Natural Human Infections with *Plasmodium cynomolgi*, *P. inui*, and 4 other Simian Malaria Parasites, Malaysia

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We detected the simian malaria parasites *Plasmodium knowlesi*, *P. cynomolgi*, *P. inui*, *P. coatneyi*, *P. inui*–like, and *P. simiovale* among forest fringe–living indigenous communities from various locations in Malaysia. Our findings underscore the importance of using molecular tools to identify newly emergent malaria parasites in humans.

Zoonotic malaria caused by *Plasmodium knowlesi*, commonly found in long-tailed macaques (*Macaca fascicularis*) and pig-tailed macaques (*M. nemestrina*), is now a major emerging disease, particularly in Malaysia (*1*,*2*). Two other simian malaria parasites, *P. cynomolgi* (*2*–*4*) and *P. inui* (*2*), have also been shown to have the potential of zoonotic transmission to humans through the bites of infected mosquitoes under natural and experimental conditions. The risk of acquiring zoonotic malaria is highest for persons living at the forest fringe and working or venturing into the forest because of their proximity with the monkey reservoir hosts and the mosquito vectors (*5*,*6*). With the aid of molecular methods, we aimed to investigate whether human infections with simian malaria parasites were present among indigenous communities in Malaysia whose villages are situated in the forest or at the forest fringe.

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The Study

We examined 645 archived blood samples that we had collected during 2011–2014 among indigenous populations of various subtribes from 14 villages in 7 states in Malaysia (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/27/8/20-4502-App1.pdf). We first screened the extracted DNA samples at Universiti Malaya (UM) for the presence of *Plasmodium* with the aid of genus-specific primers (rPLU1 and rPLU5; rPLU3 and rPLU4) (Appendix). Of the 645 indigenous community samples, 102 (15.8%) were positive for *Plasmodium*. Using species-specific nested PCR assays (Appendix), we identified these infections as monoinfections with *P. knowlesi* (n = 40), *P. vivax* (n = 21), *P. cynomolgi* (n = 9), *P. falciparum* (n = 6), *P. coatneyi* (n = 3), *P. inui* (n = 3), *P. malariae* (n = 2), and *P. ovale curtisi* (n = 1) (Table 1). In 17 samples, the species could not be identified despite repeated attempts. Our species-specific primer pairs were designed on the basis of either the asexually (A) or sexually (S) transcribed forms of *Plasmodium* small subunit (SSU) rRNA genes (*7*); the genus-specific primer pairs anneal to both asexual and sexual forms of the SSU rRNA genes, and therefore the genus-specific assay is more sensitive.

We further characterized the 55 samples that tested positive for simian malaria parasites by amplifying a longer fragment of the SSU rRNA gene (914 bp–950 bp) for direct sequencing. Phylogenetic analysis using the neighbor-joining method (Figure 1) revealed the presence of *P. knowlesi* (samples PK1–40), *P. coatneyi* (UM1–3), *P. cynomolgi* (UM9, UM11, UM12, UM14, UM15, UM17, UM18), and *P. inui* (UM5–7). Meanwhile, 2 sequences derived from

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Table 1. Human and simian *Plasmodium* malaria species identified by nested PCR at UM targeting SSU rRNA genes among indigenous community blood samples, by state, Malaysia*

State	No. samples tested	No. positive samples	Human and simian malaria species							
			P. falciparum	*P. vivax*	*P. malariae*	*P. ovale curtisi*	*P. knowlesi*	*P. coatneyi*	*P. cynomolgi*	*P. inui*
Pahang	109	5	0	2	0	1	2	0	0	0
Perak	61	55	3	10	2	0	26	3	5	0
Selangor	49	0	0	0	0	0	0	0	0	0
Negeri Sembilan	163	13	1	2	0	0	2	0	2	0
Melaka	32	13	2	3	0	0	1	0	1	1
Kelantan	32	9	0	2	0	0	6	0	1	0
Sarawak	199	7	0	2	0	0	3	0	0	2
Total/overall prevalence	645	102† (of 645; 15.8%)	6 (of 102; 5.9%)	21 (20.6%)	2 (2.0%)	1 (1.0%)	40 (39.2%)	3 (2.9%)	9 (8.8%)	3 (2.9%)

*SSU, small subunit; UM, Universiti Malaya.
†102 of 645 (15.8%) indigenous community samples were found positive with *Plasmodium* genus-specific primers; 17 *Plasmodium* genus-positive samples could not be identified up to species level despite repeated attempts.

samples UM10 and UM16 were found to be closely related to *P. simiovale*.

We then reextracted DNA from 15 blood samples that were positive for *P. coatneyi*, *P. cynomolgi*, and *P. inui* and sent these samples (blinded) together with 5 *Plasmodium*-negative samples to Universiti Malaysia Sarawak (UNIMAS) to confirm their identities by PCR and sequencing of part of the cytochrome c oxidase subunit 1 (COX1) gene. At UNIMAS, using nested PCR assays based on SSU rRNA genes, we found 1 single and 9 double species infections. We could not identify the species of *Plasmodium* for sample UM6, 4 of the *Plasmodium*-positive samples from UM were *Plasmodium* negative, and all 5 *Plasmodium*-negative samples from UM (UM4, 8, 13, 19, 20) tested negative (Table 2). Furthermore, because both laboratories at UM and UNIMAS had previously extracted DNA from macaque blood to examine for simian malaria parasites, we tested the samples for macaque DNA to rule out the possibility that the simian malaria parasites detected were the result of contamination with macaque blood. We obtained negative results using nested PCR for detection of macaque DNA for the 20 DNA samples when they were first received at UNIMAS and also when we repeated testing after completing the sequencing of COX1 genes, indicating that these samples were not contaminated with macaque blood upon receipt or during subsequent experiments at UNIMAS.

We then subjected the PCR-positive samples (UM6–7, UM9–12, UM14–18) to amplification and sequencing of partial COX1 genes. Neighbor-joining (Figure 2) phylogenetic inference of these sequences, together with available referral sequences from GenBank, indicated that 32 haplotypes from samples UM9–12 and UM14–18 were genetically indistinguishable from *P. cynomolgi*. Our phylogenetic analyses also demonstrated that sample UM7 had a single infection with *P. inui*–like parasites, whereas UM6 had a double infection with *P. simiovale* and *P. inui*–like

Table 2. Comparison between results of nested PCR and sequencing at UM and UNIMAS for identification of *Plasmodium* malaria species from indigenous community blood samples, Malaysia*

Sample ID	Identification at UM		Identification at UNIMAS	
	PCR assays based on SSU rRNA genes	Phylogenetic analysis of SSU rRNA genes	PCR assays based on SSU rRNA genes	Phylogenetic analysis of COX1 genes
UM1	*P. coatneyi*	*P. coatneyi*	Negative	ND
UM2	*P. coatneyi*	*P. coatneyi*	Negative	ND
UM3	*P. coatneyi*	*P. coatneyi*	Negative	ND
UM5	*P. inui*	*P. inui*	Negative	ND
UM6	*P. inui*	*P. inui*	Positive	*P. inui*–like, *P. simiovale*
UM7	*P. inui*	*P. inui*	*P. inui*	*P. inui*-like
UM9	*P. cynomolgi*	*P. cynomolgi*	*P. cynomolgi, P. inui*	*P. cynomolgi*
UM10	*P. cynomolgi*	*Plasmodium* spp.	*P. cynomolgi, P. inui*	*P. cynomolgi*
UM11	*P. cynomolgi*	*P. cynomolgi*	*P. cynomolgi, P. inui*	*P. cynomolgi*
UM12	*P. cynomolgi*	*P. cynomolgi*	*P. cynomolgi, P. inui*	*P. cynomolgi*
UM14	*P. cynomolgi*	*P. cynomolgi*	*P. cynomolgi, P. inui*	*P. cynomolgi*
UM15	*P. cynomolgi*	*P. cynomolgi*	*P. cynomolgi, P. inui*	*P. cynomolgi*
UM16	*P. cynomolgi*	*Plasmodium* spp.	*P. cynomolgi, P. inui*	*P. cynomolgi*, *P. inui*–like, *P. simiovale*
UM17	*P. cynomolgi*	*P. cynomolgi*	*P. cynomolgi, P. inui*	*P. cynomolgi*
UM18	*P. cynomolgi*	*P. cynomolgi*	*P. cynomolgi, P. inui*	*P. cynomolgi*

*Negative, negative for *Plasmodium* DNA and not examined by species-specific nested PCR assays; ND, not done; positive, positive for *Plasmodium* DNA but negative with species-specific nested PCR assays. SSU, small subunit; UM, Universiti Malaya; UNIMAS, Universiti Malaysia Sarawak.

parasites and UM16 had a triple infection with *P. cynomolgi, P. simiovale*, and *P. inui*–like parasites.

We generated phylogenetic trees of similar topology by the maximum-likelihood method for the SSU rRNA genes (Appendix Figure 1) and by the Bayesian maximum clade credibility method for the COX1 genes (Appendix Figure 2). There were discrepancies between the nested PCR assay results and the sequencing results between our 2 laboratories; mixed species of *Plasmodium* were identified only at UNIMAS. A possible explanation is that the DNA samples analyzed at UNIMAS were newly extracted and were different from the ones used in the experiments at UM. There might also be a compromise of the sensitivity in detecting the species with lower parasitemia in mixed infections as a result of competition for nest 1 primers by the species with higher parasite loads. Furthermore, for sequencing of the SSU rRNA genes at UM, primers that were specific for the species identified by nested PCR assays were used, whereas for the COX1 genes, both *P. cynomolgi*–specific primers and primers that could amplify other species of *Plasmodium* were used. Therefore, additional species of *Plasmodium* were identified at UNIMAS in these samples, such as *P. simiovale* and *P. inui*–like, for which no species-specific PCR primers exist.

Conclusions

The 40 *P. knowlesi* infections we detected originated from 6 states in Malaysia, thereby confirming the widespread distribution of human *P. knowlesi* malaria cases in Malaysia (*1*). We detected *P. cynomolgi* infections among indigenous communities in 4 states in Malaysia. Taken together with previous reports of naturally acquired *P. cynomolgi* infections in humans in the states of Terengganu, Sabah, and Sarawak (*3*,*8*,*9*), our findings indicate that human infections caused by *P. cynomolgi* are also widely distributed in Malaysia.

Our study highlights the occurrence of naturally acquired human infections with *P. inui, P. inui*–like, *P. coatneyi*, and *P. simiovale*. Natural human *P. inui* infections have not been described (*10*), although the parasite is experimentally transmissible to humans (*2*). For *P. coatneyi*, attempts to infect humans with blood from an infected rhesus monkey and through infected mosquitoes were unsuccessful (*2*). *P. simiovale* is a lesser-studied simian malaria parasite that was previously described only in toque macaques (*Macaca sinica*) of Sri Lanka (*2*) until it was recently identified, together with *P. inui*–like parasites, in long-tailed macaques from Sarawak in Malaysian Borneo (*11*). All these simian malaria parasites would have been diagnosed by microsco-

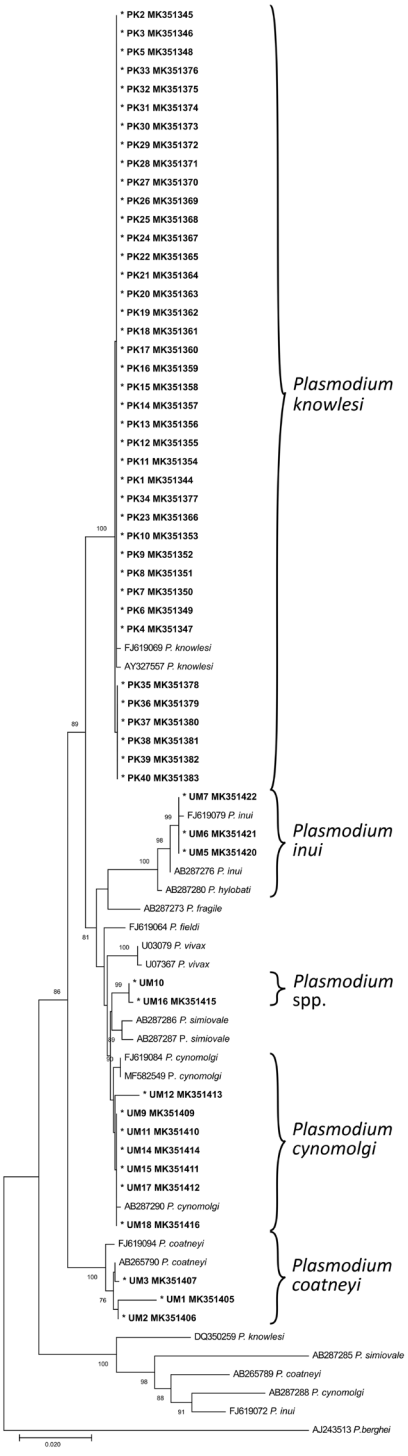

Figure 1. Neighbor-joining phylogenetic tree of *Plasmodium* species based on partial sequence of SSU rRNA genes for identification of *Plasmodium* malaria species from indigenous community blood samples, Malaysia. Nucleotide sequences generated from this study are marked with asterisks and are in bold. GenBank accession numbers are provided for all sequences. Numbers at nodes indicate percentage support of 1,000 bootstrap replicates; only bootstrap values above 70% are displayed. Scale bar indicates branch length.

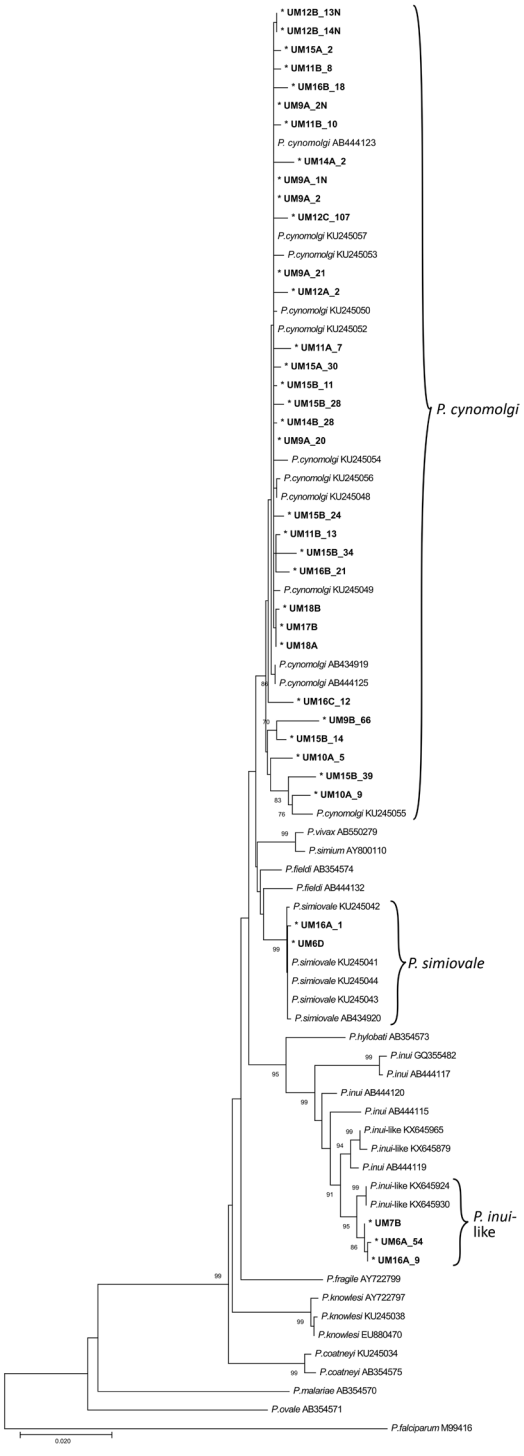

Figure 2. Neighbor-joining phylogenetic tree of *Plasmodium* species based on partial sequence of COX1 genes for identification of *Plasmodium* malaria species from indigenous community blood samples, Malaysia. Nucleotide sequences generated from this study are marked with asterisks and are in bold. GenBank accession numbers are provided for all sequences. Numbers at nodes indicate percentage support of 1,000 bootstrap replicates; only bootstrap values above 70% are displayed. Scale bar indicates branch length.

py as human malaria parasites because they share morphological similarities with human malaria parasites. The early blood stages of *P. knowlesi* resemble those of *P. falciparum*, and the other forms are similar to *P. malariae* (*2*,*6*). *P. cynomolgi* is morphologically similar to *P. vivax* (*2*), and both *P. inui* and *P. inui*–like parasites are morphologically identical to *P. malariae* (*2*,*11*), whereas *P. coatneyi* bears morphologic similarities to *P. falciparum* and *P. simiovale* bears morphologic similarities to *P. ovale* (*2*,*12*). Besides misdiagnosis of simian malaria parasites as human malaria parasites, there are other limitations of microscopy for diagnosis of malaria; thus, using molecular tools is paramount in generating accurate epidemiology data (*6*). It is envisaged that screening with molecular tools of other communities living at the forest fringes will demonstrate the widespread distribution of zoonotic malaria and uncover more newly emergent malaria parasites.

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Appendix

Materials and Methods

Ethical Considerations

The study was approved by the Medical Research Ethics Committee of University of Malaya Medical Centre (MEC Ref. No. 920.83). Approval was also obtained from the Department of Orang Asli (Indigenous) Development and the respective heads of the villages before blood sample collection from the indigenous communities. We obtained informed consents from those who agreed to participate, or from parents on behalf of their children.

Source of Archived Blood Samples

We examined 645 archived blood samples that we had collected during 2011–2014 among indigenous populations of various subtribes from 14 villages in 7 states of Malaysia: Pahang, Perak, Selangor, Negeri Sembilan, Melaka, Kelantan, and Sarawak (Appendix Table 1). These indigenous community samples were obtained during previous studies focusing on intestinal parasites. Therefore, information such as body temperature, malaria history, and malaria parasite density were not available.

The indigenous communities we studied here are a diverse group. There are ≥95 subgroups distributed in selected states throughout Malaysia, each with its own distinct language and culture. The indigenous population of peninsular Malaysia is separated into 3 main tribal groups, Negrito, Senoi, and Proto Malay (Aboriginal Malay), and consists of 18 subtribes. The largest indigenous groups in Malaysian Borneo are Ibans in Sarawak and the Kadazan Dusuns in Sabah. The indigenous communities that we studied all live in the forest fringe and are engaged with forest and agricultural activities in which there is a greater chance of being exposed to the macaque reservoirs and mosquito vectors (*1*,*2*).

Molecular Detection of *Plasmodium* Species at Universiti Malaya (UM)

We extracted genomic DNA from either blood (≈3 mL) or blood spots on filter paper using the QIAamp DNA Blood Mini Kit (QIAGEN, https://www.qiagen.com), according to the manufacturer's instructions, and stored the samples at −20°C until further analysis. We first screened the DNA samples at UM for the presence of *Plasmodium* with the aid of genus-specific primers (rPLU1, rPLU5, rPLU3 and rPLU4), as described previously (*3*). We then examined *Plasmodium*-positive samples by nested PCR assays using species-specific primers for *Plasmodium falciparum, P. vivax, P. malariae, P. ovale curtisi, P. ovale wallikeri* (*3*), *P. knowlesi* (*4*), *P. coatneyi*, *P. cynomolgi*, *P. inui*, and *P. fieldi* (*5*).

Amplification and Sequencing of SSU rRNA Genes of *Plasmodium* Species at UM

We amplified and sequenced longer fragments of SSU rRNA genes of simian *Plasmodium* species (914–950 bp) by nested PCR assays with other pairs of species-specific primers (*6*). We performed PCR amplifications in a 50 μL reaction volume consisting of 5 μL DNA template from previously amplified PCR product, 1X PCR buffer (Promega, https://www.promega.com), 0.2 mM dNTPs, 3 mM $MgCl_2$, 1.5 U Taq DNA polymerase, and 0.5 μM forward and reverse primers. The PCR was carried out in a MyCycler Thermal Cycler (Bio-Rad, https://www.bio-rad.com) under the following conditions: 94°C for 5 min; 35 cycles of 94°C for 1 min, 50–60°C for 90 sec, 72°C for 1 min; 72°C for 10 min. We examined all PCR products (1015–1050 bp) using 1.5% agarose gels before we sent amplicons to a commercial facility for bidirectional sequencing (BigDye Terminator v.3.1 chemistry; Applied Biosystems, https://www.thermofisher.com).

Molecular Detection of Simian *Plasmodium* Species at Universiti Malaysia Sarawak (UNIMAS)

We subsequently extracted DNA from 15 blood samples we had identified as having *P. cynomolgi*, *P. coatneyi* and *P. inui*, and 5 samples that were malaria-negative at UM. We then sent these samples blind to Universiti Malaysia Sarawak (UNIMAS), where they were first examined by nested PCR assays for *Plasmodium*, and the *Plasmodium*-positive ones were examined with species-specific primers as described previously (*3*,*5*,*7*).

PCR Amplification and Sequencing COX1 Genes at UNIMAS

Sequencing of the partial COX1 genes of *Plasmodium* involved a single-step PCR or a hemi-nested PCR. We amplified 3 samples (UM10, UM11, UM14) with single-step PCR and 3

(UM6, UM7, UM18) with hemi-nested PCR; we used both methods for 4 (UM9, UM12, UM15, UM16).

In the hemi-nested PCR, we amplified the complete COX1 gene using *Plasmodium*-specific primers: CYFinF1 (5′-CCTGACATGGATGGATAATACTCG-3′) and CYFinR2 (5′-CCATCCATTTAAAGCGTCTGG-3′). We performed Nest 1 PCR amplification in a 50 μL reaction mixture containing 1× Colorless GoTaq PCR buffer, 2.5 mmol of $MgCl_2$, 0.2 mmol dNTP mix (Promega, https://www.promega.com), 0.025 U GoTaq DNA polymerase, 0.25 μmol of each primer (CYFinF1 and CYFinR2), and 5 μL of purified genomic DNA under the following conditions: 94°C for 4 min; 30 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 90 sec; 5 min at 72°C. We then used the Nest 1 amplicons as a template for the hemi-nested PCR assay with *P. cynomolgi*-specific primers: cox1_F1 (5′-CCAAGCCTCACTTATTGTTAAT-3′) (*8*) and CYFinR2 and *Plasmodium*-specific primers: CYFinF3 (5′-CCAAAGTATAACCGCTGTCGC-3′) and CYFinR2. We performed the hemi-nested PCR amplification for each sample in a 30 μL reaction mixture containing 1× HF colorless PCR buffer, 0.2 mmol dNTP mix, 0.02 U Phusion Polymerase (Promega), 0.5 μmol of each primer (cox1_F1 and CYFinR2 or CYFinF3 and CYFinR2), and 3 μL of Nest 1 product under the following conditions: 98°C for 30 sec; 35 cycles at 98°C for 7 sec, 60°C (for cox1_F1 and CYFinR2) and 62°C (for CYFinF3 and CYFinR2) for 20 sec, and 72°C for 22 sec; and 72°C for 10 min.

We performed single-step PCR amplification of *P. cynomolgi* COX1 fragment using *P. cynomolgi*-specific primers: cox1_F1 (5′- CCAAGCCTCACTTATTGTTAAT-3′) and cox1_R1 (5′- ACCAAATAAAGTCATTGTTGATCC-3′) (*8*). We performed amplifications in a 30 μL reaction mixture containing similar concentrations of PCR master-mix components with cox1_F1 and CYFinR2 or with CYFinF3 and CYFinR2 primers and 3 μL of purified genomic DNA as the template, using the following parameters: 98°C for 30 sec; 35 cycles at 98°C for 7 sec, 58°C for 20 sec, and 72°C for 28 sec; and 72°C for 10 min.

We performed *Plasmodium sp.* DNA cloning and transformation of the recombinant plasmids using the Zero Blunt TOPO PCR Cloning Kit, with One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen, https://www.thermofisher.com). We extracted plasmid DNA

using the PureLink Quick Plasmid DNA Miniprep Kit (Invitrogen) and sent plasmids to a commercial facility for bidirectional DNA sequencing.

Phylogenetic Analysis

We trimmed and aligned the SSU rRNA sequences of *Plasmodium* species using the Geneious version 9.1.6 software (*9*). We constructed phylogenetic trees using the neighbor-joining method as described in MEGA v10.0.5 software (*10*) with bootstrap percentage based on 1,000 replications. We deposited the sequences in GenBank under accession nos. MK351344–MK351383, MK351405–MK351407, MK351409–MK351417, and MK351420–MK351422 (Appendix Table 2).

We used ClustalX v2 to align the partial COX1 sequences. We inferred phylogenetic relationships using the neighbor-joining method (*11*) implemented in MEGA v10.0.5. We reconstructed the neighbor-joining tree with 1,000 bootstrap percentage based on 1,000 replications. We used Tree Annotator to annotate the tree generated by BEAST (https://www.mybiosoftware.com) and visualized the maximum clade credibility tree using FigTree v1.3.1 (https://figtree-1-3-1.software.informer.com). We deposited the *Plasmodium* COX1 sequences generated in GenBank under accession nos. MT992662-MT992702 (Appendix Table 3).

Molecular Detection of Macaque DNA in the Human Samples at UNIMAS

We screened all 20 samples sent from UM to UNIMAS for the presence of macaque DNA. We amplified the cytochrome c oxidase subunit 1 (COX1) gene by PCR using *Macaca* genus-specific primers MacF (5′-CAACGTYATYGTAACGGC-3′) and MacR (5′-AGGTAGTATTGAGGTTGC-3′). We performed Nest 1 PCR amplification for each sample using the Applied Biosystems ProFlex PCR System thermocycler (Thermo Fisher Scientific, https://www.thermofisher.com) in a 20 μL reaction mixture containing 1× colorless GoTaq PCR buffer (Promega), 2 mmol of $MgCl_2$, 0.2 mmol dNTP mix, 0.25 μmol of each primer (MacF and MacR), 0.025 U GoTaq DNA polymerase, and 2 μL of purified genomic DNA under the following conditions: 94°C for 4 min; 35 cycles of 94°C for 30 sec, 59°C for 1 min, and 72°C for 30 sec; and 72°C for 5 min. We used *M. fascicularis*-specific primers MfF (5′-AGGGTTCGGGAACTGACTG-3′) and MfR (5′-TGATCAGACAAATAAAGGGGTC-3′) and *M. nemestrina*-specific primers MnF (5′-CATACCTATTATGATTGGGGGT-3′) and MnR (5′-

GGTGGAGGGAGAAGATGATTAGG-3′) for subsequent PCR amplification in a 20 μL reaction mixture containing 1× Green GoTaq PCR buffer (Promega), 2 mmol of $MgCl_2$, 0.2 mmol dNTP mix, 0.25 μmol of each primer (MfF and MfR or MnF and MnR), and 0.025 U GoTaq DNA polymerase with 2 μL of Nest 1 product under the following conditions: 94°C for 4 min; 35 cycles of 94°C for 30 sec, 57°C for 1 min, and 72°C for 30 sec; and 72°C for 5 min.

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Appendix Table 1. Distribution of indigenous community blood samples used in the study of *Plasmodium* infections in Malaysia, according to state, district, village, and subtribe (N = 645).

State	District	Village	Subtribe	n (%)	Reference
Peninsular Malaysia					
Pahang	Pekan	Chini	Proto-Malay (Jakun)	9 (1.4)	Unpublished data
	Temerloh	Paya Sendayan	Senoi (Jahut)	97 (15.0)	(*12*)
	Lanchang	Kuala Gandah	Senoi (Che Wong)	3 (0.5)	Unpublished data
Perak	Slim River	Sungail Bil	Senoi (Semai)	40 (6.2)	Unpublished data
	Tapah	Batu 7 1/2	Senoi (Semai)	7 (1.1)	Unpublished data
		Batu 8	Senoi (Semai)	14 (2.2)	Unpublished data
Selangor	Semenyih	Donglai Baru	Proto-Malay (Temuan)	49 (7.6)	(*12*)
Negeri	Jelebu	Dusun Kubur	Proto-Malay (Temuan)	100 (15.5)	(*13*)
Sembilan		Ulu Kelaka	Proto-Malay (Temuan)	63 (9.8)	(*13*)
Melaka	Alor Gajah	Bukit Sebang	Proto-Malay (Temuan)	9 (1.4)	(*12*)
		Bukit Payung	Proto-Malay (Temuan)	23 (3.6)	(*12*)
Kelantan	Gua Musang	Kuala Lah	Negrito (Mendriq)	15 (2.3)	Unpublished data
		Aring 5	Negrito (Bateq)	17 (2.6)	Unpublished data
Malaysia Borneo					
Sarawak	Sarikei	Pakan	Iban	199 (30.9)	(*14*)

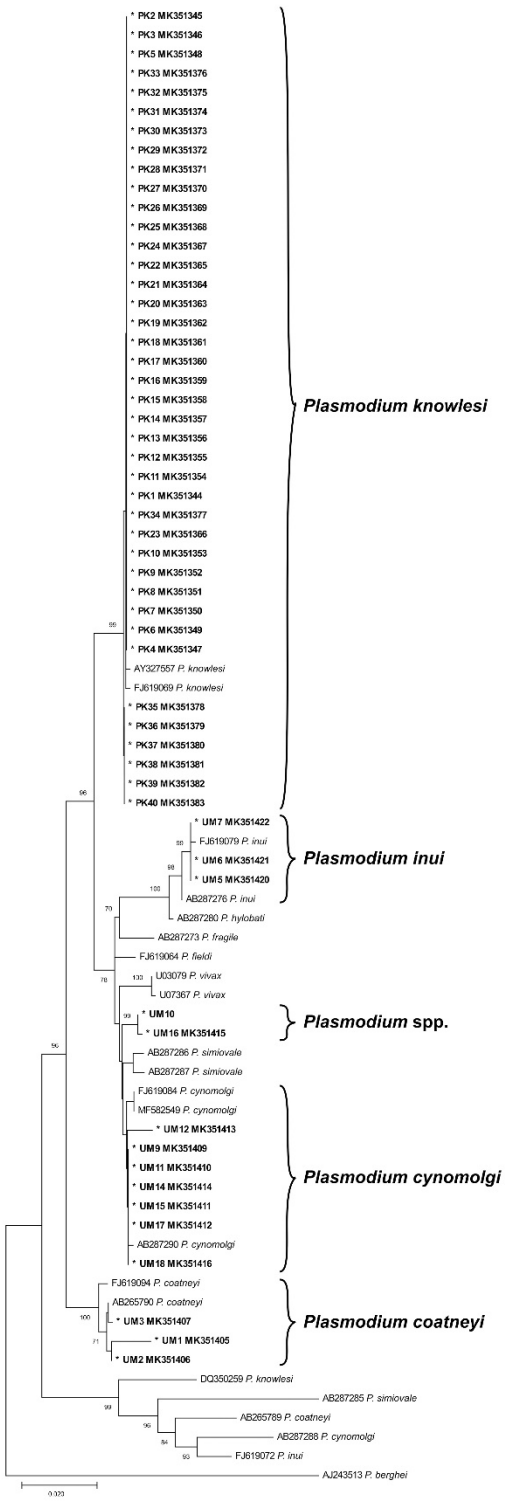
Appendix Table 2. GenBank accession numbers of partial sequence SSU rRNA gene generated from simian *Plasmodium* species found in study at Universiti Malaya

GenBank accession no.	*Plasmodium* spp.	Location	Sample ID
MK351344	*P. knowlesi*	Pakan, Sarikei, Sarawak	PK1
MK351345	*P. knowlesi*	Pakan, Sarikei, Sarawak	PK2
MK351346	*P. knowlesi*	Pakan, Sarikei, Sarawak	PK3
MK351347	*P. knowlesi*	Kg Kuala Gandah, Lanchang, Pahang	PK4
MK351348	*P. knowlesi*	Kg Chini, Pekan, Pahang	PK5
MK351349	*P. knowlesi*	Kg Ulu Kelaka, Jelebu, Negeri Sembilan	PK6
MK351350	*P. knowlesi*	Kg Ulu Kelaka, Jelebu, Negeri Sembilan	PK7
MK351351	*P. knowlesi*	Kg Sungai Bil, Slim River, Perak	PK8
MK351352	*P. knowlesi*	Kg Sungai Bil, Slim River, Perak	PK9
MK351353	*P. knowlesi*	Kg Sungai Bil, Slim River, Perak	PK10
MK351354	*P. knowlesi*	Kg Sungai Bil, Slim River, Perak	PK11
MK351355	*P. knowlesi*	Kg Sungai Bil, Slim River, Perak	PK12
MK351356	*P. knowlesi*	Kg Sungai Bil, Slim River, Perak	PK13
MK351357	*P. knowlesi*	Kg Sungai Bil, Slim River, Perak	PK14
MK351358	*P. knowlesi*	Kg Sungai Bil, Slim River, Perak	PK15
MK351359	*P. knowlesi*	Kg Sungai Bil, Slim River, Perak	PK16
MK351360	*P. knowlesi*	Kg Sungai Bil, Slim River, Perak	PK17
MK351361	*P. knowlesi*	Kg Sungai Bil, Slim River, Perak	PK18
MK351362	*P. knowlesi*	Kg Sungai Bil, Slim River, Perak	PK19
MK351363	*P. knowlesi*	Kg Sungai Bil, Slim River, Perak	PK20
MK351364	*P. knowlesi*	Kg Sungai Bil, Slim River, Perak	PK21
MK351365	*P. knowlesi*	Kg Sungai Bil, Slim River, Perak	PK22
MK351366	*P. knowlesi*	Kg Batu 7 1/2, Tapah, Perak	PK23
MK351367	*P. knowlesi*	Kg Batu 7 1/2, Tapah, Perak	PK24
MK351368	*P. knowlesi*	Kg Batu 7 1/2, Tapah, Perak	PK25
MK351369	*P. knowlesi*	Kg Batu 7 1/2, Tapah, Perak	PK26
MK351370	*P. knowlesi*	Kg Batu 8, Tapah, Perak	PK27
MK351371	*P. knowlesi*	Kg Batu 8, Tapah, Perak	PK28
MK351372	*P. knowlesi*	Kg Batu 8, Tapah, Perak	PK29
MK351373	*P. knowlesi*	Kg Batu 8, Tapah, Perak	PK30
MK351374	*P. knowlesi*	Kg Batu 8, Tapah, Perak	PK31
MK351375	*P. knowlesi*	Kg Batu 8, Tapah, Perak	PK32
MK351376	*P. knowlesi*	Kg Batu 8, Tapah, Perak	PK33
MK351377	*P. knowlesi*	Kg Bukit Sebang, Alor Gajah, Melaka	PK34
MK351378	*P. knowlesi*	Kg Kuala Lah, Gua Musang, Kelantan	PK35
MK351379	*P. knowlesi*	Kg Kuala Lah, Gua Musang, Kelantan	PK36
MK351380	*P. knowlesi*	Kg Kuala Lah, Gua Musang, Kelantan	PK37
MK351381	*P. knowlesi*	Kg Aring 5, Gua Musang, Kelantan	PK38
MK351382	*P. knowlesi*	Kg Aring 5, Gua Musang, Kelantan	PK39
MK351383	*P. knowlesi*	Kg Aring 5, Gua Musang, Kelantan	PK40
MK351405	*P. coatneyi*	Kg Sungai Bil, Slim River, Perak	UM1
MK351406	*P. coatneyi*	Kg Batu 7 1/2, Tapah, Perak	UM2
MK351407	*P. coatneyi*	Kg Batu 8, Tapah, Perak	UM3
MK351409	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM9
MK351410	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM11
MK351411	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM15
MK351412	*P. cynomolgi*	Kg Batu 8, Tapah, Perak	UM17
MK351413	*P. cynomolgi*	Kg Batu 8, Tapah, Perak	UM12
MK351414	*P. cynomolgi*	Kg Ulu Kelaka, Jelebu, Negeri Sembilan	UM14
MK351415	*Plasmodium* spp.	Kg Ulu Kelaka, Jelebu, Negeri Sembilan	UM16
MK351416	*P. cynomolgi*	Kg Bukit Sebang, Alor Gajah, Melaka	UM18
MK351420	*P. inui*	Kg Bukit Sebang, Alor Gajah, Melaka	UM5
MK351421	*P. inui*	Pakan, Sarikei, Sarawak	UM6
MK351422	*P. inui*	Pakan, Sarikei, Sarawak	UM7

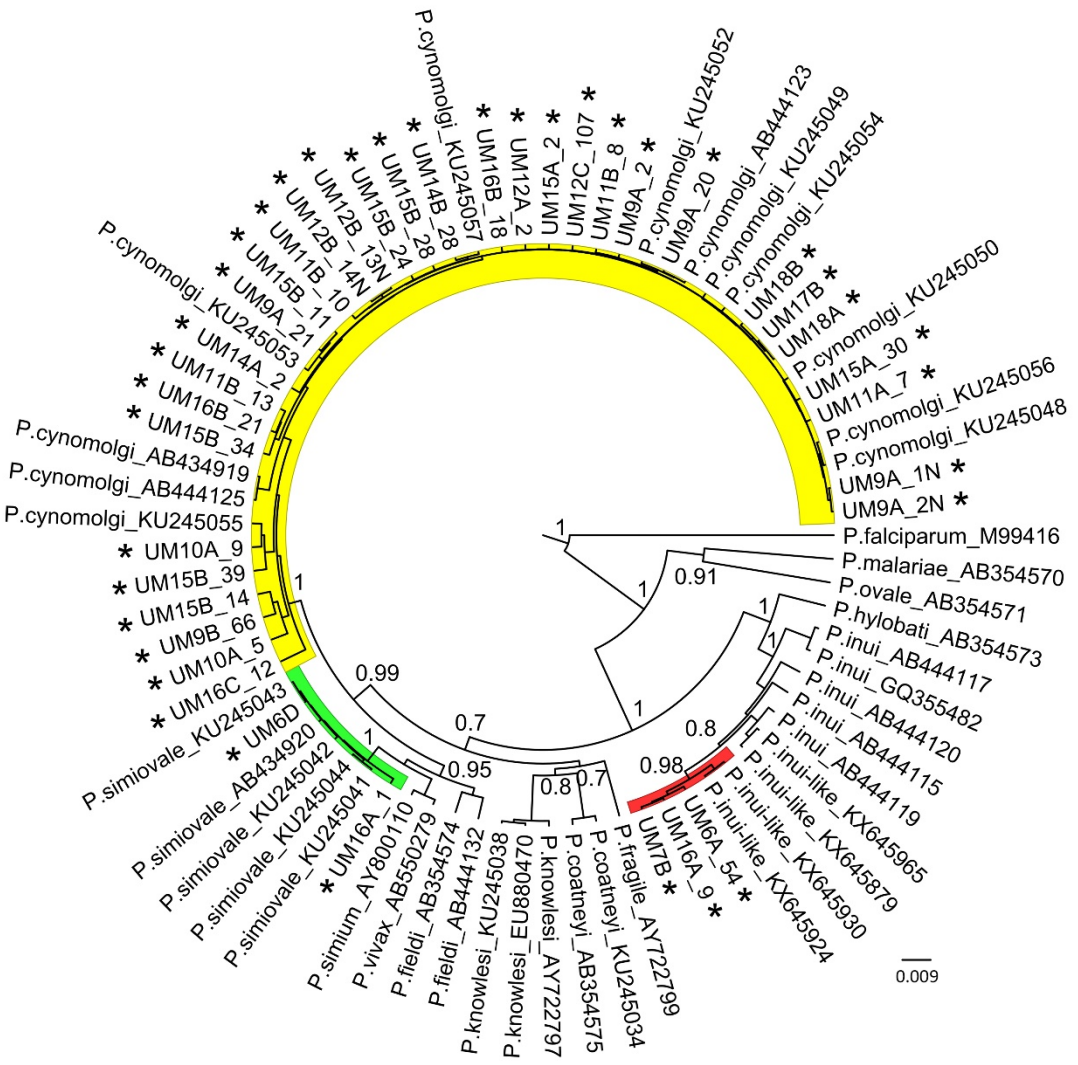
Appendix Table 3. GenBank accession numbers of partial sequence COX1 gene generated from simian *Plasmodium* species found in study at Universiti Malaysia Sarawak

GenBank accession no.	*Plasmodium* spp.	Location	Clone identity*
MT992662	*P. cf. inui*	Pakan, Sarikei, Sarawak	UM6A_54
MT992663	*P. simiovale*	Pakan, Sarikei, Sarawak	UM6D
MT992664	*P. cf. inui*	Pakan, Sarikei, Sarawak	UM7B
MT992665	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM9A_1N
MT992666	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM9A_2N
MT992667	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM9A_2
MT992668	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM9A_20
MT992669	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM9A_21
MT992670	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM9B_66
MT992671	*Plasmodium sp.*	Kg Sungai Bil, Slim River, Perak	UM9B_75
MT992672	*P. cynomolgi*	Kg Aring 5, Gua Musang, Kelantan	UM10A_5
MT992673	*P. cynomolgi*	Kg Aring 5, Gua Musang, Kelantan	UM10A_9
MT992674	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM11A_7
MT992675	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM11B_8
MT992676	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM11B_10
MT992677	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM11B_13
MT992678	*P. cynomolgi*	Kg Batu 8, Tapah, Perak	UM12A_2
MT992679	*P. cynomolgi*	Kg Batu 8, Tapah, Perak	UM12B_13N
MT992680	*P. cynomolgi*	Kg Batu 8, Tapah, Perak	UM12B_14N
MT992681	*P. cynomolgi*	Kg Batu 8, Tapah, Perak	UM12C_107
MT992682	*P. cynomolgi*	Kg Ulu Kelaka, Jelebu, Negeri Sembilan	UM14A_2
MT992683	*P. cynomolgi*	Kg Ulu Kelaka, Jelebu, Negeri Sembilan	UM14B_28
MT992684	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM15A_2
MT992685	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM15A_30
MT992686	*Plasmodium sp.*	Kg Sungai Bil, Slim River, Perak	UM15B_9
MT992687	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM15B_11
MT992688	*Plasmodium sp.*	Kg Sungai Bil, Slim River, Perak	UM15B_12
MT992689	*Plasmodium sp.*	Kg Sungai Bil, Slim River, Perak	UM15B_13
MT992690	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM15B_14
MT992691	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM15B_24
MT992692	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM15B_28
MT992693	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM15B_34
MT992694	*P. cynomolgi*	Kg Sungai Bil, Slim River, Perak	UM15B_39
MT992695	*P. simiovale*	Kg Ulu Kelaka, Jelebu, Negeri Sembilan	UM16A_1
MT992696	*P. cf. inui*	Kg Ulu Kelaka, Jelebu, Negeri Sembilan	UM16A_9
MT992697	*P. cynomolgi*	Kg Ulu Kelaka, Jelebu, Negeri Sembilan	UM16B_18
MT992698	*P. cynomolgi*	Kg Ulu Kelaka, Jelebu, Negeri Sembilan	UM16B_21
MT992699	*P. cynomolgi*	Kg Ulu Kelaka, Jelebu, Negeri Sembilan	UM16C_12
MT992700	*P. cynomolgi*	Kg Batu 8, Tapah, Perak	UM17B
MT992701	*P. cynomolgi*	Kg Bukit Sebang, Alor Gajah, Melaka	UM18A
MT992702	*P. cynomolgi*	Kg Bukit Sebang, Alor Gajah, Melaka	UM18B

*Clone identity is the identity of the clones derived from samples UM 6–7, UM 9–12, and UM 14–18.



Appendix Figure 1. Maximum-likelihood phylogenetic tree of *Plasmodium* species, based on partial sequence of SSU rRNA genes. Numbers at nodes indicate percentage support of 1,000 bootstrap replicates; only bootstrap values above 70% are displayed. Nucleotide sequences generated from our study are marked with asterisks and are in bold. Scale bar indicates branch length.



Appendix Figure 2. Maximum clade credibility phylogeny of *Plasmodium* species based on partial sequence of COX1 genes inferred using the Bayesian method. Numbers on branches are values of posterior probabilities. Sequences of *P. cynomolgi* are highlighted in yellow, *P. simiovale* in green, and *P. inui*-like in red. Nucleotide sequences generated from the present study are marked with asterisks.