Genetic analysis of polymorphic proteins of the human malaria parasite *Plasmodium falciparum*

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Summary

Five polymorphic proteins, detected by two-dimensional electrophoresis, were analysed in the parents and progeny of a cross between two clones of the malaria parasite *Plasmodium falciparum*. The information obtained showed that different forms of each protein were determined by allelic variants of each respective gene. One protein was identified as the parasite enzyme adenosine deaminase. Recombinant parasites were produced at a higher than expected frequency.

1. Introduction

Malaria is caused by the protozoan parasite *Plasmodium*. It is the most important disease of the tropics, some 200 million people being infected in Africa alone (World Health Organization, 1985). The most important species is *P. falciparum*, which may cause death when it gives rise to the condition of cerebral malaria. Control of the disease is by chemotherapy, but in recent years the widespread appearance of drug-resistant mutants has stimulated research into the development of an antimalarial vaccine. It is uncertain, however, whether this approach will be successful, as many candidate vaccine antigens have been found to exhibit considerable diversity in the parasite population.

The malaria parasite undergoes a mainly haploid life-cycle, meiosis occurring within a few hours of zygote formation. Following inoculation by mosquitoes of infective sporozoites into the vertebrate host, the parasite undergoes asexual cycles of multiplication, first in the liver and then in red blood cells. Male and female gametocytes are then formed in the blood. After ingestion by the mosquito vector, these mature as gametes and undergo fertilization. The zygotes penetrate the midgut wall and develop as oocysts, within which further divisions produce haploid sporozoites.

Genetic crossing experiments with malaria parasites are carried out by permitting mosquitoes to feed on a mixture of gametocytes of two cloned parent parasite lines. Cross-fertilization between gametes of each line produces hybrid zygotes in the mosquito midgut. Self-fertilization events between male and female gametes of the same line are also expected to take place. The parasites are allowed to develop to sporozoites, which are used to infect a new vertebrate host. The resulting blood forms are then examined, usually following cloning, for the presence of recombinants. Early crossing experiments were with the rodent malaria species *P. yoelii* and *P. chabaudi* (Walliker *et al.* 1973, 1975), and more recently with *P. falciparum* (Walliker *et al.* 1987).

Studies on isolates of *P. falciparum* from different countries have revealed a considerable degree of variability in characters such as proteins revealed by 2D-PAGE (Fenton *et al.* 1985) and electrophoretic forms of enzymes (Sanderson *et al.* 1981). Fifteen proteins have been identified by 2D-PAGE which are polymorphic in different parasite isolates. A striking feature of the variant forms of these proteins is their great variation in size, as well as in IEP. Only single forms of each of these proteins are seen in individual parasite clones, and it has been assumed that they represent allelic variants of each respective gene. This conclusion has been supported by peptide digest studies on proteins excised from 2D gels (Fenton, 1987), showing that the variants of each protein possess similar-sized fragments.

In this paper we describe the genetics of five of the polymorphic proteins and of the enzyme ADA in a cross between two *P. falciparum* clones. We show that recombination occurs between each protein marker, and that recombinant-type parasites are produced following the cross at a higher than expected frequency.

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Abbreviations: ADA, adenosine deaminase; IEP, isoelectric point; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis.

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2. Material and methods

(i) *Parasites*

(*a*) *Parasites used in crossing work*. The *P. falciparum* material used in the crossing studies were two parent clones, denoted 3D7 and HB3, and a number of clones derived from a cross between them. Parasites were maintained in *in vitro* culture using modifications of the methods of Trager & Jensen (1976) and Haynes *et al.* (1976), as described by Walliker *et al.* (1987). Details of the procedure used in making the cross are given in the latter reference, and are summarized in Fig. 1. Briefly, cultured gametocytes of 3D7 and HB3 were mixed and fed to mosquitoes to allow cross-fertilization to occur. The resulting sporozoites were used to infect a chimpanzee. The blood forms developing in this animal, denoted the progeny of the cross, were then cultured in human erythrocytes. Clones were derived from these cultures by limiting dilution (Rosario, 1981), 26 of which were used in the present work.

In cloning by dilution, it is expected that the majority of cultures will be derived from erythrocytes containing single parasites, although a minority will originate from more than one parasitized cell, or from cells containing more than one parasite (Rosario, 1981). These cultures, identified in this work as being mixed by their possession of two forms of enzyme or antigen markers (Walliker *et al.* 1987), were not examined in the present study.

(*b*) *Uncloned* P. falciparum *isolates*. A total of 60 uncloned isolates of *P. falciparum* from Brazil, Thailand and Zimbabwe, maintained in the World Health Organisation Registry of Standard Strains of Malaria Parasites in this Department, were also cultured and examined for 2D-PAGE proteins and allo-enzymes.

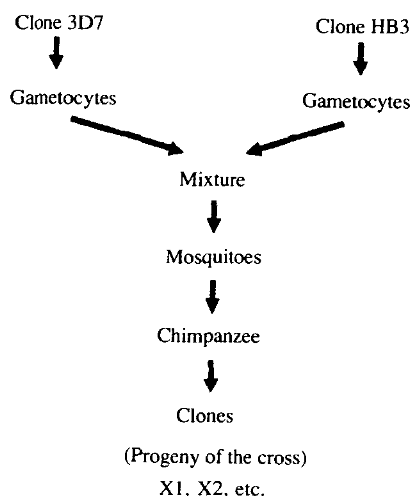

Fig. 1. Procedure for carrying out cross between *P. falciparum* clones 3D7 and HB3.

(ii) *Two-dimensional electrophoresis*

Cultured parasites were incubated with radiolabelled methionine and subjected to two-dimensional electrophoresis followed by fluorography, as described previously (O'Farrell, 1975; Fenton *et al.* 1985). Each polymorphic protein detected by 2D-PAGE is denoted by a letter, and variant forms of each by a number. Clones 3D7 and HB3 differ in their forms of five proteins, denoted C, D, G, K and P (Tables 1 and 2, Fig. 2).

(iii) *Enzyme electrophoresis*

Electrophoretic forms of the enzyme ADA in cultured parasites were detected by cellulose acetate or starch gel electrophoresis, as described previously (Sanderson *et al.* 1981).

ADA was characterized further on 2D gels as follows. Duplicate parasite cultures were set up, one of which was incubated with radiolabelled methionine. After 15 h parasites from both cultures were freed from their host erythrocytes by saponin lysis, and lysed in 0·1 ml of distilled water (Sanderson *et al.* 1981). Starch gel electrophoresis was then carried out, with unlabelled and radiolabelled parasites in adjacent tracks. The track containing unlabelled material was examined for ADA activity, which appeared as a discrete band (Sanderson *et al.* 1981). The corresponding region of the track containing labelled parasites was then excised, placed in 100 μl of 2D lysis buffer (O'Farrell, 1975), and rocked over a 12 h period to allow the enzyme to diffuse into the buffer. The starch was removed by centrifugation at 11500 *g*. The supernatant was then mixed with approximately 0·5 mg of unlabelled parasite material, to provide marker parasite proteins, and subjected to 2D-PAGE (O'Farrell, 1975). The resulting gels were stained using Coomassie blue, dried and exposed to X-ray film.

3. Results

(i) *2D-proteins and ADA forms of parent clones*

The 2D-PAGE proteins and ADA forms of clones 3D7 and HB3 are given in Fig. 2 and Table 2. Each of

Table 1. *Characteristics of the* P. falciparum *protein variants used in the present study* (*see Fenton* et al. *1985*)

Variant protein	M_r (kDa)	IEP
C7	124	5·50
3	122	5·74
D4	122	6·24
2	117	6·26
G2	58	5·31
1	57	5·35
K1	28	5·36
3	28	5·74
P2	36	5·90
1	36	5·80

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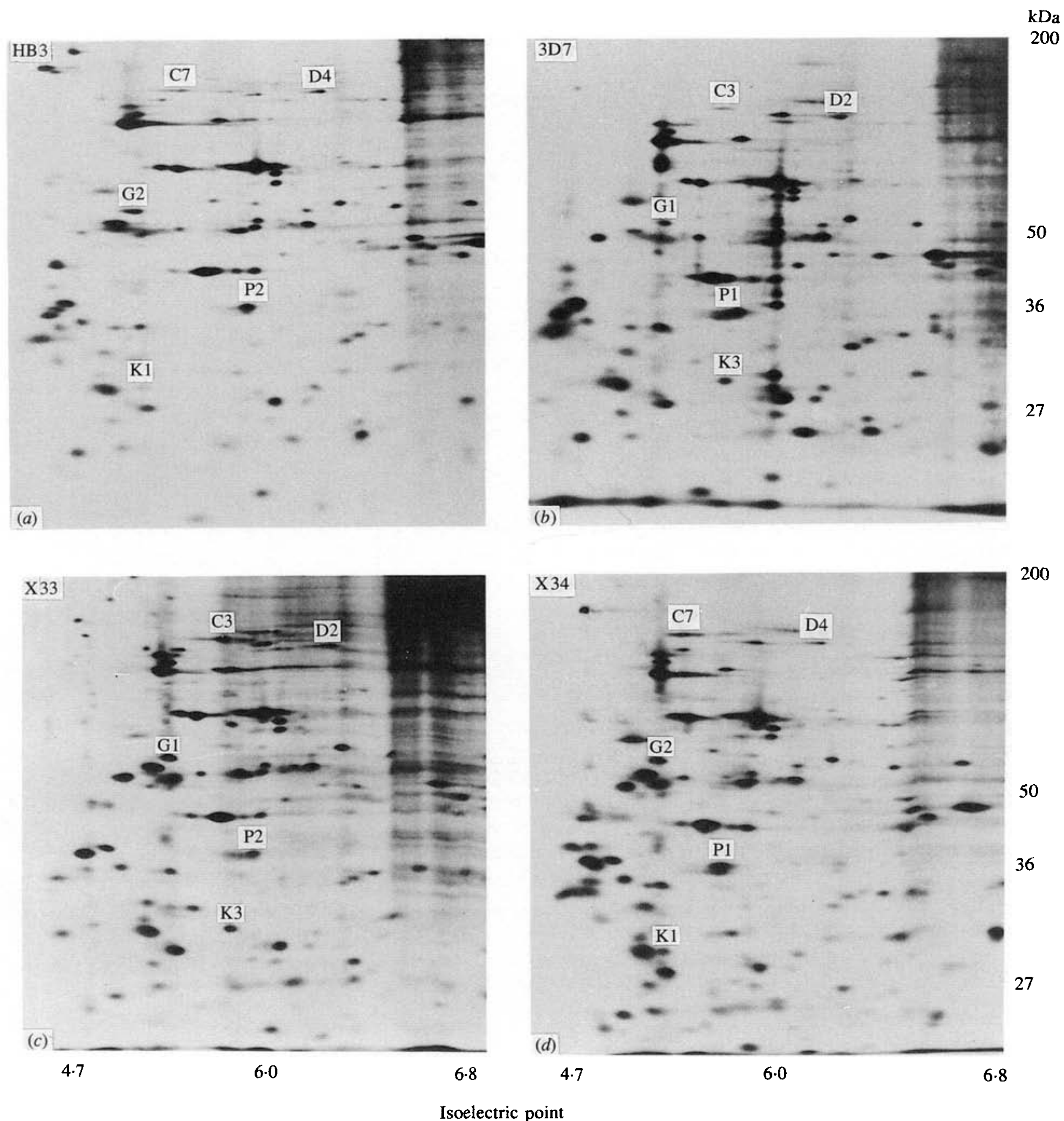

Fig. 2. 2D-PAGE proteins of parent clones HB3 (*a*), 3D7 (*b*), and progeny clones X33 (*c*) and X34 (*d*). Identification symbols (C3, C7, etc.) are printed above each respective protein. Note differences in the position of proteins C, D, G, K and P in 3D7 and HB3. X33 contains the 3D7 forms of C, D, G and K, and the HB3 form of P. X34 contains HB3 forms of C, D, G and K, and the 3D7 form of P.

the five proteins, C, D, G, K and P, possessed forms differing by molecular weight and/or IEP in each clone (Table 1). Only single forms of each protein and of ADA occurred in each clone, and they were unchanged following mosquito transmission.

(ii) *2D-proteins and ADA forms of progeny clones*

The forms of each 2D protein and of ADA found among the progeny clones are given in Table 2. The 2D-PAGE proteins of five progeny clones were identical to those of 3D7, while those of two clones were identical to HB3. The remaining 19 clones exhibited non-parental protein patterns. Each of these clones possessed single forms of each protein but in different combinations from those of the parents.

Recombination between the genes determining each 2D protein was detected. Figure 2(*c*, *d*) gives examples of two clones, X33 and X34, showing recombination between the genes for protein P and the other proteins.

With regard to ADA, each progeny clone possessed only a single ADA form, type 1 or type 2. Recombination between ADA and each of the 2D protein markers C, D, G and K was detected. Forms of ADA and protein P, however, segregated together,

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Table 2. *2D-PAGE proteins and ADA forms of parent and progeny clones*

	2D-PAGE protein					
	C	D	G	K	P	ADA type
Parental clones						
3D7A, 3D7B	3	2	1	3	1	1
HB3A, HB3B	7	4	2	1	2	2
Progeny clones						
Parental types						
XP6, X10	7	4	2	1	2	2
X8, X14, X31, X36, X37	3	2	1	3	1	1
Recombinant types						
X2	3	4	2	1	1	1
X11, XP3	7	4	1	3	1	1
X6, X35	7	4	2	3	2	2
X12, XP8	7	4	2	3	1	1
X5, XP2	7	4	1	1	1	1
XP5, X34	7	4	2	1	1	1
XP1	7	2	2	3	2	2
X4	7	2	1	3	2	2
XP4	7	2	2	3	1	1
XP7, XP9	7	2	1	1	2	2
X13	7	4	2	3	2	2
X30	3	2	1	1	2	2
X33	3	2	1	3	2	2

Variable proteins are referred to by letters (C, D, etc.) and individual variants of each protein by numbers. The enzyme adenosine deaminase (ADA) has two forms, fast (type 1) and slow (type 2) (Sanderson *et al.* 1981).

Table 3. *Forms of 2D-protein P and ADA of 60 uncloned isolates of* P. falciparum. *Figures are numbers of isolates in each category*

	Enzyme-type		
	ADA-1	ADA-2	ADA-1/ADA-2
2D-PAGE protein			
P1	36	0	0
P2	0	18	0
P1/P2	0	0	6

clones characterized by ADA-1 possessing P1, and those by ADA-2 possessing P2 (Table 2).

(iii) *2D-proteins and ADA forms of uncloned isolates*

Table 3 illustrates the forms of 2D protein P and ADA found among the 60 uncloned isolates from Brazil, Thailand and Zimbabwe. Each isolate characterized by P1 possessed ADA-1, while those which were P2 possessed ADA-2. Isolates which contained both P1 and P2 were characterized by ADA-1 and -2.

(iv) *2D-PAGE electrophoresis of ADA*

Following the identification of a band of ADA activity on a starch gel, a region of an adjacent track of the gel containing radiolabelled parasite ADA was excised, mixed with unlabelled parasite material, and prepared for 2D electrophoresis. Results of the electrophoresis are shown in Fig. 3. Figure 3*a* shows the Coomassie stained protein pattern, in which can be seen many of the proteins of the unlabelled parasite material. Figure 3*b* shows the corresponding autoradiograph of this gel. The principal spot of radioactivity corresponds exactly to protein P, when the autoradiograph is superimposed on the protein pattern of Fig. 3*a*. The protein exhibited microheterogeneity on 2D gels having two apparent molecular weights of 36·0 and 36·4 kDa. Both forms had the same IEP of 5·8.

4. Discussion

When crosses are made between two *Plasmodium* clones, recombination can be expected during meiosis

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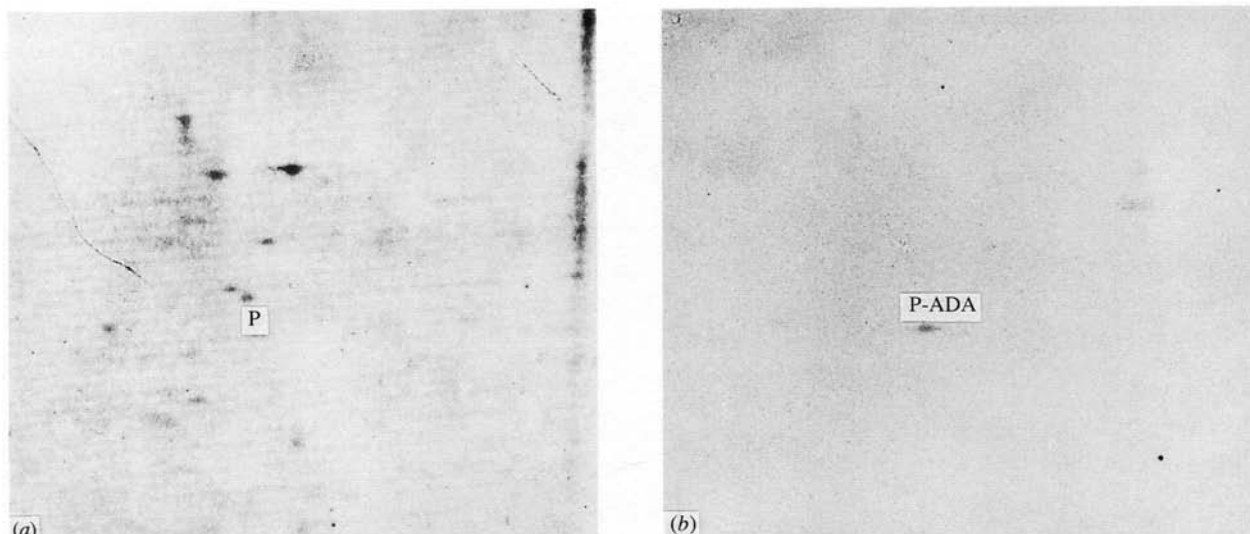

Fig. 3. Localization of *P. falciparum* adenosine deaminase (ADA) by 2D-PAGE. (*a*) *P. falciparum* marker proteins stained by Coomassie blue. (*b*) Autoradiograph of radiolabelled parasite ADA subjected to 2D-PAGE. Note that position of labelled ADA in (*b*) corresponds to position of protein P in (*a*).

of hybrid zygotes in mosquitoes. Assuming that fertilization events between male and female gametes of each parent line are random, 50% of the resulting zygotes should be hybrids. Since parent-type zygotes offer no opportunity for recombination, at least 50% of the resulting meiotic products can be expected to be parental types. In this study, 19 of the 26 progeny clones examined were recombinant for the 2D protein markers. Two of the seven clones exhibiting parental 2D patterns (X10 and XP6) have been shown to be recombinant for other markers (Wellems *et al.* 1987); thus a total of 21 of the 26 progeny clones are recombinants.

The recombinant progeny clones exhibited single forms of each protein in new, non-parental, combinations. This result is consistent with Mendelian inheritance in a haploid organism, the variant forms of each protein being determined by allelic variation of their respective genes. Table 4 shows the numbers of recombinant and parental combinations between the different gene pairs. The numbers of recombinant combinations among the 21 progeny clones vary between 6 (for C–D and D–G) and 15 (for D–P). The results thus provide evidence for independent assortment of the five markers. It is possible that some markers are linked, but the numbers of progeny examined so far are too small for firm conclusions to be drawn.

The haploid genome of *P. falciparum* consists of 14 chromosomes, as shown by pulsed-field gradient-gel electrophoresis and cytological studies (Prensier & Slomianny, 1986; Kemp *et al.* 1987; Wellems *et al.* 1987). The chromosomal locations of the genes determining the five 2D proteins studied here have not been determined yet. It is possible that each is located on a different chromosome, in which case the recombination would be due to random assortment of chromosomes at meiosis. Alternatively, if the genes are linked, crossing-over would account for the recombination. Considerable chromosomal re-arrangements occur during meiosis, resulting in the frequent production of novel-sized chromosomes, and some of these are known to involve crossing-over events (Sinnis & Wellems, 1988).

Forms of protein P and of ADA do not undergo recombination during the cross. In addition, in the survey of 60 isolates of *P. falciparum* from three countries, parasites characterized by P1 invariably possessed ADA-1, and those by P2 possessed ADA-2. These findings can be explained either by close linkage between two genes, or by a single gene determining both characters. The latter explanation has been shown here to be the correct one by the 2D electrophoretic study on ADA extracted from starch gel. Figure 2 shows that the most strongly radio-labelled protein in this extract, following 2D-PAGE, corresponds to protein P. These independent pieces of evidence make a compelling case for the two markers being the same gene product.

The molecular weight of parasite ADA has been

Table 4. *Numbers of parental and recombinant combinations of different gene pairs in progeny clones*

Gene pairs	Parental	Recombinant
C–D	15	6
C–G	13	8
C–K	9	12
C–P	10	11
D–G	15	6
D–K	11	10
D–P	6	15
G–K	9	12
G–P	10	11
K–P	10	11

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calculated previously as 36 kDa (Dadonna *et al.* 1984), which is in close agreement with present results. These authors also calculated the IEP of ADA to be 4·5. This is a lower pH than that found here for either ADA-1 or -2, and is probably due to the different techniques used in the two studies. The micro-heterogeneity in this molecule has not been detected previously, and the cause of this is unknown at present.

These findings confirm the conclusions drawn from previous work on peptide digest studies of 2D proteins of cloned parasites, that the variant forms of each protein are allelic. The forms of each respective protein C, D, G and P excised from 2D gels possess similar sized peptide fragments following digestion with specific proteases (Fenton, 1987, and unpublished results). These findings are expected for allelic variants which differ by minor amino-acid substitutions. The fragments resulting from digests of K1 and K3 showed several differences, which suggested more extensive amino-acid divergence between the two alleles of this protein than between those of the other proteins.

Approximately equal numbers of progeny clones possessed the alternative forms of proteins D, G, K and P (Table 2). There was thus no evidence that either form of any of these proteins was selectively favoured following the cross. For protein C, however, the parental HB3 form C7 predominated among the recombinant progeny. In other studies, a form of a 40 kDa merozoite antigen present in HB3 was characteristic of most progeny clones examined (Walliker *et al.* 1987). As no recombination between these proteins has been detected it is possible that they are genetically linked, but confirmation of this awaits determination of the chromosomal location of their genes.

A finding of interest is that the numbers of recombinants present among the progeny (21 out of 26) is higher than expected. This disproportion could be due to unequal numbers of male and female gametes produced by each parent, to cross-fertilization being favoured over self-fertilization, or to selection favouring certain recombinants during development in the mosquito host or in the chimpanzee. The high frequency of recombinant progeny illustrates the ease with which this parasite can produce novel genotypes during mosquito transmission of parasite mixtures. Since such mixed infections are common in nature (Thaithong *et al.* 1984), genetic recombination must clearly be taken into account in future work to develop vaccines or new chemotherapeutic agents.

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