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Spontaneous Mutations in the *Plasmodium falciparum* Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase (PfATP6) Gene among Geographically Widespread Parasite Populations Unexposed to Artemisinin-Based Combination Therapies▽†

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Recent reports on the decline of the efficacy of artemisinin-based combination therapies (ACTs) indicate a serious threat to malaria control. The endoplasmic/sarcoplasmic reticulum Ca^{2+}-ATPase ortholog of *Plasmodium falciparum* (PfSERCA) has been suggested to be the target of artemisinin and its derivatives. It is assumed that continuous artemisinin pressure will affect polymorphism of the PfSERCA gene (*serca*) if the protein is the target. Here, we investigated the polymorphism of *serca* in parasite populations unexposed to ACTs to obtain baseline information for the study of potential artemisinin-driven selection of resistant parasites. Analysis of 656 full-length sequences from 13 parasite populations in Africa, Asia, Oceania, and South America revealed 64 single nucleotide polymorphisms (SNPs), of which 43 were newly identified and 38 resulted in amino acid substitutions. No isolates showed L263E and S769N substitutions, which were reportedly associated with artemisinin resistance. Among the four continents, the number of SNPs was highest in Africa. In Africa, Asia, and Oceania, common SNPs, or those with a minor allele frequency of ≥0.05, were less prevalent, with most SNPs noted to be continent specific, whereas in South America, common SNPs were highly prevalent and often shared with those in Africa. Of 50 amino acid haplotypes observed, only one haplotype (3D7 sequence) was seen in all four continents (64%). Forty-eight haplotypes had frequencies of less than 5%, and 40 haplotypes were continent specific. The geographical difference in the diversity and distribution of *serca* SNPs and haplotypes lays the groundwork for assessing whether some artemisinin resistance-associated mutations and haplotypes are selected by ACTs.

Artemisinin-based combination therapies (ACTs) are currently the first-line treatment for uncomplicated falciparum malaria in most areas of endemicity (23, 34). The deployment of ACT has greatly reduced malaria morbidity and mortality (8). However, recently there has been accumulating evidence which suggests a decline of the efficacy of ACTs and artemisinin monotherapy in western Cambodia (7, 22). Although the molecular mechanism of the antimalarial action of artemisinin and its derivatives (artemisinins) remains to be clarified, the endoplasmic and sarcoplasmic reticulum Ca^{2+}-ATPase ortholog of *Plasmodium falciparum* (PfSERCA or PfATP6) has been suggested to be the target of artemisinins (9, 15). A replacement of L at codon 263 of the PfSERCA gene (*serca*) with E (L263E) results in abrogation of inhibition of PfSERCA by artemisinin (32). A recent allelic exchange study also showed reduced (though not significantly) susceptibility to artemisinins in parasites expressing the L263E allele (33). Mutations(s) of *serca* has also been associated with *in vitro* artemether resistance, with field isolates from French Guiana having an artemether 50% inhibitory concentration (IC_{50}) of 1.7 nM whereas that for parasites having an S769N substitution was 79.4 nM (13, 15, 16). The association of these mutations with artemisinin resistance, however, has not been confirmed for other geographic areas (2, 3, 5–7, 11, 12, 14, 18, 20, 26, 35).

Limited sequence analyses have previously shown that *P. falciparum serca* contains a number of single nucleotide polymorphisms (SNPs) (6, 14, 28). Thus, a large number of field isolates would be required to properly assess whether drug pressure imposed by continuous deployment of ACTs causes a potential selection of a mutation(s) in *serca* that is associated

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with artemisinin resistance. We consider that baseline information on *serca* polymorphism occurring before the implementation of ACTs would provide necessary information to infer whether some *serca* mutations are likely selected by ACTs. We have recently used *serca* as a genetic marker to study the geographical distribution of genetic diversity of *P. falciparum* and obtained 514 full-length *serca* sequences from nine *P. falciparum* populations in Africa, Asia, Oceania, and South America (30). In this study we newly obtained 139 full-length *serca* sequences, mostly from Malawi, Madagascar, Iran, and Bangladesh. Together with published sequences, a total of 656 sequences were analyzed for *serca* polymorphism. Importantly, all parasite isolates examined hereto were unexposed to ACTs. The results show that *serca* has many spontaneous mutations and haplotypes, which are geographically distinctive. It is predicted that further studies will reveal more SNPs/haplotypes. This information has several implications for inferring whether some *serca* mutations and haplotypes are selected by the current continuous deployment of ACTs.

MATERIALS AND METHODS

Parasite isolates. We collected *P. falciparum* isolates from Malawi, Madagascar, Iran, and Bangladesh. In Malawi, samples were collected from infected individuals in all age groups during cross-sectional surveys in June and July 2000 at two primary schools in the Salima District (4). The study was approved by the local ethics committee of the Malaria Control Programme and the Malawi Ministry of Health. In Madagascar, blood samples containing parasites were collected from consenting symptomatic outpatients in 2005 as part of the national network activities for the surveillance of drug-resistant *Plasmodium* spp. (24). Administrative authorizations and ethical clearances were provided by the Ministry of Health and the national ethics committee. Samples from rural areas of Ampasimpotsy and Saharevo were sent to the malaria research unit of the Institut Pasteur de Madagascar and kept frozen at −20°C until use. In Iran, blood samples were collected from *P. falciparum*-infected individuals, 1 to 70 years old, with symptomatic uncomplicated malaria attending the Malaria Health Center in Chabahar and the Public Health Department in Sistan and Baluchistan province, southeastern Iran, during 2001 and 2002. The study was approved by the Ethical Review Committee of Research of the Pasteur Institute of Iran. In Bangladesh, samples were collected from symptomatic malaria patients in all age groups at Bandarban district hospital from October to December 2007 (17). Approval of the study was obtained from the Bangladesh Medical Research Council and the local health regulatory body in Bandarban, Bangladesh. All isolates examined in this study were from parasite populations not exposed to ACTs (including rural areas in Bangladesh and Papua New Guinea [PNG] which have had no previous access to ACT, despite the countries' support for the WHO policy). We also used three cultured strains originally isolated from Sudan (29).

DNA sequences. Parasite genomic DNA was extracted using the QIAamp DNA blood minikit (Qiagen, Hilden, Germany). Full-length *serca* was amplified by PCR using Takara LA *Taq* polymerase (Takara Bio, Japan) in a 20-μl reaction mixture as previously described (28) with slight modifications: Primers U1 and 4099R were used (see Table S1 in the supplemental material). Forty cycles of amplification (20 s at 93°C and 5 min at 62°C) were preceded by denaturation at 93°C for 1 min and followed by a final elongation at 72°C for 10 min. The PCR product was diluted 10-fold, and a 2-μl aliquot was used as the template for a second PCR amplification of 20 cycles in a 50-μl reaction mixture using primers U1 and 4094R. The PCR products were purified using the QIAquick PCR purification kit (Qiagen). DNA sequencing was performed directly from two independent PCR products, using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and an ABI 3130 Genetic Analyzer (Applied Biosystems). Sequencing primers were designed to cover target regions in both directions (see Table S1 in the supplemental material). Mixed-genotype infections, judged from superimposed electropherogram peaks, were excluded from further analysis.

Sequence analyses. We obtained a total of 139 full-length sequences of *serca* coding regions from Malawi ($n = 38$), Madagascar ($n = 19$), Iran ($n = 35$), Bangladesh ($n = 44$), and Sudan ($n = 3$). Multiple infections were detected for 36 isolates (18.0%). Also, 514 full-length sequences that we recently published (30) were included for analysis; these were from Ghana ($n = 38$), Tanzania ($n = 69$), Thailand ($n = 82$), Philippines ($n = 53$), Papua New Guinea (PNG) ($n = 89$), Solomon Islands ($n = 51$), Vanuatu ($n = 80$), Brazil ($n = 42$), and Venezuela ($n = 10$). Table S2 in the supplemental material summarizes all *serca* sequences used in this study. Three additional sequences from cultured strains (3D7, Dd2, and HB3) (28) were also included. Nucleotide diversity was estimated by $\theta\pi$, the average pairwise nucleotide distance, and θ_S, the standardized number of polymorphic sites per site (Watterson's estimator), using DnaSP version 4.10 (25). Sequences were aligned using Clustal W (31) implemented in MEGA version 4.0 (27). Polymorphic sites and synonymous and nonsynonymous substitutions were determined using DnaSP.

The allele frequency of SNPs was calculated using Arlequine version 3.1 (10). We categorized SNPs as either common or uncommon, defined as those with a minor allele frequency of ≥ 0.05 or with a minor allele frequency of < 0.05, respectively (19). (Excluding samples with multiple infection did not affect the frequency of common SNPs in this study, because analysis of 36 multiply infected samples showed a frequency of common SNPs [27/39 = 69%] that was comparable to that for 139 singly infected samples [103/139 = 74%] [$P = 0.88$, chi-square test].) Nucleotide and amino acid positions were numbered according to the 3D7 sequence (PlasmoDB gene identification no. PFA0310c). Amino acid haplotype diversity (h) was calculated using the formula $h = [n/(n-1)] \times (1 - \Sigma p_i^2)$, where p_i is the frequency of the ith *serca* amino acid haplotype (21). The variance (V) of h was calculated using a formula modified from Nei's formula for a haploid genome: $V = [2/n(n-1)]\{2[n-2][\Sigma p_i^3 - (\Sigma p_i^2)^2] + \Sigma p_i^2 - [\Sigma p_i^2]^2\}$.

Nucleotide sequence accession numbers. The sequences reported in this study have been deposited in the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank database (accession numbers AB576210 to AB576348).

RESULTS

An alignment of 656 sequences (3,687 to 3,693 bp) revealed 62 polymorphic nucleotide sites with 64 SNPs, of which 38 resulted in amino acid substitutions (Fig. 1; see Table S3 in the supplemental material). Among the 64 SNPs, 43 SNPs (20 synonymous SNPs and 23 nonsynonymous SNPs) were newly identified in this study. Together with partial *serca* sequences obtained by other investigators, there were 110 SNPs (42 synonymous SNPs and 68 nonsynonymous SNPs) in total. In addition to SNPs, a variation in the number of Asn residues (9 to 11) in the Asn-tandem repeat region at codons 457 to 465 (of the 3D7 sequence) and a deletion of the Gly residue at codon 844 were also noted (Fig. 1; see Table S3 in the supplemental material). In our samples, we found no polymorphism at codons 263 and 769; these polymorphisms have been shown to affect the PfSERCA activity or to be associated with increased artemisinin IC_{50}s (13, 32). An E431K SNP, which has been reported to be associated with increased artesunate IC_{50}s in Senegal (13), was detected in Africa, Asia, and South America. I89T and N465K, which have been inferred not to be associated with artemisinin resistance (7), were observed only in Asia and Oceania. Amino acid replacements were largely clustered in cytoplasmic domain 3 (Fig. 1). In contrast, 10 transmembrane domains contained only two amino acid changes (at codons 67 and 1169), which had similar residue properties (i.e., basic residues K67R and hydrophobic residues V1169I).

The number of SNPs was relatively high in Africa (44 SNPs with 27 nonsynonymous SNPs) compared to other continents (10 to 21 SNPs with 5 to 10 nonsynonymous SNPs) (Table 1 and Fig. 2a). Two nucleotide diversity indices, i.e., θ_S, the number of polymorphic sites per site, and $\theta\pi$, the average number of pairwise nucleotide differences, showed substantially different levels in the four continents. Overall, θ_S was higher than $\theta\pi$ in Africa, Asia, and Oceania, whereas $\theta\pi$ was somewhat higher than θ_S in South America. This indicates that the majority of the alleles in Africa, Asia, and Oceania are

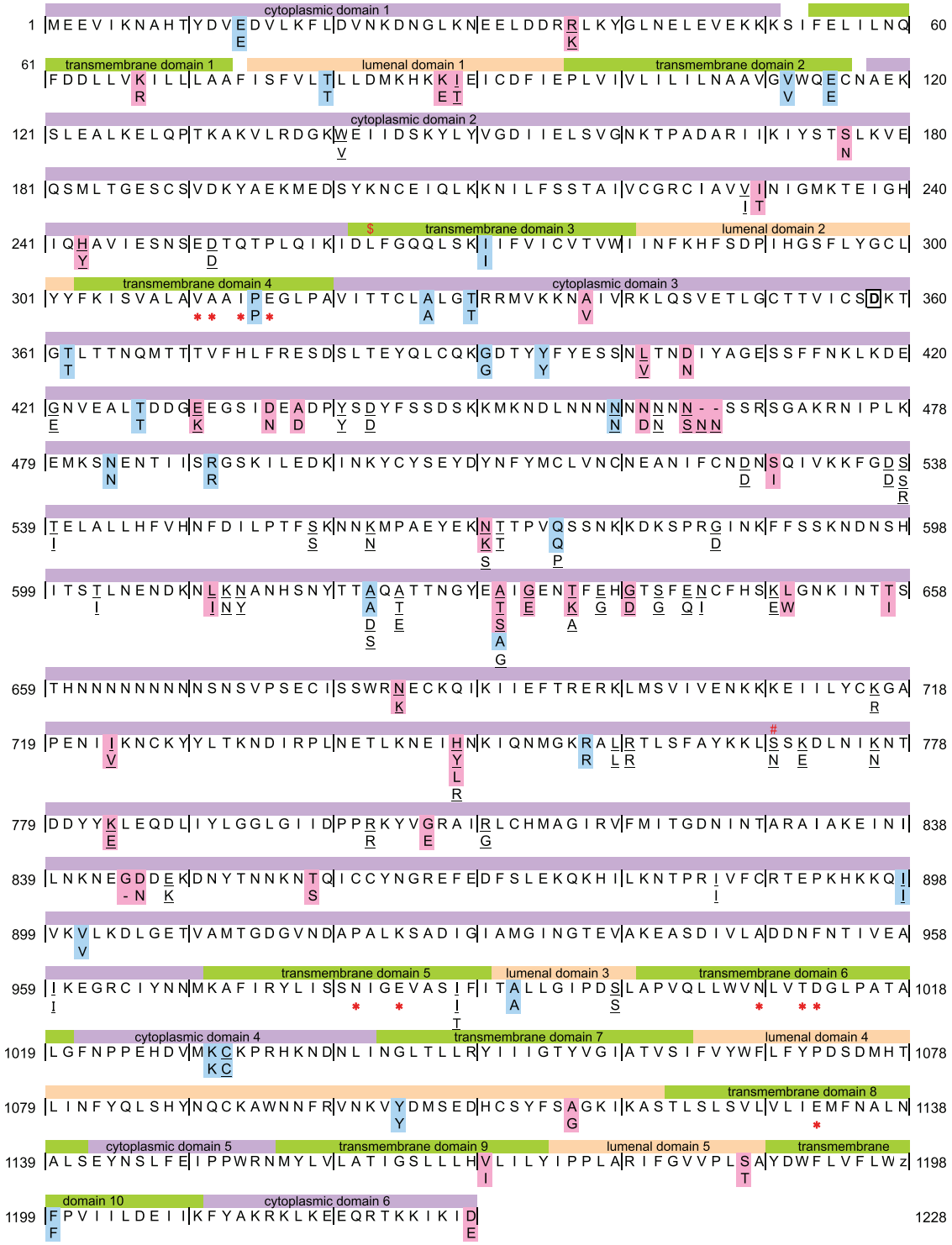

FIG. 1. Polymorphism in the *P. falciparum* SERCA (PfATP6) genes from worldwide parasite populations unexposed to artemisinin-based combination therapies. Nonsynonymous substitutions and synonymous substitutions observed in 656 samples examined in this study are highlighted in pink and blue, respectively, alongside the 3D7 sequence (PlasmoDB, PFA0310c). Substitutions reported by other investigators are underlined. Sequence regions for 10 transmembrane domains, 6 cytoplasmic domains, 5 luminal domains, putative calcium-binding sites (*), and a phosphorylation site (boxed D at 358) were inferred from the rabbit *serca* gene (Swiss-Prot Protein Data Bank [PDB] code P04191). The L at position 263, where experimental substitution to E results in abrogation of inhibition of PfSERCA by artemisinin (32), is indicated by $. The S769N substitution, which has been shown to be associated with an increased artemether IC_{50} in French Guiana (13), is indicated by #. Dashes between positions 465 and 466 and at position 844 denote deletions.

TABLE 1. SNPs and amino acid haplotypes in 656 full-length *serca* sequences from 13 *P. falciparum* populations (656 isolates)

Geographic area	*n*	No. of SNPs				Nucleotide diversity (mean ± SD)		No. of amino acid haplotypes			Haplotype diversity, *h* (mean ± SD)
		Total	Nonsynonymous	Common[b]	Continent specific	$\theta\pi$	θ_S	Total	Common[b]	Continent specific	
Worldwide[a]	656	64	38	61	50	0.00045 ± 0.00002	0.00238 ± 0.00030	50	48	40	0.579 ± 0.023
Africa	164	44	27	38	35	0.00061 ± 0.00003	0.00206 ± 0.00031	34	30	27	0.760 ± 0.033
Ghana	38	13	7	9		0.00041 ± 0.00006	0.00084 ± 0.00023	9	5		0.563 ± 0.094
Tanzania	69	31	18	26		0.00067 ± 0.00005	0.00170 ± 0.00031	19	15		0.771 ± 0.051
Malawi	38	14	10	7		0.00056 ± 0.00006	0.00090 ± 0.00024	13	8		0.801 ± 0.058
Madagascar	19	10	6	NA		0.00074 ± 0.00010	0.00078 ± 0.00025	10	NA		0.918 ± 0.036
Asia + Oceania	434	24	11	21	16	0.00028 ± 0.00002	0.00098 ± 0.00020	19	17	12	0.420 ± 0.030
Asia	214	21	10	15	9	0.00037 ± 0.00003	0.00096 ± 0.00021	17	15	8	0.581 ± 0.036
Iran	35	11	5	4		0.00070 ± 0.00005	0.00073 ± 0.00022	7	2		0.805 ± 0.032
Bangladesh	44	8	5	4		0.00025 ± 0.00004	0.00050 ± 0.00018	7	4		0.432 ± 0.091
Thailand	82	11	7	8		0.00027 ± 0.00004	0.00060 ± 0.00018	9	7		0.404 ± 0.068
Philippines	53	4	2	1		0.00024 ± 0.00003	0.00024 ± 0.00012	4	1		0.565 ± 0.040
Oceania	220	11	5	8	3	0.00018 ± 0.00003	0.00050 ± 0.00015	6	4	2	0.217 ± 0.036
PNG	89	9	5	7		0.00016 ± 0.00004	0.00048 ± 0.00016	6	5		0.211 ± 0.058
Solomon Islands	51	4	2	2		0.00010 ± 0.00004	0.00024 ± 0.00012	2	1		0.077 ± 0.050
Vanuatu	80	3	2	0		0.00024 ± 0.00005	0.00016 ± 0.00009	2	0		0.292 ± 0.055
South America	52	10	7	3	3	0.00083 ± 0.00005	0.00060 ± 0.00019	7	2	3	0.798 ± 0.024
Brazil	42	9	6	2		0.00072 ± 0.00006	0.00057 ± 0.00019	6	1		0.783 ± 0.035
Venezuela	10	4	2	NA		0.00026 ± 0.00013	0.00038 ± 0.00019	2	NA		0.200 ± 0.154

[a] Three sequences from Sudan and three sequences from cultured parasites are included. See Materials and Methods for details.
[b] Common SNPs are those with a minor allele frequency of ≥5%. Common haplotypes are those with a frequency of ≥5%. Countries for which there were fewer than 20 isolates are excluded (NA).

uncommon (allele frequency of <5%) but that this is not so in South America. The rank order of nucleotide diversity (θ_S and $\theta\pi$) was Africa > Asia > Oceania ~ South America, with the exception of $\theta\pi$ in South America (Table 1).

The geographical distribution of SNPs in *serca* was remarkably different, particularly between Africa/Asia/Oceania and South America; there were two notable features. First, common SNPs with allele frequencies of ≥0.05 were less prevalent in Africa, Asia, and Oceania, at 6/44 SNPs (14%), 6/21 SNPs (29%), and 3/11 SNPs (27%), respectively (Table 1 and

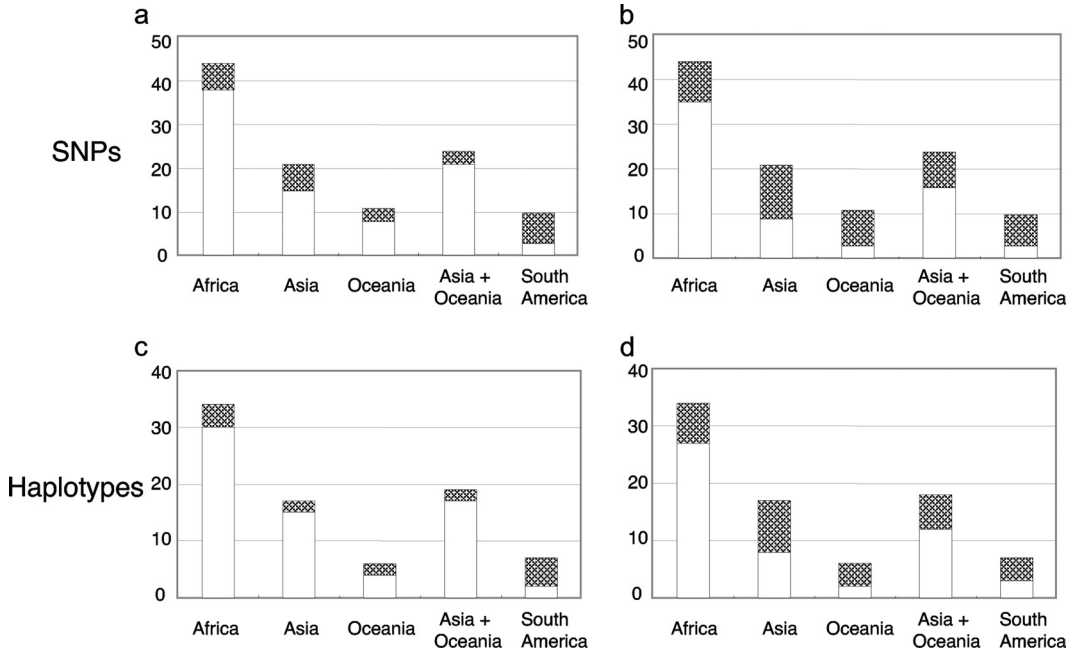

FIG. 2. Frequency distributions of SNPs and amino acid haplotypes in *serca* of *P. falciparum* isolates from Africa, Asia, Oceania, and South America. (a) Alleles in SNPs are divided into those with a minor allele frequency of <5% (open bars) and those with a minor allele frequency of ≥5% (shaded bars). (b) SNPs are divided into those that are continent specific (open bars) or not (shaded bars). (c) Amino acid haplotypes are divided into those with a minor haplotype frequency of <5% (open bars) and those with a minor haplotype frequency of ≥5% (shaded bars). (d) Amino acid haplotypes are divided into those that are continent specific (open bars) or not (shaded bars). The vertical axes represent the number of isolates.

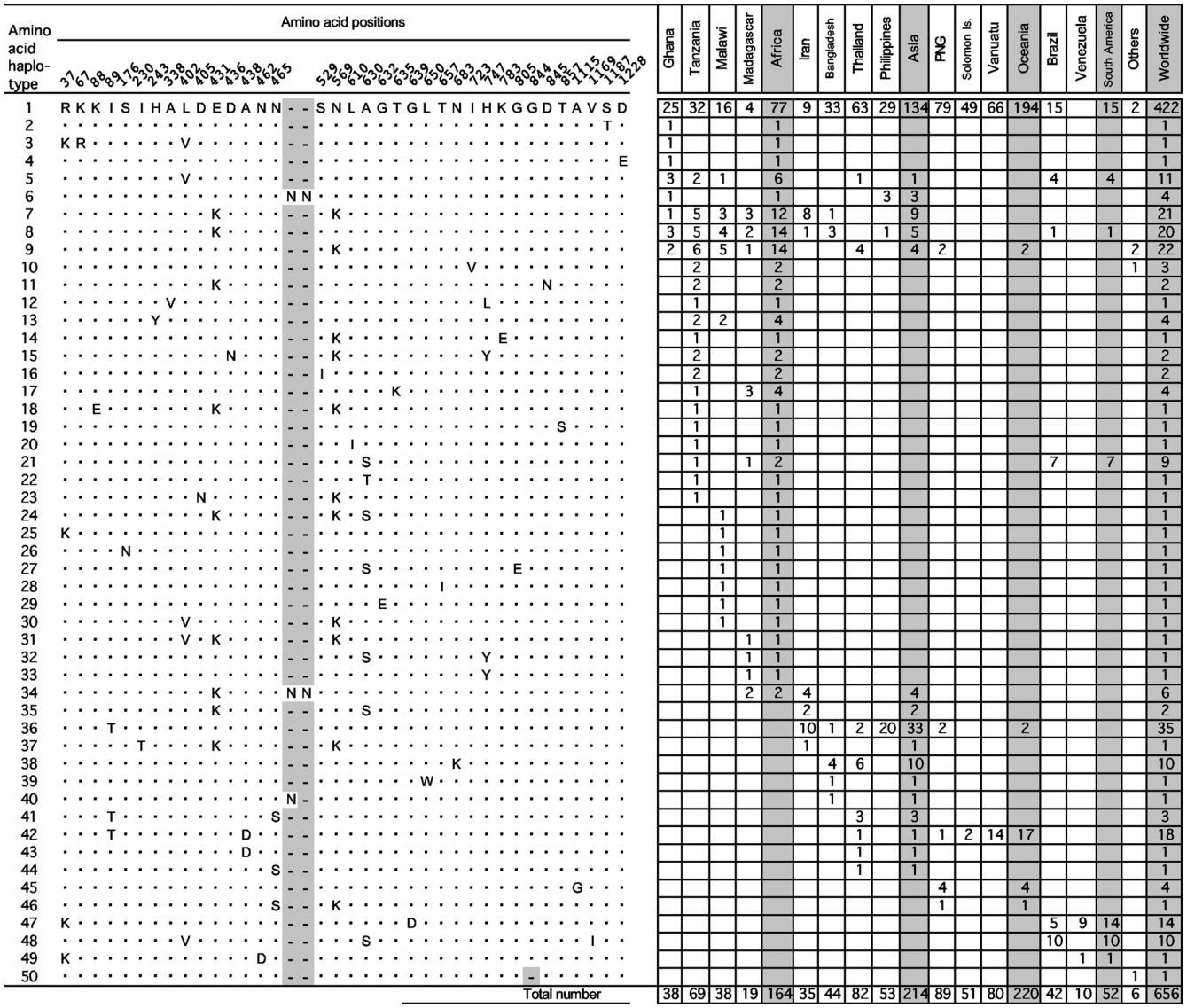

FIG. 3. Amino acid haplotypes of *P. falciparum serca* observed in 656 worldwide samples. Amino acid positions are numbered according to the 3D7 *serca* sequence (PFA0310c). Deletions of N next to position 465 are dashed. "Others," haplotypes of cultured parasites: 1, 3D7 and Dd2; 9, two Sudanese isolates; 10, one Sudanese isolate; 50, HB3.

Fig. 2a). However, in South America the prevalence of common SNPs was high (7/10 SNPs [70%]). Second, substantial numbers of SNPs were continent specific in Africa and Asia: 35/44 SNPs (80%) were found only in Africa and 9/21 SNPs (43%) in Asia (Table 1 and Fig. 2b). Most SNPs found in Oceania (8/11 [73%]) were also found in Asia, and thus when SNPs in Asia and Oceania are combined, 16/24 SNPs (67%) are specific to these geographic areas. Limited numbers of SNPs are shared between Africa and Asia/Oceania: of 24 SNPs (33%) found in Asia/Oceania, only 8 are shared with Africa. In contrast, in South America, only 3/10 SNPs (30%) found are specific to the continent, and the remaining 70% are shared with Africa.

In total, there are 50 *serca* amino acid haplotypes in our sample set (Fig. 3). Haplotype 1 (3D7 strain) is the most prevalent (422/656 [64%]) and is the only haplotype found in all four continents. Next was haplotype 36 (35/656 [5%]), which was found in Asia and Oceania. All other haplotypes are rare (<5%), with numerous singleton haplotypes ($n = 28$), and 40 haplotypes are continent specific (Fig. 2c and d). These results indicate that there are unexpectedly numerous *serca* haplotypes that are specific to a geographic area. The frequencies and geographical distribution of haplotypes differ greatly among geographic areas (Fig. 3). The number of haplotypes and haplotype diversity (h) are high in Africa (34 and 0.760, respectively) compared to Asia and Oceania (17 haplotypes and $h = 0.581$ in Asia; 6 haplotypes and $h = 0.217$ in Oceania) (Table 1). In South America, the value for h (0.798) was comparable to that in Africa.

DISCUSSION

Continuous implementation of ACTs as first-line treatment for uncomplicated malaria is likely imposing artemisinin pres-

sure to *P. falciparum* populations in wide geographic areas. At present, however, no alternative classes of antimalarial drugs are available to replace the artemisinin derivatives. We therefore believe it important to see whether and how the polymorphism profiles of *serca* change over time and spatially and in particular whether specific mutations/haplotypes are selected for under artemisinin pressure. The present sequence data provide baseline information on spontaneous mutations in *serca* from global *P. falciparum* populations that were unexposed to artemisinin or its derivatives. Natural variations in *serca* are characterized by abundance of geographic area-specific SNPs, the majority of which are observed at frequencies of less than 5%. The highest diversity of geographic area-specific haplotypes is observed in African parasite populations. The low prevalence of common SNPs in all parasite populations, except in South America, underscores several implications for detecting potential artemisinin-driven selection of resistant parasites and lends valuable insight on whether some *serca* haplotypes are selected by ACTs in natural parasite populations.

First, the occurrence of numerous SNPs in *serca* may make the rapid detection of artemisinin-driven selection of resistant parasites difficult. Resistant parasites, when they appear, would be geographically restricted, and their prevalence would initially be low. It is therefore rather difficult to distinguish a mutation associated with artemisinin resistance from abundantly present resistance-unrelated mutations, which are low in frequency and also geographic area specific. This is particularly true for African parasite populations. Identification of a resistance-associated mutation(s) is likely to be missed until such a time that the resistance-associated mutation becomes considerably prevalent. In this context, it should also be mentioned that some SNPs, though limited in number, are already highly prevalent. Those SNPs are probably unrelated to artemisinin resistance; for example, an I89T SNP was common in Asia, but the mutation has been suggested to be unrelated to artemisinin resistance (7). Such highly prevalent SNPs found in populations unexposed to ACTs therefore must be excluded from candidate mutations for consideration.

Second, the detection of potential artemisinin-driven selection would require a better understanding of the parasite population structure. This is particularly true for South America. The characteristics of *serca* polymorphism in South America are distinct from those in other continents: overall, the number of SNPs (and haplotypes) is limited, and SNPs with a minor allele frequency of ≥ 0.05 are prevalent. In such a scenario, distinguishing between an increase in allele frequency of a mutation due to artemisinin resistance and an increase of spontaneous mutations unrelated to artemisinin resistance would not be straightforward. South American parasite populations are strongly structured and genetic differentiation among local populations is remarkably high, probably due to epidemic expansion of some parasite genotypes (1). Consistently, *serca* SNPs/haplotypes were found to be limited in numbers but multiply represented (Table 1 and Fig. 3). Thus, the population structure must be taken into account to properly assess artemisinin-driven selection, preferably using neutral markers such as microsatellites and synonymous SNPs.

Third, the abundance of minor haplotypes may confound potential artemisinin-driven selection in some cases. If an artemisinin resistance-associated mutation(s) was generated in the major wild genotype (3D7 type), the identification of that mutation would not be difficult. A selective sweep of resistance parasites during continuous exposure to artemisinins would greatly reduce within-population diversity of *serca* haplotypes, with a fixation or predominance of a resistance-associated haplotype. A simple comparison of haplotypes from artemisinin-sensitive and -resistant parasites should reveal the resistance-associated mutation(s). However, if the resistance-associated mutation(s) was selected from one of the less prevalent haplotypes, the identification could not be readily made. Of the 48 minor haplotypes observed in this study (Table 1), 21 haplotypes have two to four amino acid substitutions compared with the major (3D7) haplotype. Since these haplotypes are in most cases continent specific, if resistant haplotypes were generated independently in several geographic areas, minor haplotypes would possess multiple SNPs, most of which might be unrelated to resistance (in some cases continent specific) but possibly selected by genetic hitchhiking linked to a nearby resistance-affording mutation. In these cases, comparison of resistance haplotypes originating from different continents with different evolutionary histories of *serca* would lead to identification of resistance-conferring mutations with high confidence.

In summary, the present analysis of a total of 656 full-length *serca* sequences identified numerous SNPs and haplotypes from geographically widespread *P. falciparum* populations that were unexposed to ACTs. The SNPs and haplotypes observed were in most cases present at a low frequency and were geographic area specific, with the exception of sequences from South America. The geographical difference in the diversity and distribution of *serca* SNPs and haplotypes observed in this study lays the groundwork for assessing whether some artemisinin resistance-associated mutations and haplotypes are selected by the current continuous and increasing deployment of ACTs.

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