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Dimorphism in genes encoding sexual-stage proteins of *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* ☆

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ABSTRACT

Plasmodium ovale curtisi and *Plasmodium ovale wallikeri* are distinct species of malaria parasite which are sympatric throughout the tropics, except for the Americas. Despite this complete overlap in geographic range, these two species do not recombine. Although morphologically very similar, the two taxa must possess distinct characters which prevent recombination between them. We hypothesised that proteins required for sexual reproduction have sufficiently diverged between the two species to prevent recombination in any mosquito blood meal in which gametocytes of both species are ingested. In order to investigate possible barriers to inter-species mating between *P. ovale curtisi* and *P. ovale wallikeri*, homologues of genes encoding sexual stage proteins in other plasmodia were identified and compared between the two species. Database searches with motifs for 6-cysteine, *Limulus* Coagulation factor C domain-containing proteins and other relevant sexual stage proteins in the genus *Plasmodium* were performed in the available *P. ovale curtisi* partial genome database (Wellcome Trust Sanger Institute, UK). Sequence fragments obtained were used as the basis for PCR walking along each gene of interest in reference isolates of both *P. ovale curtisi* and *P. ovale wallikeri*. Sequence alignment of the homologues of each gene in each species showed complete dimorphism across all isolates. In conclusion, substantial divergence between sexual stage proteins in the two *P. ovale* spp. was observed, providing further evidence that these do not recombine in nature. Incompatibility of proteins involved in sexual development and fertilisation thus remains a plausible explanation for the observed lack of natural recombination between *P. ovale curtisi* and *P. ovale wallikeri*.

1. Introduction

Ovale malaria in humans is caused by *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri*, which were recently differentiated (Sutherland et al., 2010) and found to be sympatric in their distribution across several communities in Africa and Asia (Oguike et al., 2011; Bauffe et al., 2012; Fuehrer et al., 2012; Putaporntip et al., 2013). Although occurring together, these two species do not recombine in nature and are genetically distinct at all loci examined to date. Phylogenetic analysis based on only a few loci suggests that divergence between *P. ovale curtisi* and *P. ovale wallikeri* is likely to have occurred 1–5 million years ago. This genetic distance may reflect evolutionary isolation (Sutherland and Polley, 2011) as the most likely explanation for lack of recombination. *Plasmodium ovale curtisi* and *P. ovale wallikeri* are sometimes found co-infecting a single individual (Fancony et al., 2012; Fuehrer et al., 2012), thus providing compelling evidence for true biological separation and consequently their inability to mate. *Plasmodium ovale* spp. often circulate in the population at submicroscopic levels, frequently as mixed infections with *Plasmodium falciparum*, *Plasmodium vivax* or *Plasmodium malariae*, and so pose a challenge to malaria control and elimination (Fançony et al., 2012; Dinko et al., 2013). These usually asymptomatic infections make a contribution to the overall malaria transmission burden, and thus a better understanding of the sexual stage biology of ovale malaria is needed to explain the observed pattern of transmission of these parasites.

Fertilisation and development of the malaria parasite in the mosquito midgut is a crucial process for malaria transmission,

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers **KP690796**, **KP690797**, **KP725066**, **KP725067**, **KP749432**–**KP749437**.

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which in *P. falciparum* can be interrupted by human antibodies against a number of sexual stage proteins (Ouedraogo et al., 2011), and several of these have been investigated as potential malaria transmission-blocking vaccine antigens. Pfs230 and Pfs48/45 belong to the 6-cysteine family and are proteins expressed on the surface of gametes (Carter et al., 1995; van Dijk et al., 2001, 2010). CCP/LAP proteins are another group of antigens expressed during gametocyte differentiation and gametogenesis (Delrieu et al., 2002; Pradel et al., 2004; Scholz et al., 2008) which, when disrupted, lead to blockage of the translation of sporozoites from the oocyst on the mosquito midgut wall to the salivary gland. These proteins are highly conserved in apicomplexan parasites and consist of multiple predicted adhesive domains, including a common *Limulus* Coagulation factor C (LCCL) domain (Dessens et al., 2004; Pradel et al., 2004; Templeton et al., 2004). Pfg377 is a gametocyte-specific protein expressed during the development of osmiophilic bodies, which are thought to be essential for the emergence of female gametocytes from red blood cells to allow fertilisation with the short-lived male gametes in the mosquito midgut (Severini et al., 1999; de Koning-Ward et al., 2008). The gene is located on chromosome 12 in *P. falciparum* and is expressed as protein in stage III gametocytes. *Plasmodium* meiosis occurs in the mosquito after fertilisation between male and female gametes, and requires a variety of well-conserved components. Rad51 and Dmc1 (disrupted meiotic cDNA), eukaryotic homologues of *Escherichia coli* RecA protein, play a critical role in homologous DNA strand exchange reaction during meiotic recombination and repair (Gopalakrishnan and Kumar, 2013) in malaria parasites.

The publication of complete genome sequences for certain *Plasmodium* spp. (Gardner et al., 2002; Hall et al., 2005) has provided an opportunity for better understanding of parasite biology. Thus far, only partial genome sequence data based on a single Nigerian isolate of *P. ovale curtisi*, collected in the 1970s and propagated in captive chimpanzees, is publicly available. This incomplete dataset can be accessed for BLAST searches on the Wellcome Trust Sanger Institute server (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/p_ovale). We hypothesised that the apparent genetic isolation between *P. ovale curtisi* and *P. ovale wallikeri* will be strongly reflected in sexual stage genes, which encode proteins involved in the processes leading to fertilisation and meiosis. Incompatibility between *P. ovale curtisi* and *P. ovale wallikeri* caused by divergence in these molecules is one possible component of the hypothetical barrier to genetic recombination. Therefore, we combined in silico and laboratory investigations to identify and characterise genes encoding sexual stage proteins from both species.

2. Materials and methods

2.1. Patients

Initial characterisation of loci of interest was performed on two parasite isolates previously obtained with written informed consent from patients treated for ovale malaria at University College London Hospital, UK in 2010 (UK Health Research Authority National Research Ethics reference 07/Q0505/60).

Plasmodium ovale curtisi isolate HL1002*Poc* was obtained from a Ugandan-born woman of 51 years, permanently resident in the UK, who reported several overseas journeys in the 3 years from 2007 to 2010. The patient presented with symptoms of malaria at the beginning of October, 2010, after having spent 1 week in Sierra Leone in September 2010. No conventional chemoprophylaxis was used, but a "herbal formula" (not further specified) was taken for malaria prevention while travelling.

Plasmodium ovale wallikeri isolate HL1001*Pow* was obtained from a British male, aged 18 years, who returned to the UK in August 2010 from 1 month charity work in coastal Ghana. He reported good compliance with atovaquone-proguanil prophylaxis including the complete 7-day post-exposure period and use of mosquito protection, but experienced the first malaria symptoms 5 weeks after arriving back in the UK. This suggests the isolate came from a liver-stage relapse without a prior primary blood-stage infection due to effective chemoprophylaxis, as previously described (Nolder et al., 2013).

Table 1
Origin of samples evaluated in this study and their speciation.

Country	*Plasmodium ovale curtisi*	*Plasmodium ovale wallikeri*
Cameroon	2	4
Ghana	10	9
Nigeria	14	12
Madagascar		1
Ivory Coast	1	
Malawi	2	2
Sierra Leone	3	4
Uganda	5	5
East Africa		2
Mozambique	1	
Kenya	1	
Total	39	39

Genomic DNA preparations from archived *P. ovale* spp. blood samples from the Public Health England Malaria Reference Laboratory (PHE MRL), UK, previously confirmed as either *P. ovale curtisi* ($n = 39$) or *P. ovale wallikeri* ($n = 39$) by Nolder et al. (2013), were also available for this study (Table 1).

2.2. Informatic and laboratory procedures

BLASTX searches using amino acid sequences from LCCL domain-containing proteins, Pfs48/45, Pfs230, Pfg377 and DMC1-like protein, were performed in the *P. ovale curtisi* fragment database (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/p_ovale) to find *P. ovale*-specific homologues. Primers were designed to target specific *P. ovale* sequences obtained (Table 2). For a putative LCCL domain-containing protein, after obtaining approximately the first 2 kb fragment, a further search was done with the sequence in the ovale database resulting in an extended ~5 kb contig assembled from various partial sequences. Since the available ovale sequence database is from *P. ovale curtisi*, corresponding *P. ovale wallikeri* sequences were obtained after amplification of genomic DNA from isolates of both species and direct sequencing. Comparison between *P. ovale curtisi* and *P. ovale wallikeri* sequences generated from all putative fertilisation genes was performed using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

3. Results

We identified the *P. ovale curtisi* and *P. ovale wallikeri* homologues of genes encoding known sexual-stage proteins in other members of the genus *Plasmodium* and determined their DNA sequence in order to quantify any divergence between the two ovale species. Using BLASTX searches, homologues of five genes encoding known sexual-stage proteins in the genus *Plasmodium* were identified in *P. ovale curtisi*. Corresponding orthologues of each gene were successfully amplified from *P. ovale wallikeri* genomic DNA and sequenced as previously described (Sutherland et al., 2010). Data obtained are summarised in Table 3. All novel sequences have been deposited in GenBank with accession numbers **KP690796**, **KP690797**, **KP725066**, **KP725067**, **KP749432**–**KP749437**.

Table 2
Primers and cycling conditions. A list of all five genes with primer sequences used for PCR and sequencing are shown, including PCR product sizes and cycling conditions.

Gene	Primers	Primer sequence	PCR product size	PCR conditions
Pos230	Po-398 F1	5′-AAT GGA GGA GAA ACA GGA GG-3′	643 bp	$95°C^{3min}/(95°C^{15s}–52°C^{20s}–68°C^{50s} \times 34)/68°C^{5min}$
	Po-398 R1	5′-GTT GTT CAT GAT AGT ATA ATG C-3′		
	Po-398 F3	5′-TCA AGA GAT AAA TAT GTC-3′	290 bp	$95°C^{3min}/(95°C^{15s}–48°C^{20s}–68°C^{50s} \times 30)/68°C^{5min}$
	Po-398 R3	5′-GAA GAT CTA ACA TAA GTA-3′		
Po12	Po-161 F1	5′-GTA TCA TTT AGA GTA CCT CC-3′	598 bp	$95°C^{3min}/(95°C^{30s}–52°C^{45s}–72°C^{1min} \times 34)/72°C^{5min}$
	Po-161 R1	5′-GAC TTT CAC TAC CCT GCA A-3′		
	Po-161 F1	5′-GTA TCA TTT AGA GTA CCT CC-3′	343 bp	$95°C^{3min}/(95°C^{30s}–52°C^{45s}–72°C^{1min} \times 29)/72°C^{5min}$
	Po-161 newR3	5′-AAC ATT CTT CTG GTT CTA CTA-3′		
LCCL Domain	LCF1	5′-AGC AAG TGG AGA TGG AAG AG-3′	2408 bp	$94°C^{3min}/(94°C^{30s}–55°C^{50s}–68°C^{2min\ 30s} \times 39)/68°C^{8min}$
-containing	LCR1	5′-CCG TAT CAA CAT ACC CCC AA-3′		
protein	LCF2	5′ –GCT TTG GAA GAT GGG GAT G-3′	1934 bp	$94°C^{3min}/(94°C^{30s}–52°C^{50s}–68°C^{2min\ 30s} \times 34)/68\ °C^{8min}$
(CCp1)	LCR3	5′-TGA ACG ACG GAA ATT ACT GAT G-3′		
LCCL sequencing primers	LCSeq F1	5′-GAT GCC GCT AAC TCA GAT G-3′		
	LCSeq R1	5′-ATC GCA TTG AAG TAA TCC TCC-3′		
	LCSeq R2	5′-CCA CTA CAA CTA CCA TGA TCA C-3′		
LCCL fragments 1 & 3 primers	POMF1 Fwd1	5′-TTC CTC CAG CGT CGA AAG AA-3′	944 bp	$95°C^{3min}/(95°C^{30s}–52°C^{50s}–68°C^{1min\ 30s} \times 39)/68°C^{8min}$
	POMF1 Rev1 Seminest	5′-ACT AGA ATC AAC AGA TAC ATC C-3′		
	POMF1 Rev2	5′-GAG ATG CAT AAA GGT TCC CCA-3′	868 bp	$95°C^{3min}/(95°C^{30s}–52°C^{50s}–68°C1^{min\ 30s} \times 34)/68°C^{8min}$
	POMF3 fwd1	5′-CCA AGT GGA TTA TTG GAT ATA-3′	2308 bp	$95°C^{3min}/(95°C^{30s}–58°C^{50s}–68°C^{2min\ 30s} \times 39)/68°C^{8min}$
	POMF3 rev1	5′-TTT CCG TAC ATC CTT TTG C-3′		$95°C^{3min}/(95°C^{30s}–58°C^{50s}–68°C^{2min\ 30s} \times 34)/68°C^{8min}$
	POMF3 Fwd2	5′-TGG CAC AAA AGA GGA CAA A-3′		
	POMF3 Rev2	5′-ATT GCA CTG AAA TTG ACA TTC-3′	2227 bp	$95°C^{3min}/(95°C^{30s}–58°C^{50s}–68°C^{2min\ 30s} \times 34)/68°C^{8min}$
LCCL F1 & F3 sequencing primers	POMF1 SeqR1	5′-GCC CTT CTT CTA CCT GTA AGC A-3′		
	SeqF3 midRev	5′-ATG AAC CCT GTC CAT GTA TTT G-3′		
	SeqF3 midFwd	5′-CAA ATA CAT GGA CAG GGT TCA T-3′		
PoRad51	PoRad F1	5′-TGT CTA ACA ATG AAA CCA G-3′	710 bp	$95°C^{3min}/(95°C^{30s}–50°C^{30s}–65°C^{1min} \times 35)/65°C^{5min}$
	PoRad R1	5′-GCA GAA TCC ACT ATT AGT A-3′		
	PoRad F2	5′-AGG AAG ATG TAG TCC CGA AGG-3′	663 bp	$95°C^{3min}/(95°C^{30s}–60°C^{30s}–65°C^{1min} \times {}^{30})/65°C^{5min}$
	PoRad R2	5′-AGG CAA ATC TGG CAT CAG CCA-3′		
Po419 (Pog377)	po-419 F1	5′-GTA ATA TAA AAT ACA TAC CAT C-3′	670 bp	$95°C^{3min}/(95°C^{30s}–47°C^{30s}–65°C^{50s} \times 35)/65°C^{5min}$
	po-419 R1	5′-TCA ATC TGG AAA AGA CTA ATA A-3′		
	po-419 F2	5′-ATG TTA GAA TGA TTG ACA AAG-3′	609 bp	$95°C^{3min}/(95°C^{30s}–54°C^{30s}–65°C^{50s} \times 30)/65°C^{5min}$
	po-419 R2	5′-GTT CGT TAA AAA AAT GTT CAA G-3′		

LCCL, *Limulus* Coagulation factor C domain.

Table 3
Five sexual stage genes were sequenced in *Plasmodium ovale curtisi* (*Poc*) and *Plasmodium ovale wallikeri* (*Pow*). The number of isolates sequenced for each gene and their codons relative to that of *Plasmodium falciparum* 3D7 are shown. For the purposes of this study only the "Reference" sequence for these sexual stage loci was that obtained for each gene from the first isolate sequenced. This was isolate OX015 for *P. ovale curtisi* and OX014 for *P. ovale wallikeri*.

		Genes sequenced				
		PoCCp1	Pog377	Pos230	Po12	PoRad51
Sequence length analysed		1586 aa (4758 nt)	192 aa (576 nt)	97 aa (291 nt)	114 aa (142 nt)	233 aa (699 nt)
P. falciparum homologue (codons)		PF3D7_1475500 (1–1620)	PF3D7_1250100 (367–557)	PF3D7_0209000 (885–986)	PF3D7_0612700 (120–236)	PF3D7_1107400 (1–233)
Full-length		Yes	No	No	No	No
Observed non-synonymous differences *Poc* vs *Pow*		35 aa	18 aa	4 aa	4 aa	0 aa
No. identical to reference (No. sequenced)	Poc	4 (4)	6 (6)	40 (40)	40 (40)	7 (7)
	Pow	4 (4)	6 (6)	40 (40)	40 (40)	6 (6)

aa, amino acids; nt, nucleotides.

Sequence alignment of *P. ovale curtisi* and *P. ovale wallikeri* for each gene demonstrated perfect dimorphism between the two species, with up to 40 independent isolates of each species analysed (Table 3). Best scoring homologues by sequence identity for *PoCCp1*, *Pos230*, *Pog377* and *PoRad51* were *Plasmodium knowlesi* LCCL domain-containing protein, *Plasmodium chabaudi* 6-cysteine protein (230), *Plasmodium yoelli* osmophilic body protein (G377) and *P. falciparum* Rad51, respectively.

3.1. Poc_ccp1 and Pow_ccp1

Full-length sequences for *poc_ccp1* and *pow_ccp1*, encoding amino acids (aa) 1–1586 of the LCCL-domain protein CCP1, were isolated by a combination of draft sequence contig assembly and PCR "walking" along the gene for both parasite species. A total of eight samples were fully sequenced. *Poc_ccp1* and *Pow_ccp1* are similar to each other and show good sequence identity to

orthologues in six other species in the genus (Supplementary Fig. S1). Despite their sequence similarity, the genes from the two ovale species differed from each other at 35 of 1586 aa positions (2.2%) and the four isolates of each species from which we derived full-length sequence were identical. For comparison, the two rodent malaria orthologues most similar to each other, *P. yoelli* and *Plasmodium berghei*, differed by 55 aa over a total of 1615 (3.4%) (Supplementary Fig. S1).

3.2. Poc_g377 and Pow_g377

A contig encoding 193 aa of both ovale species was obtained, corresponding to codons 367–557 of the *P. falciparum* (3D7 reference genome) orthologue PF3D7_1250100, which has a coding region of 3119 aa. A total of 12 samples were evaluated. Nineteen of the 193 amino acids sequenced (9.8%) differed between *P. ovale curtisi* and *P. ovale wallikeri*, suggesting substantial inter-species heterogeneity (Fig. 1). *Plasmodium yoelli* and *P. berghei* also differ from each other by 19 aa in this region of the gene. To understand how this compares to intra-species diversity, we examined publicly available whole genome data for over 600 *P. falciparum* isolates across this 200 aa segment, using the PlasmoView freeware program (Preston et al., 2014). We could identify only eight non-synonymous nucleotide substitutions across these 200 codons in *P. falciparum* and most were at low frequency, occurring in only a single isolate. These data suggest that this portion of the gene encoding the 377 kD sexual stage protein is more diverse between *P. ovale curtisi* and *P. ovale wallikeri* than between any two isolates of *P. falciparum*.

3.3. Poc_s230 and Pow_s230

Pfs230, *a* member of the 6-cysteine gene family, plays a crucial role in the sexual stages of *Plasmodium* development (van Dijk et al., 2010) and shows significant inter-species divergence apart from the conserved cysteine-rich domains (Fig. 2A). A total of 80 samples were evaluated. The orthologues in *P. ovale curtisi* and *P. ovale wallikeri* were more similar in sequence to each other than to other members of the genus, but four non-synonymous differences between the ovale species were observed in the 97 codon segment analysed here.

3.4. Poc_p12 and Pow_p12

P12 is a member of the 6-cysteine gene family but not implicated in the sexual stages of *Plasmodium* development (Li et al., 2012). It also shows significant inter-species divergence in between the conserved cysteine-rich domains (Fig. 2B). A total of 80 samples were evaluated. As for Po_s230, the P12 orthologues in *P. ovale curtisi* and *P. ovale wallikeri* were more similar in sequence to each other than to other members of the genus, with four non-synonymous differences in the 114 codon segment analysed here.

3.5. Poc_rad51 and Pow_rad51

A total number of 13 samples were evaluated. The Rad51 protein, which contributes to maintenance of chromosome strand sequence fidelity during replication, is highly conserved across the genus, and there were no differences between the 233 aa predicted from the DNA segment sequenced in *P. ovale curtisi* and those predicted by the corresponding segment of the gene in *P. ovale wallikeri* (Table 3). However, differences were present at the nucleotide level, with seven synonymous nucleotide differences between the two species present in this partial gene sequence.

4. Discussion

In this study, we identified significant dimorphism in genes of *P. ovale curtisi* and *P. ovale wallikeri* encoding protein orthologues to sexual stage proteins in other *Plasmodium* spp. All *P. ovale curtisi* sequences were identical to each other across the five gene segments studied; the same was true for *P. ovale wallikeri*. Previous studies (Pradel et al., 2004; de Koning-Ward et al., 2008; van Dijk et al., 2010; Gopalakrishnan and Kumar, 2013) have shown the role of Pfs230, Pfs48/45, LCCL domain-containing proteins, Pfg377 and Rad51 in sexual development, fertilisation and ookinete formation. Although these have been identified and studied in other *Plasmodium* spp. we believe this is the first study of these particular genes in *P. ovale* spp. An ~5 kb region of the gene encoding the Ccp1 protein was sequenced in *P. ovale curtisi* and *P. ovale wallikeri* and 36 single nucleotide polymorphisms (SNPs) were observed between them. Although the other loci investigated were smaller in size, substantial dimorphisms were also obtained between the *P. ovale* spp. particularly in the gene for Pos377, which exhibited only 90% sequence identity at the amino acid level between the two ovale species. This striking dimorphism between *P. ovale curtisi* and *P. ovale wallikeri* is consistent with previous studies of a variety of loci (reviewed by Fuehrer and Noedl, 2014). Although some intra-species heterogeneity has been

Fig. 1. Inter-specific diversity among Pgs377 sequences in seven *Plasmodium* spp. A 192 codon portion of the homologous locus was successfully amplified and sequenced in both *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri*. Other species included in the Clustal W alignment are: *Plasmodium falciparum* reference genome (Pf), *Plasmodium knowlesi* (Pk), *Plasmodium yoelli* (Py), *Plasmodium berghei* (Pb) and *Plasmodium chabaudi* (Pch). White shading denotes substitution of the consensus residue with a dissimilar amino acid; grey shading denotes a similar (conservative) substitution.

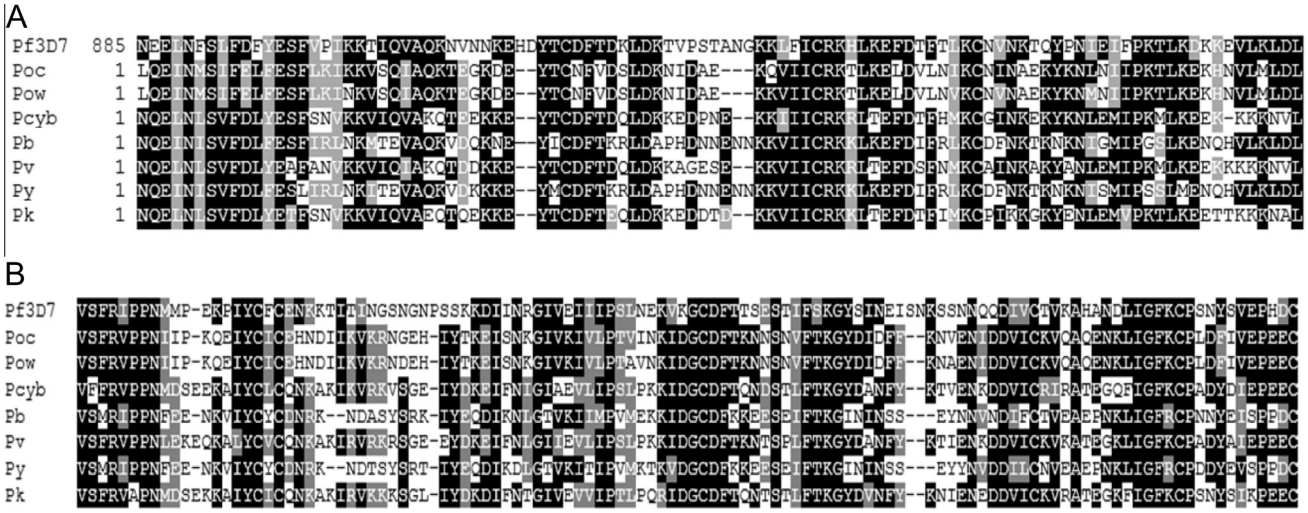

Fig. 2. Inter-specific diversity in two members of the *Plasmodium* 6-cysteine protein family. Partial amino acid (aa) sequences of 97 and 114 aa, respectively, were deduced by direct sequencing of amplified DNA sequence for the (A) Pgs230 and (B) PgP12 proteins ("g" here representing the word "genus"). Other species included in the Clustal alignment are: *Plasmodium falciparum* reference genome (Pf3D7), *Plasmodium cynomolgi* (Pcyb), *Plasmodium berghei* (Pb), *Plasmodium vivax* (Pv), *Plasmodium yoelli* (Py) and *Plasmodium knowlesi* (Pk). White shading denotes substitution of the consensus residue with a dissimilar amino acid; grey shading denotes a similar (conservative) substitution.

observed in genes encoding Cox1, TRA, MSP1 and the ssrRNA, from one or both species, in each case the alleles sequenced belonged clearly to the appropriate dimorph (Sutherland et al., 2010; Fuehrer et al., 2012; Putaporntip et al., 2013; Tanomsing et al., 2013).

Recent evidence (Nolder et al., 2013) has indicated a significant difference in relapse patterns between *P. ovale curtisi* and *P. ovale wallikeri* in ovale malaria cases imported to the UK. This is the first known description of a clinically important feature which differs between these two genetically distinct species, but does not necessarily explain the separation between them. It is accepted that for true speciation to occur, two populations from an ancestral species must become reproductively isolated, thereby preventing gene flow between them (Sutherland and Polley, 2011). Presumably, this is the case for *P. ovale curtisi* and *P. ovale wallikeri*, which are sympatric across their range and thus are not separated by geographical barriers. Biological barriers to genetic recombination could arise by various mechanisms; for example temporal segregation could occur through a shift in the timing of gamete release, and genetic segregation could occur through accrual of mutations in gamete surface receptors that might prevent viable fertilisation. It is likely that the separation of the two ovale lineages was further exacerbated by the timing of ancestral host transitions into hominids, allowing a lengthy period in different primate hosts during which incompatibility in fertilisation biology could have developed (Sutherland and Polley, 2011). The most striking divergence between *P. ovale curtisi* and *P. ovale wallikeri* in our analysis was in the gene variants encoding Po_g377, a protein expressed only in female gametocytes (in other *Plasmodium* spp.) and localised to the osmiophilic bodies (de Koning-Ward et al., 2008). The divergence of these putative sexual stage molecules between the two *P. ovale* spp. may have contributed to their inability to mate. Mutations within incompatible loci are known to produce genetic isolations within sympatric populations of animals and plants (Palumbi and Metz, 1991; Amato et al., 2007). As discussed above, ovale parasites exhibit minimal intra-species heterogeneity and it remains possible that *P. ovale curtisi* and *P. ovale wallikeri* may essentially propagate clonally with frequent self-fertilization (Sutherland and Polley, 2011; Putaporntip et al., 2013).

Not all of the protein coding genes investigated showed great diversity between the two ovale species. Ps230 is the largest representative, and P12 the smallest, of a 10-member family of proteins found in all *Plasmodium* spp. The family is defined by partially conserved, cysteine-rich "double domains" that are approximately 350 aa in length and have one to three predicted disulfide bridges in each half. These domains are important features for protein folding in this family and are characterised by motifs of two cysteine molecules spanned by two other amino acids (Dessens et al., 2004). This results in a specific tertiary structure which is conserved among species. Pfs230, with seven double domains, is expressed on the gamete surface and is described as a transmission-blocking vaccine candidate antigen (Gerloff et al., 2005). Comparison based on sequence identities in the putative Pos230 and other gene loci investigated suggest a closer relationship between *P. ovale* spp. and the rodent species *P. yoelli*, *P. berghei*, *P. chaubadi*, but less similarity to *P. falciparum*. Neither of these genes were as divergent between the ovale species as the *Pg_s377* genes. Similarly, for Rad51, a highly conserved chromosome repair protein associated with DNA replication at mitosis and meiosis, only a handful of synonymous sequence differences were observed between *P. ovale curtisi* and *P. ovale wallikeri*. Recent advances in molecular diagnostics and species discrimination have provided further insight on the global distribution of these two species and all evidence to date is wholly consistent with non-recombination between them (Fuehrer et al., 2012; Putaporntip et al., 2013).

In conclusion, we have observed that substantial dimorphism exists between putative sexual stage proteins in the two *P. ovale* spp. adding yet further evidence that these two species do not recombine in nature. Gametocytes are often observed in peripheral blood smears in ovale malaria patients but it is unclear how this relates to *P. ovale curtisi* and *P. ovale wallikeri* transmissibility. The data herein provide a platform for gene expression studies to further investigate the transmission biology of ovale malaria.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2015.02.004.

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