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Nationwide Surveillance of *Pfhrp2* Exon 2 Diversity in *Plasmodium falciparum* Circulating in Symptomatic Malaria Patients Living in Ghana

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Abstract. Reports of increasing false-negative HRP2-based rapid diagnostic test results across Africa require constant monitoring of factors associated with these false-negative outcomes, as failure of this diagnostic tool will have severe consequences on malaria treatment and control programs. This study characterized the extent of genetic diversity in the *Plasmodium falciparum* histidine-rich protein 2 (*Pfhrp2*) gene in *P. falciparum* isolates from symptomatic malaria patients across the regions of Ghana. Exon 2 of *Pfhrp2* was amplified from gDNA using polymerase chain reaction. All *Pfhrp2*-negative samples were subjected to Pf*18S rRNA* and *Pfmsp2* gene amplifications. The amplified *Pfhrp2* exon 2 fragments from clonal samples were sent for commercial Sanger sequencing. The type and number of *Pf*HRP2 repeats, classified based on repeat types previously reported, were estimated from the sequence data and compared among geographical regions. About 81% (2,333/2,890) of the original microscopy positive dried blood spot (DBS) samples were available and used in this study. The *Pfhrp2* exon 2 amplification was successful in 98.5% (2,297/2,333) of the tested samples, with band size ranging from 400 bp to 1,050 bp. A total of 13 out of the 24 previously reported repeat types were identified among the samples, with three samples lacking both type 2 and type 7 repeat motifs. This study suggested that the genetic diversity of *Pfhrp2* exon 2 identified in *P. falciparum* circulating in symptomatic malaria patients in Ghana is unlikely to influence the sensitivity and specificity of HRP2 RDT-based diagnosis.

INTRODUCTION

Malaria remains one of the most important infectious diseases in humans particularly in Africa, causing millions of illnesses and thousands of deaths each year, with most deaths occurring in young children.[1] The ability to reliably diagnose malaria infections accurately and rapidly is essential to both the management of individual patients as well as public health efforts to control and eliminate the disease.[2]

Microscopy remains the gold standard for malaria diagnosis, however, it is not a rapid diagnostic tool and requires well-trained microscopists to read the blood smears. Thus, there is a need for rapid diagnostic tools with higher sensitivities than microscopy that are user friendly.[3] Polymerase chain reaction (PCR) is a highly sensitive molecular tool that can detect ultra-low parasite densities, but it requires sophisticated and expensive equipment and reagents, highly trained staff, and a reliable source of power.[4] In Africa and most malaria endemic countries, PCR is used primarily for research in advanced facilities but not as a point of care diagnostic tool. Rapid diagnostic tests (RDT) for malaria are user-friendly point of care devices that do not require electricity and highly skilled personnel to operate. Although they are not as sensitive as PCR, RDT are ideal for use in remote areas where accurate diagnosis by microscopy is not available.[4,5]

To date, there are many commercially available RDT for malaria, with the most used ones detecting *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2), parasite lactate dehydrogenase enzyme (LDH), or aldolase.[2] Malaria in Ghana and most parts of the WHO Africa Region is primarily caused by *P. falciparum* and as such the most used malaria RDT detects primarily PfHRP2 or in some cases PfLDH antigens, both of which are specific to detecting *P. falciparum*. The PfHRP2-based malaria RDTs are considered the most sensitive for *P. falciparum* detection whereas the PfLDH RDTs are most specific.[6] Malaria diagnosis by RDT can be affected by the parasite density of the sample, misinterpretation of test results, and/or improper handling and storage of test, resulting in false-negative or false-positive diagnoses.[6,7] False-negative results by PfHRP2-based RDT can also occur due to the deletion of the *Pfhrp2* and *Pfhrp3* genes in some parasite isolates, low levels of circulating HRP2 antigens, and the prozone effect.[8] An earlier study conducted in Ghana reported the prevalence of false-negative PfHRP-2 based RDT results at about 1%, out of which low parasite density accounted for about 9% and parasites with *Pfhrp2* exon2 deletions accounting for about 39%.[9]

The deletion of *Pfhrp2/3* genes and *Pfhrp2* gene diversity in *P. falciparum* infections have been reported to undermine the accurate RDT diagnosis.[4–6,9] Although there have been a few reports on the prevalence and distribution of *P. falciparum* parasites with *Pfhrp2* gene deletions in Ghana, there are very limited reports on the distribution and extent of diversity in the *Pfhrp2* gene. Therefore, this study investigated the distribution and extent of *Pfhrp2* gene diversity in *P. falciparum* from symptomatic malaria patients across all the regions of Ghana.

MATERIALS AND METHODS

Ethical clearance and approval. Ethical approval for the study was obtained from the Institutional Review Board of the Noguchi Memorial Institute for Medical Research (CPN #068/17-18). Participants of all age groups were

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eligible to be enrolled into the study. Written informed consent was obtained from all adults and parental consent was obtained from the parent/guardian of all children below the age of 17 years and child assent was obtained from all children aged between 12 and 17 years.

Study site and sample collection. The study used samples from an earlier cross-sectional study that recruited 19,787 suspected malaria patients aged between 1 and 94 years old from 100 health facilities distributed across Ghana, (Supplemental Table 1), from May to August (during the peak malaria season) of 2018.[9] The original study collected 2 mL of peripheral blood from each study participant into an ethylenediaminetetraacetic acid (EDTA) Vacutainer tube. An aliquot of the whole blood was used to spot a commonly used malaria RDT supplied by the National Malaria Control Program (NMCP), the CareStart® malaria HRP2 RDT kit (Assess Bio, Inc, USA) according to the manufacturer's instructions, and to prepare a thick and thin blood smear using a standard procedure.[10] Four separate 50 μL drops of whole blood was dropped onto the filter paper strip (labeled with the same ID as the whole blood sample), which was air dried and kept individually in a Ziploc® bag containing a desiccant. The Ziploc bags containing the individual blood spots (DBS) were then stored in a +4°C cold room until used. The remaining whole blood sample was separated into packed cells and plasma and independently stored at −20°C until needed. Each time blood spots were punched out of a filter paper strip; it was returned to the +4°C cold room for storage. Samples selected for this study included all samples that were confirmed in the original study to contain *P. falciparum* by microscopy. The *Plasmodium* parasite density of each sample was estimated from the thick blood smears as the number of parasites counted against 200 white blood cells (WBCs) multiplied by 40, based on the assumption that 1 μL of blood contains 8,000 WBCs. The demographic data for all the selected samples, including the microscopy data was obtained from the original study database.

DNA extraction. Genomic DNA (gDNA) was extracted from two 3-mm diameter discs of dried blood that was punched out of the DBS, which had been stored at +4°C for about 18 months using the Saponin-Chelex method previously described.[9] The supernatant containing the gDNA was stored in a sterile 0.5-mL microfuge tube and used immediately or stored at −20°C.

***Plasmodium* species identification.** The conserved regions of the 18S *rRNA* gene of *P. falciparum* was PCR amplified using a protocol previously described by Amoah et al.[9] and others.[11,12] Briefly, the 15 μL total reaction volume of the primary reaction contained 1X PCR buffer, 167 nM dNTPs, 2.5 mM $MgCl_2$, 80 nM of the genus-specific primer rPLU1 (forward) and rPLU5 (reverse), 1 U of OneTaq DNA polymerase, and about 3 μL of DNA template. The secondary (nested) reaction contained similar composition of reagents as the primary reaction; however, the species-specific primer set rFAL1 (forward) and rFAL2 (reverse) and 0.5 μL of the primary reaction product were used as the template. The nested PCR products were separated on a 2% agarose gel prestained with ethidium bromide and visualized using a Vilber gel documentation system (Vilber, France).

Amplification of *Pfhrp2* exon 2. Dreamtaq Master Mix was used to PCR amplify exon 2 of the *pfhrp2* gene from 3 μL of extracted gDNA (DNA template). The 15 μL primary reaction mixture contained 0.56 μM of forward primer, PfHRP2-F1 and reverse primer, PfHRP2-R1.[9] All samples that failed to yield an amplicon after the primary PCR reaction were subjected to a secondary semi-nested PCR using a similar reaction mix and PCR conditions as stated earlier with the exception that forward primer, PfHRP2-F2 and reverse primer, PfHRP2-R1 were used. Genomic DNA from the NF54 *P. falciparum* isolate (MRA 1000G) was used as a positive control. The sequence and melting temperature of all primers used in this study are listed in Supplemental Table 2.

The *PfHRP2* exon 2 gene sequencing. Genomic DNA from all samples that yielded a single band after amplification of the *Pfhrp2* exon 2 from both the primary and semi-nested PCR were selected for *Pfhrp2* exon 2 gene sequencing. Briefly, 3 μL of extracted gDNA was amplified using Amplitaq Gold® Fast PCR Master Mix (2X) (Applied Biosystems, USA) in a 25 μL reaction volume, using primers PfHRP2-F1 and PfHRP2-R1 (for nest 1 products) or PfHRP2-F2 and PfHRP2-R1 (for nest 2 products). The PCR products were subsequently sent for commercial Sanger sequencing (Macrogen Europe Laboratory, The Netherlands). Bidirectional sequencing was performed on all the samples, using PfHRP2-R1 and PfHRP2-F1 for nest 1 products and PfHRP2-R1 and PfHRP2-F2 for nest 2 products. The amplified *PfHRP2* exon 2 from NF54 *P. falciparum* isolate (MRA 1000G) was used as a positive control for the sequencing reactions.

Merozoite surface protein 2 genotyping. The polymorphic region of merozoite surface protein 2 (*msp2)* block 3 was genotyped using a previously described nested PCR[10,13,14] to further confirm the presence or absence of *P. falciparum DNA* in samples that remained negative for *pfhrp2* after the semi-nested reaction gDNA from K1 (MRA-155G) and 3D7 (MRA-102G) *P. falciparum* strains were used as positive controls and nuclease-free water was used as the negative control (no template control).

Analysis of *Pfhrp2* exon 2 gene sequence. The sequence data (ab1 files) obtained from Sanger sequencing were processed and cleaned of the noisy and unevenly spaced traces of nucleotides and analyzed using CodonCode aligner program v. 6.0.2 (CodonCode Corporation, Centerville, MA). The nucleotide sequences were translated into the corresponding amino acid sequence with correct open reading frame using online software ExPASy Translate Tool (Swiss Institute of Bioinformatics Resource Portal; www.expasy.org). The correct reading frame was determined by selecting the frame that resulted in the most histidine residues and no stop codons in the continuous sequence (Supplemental Table 3). The translated amino acid sequences data were aligned to PfHRP2 exon 2 amino acid sequence from the Pf3D7 reference strain (PF3D7_0831800) using MAFFT version 7 for multiple alignment program for amino acid or nucleotide sequences.[15] The PfHRP2 repeats were classified based on previously reported repeat types.

Data analysis. Statistical analyses were performed using IBM SPSS v. 26 (IBM Corp., Armonk, NY) to calculate the frequency, median, mean, standard error, and other descriptive statistics of the demographic as well as the *Pfhrp2* sequence data. The R package was used to locate and count each of the 24 previously reported repeat types in each *PfHRP2* gene sequence. The data were then combined with the location and demographic metadata such that the

occurrence of each motif type was compared regionally and by gender.

RESULTS

Out of the 2,890 microscopic-positive samples, 2,333 DBS were identified to contain enough blood sample and selected for further molecular screening (Figure 1). A total of 20.3% (465/2,286) of the microscopic-positive samples had low parasitemia < 5,000 parasites/μL (mean ± SD = 1,789 ± 1,378, range = 16–4,983 parasites/μL) (Table 1). There were generally more samples from females (55.4%) than males and samples from the Greater Accra region were from significantly older participants than those from the other nine regions (analysis of variance [ANOVA], $P = 0.037$). The highest number of samples were from the Ashanti region ($N = 364$), whereas the smallest number of samples ($N = 111$) were from the Eastern region (Table 1). Overall, 94.7% ($N = 2,199$) of the samples tested positive by the HRP2 RDT used in the study (Table 1). A total of 36/2,333 (1.5%) samples were identified that lacked exon 2 of the *Pfhrp2* gene; however, all except 2 tested positive for *P. falciparum* using theHRP2 RDT. These samples were distributed in all the regions except for the Upper East region (Table 2). Of the 36 samples that tested negative based on the *PfHRP2* exon 2 amplification, 29 (80.6%) tested positive for *P. falciparum* based on amplification of the *18S rRNA* gene and the *msp2* gene.

***Pfhrp2* exon 2 gene size diversity.** The *Pfhrp2* gene fragments among the *Pfhrp2*-positive samples ranged from 400 bp to 1,050 bp and were categorized into groups varying by about 50 bp (Figure 2). The *Pfhrp2* variants of size between 650 bp and 1,000 bp were identified in samples from all the 10 regions. By contrast, variants including the 400 bp, 450 bp, 500 bp, 550 bp, and the 1,050 bp were identified only in samples from selected regions. For example, the 400 bp variant was only identified in the Brong Ahafo, Central, and Upper East regions, whereas the 500 bp variant was only identified in the Ashanti, Brong Ahafo, Central and Northern regions. The 550 bp and 1,050 bp variants were found in six and eight regions, respectively. Four of the five least common variants were present in samples from the Central and Volta regions (Figure 2). The Eastern Region had the lowest number of *Pfhrp2* exon 2 size variants.

Genetic diversity in exon 2 of the *Pfhrp2* gene. Out of the 2,295 samples from which *Pfhrp2* exon 2 was successfully amplified, only 286 yielded a single *Pfhrp2* band, and were thus classified as clonal at the *Pfhrp2* locus and selected for sequencing. Among the sequences obtained, 208 (72%) were of high enough quality to derive consensus sequences for subsequent analyses. The nucleotide sequences were translated into amino acid sequences using the standard translation table, utilizing the reading frame that produced the most histidine residues. Among the 24 *PfHRP2* repeat types,[4] 13 types were observed in the 208 samples. Types 2, 4, and 7 were most commonly among all samples whereas type 14 was only observed in six of the samples (Supplemental Table 4). The number of repeats was shown to be the same for some repeat types across all samples. For example, there were nine repeats of types 1, 2, 3, 5, and 14, three repeats of types 4 and 9, six repeats of types 6, 7, 8, and 13, and five repeats of type 19 found consistently across the samples (Supplemental Table 4). There were

TABLE 1
Demographic characteristics of study participants

	AS	BA	C	E	GA	N	UE	UW	V	W	Total
Sex (F)											
n	197	163	184	63	87	172	72	100	125	110	1,273
(%)	54.40	56.60	63.00	56.80	49.40	55.00	55.40	53.20	55.30	52.10	55.40
Parasitemia, n (mean ± SD, range)											
< 5,000	119 (1,756 ± 1,390, 34–4,983)	42 (2,502 ± 1,360, 286–4,710)	99 (1,635 ± 1,436, 16–4,920)	3 (2,512 ± 993.2, 1,852–3,654)	39 (1,721 ± 1,095, 160–4,272)	38 (1,514 ± 1,307, 95–4,660)	22 (1,866 ± 1,495, 32–4,388)	14 (1,998 ± 1,152, 561–4,527)	56 (1,855 ± 1,471, 48–4,941)	33 (1,540 ± 1,238, 127–4,368)	465 (1,789 ± 1,378, 16–4,983)
Parasites/μL											
>5,000	293 (90,462 ± 122,974, 5,785–1,096,867)	293 (103,417 ± 123,574, 5,242–667,142)	196 (71,031 ± 101,991, 5,000–675,000)	38 (176,476 ± 200,494, 5,663–662,500)	93 (45,079 ± 42,965, 5,231–230,088)	135 (170,647 ± 190,537, 5,463–930,000)	133 (148,459 ± 199,460, 5,000–1,037,500)	175 (146,697 ± 180,329, 5,245–922,500)	221 (68,134 ± 79,835, 5,240–537,500)	244 (64,687 ± 95,577, 5,015–1,030,000)	1,821 (99,354 ± 137,780, 5,000–1,096,867)
Parasites/μL											
RDT											
n	344	281	266	106	170	291	124	172	222	223	2,199
(%)	95.00	97.20	91.10	95.50	96.60	93.00	92.50	91.00	98.20	97.00	94.70
Age (years)											
Mean (SEM)	16.13 (0.878)	11.39 (0.841)	17.92 (1.037)	14.66 (1.613)	20.26 (1.21)	12.51 (0.796)	18.01 (1.519)	15.69 (1.188)	15.03 (1.013)	11.77 (0.733)	15.08 (0.329)
Min–Max	1–92	1–85	1–85	1–85	1–76	1–86	1–88	1–85	1–73	1–60	1–92

Yrs = years; n = exact count; AS = Ashanti region; BA = Brong Ahafo region; C = Central region; E = Eastern region; GA = Greater Accra region; N = Northern region; RDT = rapid diagnostic test; SEM = standard error of the mean; UE = Upper East region; UW = Upper West region; V = Volta region; W = Western region. The numbers represent the exact count (n) as a % of the total number of participants in the group (%). About 1.2% of the participants did not have gender data recorded in the database.

TABLE 2
Distribution of P*fhrp2* exon 2 negative samples

	AS	BA	C	E	GA	N	UW	V	W
F/M (n)	(1/0)	(1/2)	(4/5)	(2/1)	(1/1)	(5/3)	(0/2)	(4/2)	(2/0)
Age (mean, SEM)	75	2.67 (1.20)	13.22 (1.67)	6 (2.89)	8.5 (0.5)	9.38(2.73)	12.50 (2.5)	11.8 (5.34)	7.50 (8.5)
PD (median, Range)	4,057	72,722.67 (42,857–157,243)	36,690.67 (1,440–128,400)	303,346.67 (25,040–490,000)	20,671 (15,844–25,498)	116,628.13 (4,060–390,000)	18,334.5 (10,192–26,477)	37,334.5 (288–185,000)	16,829.5 (17,059–16,600)

AS = Ashanti region; *BA* = Brong Ahafo region; C = Central region; E = Eastern region; F = female; GA = Greater Accra region; n = count; N = Northern region; PD = parasite density; *Pfhrp2* = *Plasmodium falciparum* histidine-rich protein 2; SEM = standard error of the mean; UW = Upper West region; V = Volta region; W = Western region. All scripts used in this work are available on GitHub at https://github.com/colbyford/amoah_ghana_phylo.

110 samples that lacked both repeat type 2 and type 7; 99 of these tested positive by the HRP2 RDT kit (Supplemental Table 4).

The total number of repeats, regardless of the type, ranged from 3 to 91 (Supplemental Table 4). Compared with the reference 3D7, which has 95 total repeats, one sample from the Central region and the other from Western region had the largest number of repeats ($N = 91$), giving the longest *Pfhrp2* exon 2. By contrast, two samples (one from Greater Accra region and the other from Western region) had the smallest number of repeats and shortest *Pfhrp2* exon 2 fragments. The 13 repeat types were found in all 10 regions of Ghana without clear geographical differences (Figure 3). Although type 14 was only found in six *P. falciparum* isolates, these isolates were distributed broadly across Ghana. In each region, the number of repeat types varied widely, despite the differences in sample size among regions (Figure 3). Further comparison between genders indicated slight but non-significant difference in the variation of repeat number (Supplemental Figure 2).

DISCUSSION

Genetic diversity in the malaria parasites is a major challenge to malaria control interventions and diagnosis, as such, continued surveillance activities in expanded settings could help inform the implementation of appropriate control interventions. There have been a number of reports on parasite diversity as a whole in Ghana based on parasite surface proteins such as MSP1 and MSP2,[1] and microsatellite markers.[14] Deletions as well as variations in the *Pfhrp2* gene sequence may interfere with effective malaria diagnosis using PfHRP2-based RDT.[16] The pLDH RDT are not routinely used for malaria diagnosis in Ghana, mainly because the combo RDTs have proven in a number of studies to have much lower sensitivities relative to the HRP2-based RDT. Despite the advent of parasites with *Pfhrp2* exon 2 gene deletions, the superior sensitivities of PfHRP2-based malaria

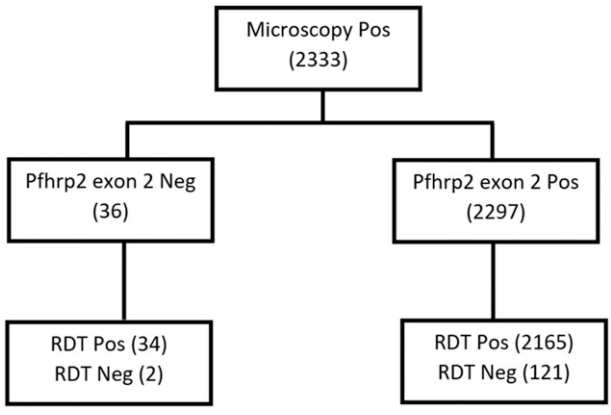

FIGURE 1. Flow chart of sample processing for the samples that tested negative for exon 2 of the *Pfhrp2* gene. The integrity of the DNA was further evaluated using msp2 genotyping (Supplemental Figure 1). Out of the total samples, RDT data were not available for 11 samples. msp2 = merozoite surface protein 2; Neg = negative; *Pfhrp2* = *Plasmodium falciparum* histidine-rich protein 2; Pos = positive; RDT = rapid diagnostic test.

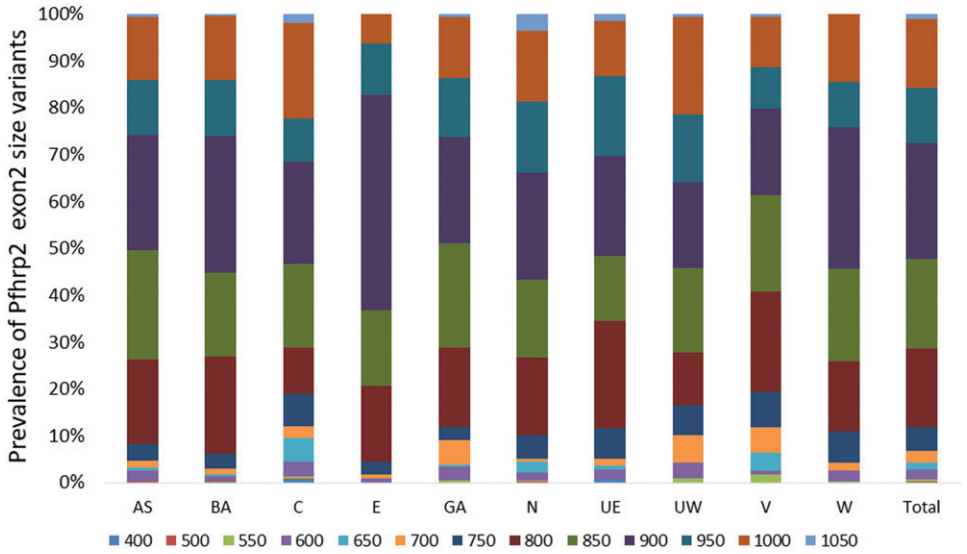

FIGURE 2. Regional distribution of *Pfhrp2* exon 2 size variants. AS =Ashanti region; *BA* = Brong Ahafo region; C = Central region; E = Eastern region; GAR = Greater Accra region; N = Northern region; *Pfhrp2* = *Plasmodium falciparum* histidine-rich protein 2; UE = Upper East region; UW = Upper West region; V = Volta region; *W* = Western region. The data are represented as a percent of the total number of fragments obtained from each region and the different colors as described by the legend represent varying *Pfhrp2* exon 2 variants. This figure appears in color at www.ajtmh.org.

RDT relative to presently available pLDH-based malaria RDT have prevented the large-scale use of pLDH-based RDT for routine malaria diagnosis. Challenges with the use of microscopy, the gold standard for malaria diagnosis in many rural settings in Africa, including Ghana where malaria incidence is high, such as the unstable nature of power supply and the slow turnaround time, both of which make the use of the PfHRP2-based malaria RDT still a very attractive diagnostic tool for diagnosing clinical malaria cases and make the nationwide surveillance of the efficiency of the PfHRP2 RDT cost effective.

There are a few reports on *Pfhrp2* gene deletions in Ghana, but so far, only one of them examined the diversity of the *Pfhrp2* gene among parasites from one region (the Volta region) of the country.[6] This study provided comprehensive *Pfhrp2* gene diversity data from *P. falciparum* isolates harbored in suspected malaria patients attending several health facilities across all the regions of the country.

Extensive diversity was observed in the *Pfhrp2* exon 2 gene sequence within and between the different regions of the country, with the Eastern region having parasites with the least number of *Pfhrp2* exon 2 size variants. Similar observations have been reported among *P. falciparum* isolates from Ethiopia, Kenya, Mali, Uganda, and Senegal.[17–19] The range of *Pfhrp2* exon 2 size variants identified in this study is similar to that reported in the China–Myanmar boarder area and Papua New Guinea,[6,20–22] but much greater than previously reported in the Volta region of Ghana[6] as well as in Yemen.[23] Such discrepancy is likely due to the difference in sample size and collection sites. An earlier report from the Volta region of Ghana identified 11 different *PfHRP2* repeat types,[6] whereas 13 *PfHRP2* repeat types were identified in this study, with only eight repeat types identified from the two sequenced samples from the Volta region. No sample was identified with the Type 11 and 12 repeat. The type 11 repeat seems to be very rare among *P. falciparum isolates* from Africa as it was not observed in Ghana, Ethiopia, Kenya, Mali, Uganda, and Senegal,[6,17–19] similar to the current study. Also, the type 11 repeat was absent among Yemen *P. falciparum* isolates.[24] The frequency of type 12 repeat varies from country to country, with isolates from Ethiopia harboring 10.4%[17] and isolates from Kenya identified to harbor between 80% and 99%,[18] however, no type 12 repeat was detected in samples from this study.

The type 2 and type 7 have been suggested to be predictive of the sensitivity of PfHRP2 RDT, with a low number of these two repeats increasing the likelihood of obtaining a false-negative HRP2 RDT result.[15,25] In this study, there were only three samples that lacked both the type 2 and type 7 repeats but no sample lacked only one of the two repeat types, supporting the notion that these two repeat types may have complementary function in HRP2 antigen production. However, the absence of the type 2 and type 7 repeats in a sample did not always result in a negative HRP2 RDT result, consistent with previous reports that showed these two repeat types are unlikely to be responsible for altering the sensitivity of HRP2 RDT.[4]

A few msp2-positive samples did not yield amplicons after the *Pfhrp2* exon 2 amplifications, suggesting possible *Pfhrp2* gene deletions or mutations in priming sites or a reduced efficiency of the PCR used. Majority of these samples, however, tested positive by the HRP2 RDT test. One possible explanation is that the HRP2 antigen detected by the RDT was from a recently cleared malaria infection or that the parasites in these samples had high levels of PfHRP3, which is recognized by some PfHRP2 antibodies and can compensate for the absence of the *Pfhrp2* gene in a sample.[26] The sensitivity of PCR is higher than conventional malaria RDTs given that some samples that tested negative by the HRP2 RDT test were identified as positive by different types of PCR, including the *Pfhrp2* exon 2 PCR. This could be explained by

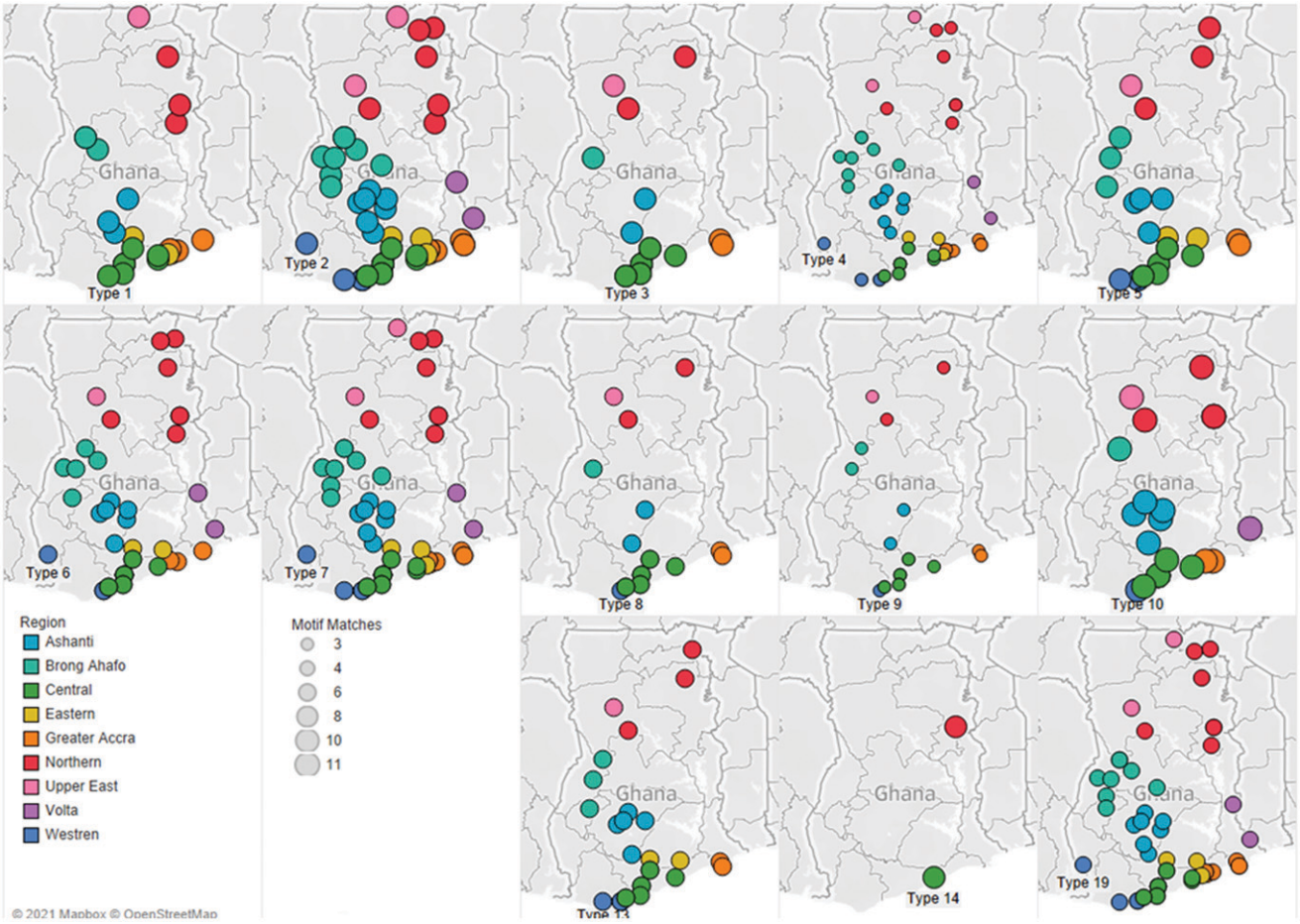

FIGURE 3. Distribution of the 13 *Pfhrp2* exon 2 repeat types observed in the Ghanaian *Plasmodium falciparum*. Point size represents the number of motifs matches at a given location and each point represents a sample. No samples were identified with the Type 11 and Type 12 repeat motifs. *Pfhrp2* = *Plasmodium falciparum* histidine-rich protein 2. This figure appears in color at www.ajtmh.org.

low-density *P. falciparum* infections and consequently, low circulating PfHRP2 antigens in the infections.

Limitations. The analysis and the determination of *PfHRP2* exon 2 diversity in *P. falciparum* circulating in Ghana require a large sample set and high-quality sequencing data. Although the study started with large sample size, only 12% of the samples were clonal at the *Pfhrp2* locus and could be sequenced using Sanger sequencing as mixed clone samples tend to produce Sanger sequence results with ambiguities. Also, this study did not perform genetic analysis on the *Pfhrp3* gene, which is known to play a role in the outcome of PfHRP2 RDTs. In addition, the limit of detection for the *Pfhrp2* PCR used in this study was not evaluated, suggesting that some *Pfhrp2* exon 2 fragments could have been missed.

CONCLUSION

This study is the first to report genetic diversity in the *Pfhrp2* gene in samples collected from multiple regions of Ghana and provides baseline information of the prevalence and distribution of parasites with *Pfhrp2* gene variants. The study shows that diversity in the *Pfhrp2* gene among the Ghanaian *P. falciparum* isolates used in this study does not influence the sensitivity and specificity of HRP2 RDT diagnosis in Ghana. However, there is still the need to continue to monitor *Pfhrp2* exon 2 gene diversity, especially exon 2 gene deletions as these have the potential to result in the failure of routine malaria diagnosis in Ghana.

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Note: Supplemental tables and figures appear at www.ajtmh.org.

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