Molecular characterization and phylogenetic analysis of *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium cynomolgi*

Soumendranath Chatterjee¹ · Priyanka Mukhopadhyay¹ · Raktima Bandyopadhyay² · Paltu Dhal¹ · Debraj Biswal¹ · Prabir Kumar Bandyopadhyay³

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Abstract 18S ribosomal RNA gene sequences of different species of *Plasmodium* were aligned and analyzed to determine the molecular diversity among different species of *Plasmodium*. AT content of *P. cynomolgi*, *P. ovale*, *P. falciparum*, *P. vivax* and *P. malariae* ranged from 62.30 to 63.15, 63.90 to 65.29, 66.67 to 68.40, 61.66 to 63.25 and 64.09 to 76.36 in case respectively. GC content of *P. cynomolgi*, *P. ovale*, *P. falciparum*, *P. vivax* and *P. malariae* ranged from 36.85 to 37.70, 34.71 to 36.43, 31.60 to 33.27, 23.64 to 35.91 and 36.75 to 38.34 respectively. Molecular weight (Da) of *P. cynomolgi*, *P. ovale*, *P. falciparum*, *P. vivax* and *P. malariae* ranged from 1,160,127.00 to 1,400,332.00, 437,273.00 to 481,438.00, 668,021.00 to 685,243.00, 540,378.00 to 1,176,962.00 and 132,788.00 to 305,211.00 respectively. Relative melting temperature (T_m) of *P. cynomolgi* and *P. ovale* varied from 0.36799 to 0.36837 and 0.36712 to 0.36814 respectively. In *P. falciparum* and *P. vivax* relative T_m ranged from 0.36545 to 0.36626 and 0.36802 to 0.36866 respectively. Relative T_m of *P. malariae* varied from 0.36174 to 0.36790.

Keywords Molecular phylogeny · Different species of *Plasmodium* · AT content · GC content · Relative melting temperature (T_m)

Introduction

Protozoan parasites belonging to the genus *Plasmodium* cause malaria and are usually host specific. Species of *Plasmodium* are different as they undergo asexual reproduction in the blood which is lacking in the life cycle of other haemosporidian lineages (Perkins and Schall 2002). Humans are natural hosts for *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, and orangutans are naturally infected with *P. pitheci* and *P. silvaticum* (Garnham 1966; Garnham et al. 1972). In India, *P. vivax* causes about 60–65 % of all malaria infections (Maestre et al. 2004; Nandy et al. 2003; Adak et al. 1998). *P. vivax* is the most frequent malarial parasite infecting humans and distributed mainly in Asia and America (Price et al. 2007). *Plasmodium falciparum* is more virulent than other malarial parasites. *Plasmodium ovale* causes tertian malaria in human and is closely related to *Plasmodium falciparum* and *Plasmodium vivax*. *Plasmodium ovale* has been reported from different parts of the world most importantly being sub-Saharan Africa (Collins and Jeffery 2005). It is substantially less dangerous than *P. falciparum* (McMillan and Kelly 1967). It has recently been reported that it consists of two subspecies, *P. ovalecurtisi* and *P. ovalewallikeri* (Sutherland et al. 2010). *Plasmodium malariae* has been recognized since the Greek and Roman civilizations with different patterns of fever described by the early Greeks (Collins and Jeffery 2007). *P. malariae* is the least studied of the four species that infect humans because of its low prevalence and milder clinical manifestations as compared to the other three species. It is widespread throughout sub-Saharan Africa, much of Southeast Asia, Indonesia, on many of the islands of the western Pacific and in areas of the Amazon Basin of South America (Westling et al. 1997). In

✉ Prabir Kumar Bandyopadhyay
prabir0432@hotmail.com

¹ Parasitology and Microbiology Research Laboratory,
Department of Zoology, Burdwan University, Burdwan,
West Bengal, India

² Department of Nutrition, A.K.P.C Mahavidyalaya,
Bengai, Hooghly, West Bengal, India

³ Parasitology Laboratory, Department of Zoology,
University of Kalyani, Nadia, Kalyani, West Bengal, India

endemic regions, prevalence ranges from less than 4 % to more than 20 % (Bruce et al. 2007), but *P. malariae* infections are vastly underreported (Mohapatra et al. 2008). Another important species of *Plasmodium* is *Plasmodium cynomolgi*, a non-human primate malaria parasite infecting a large range of Old and New World monkeys and very closely related to the human parasite *P. vivax* (Collins et al. 1988). *Plasmodium cynomolgi* has also been proved to be an excellent model for the study of *P. vivax*-like malaria (Collins et al. 1988; Waters et al. 1993).

Phylogenetic analysis of genomic data are important tools for studying the evolutionary history of pathogens including the events of shifting of hosts and attacking new hosts together with important insights into life-history traits (Martinsen et al. 2007). Malarial parasites affect a vast diversity of animals including reptiles, birds and mammals (Perez-Tris et al. 2005) but the evolutionary events behind this diversification and success still remains unentangled (Martinsen et al. 2007). According to Bensch et al. (2004) there are thousands of undescribed cryptic species of malaria sharing convergent morphology with known taxa. Recent developments in the field of molecular systematic studies are a challenge to earlier systematic largely based on morphological studies, study of the life-cycle along with the vertebrate and insect host taxa (Martinsen et al. 2007). Studies in this field are new and limited. The aim of the present work is to study the nucleotide diversity and molecular characters of *Plasmodium* species on the basis of 18S ribosomal RNA gene sequence of different species of *Plasmodium* downloaded from NCBI (www.ncbi.nlm.nih.gov).

Materials and methods

18S ribosomal RNA gene sequence of different species of *Plasmodium* was downloaded from NCBI (www.ncbi.nlm.nih.gov). The sequence data were aligned using the ‘ClustalW submission form’ (<http://www.ebi.ac.uk/clustalw/>) and analyzed by ClustalW (Thompson et al. 1994). Sequences were analyzed to determine the diversity in respect to percentage of Adenine, Thymine, Guanine and Cytosine, AT content, G+C content, relative melting temperature of 18S ribosomal RNA gene sequence of different species of *Plasmodium*. Determination of Relative melting temperature (T_m) based on nearest-neighbour thermodynamics (Santalucia 1998) by $T_m = \text{SumDeltaH} / \text{SumDeltaS}$, where SumDeltaH and SumDeltaS are the summation of the dinucleotide enthalpy and entropy, respectively.

Results and discussion

The nucleotide base composition (mol%) of the sequences of *P. cynomolgi*, *P. ovale*, *P. falciparum*, *P. vivax* and *P. malariae* have been depicted in Tables 1, 2, 3, 4 and 5 respectively. Length of DNA, molecular weight (Da), AT and GC content of *P. cynomolgi*, *P. ovale*, *P. falciparum*, *P. vivax* and *P. malariae* have been shown in Tables 6, 7, 8, 9 and 10 respectively. AT and GC content of *P. cynomolgi* ranged from 62.30 to 63.15 and 36.85 to 37.70 respectively in Table 6. In *P. ovale* AT and GC content varied from 63.90 to 65.29 and 34.71 to 36.43 respectively (Table 7). AT and GC content ranged from

Table 1 Nucleotide base composition (mol%) of the 18S ribosomal RNA gene sequence of *P. cynomolgi*

Accession no.	Adenine (mol%)	Cytosine (mol%)	Guanine (mol%)	Thymine (mol%)
AB287290	30.18	16.40	21.20	32.22
L08241	30.97	15.92	20.93	32.18
L07559	30.19	16.46	21.20	32.14
AB287289	30.13	16.55	21.15	32.17

Table 2 Nucleotide base composition (mol%) of the 18S ribosomal RNA gene sequence of *P. ovale*

Accession no.	Adenine (mol%)	Cytosine (mol%)	Guanine (mol%)	Thymine (mol%)
EU935736	31.95	15.09	21.01	31.95
GU815532	32.27	15.37	21.05	31.30
GQ231515	32.42	15.11	19.78	32.69
GQ183063	32.60	15.75	19.48	32.18
GQ183062	32.64	15.84	18.87	32.64

Table 3 Nucleotide base composition (mol%) of the 18S ribosomal RNA gene sequence of *P. falciparum*

Accession no.	Adenine (mol%)	Cytosine (mol%)	Guanine (mol%)	Thymine (mol%)
HQ283222	30.74	13.69	19.08	36.48
HQ283221	31.16	12.89	18.71	37.25
HQ283220	31.36	12.93	18.69	37.02
HQ283217	31.36	12.93	18.69	37.02
HQ283218	30.83	13.92	19.35	35.90
HQ283212	30.89	13.95	19.29	35.78

Table 4 Nucleotide base composition (mol%) of the 18S ribosomal RNA gene sequence of *P. vivax*

Accession no.	Adenine (mol%)	Cytosine (mol%)	Guanine (mol%)	Thymine (mol%)
HQ283223	29.02	16.16	20.98	33.84
HQ283225	28.99	16.15	21.06	33.81
HQ283224	29.06	16.23	20.94	33.77
U93233	29.69	16.78	21.56	31.97
X13926	30.42	16.16	21.05	32.37
U83877	29.76	16.59	21.47	32.18
GU233451	28.70	15.92	21.52	33.63
AF145335	28.46	15.52	21.23	34.79

Table 5 Nucleotide base composition (mol%) of the 18S ribosomal RNA gene sequence of *P. malariae*

Accession no.	Adenine (mol%)	Cytosine (mol%)	Guanine (mol%)	Thymine (mol%)
GU815530	31.94	14.68	21.23	32.14
EF467838	32.73	11.36	12.27	43.64
EF467839	32.73	11.36	12.27	43.64
EF467837	32.73	11.36	12.27	43.64
EF467836	32.73	11.36	12.27	43.64

Table 6 Lengths of DNA, Molecular weight, AT and GC content of the 18S ribosomal RNA gene sequence of *P. cynomolgi*

Accession no.	Length of DNA (bp)	Mol. Wt. (Da)	AT content (%)	GC content (%)	Relative Tm (%)
AB287290	1915	1,160,127.00	62.40	37.60	0.36829
L08241	2312	1,400,332.00	63.15	36.85	0.36799
L07559	2047	1,240,114.00	62.34	37.66	0.36832
AB287289	1915	1,160,161.00	62.30	37.70	0.36837

66.67 to 68.40 and 31.60 to 33.27 respectively in *P. falciparum* (Table 8). In *P. vivax* AT and GC content varied from 61.66 to 63.25 and 36.75 to 38.34 respectively (Table 9). AT and GC content of *P. malariae* ranged from 64.09 to 76.36 and 23.64 to 35.91 respectively (Table 10). Molecular Weight (Da) of *P. cynomolgi*, *P. ovale*, *P. falciparum*, *P. vivax* and *P. malariae* ranged from 1,160,127.00 to 1,400,332.00, 437,273.00 to 481,438.00, 668,021.00 to 685,243.00, 540,378.00 to 1,176,962.00 and

132,788.00 to 305,211.00 respectively (Tables 6, 7, 8, 9, 10). Relative Tm of *P. cynomolgi* and *P. ovale* varied from 0.36799 to 0.36837 and 0.36712 to 0.36814 respectively (Tables 6, 7). In *P. falciparum* and *P. vivax* Relative Tm ranged from 0.36545 to 0.36626 and 0.36802 to 0.36866 respectively (Tables 8, 9). Relative Tm of *P. malariae* varied from 0.36174 to 0.36790 (Table 10).

Approximately 2 million people are killed each year and a half billion others are affected by human malaria

Table 7 Lengths of DNA, Molecular weight, AT and GC content of the 18S ribosomal RNA gene sequence of *P. ovale*

Accession no.	Length of DNA (bp)	Mol. Wt. (Da)	AT content (%)	GC content (%)	Relative Tm (%)
EU935736	795	481,438.00	63.90	36.10	0.36771
GU815532	722	437,273.00	63.57	36.43	0.36814
GQ231515	728	440,718.00	65.11	34.89	0.36723
GQ183063	724	438,337.00	64.78	35.22	0.36737
GQ183062	726	439,484.00	65.29	34.71	0.36712

Table 8 Lengths of DNA, Molecular weight, AT and GC content of the 18S ribosomal RNA gene sequence of *P. falciparum*

Accession no.	Length of DNA (bp)	Mol. Wt. (Da)	AT content (%)	GC content (%)	Relative Tm (%)
HQ283222	1132	684,863.00	67.23	32.77	0.36598
HQ283221	1133	685,243.00	68.40	31.60	0.36547
HQ283220	1129	682,828.00	68.38	31.62	0.36545
HQ283217	1129	682,828.00	68.38	31.62	0.36545
HQ283218	1106	669,228.00	66.73	33.27	0.36626
HQ283212	1104	668,021.00	66.67	33.24	0.36624

Table 9 Lengths of DNA, molecular weight, AT and GC content of the 18S ribosomal RNA gene sequence of *P. vivax*

Accession no.	Length of DNA (bp)	Mol. Wt. (Da)	AT content (%)	GC content (%)	Relative Tm (%)
HQ283223	1058	640,883.00	62.85	37.15	0.36818
HQ283225	1059	641,499.00	62.80	37.20	0.36821
HQ283224	1060	642,098.00	62.83	37.17	0.36819
U93233	1883	1,140,981.00	61.66	38.34	0.36866
X13926	1943	1,176,962.00	62.79	37.21	0.36817
U83877	1700	1,030,015.00	61.94	38.06	0.36854
GU233451	892	540,378.00	62.33	37.44	0.36832
AF145335	1121	678,967.00	63.25	36.75	0.36802

Table 10 Lengths of DNA, molecular weight, AT and GC content of 18S ribosomal RNA gene sequence of *P. malariae*

Accession no.	Length of DNA (bp)	Mol. Wt. (Da)	AT content (%)	GC content (%)	Relative Tm (%)
GU815530	504	305,211.00	64.09	35.91	0.36790
EF467838	220	132,788.00	76.36	23.64	0.36174
EF467839	220	132,788.00	76.36	23.64	0.36174
EF467837	220	132,788.00	76.36	23.64	0.36174
EF467836	220	132,788.00	76.36	23.64	0.36174

(Teklehaimanot et al. 2005). Four species of *Plasmodium* have already been known to be the causative agents of human malaria (Singh et al. 2004; Win et al. 2004). Approximately 15 genera have been erected within the order Haemosporidia (Phylum-Apicomplexa) containing more than 500 described species from reptiles, turtles, birds and mammals using at least seven families of dipteran vectors (Levine 1988). There exists variation in the life

history of malarial parasites. Boyd (1939) had hypothesized that these variations in *Plasmodium* spp. help them adapt to the host conditions. Study of variations on the life history traits of *Plasmodium* spp. namely the rat/peak, time and maturity has been proposed to have a genetic factor (Eisen and Schall 2000). Studies of such host- depended variations seem to form the basis of speciation due to host-switching. Phylogenetic studies on malarial parasites are

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HQ283220 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
HQ283217 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
HQ283221 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
HQ283222 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
HQ283218 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
HQ283212 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
PVU9323 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
PVU8387 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
AB287290 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
AB287289 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
L07559 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
HQ283223 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
HQ283225 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
HQ283224 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
GU233451 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
AF145335 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
EF467838 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
EF467839 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
EF467837 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
EF467836 TTAACCTGCTAATT-AGCGGTAAGTACACTATATTTTATTGAAATT-G
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HQ283220 TAGTTCGTGAATATGATTGTCTGGTTAATCCGATAACGAACGAG-ATC
HQ283217 TAGTTCGTGAATATGATTGTCTGGTTAATCCGATAACGAACGAG-ATC
HQ283221 TAGTTCGTGAATATGATTGTCTGGTTAATCCGATAACGAACGAG-ATC
HQ283222 TAGTTCGTGAATATGATTGTCTGGTTAATCCGATAACGAACGAG-ATC
HQ283218 TAGTTCGTGAATATGATTGTCTGGTTAATCCGATAACGAACGAG-ATC
HQ283212 TAGTTCGTGAATATGATTGTCTGGTTAATCCGATAACGAACGAG-ATC
U93233 TAGTTCGTGAATATGATTGTCTGGTTAATCCGATAACGAACGAG-ATC
U83877 TAGTTCGTGAATATGATTGTCTGGTTAATCCGATAACGAACGAG-ATC
AB287290 TAGTTCGTGAATATGATTGTCTGGTTAATCCGATAACGAACGAG-ATC
AB287289 TAGTTCGTGAATATGATTGTCTGGTTAATCCGATAACGAACGAG-ATC
L07559 TAGTTCGTGAATATGATTGTCTGGTTAATCCGATAACGAACGAG-ATC
HQ283223 TAGTTCGTGAATATGATTGTCTGGTTAATCCGATAACGAACGAG-ATC
HQ283225 TAGTTCGTGAATATGATTGTCTGGTTAATCCGATAACGAACGAG-ATC
HQ283224 TAGTTCGTGAATATGATTGTCTGGTTAATCCGATAACGAACGAG-ATC
GU233451 TAGTTCGTGAATATGATTGTCTGGTTAATCCGATAACGAACGAG-ATC
AF145335 TAGTTCGTGAATATGATTGTCTGGTTAATCCGATAACGAACGAG-ATC
EF467838 -----TAACGAACGAG-ATC
EF467839 -----TAACGAACGAG-ATC
EF467837 -----TAACGAACGAG-ATC
EF467836 -----TAACGAACGAG-ATC
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HQ283220 ATAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
HQ283217 ATAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
HQ283221 ATAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
HQ283222 ATAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
HQ283218 A - - AAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
HQ283212 A - - AAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
U93233 GTAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
U83877 GTAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
AB287290 GTAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
AB287289 GTAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
L07559 GTAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
HQ283223 GTAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
HQ283225 GTAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
HQ283224 GTAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
GU233451 GTAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
AF145335 TTAAGGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
EF467838 GTAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
EF467839 GTAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
EF467837 GTAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
EF467836 GTAAAGCTTCTTAGAGGAACAGTGTGTCTAACACAAGGAAGTTAAGG
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Fig. 1 Multiple sequence alignment of the 18S rRNA gene sequences of *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium cynomolgi* under our study. Result indicates three most conserved regions are indicated

indeed challenging because they show specializations not only to the vertebrate host but also to the vectors that transmit them (Charleston and Perkins 2002). Studies on the role of insect vectors in moving malarial parasites from one place to another or introducing new species to novel hosts are very limited (Charleston and Perkins 2002). Phylogenetic studies using molecular markers may be useful in this field and help to hypothesize new concepts that may create a revolution in the field of malaria-medicine. Infection by malarial parasites cause energy drain and may thereby affect the development of a community (Fallon et al. 2004). Studies on malarial parasite diversity may help in the formulation of hypothesis on the development of community structure. Studies of Bell et al. (2006) on *Plasmodium chabaudi* shows that more virulent pathogen lineages have competitive advantages within co-infected hosts and thus pathogens may evolve to become more virulent in genetically diverse infections. This may serve as a clue in the development of more virulent strains of malarial parasites and a selection backed speciation resulting therein.

Morphological features for taxonomic and systematic study have some limitations such as length, width and shape of different life history stages in the host erythrocyte (Martinsen et al. 2006). The rapidly developing concept of molecular taxonomy based on gene sequence data have already brought revolution in the field of taxonomy challenging several well-established hypotheses based on classical taxonomy of malarial parasites (Martinsen et al. 2007). Such tools are very important in the study of the diversity of malarial parasites and trace back their origins co-relating them with the implications both at present and in future.

Phylogenetic relationship among five different *Plasmodium* species is shown by phylogenetic tree (Fig. 2) which is constructed using total 25 different 18S rRNA gene sequences. Multiple sequence alignment of 18S rRNA gene sequences of the five *Plasmodium* species is shown in Fig. 1. Six 18S rRNA gene sequences of *Plasmodium vivax* were used; all of them formed a single clade with 99 bootstrap values, whereas clade two composed with four different 18S rRNA gene sequences of *Plasmodium cynomolgi* with 82 bootstrap values. Interestingly both the node is attached with 100 % bootstrap values. The other node containing four sequences of *Plasmodium malariae* showed a strong phylogenetic relation among themselves with 100 % bootstrap values.

Fig. 2 Neighbor-joining tree showing the relationship of selected representatives of different *Plasmodium* species based on 18S rRNA gene sequences. Five different *Plasmodium* species are indicated by bracket. Nodal values represent bootstrap probabilities based on 500 replicates

It is noted that above mentioned three *Plasmodium* species formed a single node with 98 % bootstrap support. Therefore, it indicates their phylogenetic relatedness with the sequences taken consideration than others under this study. Six *Plasmodium falciparum* sequences formed two nodes, one with two sequences and another with four sequences. Another interesting observation was that the remaining five sequences from *Plasmodium ovale* formed a distinct cluster from the above four clusters. The phylogenetic tree shows that most probably *Plasmodium vivax* have evolved from the non-human primate malarial parasite, *Plasmodium cynomolgi* and it probably took place by host-switching from monkeys to humans. It may be inferred that *Plasmodium cynomolgi* is probably the oldest of the five species of *Plasmodium* studied in the current investigation. *Plasmodium malariae* seems to have relatedness to *P. cynomolgi* and *P. vivax* in that *P. malariae* seems to share common ancestry with the ancestor of *P. cynomolgi*–*P. vivax* group. Studies like the current one is important as it helps to identify the

relatedness of human malarial parasite with other non-human parasites (e.g. *Plasmodium cynomolgi* and *Plasmodium vivax*) which may be used as models for studying the physiology, biochemistry and immunology of malarial parasite and to use the information in drug-designing and development of vaccines. They also help to untangle the unresolved complex mysteries of evolution as well.

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Compliance with ethical standards

Conflict of interest None.

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