates from Begoro (65.5%, 19/29) had more than 3 mutations per isolate for the *pfubp1* gene. There were 15 common SNPs detected in isolates from all three sites. These include D1539D, E1528D, I1487I, I1552M, K1502\*, K1537L, N1542D, N1551G, N1560I, N1560K, P1547P, Q1543H, Y1501E,



**Fig. 4** Proportion of isolates from the three sites with shared *pfap2mu* and *pfubp1* mutations. **a** *pfap2mu*. **b** *pfubp1*

**Table 2** Shared *pfap2mu* mutations observed in the isolates from the three sites. Mutations indicated in bold are known delayed clearance genotypes, underlined nucleotides are the changed bases

Nucleotide position	Nucleotide change	Amino acid position and change
446	<u>C</u> AG to <u>C</u> GG	Q149R
479	<u>AG</u> T to <u>A</u> AT	<b>S160N</b>
481	<u>G</u> TG to <u>A</u> AG	V161K
482	<u>G</u> TG to <u>G</u> AG	V161E
504	<u>GAT</u> to <u>GAA</u>	D168E
564	<u>AGA</u> to <u>AGG</u>	R188R
607	<u>GAT</u> to <u>TAT</u>	D203Y
607, 608	<u>GAT</u> to <u>TTT</u>	D203F
616	<u>GAA</u> to <u>TAA</u>	E206 <sup>a</sup>
705	<u>ACA</u> to <u>ACG</u>	T235T
718	<u>AAT</u> to <u>TAT</u>	N240Y
767	<u>AAG</u> to <u>TAG</u>	K256 <sup>a</sup>
789	<u>GAT</u> to <u>GTT</u>	D263V
810	<u>GTA</u> to <u>GTT</u>	V270V
852	<u>GGA</u> to <u>GGG</u>	G284G
855	<u>AAG</u> to <u>GAG</u>	K285E
951	<u>AAC</u> to <u>AGC</u>	N317S
954	<u>ACA</u> to <u>ACC</u>	T318T

<sup>a</sup>Stop codon

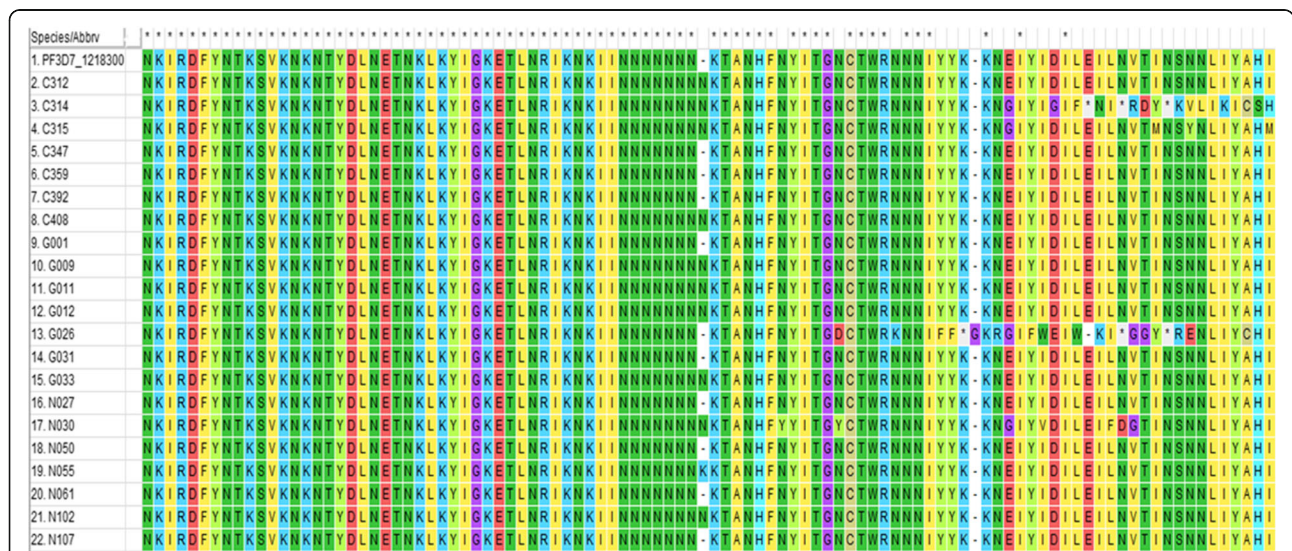
Y1549\* and Y1549L. The proportion of isolates with these mutations from each of the study sites is shown in Fig. 4b. There were 36 other shared SNPs detected in isolates from two out of the three sites. The nucleotide changes for the commonly shared mutations are also shown in Table 3. The amino acid sequence alignment is shown in Fig. 7.

Two samples, G005 and C329, had wildtype sequences for both *pfap2mu* and *pfubp1*. Of the 5 isolates with many *pfap2mu* SNPs, 2 were wildtype for *pfubp1* (G022 and G034). For the other three, G020 had 4 SNPs, G025 had 2 SNPs and G029 had 11 SNPs for the *pfubp1*.

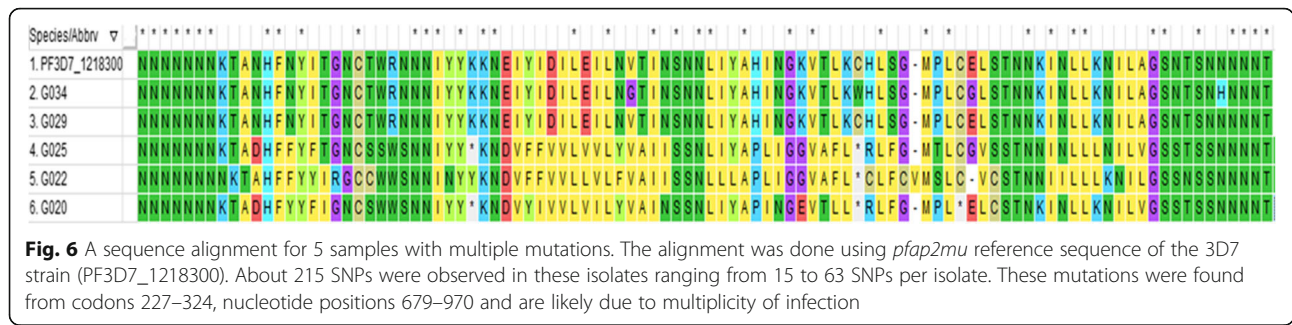
**Prevalence of *pfap2mu* and *pfubp1* SNPs from the three sentinel sites**

The *pfap2mu* SNPs were detected in 92.9% (26/28), 85.7% (24/28) and 88% (22/25) from Begoro, Cape Coast and Navrongo respectively. There was no significant difference in the prevalence of *pfap2mu* SNPs in the isolates from the three sites ( $\chi^2 = 6.905, df = 2, P = 0.546$ ). The most prevalent mutation was R188R and was observed in 25.9% (21/81) of the isolates. The D168E was also observed in 22.2% (18/81) of the isolates. S160N which have been reported to be associated with ACT delayed clearance was prevalent in 7.4% (6/81) of the isolates whilst the V161K was seen in 6.2% (5/81). The S160N genotypes occurred mostly in isolates from Cape Coast (24.7%) as compared to the other two sites. In all, 35 indels were identified with 17.1% (6/35) causing a frame shift in the sequence reading frame (Fig. 5).

For *pfubp1*, mutations were detected in 86.7% (26/30) of the Cape Coast isolates, 86.2% (25/29) for Begoro and



**Fig. 5** A sequence alignment of *pfap2mu* gene showing amino acid changes due to single nucleotide polymorphisms. The alignment was done using *pfap2mu* reference sequence of the 3D7 strain (PF3D7\_1218300). Mutations present at codons 185–282, nucleotide positions 553–849 of *pfap2mu* for 21 samples. Samples C314 and G026 had a frameshift, samples C312, C314, C315, C408, G009, G011, G012, G033, N030 and N102 had an asparagine (N) insertion at codon 233, as well as a lysine (K) insertion at the same position for sample N055



95.5% (21/22) for Navrongo. There was no significant difference in the prevalence of SNPs in the isolates from the three sites ( $\chi^2 = 4.883$ ,  $df = 2$ ,  $P = 0.769$ ). The most prevalent NS mutation was K1502\* which was observed in 18.5% (15/81) of the isolates. The reported SNPs linked to delayed clearance of parasite with ACT use, E1528D and D1525E, were observed in 7.4% (6/81) and 4.9% (4/81) of the isolates, respectively. The predominant SYN mutation I1487I was observed in 11.1% (9/81) of the isolates. In all, 20 indels were identified with 25% (5/20) causing a frame shift in the sequence reading frame (Table 4).

The results showed a number of SNPs that were being inherited together on the gene as haplotypes in some of the isolates. However, the prevalence of the haplotypes was low (Table 5). Twenty-two different haplotypes were observed: 3 for *pfap2mu* and 19 for *pfubp1*. The most prevalent haplotype for the *pfap2mu* gene was V161K-D168E, which was observed in 8.6% (7/81) of the isolates with mutations. For the *pfubp1* gene, the haplotypes Y1548L-N1560I, Y1549L-I1552M and N1560I-L1563 were each observed in 3.7% (3/81) of the isolates. Most of the haplotypes for both genes were observed in isolates from Begoro.

**Discussion**

The search for a molecular marker for the early detection of ART resistance by the malaria parasite is ongoing. The use of molecular markers to track and identify early development of parasite resistance to drugs is a powerful tool that should be available in all malarious regions of the world. With the implementation of ACTs in Africa, studies to identify possible markers of resistance have not been conducted extensively in the continent. The key *k13* molecular marker, which has been linked to drug resistance in SEA has not been observed in African isolates, partly because there is no ‘true resistance’ to ARTs except delayed clearance of parasites. New markers that have recently been discovered, such as the *pfap2mu* and *pfubp1* gene mutations [7], need further validation for their role in delayed parasite clearance. This study detected ART delayed clearance

associated polymorphisms of the *pfap2mu* and *pfubp1* genes in Ghanaian isolates from three sites located in three distinct ecological zones. Majority of the isolates had mutations of both genes and were mostly NS mutations. The known delayed clearance genotypes, *pfap2mu* N160 and *pfubp1* D1528 and E1525, were observed in 7.4, 7.4 and 4.9% of the isolates, respectively. It is interesting to note that although these were not the predominant mutations, it is an indication of the presence of these drug resistance genotypes in Ghanaian parasite populations and in the long term their selection with drug use will enhance the emergence of ART resistance in Ghana.

The observation that minority of the isolates were wildtype with no mutations like the reference 3D7 strain (11.1% for both *pfap2mu* and *pfubp1*) is indicative of the high rate of spontaneous mutations in the two genes. For *pfap2mu*, a study by Henriques et al. [7] reported that 41.5% of Kenyan isolates had wildtype gene sequence whilst another study reported 92.8% and 30.9% for Ethiopian and Tanzanian isolates, respectively [17]. Comparatively, the Ghanaian isolates had low levels of wildtype strains portraying a rapid genetic recombination of different parasite clones (multiclonal infections observed in Ghana) during the sexual stage in the vector resulting in gene shuffling [18]. About 64% and 93% of the isolates, respectively, had more than one mutation for the *pfap2mu* and *pfubp1* genes.

The results revealed a high proportion of NS mutations, 78% and 92% for *pfap2mu* and *pfubp1*, respectively, in the Ghanaian isolates. The NS mutations are of much importance because each amino acid substitution may affect protein conformation and function [19]. Most of the NS mutations were single base or double base substitutions. For SYN mutations, it was initially assumed that since the resultant change of the nucleotide does not affect the amino acid, the change may go undetected as the gene function may not necessarily be affected [20]. However, this perception has since changed due to the evidence that SYN mutations in the *pfmdr1* gene resulted in alterations in the functions of the P-glycoprotein (P-gp), a product which affects drug



**Table 3** Shared *pfubp1* mutations observed in the isolates from the three sites. Mutations indicated in bold are known delayed clearance genotypes

Nucleotide position	Nucleotide change	Amino acid position and change
4383	<u>CCT</u> to <u>ACC</u>	P1461T
4386	<u>TAT</u> to <u>TTA</u>	Y1462L
4389	<u>CGT</u> to <u>TCG</u>	R1463S
4392	<u>AAA</u> to <u>TAA</u>	K1464 <sup>a</sup>
4461	<u>ATA</u> to <u>ATC</u>	I1487I
4461	<u>ATA</u> to <u>ACC</u>	I1487T
4466	<u>ATG</u> to <u>ACG</u>	M1489T
4502	<u>TAT</u> to <u>TTT</u>	Y1501F
4504	<u>AAA</u> to <u>TAA</u>	K1502 <sup>a</sup>
4509	<u>AAT</u> to <u>ATT</u>	N1503I
4509	<u>AAT</u> to <u>ATG</u>	N1503M
4527	<u>GAA</u> to <u>GAC</u>	E1509D
4554	<u>AAC</u> to <u>TAT</u>	N1518Y
4557	<u>GAA</u> to <u>GAC</u>	E1519D
4561	<u>TAT</u> to <u>AAT</u>	Y1521N
4575	<u>GAC</u> to <u>GAA</u>	<b>D1525E</b>
4581	<u>TAT</u> to <u>TTT</u>	Y1527F
4581	<u>TAT</u> to <u>AAT</u>	Y1527N
4584	<u>GAA</u> to <u>GAC</u>	<b>E1528D</b>
4599	<u>TAT</u> to <u>TAC</u>	Y1533Y
4602	<u>GAT</u> to <u>GAA</u>	D1534E
4608	<u>TAC</u> to <u>TCC</u>	Y1536S
4611	<u>AAA</u> to <u>TTT</u>	K1537F
4609	<u>AAA</u> to <u>TTA</u>	K1537L
4611	<u>AAA</u> to <u>TAC</u>	K1537Y
4617	<u>GAT</u> to <u>GAC</u>	D1539D
4623	<u>AAA</u> to <u>CIT</u>	K1541L
4623	<u>AAA</u> to <u>AAT</u>	K1541N
4626	<u>AAT</u> to <u>GAT</u>	N1542D
4629	<u>CAA</u> to <u>CAT</u>	Q1543H
4632	<u>CAT</u> to <u>CTT</u>	H1544L
4632	<u>CAT</u> to <u>CCT</u>	H1544P
4632	<u>AAA</u> to <u>AAT</u>	K1544N
4641	<u>CCA</u> to <u>CCT</u>	P1547P
4647	<u>TAT</u> to <u>TAG</u>	Y1549 <sup>a</sup>
4647	<u>TAT</u> to <u>TTT</u>	Y1549F
4646	<u>TAT</u> to <u>TTG</u>	Y1549L
4650	<u>GAT</u> to <u>ATT</u>	D1550I
4650	<u>GAT</u> to <u>AAT</u>	D1550N
4651	<u>AAT</u> to <u>GGT</u>	N1551G
4653	<u>AAT</u> to <u>ATT</u>	N1551I
4653	<u>AAT</u> to <u>CIT</u>	N1551L

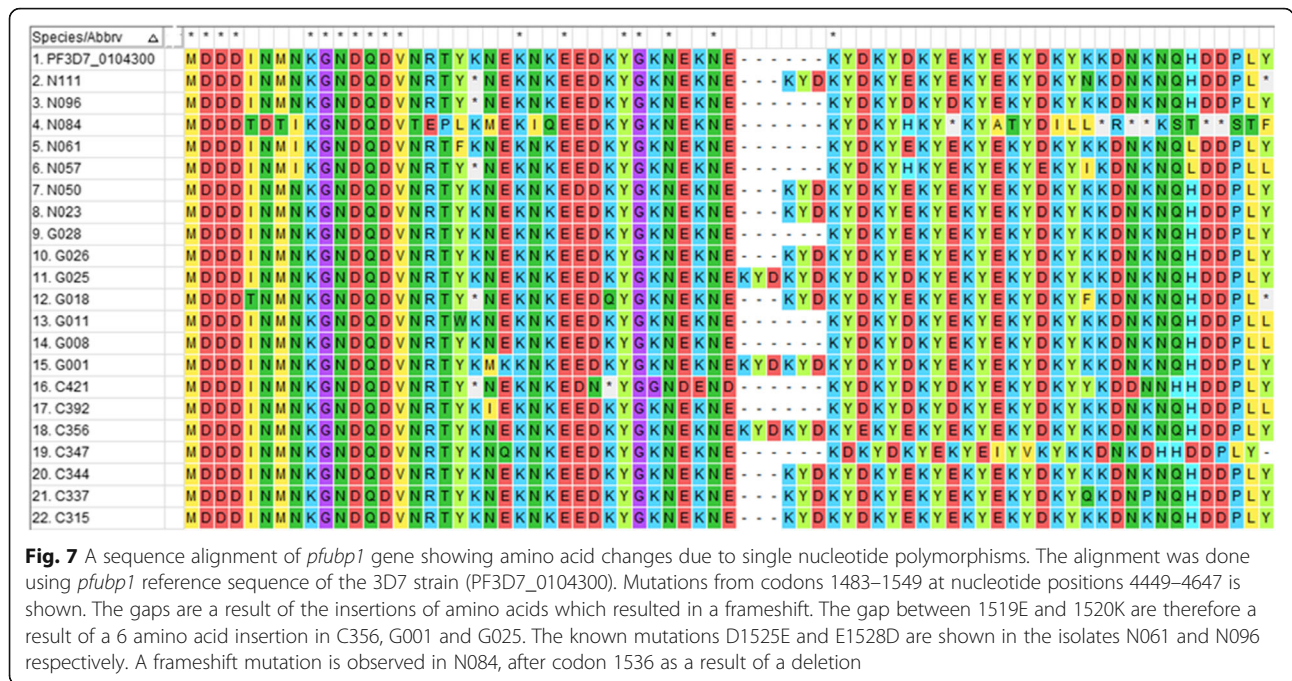
**Table 3** Shared *pfubp1* mutations observed in the isolates from the three sites. Mutations indicated in bold are known delayed clearance genotypes (Continued)

Nucleotide position	Nucleotide change	Amino acid position and change
4656	<u>ATT</u> to <u>ATG</u>	I1552M
4659	<u>AAT</u> to <u>AAC</u>	N1553N
4667	<u>TAC</u> to <u>TGC</u>	Y1556C
4674	<u>AAT</u> to <u>GAT</u>	N1558D
4679	<u>AAT</u> to <u>ATA</u>	N1560I
4680	<u>AAT</u> to <u>AAA</u>	N1560K
4683	<u>AAA</u> to <u>AAG</u>	K1561K
4692	<u>GAG</u> to <u>GAC</u>	E1564D
4695	<u>TTC</u> to <u>GGC</u>	F1565G
4694	<u>TTC</u> to <u>CAA</u>	F1565Q
4765	<u>AAA</u> to <u>TAA</u>	K1589 <sup>a</sup>

<sup>a</sup>Stop codon

interactions [21]. A high prevalence of novel *ap2mu* mutation, D168E (25%), was observed in the Ghanaian isolates from all three sites followed by V161K (7%). The codons for the common mutations found among the isolates from all the three sites were between codons 160 and 170. The known SNP, *pfap2mu* S160N, found in Kenyan isolates with delayed clearance [7] was also found in all three sites (7.2%). In addition, isolates from a UK patient who arrived from Angola and failed AL treatment had the S160N thereby strengthening the role of that mutation in recurrent parasitemia with AL use [8]. The observed *pfubp1* E1528D in Ghanaian samples was also seen in Kenyan and Tanzanian isolates [7, 17]. Borrmann et al. [22] first identified this mutation in Kenyan isolates. The prevalence of E1528D in Ghanaian isolates was 7.4%, which is lower than that of Kenya's 17.1% but higher than that of Tanzania (4.8%) [7, 17]. However, with the continuing use of ACTs, the mutation may be selected and the prevalence may increase with time as observed in Kenya. The SYN mutation N1518 found in the Kenyan and Burkinabe isolates was also seen in one of the Ghanaian isolates from Begoro. Pre- and post-treatment samples from Burkina Faso, had the D1525E mutation which was seen in four Ghanaian isolates. Henriques et al. [7] compared South East Asian and African phenotypes of the *pfubp1* gene and detected significant differences in the genetic signatures. The recent implication of SNPs in these genes, especially *pfap2mu* S160N and *pfubp1* E1528D and D1525E in ART resistance, raises a genuine concern due to their presence in Ghanaian isolates.

Of the three sites, Begoro (forest) had the most *pfap2mu* mutations (93%) followed by Cape-Coast (coastal savannah) (86%) and Navrongo (guinea savannah) (88%). Despite this observation, there is comparatively higher



diversity in the mutations in Navrongo isolates with 72 different SNPs. Most of the isolates (63%) had more than one mutation. The high diversity of SNPs in Navrongo, where there is intense seasonal transmission of malaria, is expected. For *pfubp1*, Navrongo had the most mutations (96%), followed by Cape Coast (87%) and Begoro (86%). It is quite interesting to observe diversity of mutations from both genes from the guinea savannah zone. However, it must be emphasised that transmission intensity does not affect the evolution of resistance but plays a major role in

the spread of resistance genotypes [18]. Therefore, discussing our observations along the line of different transmission intensities from the three distinct ecological zones will be premature.

Insertions/deletions (indels) identified in the Ghanaian isolates were similar to those observed in Burkina Faso, Kenya and the UK patients from Liberia and Uganda [7, 8]. The most common indels, observed in the isolates from the Ghanaian and the other African countries resulted from an insertion of AAT which resulted in an

**Table 4** Genetic insertions in the *pfubp1* gene of isolates from the study sites

Sample ID	Amino acids	Insertion	Nucleotide position
C315	KYE	<u>AAA TAT GAA</u>	4576 to 4584
C337	KYE	<u>AAA TAT GAA</u>	4583 to 4591
C344	KYE	<u>AAA TAT GAA</u>	4548 to 4556
C356	KYEKYE	<u>AAA TAT GAA AAA TAT GAA</u>	4588 to 4605
C360	KYE	<u>AAA TAT GAA</u>	4583 to 4591
C321	EKY	<u>GAA AAA TAT</u>	4590 to 4598
G001	DKYDKY	<u>GAC AAA TAT GAC AAA TAT</u>	4563 to 4580
G018	EKY	<u>GAA AAA TAT</u>	4598 to 4606
G025	KYDKYE	<u>AAA TAT GAC AAA TAT GAA</u>	4585 to 4602
G026	EKY	<u>GAA AAA TAT</u>	4580 to 4588
G034	YDKYDK	<u>TAT GAC AAA TAT GAC AAA</u>	4579 to 4596
N023	EKY	<u>GAA AAA TAT</u>	4582 to 4590
N031	EKY	<u>GAA AAA TAT</u>	4582 to 4590
N050	KNE	<u>AAA AAC GAA</u>	4549 to 4557
N111	YEK	<u>TAT GAA AAA</u>	4597 to 4605

**Table 5** Haplotypes of the *pfap2mu* and *pfubp1* mutations in Ghanaian isolates

Gene	Haplotype	No. of isolates
<i>pfap2mu</i>	V161K-D168E	7
	V161E-R188R	6
	D168E-R188R	6
<i>pfubp1</i>	I1487I-N1488D	2
	I1487I-N1490I	2
	Y1549L-I1552M	3
	N1540H-K1541N	2
	N1540H-H1544P	3
	N1540H-N1542D	2
	N1488D-D1525H	2
	I1487I-Y1501F	2
	N1490I-Y1501F	2
	N1560L-L1563A	3
	E1528D-N1560K	2
	Y1549L-N1560I	3
	Y1549L-I1552M	2
	Y1501W-Y1549L	2
	N1542D-Q1543H	2
	N1488D-G1492G	2
	Q1543H-P1547P	2
I1487I-N1490I-H1544L	2	
N1490I-Y1501F-Y1533Y-P1547P-K1554N-N1555I-D1557N	2	

asparagine and lysine residues at codons 226 and 233, respectively [7, 8]. Indels of one to six amino acids were observed for the *pfubp1* gene in the Ghanaian isolates and these were similar to those seen in Kenya and Burkina Faso [7, 8]. These resulted from an insertion of a KYD, KYE or KNE amino acids at codons ranging from 1516 to 1535. Most of the indels that resulted in a frameshift were caused by a deletion or insertion of either a guanosine or thymidine nucleotide.

The presence of the molecular markers implicated in drug resistance is of great importance in their role in modulating drug susceptibility and subsequently the prediction of the dynamics of resistance [23]. The spread of *P. falciparum* resistance to ARTs is a real global challenge and therefore insights into the mechanisms of drug action and resistance are critical for early detection of resistance. The detection and characterisation of these mutations in post-treatment Ghanaian isolates is the way forward for further validation of their roles in conferring resistance. This study has, therefore, highlighted the existence of delayed clearance markers of the *pfap2mu* and *pfubp1* genes in circulating parasites in Ghana and will serve as baseline data for future surveillance studies.

## Conclusions

The identification of molecular markers of ART resistance in Ghanaian isolates has implications for the development of ACT resistance especially for AL use. The findings from this study give first-hand information on potential molecular markers of ART resistance in Ghana and as such highlight the possibility of circulating parasites with reduced susceptibility to ACTs in use. Further investigations are underway to ascertain their contribution to the less than 100% cure rates observed, particularly for AL.

## Additional file

**Additional file 1: Table S1.** The list of all observed *pfap2mu* SNPs in Ghanaian isolates from the three study sites. **Table S2.** The list of *pfap2mu* SNPs from five isolates with at least 20 SNPs per sample. **Table S3.** The list of all observed *pfubp1* SNPs in Ghanaian isolates from the three study sites. (XLSX 41 kb)

## Abbreviations

ACT: artemisinin-based combination therapy; AL: artemether-lumefantrine; ART: artemisinin; AS-AQ: artesunate-amodiaquine; DUB: deubiquitinating enzyme; IRB: Institutional Review Board; IRS: indoor residual spraying; ITNS: insecticide-treated mosquito nets; NMCP: National Malaria Control Programme; nPCR: nested polymerase chain reaction; NS: non-synonymous mutations; *pfap2mu*: *P. falciparum* adaptor protein complex 2 mu subunit gene; *pfk13*: *P. falciparum* kelch propeller domain on chromosome 13; *pfubp1*: *P. falciparum* ubiquitin specific protease 1 gene; SEA: Southeast Asia; SNPs: single nucleotide polymorphisms; SYN: synonymous mutations; WHO: World Health Organization

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## Availability of data and materials

The observed SNPs from the sequence data from which the conclusions of this manuscript were drawn are available as Additional file 1: Table S1. The list of all observed *pfap2mu* SNPs in Ghanaian isolates from the three study sites. Table S2. The list of *pfap2mu* SNPs from five isolates with at least 20 SNPs per sample. Table S3. The list of all observed *pfubp1* SNPs in Ghanaian isolates from the three study sites.

## Authors' contributions

NOD, NBQ, GF, BA and KAK conceived and designed the study. TA, NAAE and SM did the laboratory analysis and generated molecular data. TA, NAAE and OCKH conducted data analysis. TA and NAAE drafted the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Ethical approval for the study was given by the NMIMR Institutional Review Board (IRB). The samples were taken after parents or guardians of the children gave their consent. This work is part of an ongoing surveillance of antimalarial drug efficacy studies in Ghana approved by the NMIMR IRB (CPN032/05-06a).

## Consent for publication

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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