

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

Nan Jiun Yap,<sup>1</sup> Hanisah Hossain,<sup>1</sup> Thamayanthi Nada-Raja, Romano Ngui, Azdayanti Muslim, Boon-Peng Hoh, Loke Tim Khaw, Khamisah Abdul Kadir, Paul Cliff Simon Divis, Indra Vythilingam, Balbir Singh, Yvonne Ai-Lian Lim

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.

Author affiliations: Universiti Malaya, Kuala Lumpur, Malaysia (N.J. Yap, R. Ngui, A. Muslim, I. Vythilingam, Y.A.-L. Lim); Universiti Malaysia Sarawak, Kota Samarahan, Sarawak, Malaysia (H. Hossain, T. Nada-Raja, K.A. Kadir, P.C.S. Divis, B. Singh); Universiti Teknologi MARA (Sungai Buloh Campus), Selangor, Malaysia (A. Muslim); UCSI University, Kuala Lumpur, Malaysia (B.-P. Hoh); International Medical University, Kuala Lumpur, Malaysia (L.T. Khaw)

DOI: <https://doi.org/10.3201/eid2708.204502>

## 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 mono-infections 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

<sup>1</sup>These authors contributed equally to this article.

**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 |                 |                    |                         |                    |                    |                     |                |
|--------------------------|--------------------|----------------------|----------------------------------|-----------------|--------------------|-------------------------|--------------------|--------------------|---------------------|----------------|
|                          |                    |                      | <i>P. falciparum</i>             | <i>P. vivax</i> | <i>P. malariae</i> | <i>P. ovale curtisi</i> | <i>P. knowlesi</i> | <i>P. coatneyi</i> | <i>P. cynomolgi</i> | <i>P. inui</i> |
| 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       | <i>P. coatneyi</i>                 | <i>P. coatneyi</i>                      | Negative                             | ND  |
| UM2       | <i>P. coatneyi</i>                 | <i>P. coatneyi</i>                      | Negative                             | ND  |
| UM3       | <i>P. coatneyi</i>                 | <i>P. coatneyi</i>                      | Negative                             | ND  |
| UM5       | <i>P. inui</i>                     | <i>P. inui</i>                          | Negative                             | ND  |
| UM6       | <i>P. inui</i>                     | <i>P. inui</i>                          | Positive                             | <i>P. inui</i> -like, <i>P. simiovale</i>                       |
| UM7       | <i>P. inui</i>                     | <i>P. inui</i>                          | <i>P. inui</i>                       | <i>P. inui</i> -like  |
| UM9       | <i>P. cynomolgi</i>                | <i>P. cynomolgi</i>                     | <i>P. cynomolgi</i> , <i>P. inui</i> | <i>P. cynomolgi</i>   |
| UM10      | <i>P. cynomolgi</i>                | <i>Plasmodium</i> spp.                  | <i>P. cynomolgi</i> , <i>P. inui</i> | <i>P. cynomolgi</i>   |
| UM11      | <i>P. cynomolgi</i>                | <i>P. cynomolgi</i>                     | <i>P. cynomolgi</i> , <i>P. inui</i> | <i>P. cynomolgi</i>   |
| UM12      | <i>P. cynomolgi</i>                | <i>P. cynomolgi</i>                     | <i>P. cynomolgi</i> , <i>P. inui</i> | <i>P. cynomolgi</i>   |
| UM14      | <i>P. cynomolgi</i>                | <i>P. cynomolgi</i>                     | <i>P. cynomolgi</i> , <i>P. inui</i> | <i>P. cynomolgi</i>   |
| UM15      | <i>P. cynomolgi</i>                | <i>P. cynomolgi</i>                     | <i>P. cynomolgi</i> , <i>P. inui</i> | <i>P. cynomolgi</i>   |
| UM16      | <i>P. cynomolgi</i>                | <i>Plasmodium</i> spp.                  | <i>P. cynomolgi</i> , <i>P. inui</i> | <i>P. cynomolgi</i> , <i>P. inui</i> -like, <i>P. simiovale</i> |
| UM17      | <i>P. cynomolgi</i>                | <i>P. cynomolgi</i>                     | <i>P. cynomolgi</i> , <i>P. inui</i> | <i>P. cynomolgi</i>   |
| UM18      | <i>P. cynomolgi</i>                | <i>P. cynomolgi</i>                     | <i>P. cynomolgi</i> , <i>P. inui</i> | <i>P. cynomolgi</i>   |

\*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.

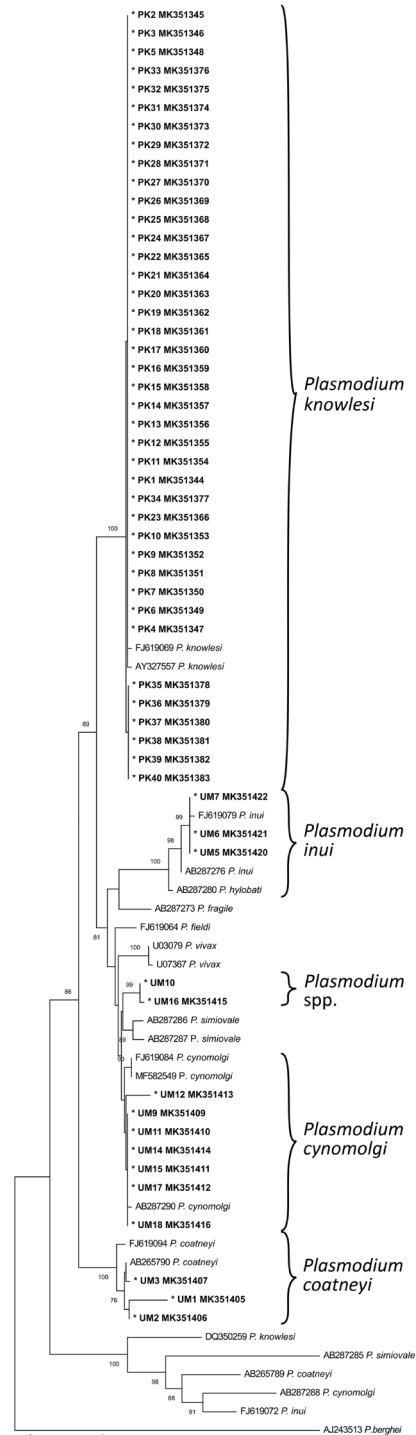
parasites and UM16 had a triple infection with *P. cynomolgi*, *P. simiovale*, and *P. inui*-like parasites.

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.

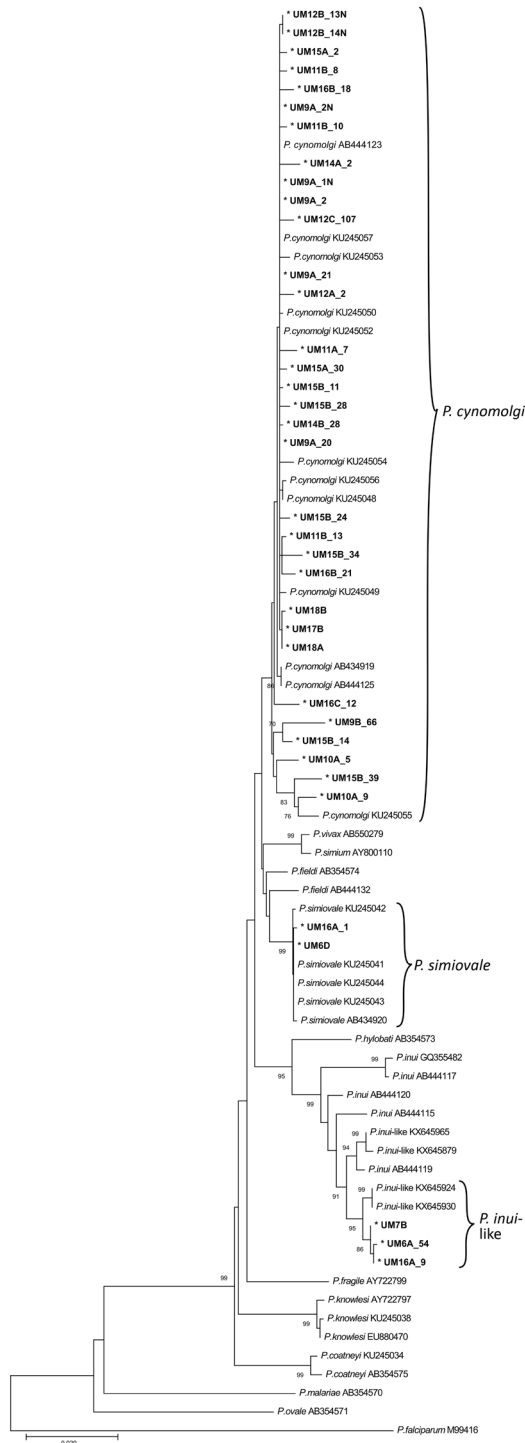
**Conclusions**

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 microscop-



**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.

### Acknowledgments

We thank the staff from the Department of Orang Asli Development, Ministry of Rural Development, Malaysia, for assisting in the collection of blood samples from communities in rural areas.

The research was supported by funding from Universiti Malaya Student Grant (no. PG056-2013A to N.J.Y.), UM/MoHE High Impact Research Grant (no. H-20001-00-E000061 to Y.A.L.), and UNIMAS Special Top Down Grant (no. F05/TDG/1734/2018 to B.S.).

### About the Author

Dr. Yap is a postdoctoral researcher at the Department of Parasitology, Faculty of Medicine, Universiti Malaya, Kuala Lumpur, Malaysia. Her primary research interests include epidemiology and molecular characterization of zoonotic malaria species.

### References

- Jeyaprakasam NK, Liew JWK, Low VL, Wan-Sulaiman WY, Vythilingam I. *Plasmodium knowlesi* infecting humans in Southeast Asia: what's next? PLoS Negl Trop Dis. 2020;14:e0008900. <https://doi.org/10.1371/journal.pntd.0008900>
- Coatney GR, Collins WE, Warren M, Contacos PG. The primate malarial. Washington (DC): US National Institute of Allergy and Infectious Diseases; 1971
- Ta TH, Hisam S, Lanza M, Jiram AI, Ismail N, Rubio JM. First case of a naturally acquired human infection with *Plasmodium cynomolgi*. Malar J. 2014;13:68. <https://doi.org/10.1186/1475-2875-13-68>
- Imwong M, Madmanee W, Suwannasin K, Kunasol C, Peto TJ, Tripura R, et al. Asymptomatic natural human infections with the simian malaria parasites *Plasmodium*

- cynomolgi* and *Plasmodium knowlesi*. J Infect Dis. 2019;219:695–702. <https://doi.org/10.1093/infdis/jiy519>
5. Anstey NM, Grigg MJ. Zoonotic malaria: the better you look, the more you find. J Infect Dis. 2019;219:679–81. <https://doi.org/10.1093/infdis/jiy520>
  6. Singh B, Daneshvar C. Human infections and detection of *Plasmodium knowlesi*. Clin Microbiol Rev. 2013;26:165–84. <https://doi.org/10.1128/CMR.00079-12>
  7. Li J, Gutell RR, Damberger SH, Wirtz RA, Kissinger JC, Rogers MJ, et al. Regulation and trafficking of three distinct 18 S ribosomal RNAs during development of the malaria parasite. J Mol Biol. 1997;269:203–13. <https://doi.org/10.1006/jmbi.1997.1038>
  8. Grignard L, Shah S, Chua TH, William T, Drakeley CJ, Fornace KM. Natural human infections with *Plasmodium cynomolgi* and other malaria species in an elimination setting in Sabah, Malaysia. J Infect Dis. 2019;220:1946–9. <https://doi.org/10.1093/infdis/jiz397>
  9. Raja TN, Hu TH, Kadir KA, Mohamad DSA, Divis PCS, Wong LL, et al. Naturally acquired human *Plasmodium cynomolgi* and *P. knowlesi* infections, Malaysian Borneo. Emerg Infect Dis. 2020;26:1801–9. <https://doi.org/10.3201/eid2608.200343>
  10. Siner A, Liew ST, Kadir KA, Mohamad DSA, Thomas FK, Zulkarnaen M, et al. Absence of *Plasmodium inui* and *Plasmodium cynomolgi*, but detection of *Plasmodium knowlesi* and *Plasmodium vivax* infections in asymptomatic humans in the Betong division of Sarawak, Malaysian Borneo. Malar J. 2017;16:417. <https://doi.org/10.1186/s12936-017-2064-9>
  11. Nada Raja T, Hu TH, Zainudin R, Lee KS, Perkins SL, Singh B. Malaria parasites of long-tailed macaques in Sarawak, Malaysian Borneo: a novel species and demographic and evolutionary histories. BMC Evol Biol. 2018;18:49. <https://doi.org/10.1186/s12862-018-1170-9>
  12. Dissanaikie AS. Simian malaria parasites of Ceylon. Bull World Health Organ. 1965;32:593–7.

Addresses for correspondence: Yvonne Ai-Lian Lim, Department of Parasitology, Faculty of Medicine, Universiti Malaya, Kuala Lumpur, Malaysia; email: limailian@um.edu.my; Balbir Singh, Malaria Research Centre, Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, Kota Samarahan, Sarawak, Malaysia; email: bsingh@unimas.my

## EID Podcast: Tracking Canine Enteric Coronavirus in the UK

**Dr. Danielle Greenberg, founder of a veterinary clinic near Liverpool, knew something was wrong. Dogs in her clinic were vomiting—and much more than usual. Concerned, she phoned Dr. Alan Radford and his team at the University of Liverpool for help.**

**Before long they knew they had an outbreak on their hands.**

**In this EID podcast, Dr. Alan Radford, a professor of veterinary health informatics at the University of Liverpool, recounts the discovery of an outbreak of canine enteric coronavirus.**

Visit our website to listen: <https://go.usa.gov/xsMcP> **EMERGING  
INFECTIOUS DISEASES™**

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

## 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  $\geq 95$  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 ( $\approx 3$  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  $-20^{\circ}\text{C}$  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  $\mu\text{L}$  reaction volume consisting of 5  $\mu\text{L}$  DNA template from previously amplified PCR product, 1X PCR buffer (Promega, <https://www.promega.com>), 0.2 mM dNTPs, 3 mM  $\text{MgCl}_2$ , 1.5 U Taq DNA polymerase, and 0.5  $\mu\text{M}$  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:  $94^{\circ}\text{C}$  for 5 min; 35 cycles of  $94^{\circ}\text{C}$  for 1 min,  $50\text{--}60^{\circ}\text{C}$  for 90 sec,  $72^{\circ}\text{C}$  for 1 min;  $72^{\circ}\text{C}$  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<sub>2</sub>, 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<sub>2</sub>, 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'-AGGGTTCGGGAAGTACTG-3') and MfR (5'-TGATCAGACAAATAAAGGGGTC-3') and *M. nemestrina*-specific primers MnF (5'-CATACTATTATGATTGGGGGT-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<sub>2</sub>, 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.

## References

1. Jiram AI, Hisam S, Reuben H, Husin SZ, Roslan A, Wan Ismail WR. Submicroscopic evidence of the simian malaria parasite, *Plasmodium knowlesi*, in an Orang Asli community. *Southeast Asian J Trop Med Public Health*. 2015;47:4.
2. Kaur G. Prevalence of clinical malaria among an Orang Asli community in Malaysia. *Southeast Asian J Trop Med Public Health*. 2009;40:665–73. [PubMed](#)
3. Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. *Am J Trop Med Hyg*. 1999;60:687–92. [PubMed](#) <https://doi.org/10.4269/ajtmh.1999.60.687>
4. Singh B, Sung LK, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, et al. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet*. 2004;363:1017–24. [PubMed](#) [https://doi.org/10.1016/S0140-6736\(04\)15836-4](https://doi.org/10.1016/S0140-6736(04)15836-4)
5. Lee KS, Divis PC, Zakaria SK, Matusop A, Julin RA, Conway DJ, et al. *Plasmodium knowlesi*: reservoir hosts and tracking the emergence in humans and macaques. *PLoS Pathog*. 2011;7:e1002015. [PubMed](#) <https://doi.org/10.1371/journal.ppat.1002015>
6. Chua TH, Manin BO, Daim S, Vythilingam I, Drakeley C. Phylogenetic analysis of simian *Plasmodium* spp. infecting *Anopheles balabacensis* Baisas in Sabah, Malaysia. *PLoS Negl Trop Dis*. 2017;11:e0005991. [PubMed](#) <https://doi.org/10.1371/journal.pntd.0005991>
7. Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do Rosario VE, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol Biochem Parasitol*. 1993;61:315–20. [PubMed](#) [https://doi.org/10.1016/0166-6851\(93\)90077-B](https://doi.org/10.1016/0166-6851(93)90077-B)
8. Raja TN, Hu TH, Kadir KA, Mohamad DSA, Divis PCS, Wong LL, et al. Naturally acquired human infections with *Plasmodium cynomolgi* and *Plasmodium knowlesi* infections, Malaysian Borneo. *Emerg Infect Dis*. 2020;26:1801–9. [PubMed](#) <https://doi.org/10.3201/eid2608.200343>

9. Kearsse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*. 2012;28:1647–9. [PubMed](#)  
<https://doi.org/10.1093/bioinformatics/bts199>
10. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol*. 2018;35:1547–9. [PubMed](#)  
<https://doi.org/10.1093/molbev/msy096>
11. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*. 1987;4:406–25. [PubMed](#)
12. Choy SH, Al-Mekhlafi HM, Mahdy MA, Nasr NN, Sulaiman M, Lim YA, et al. Prevalence and associated risk factors of *Giardia* infection among indigenous communities in rural Malaysia. *Sci Rep*. 2014;4:6909. [PubMed](#) <https://doi.org/10.1038/srep06909>
13. Lee SC, Tang MS, Lim YA, Choy SH, Kurtz ZD, Cox LM, et al. Helminth colonization is associated with increased diversity of the gut microbiota. *PLoS Negl Trop Dis*. 2014;8:e2880. [PubMed](#)  
<https://doi.org/10.1371/journal.pntd.0002880>
14. Rajoo Y, Ambu S, Lim YA, Rajoo K, Tey SC, Lu CW, et al. Neglected intestinal parasites, malnutrition and associated key factors: a population based cross-sectional study among indigenous communities in Sarawak, Malaysia. *PLoS One*. 2017;12:e0170174. [PubMed](#)  
<https://doi.org/10.1371/journal.pone.0170174>

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

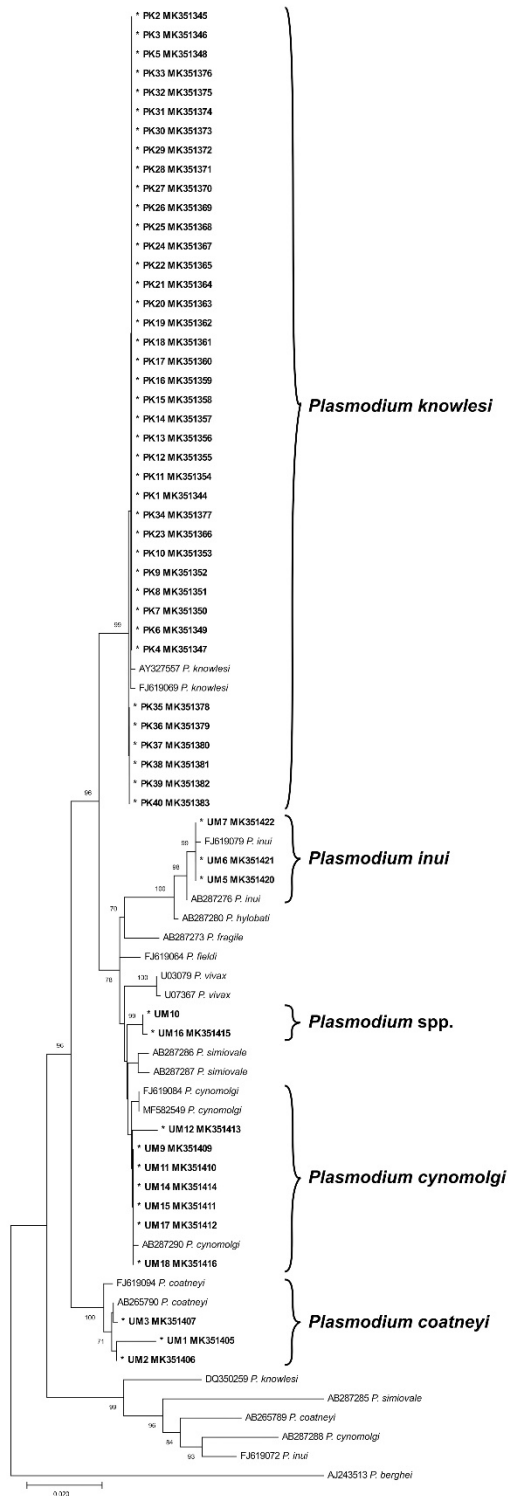
**Appendix Table 2.** GenBank accession numbers of partial sequence SSU rRNA gene generated from simian *Plasmodium* species found in study at Universiti Malaya

| GenBank accession no. | <i>Plasmodium</i> spp. | Location                               | Sample ID |
|-----------------------|------------------------|--|-----------|
| MK351344              | <i>P. knowlesi</i>     | Pakan, Sarikei, Sarawak                | PK1       |
| MK351345              | <i>P. knowlesi</i>     | Pakan, Sarikei, Sarawak                | PK2       |
| MK351346              | <i>P. knowlesi</i>     | Pakan, Sarikei, Sarawak                | PK3       |
| MK351347              | <i>P. knowlesi</i>     | Kg Kuala Gandah, Lanchang, Pahang      | PK4       |
| MK351348              | <i>P. knowlesi</i>     | Kg Chini, Pekan, Pahang                | PK5       |
| MK351349              | <i>P. knowlesi</i>     | Kg Ulu Kelaka, Jelebu, Negeri Sembilan | PK6       |
| MK351350              | <i>P. knowlesi</i>     | Kg Ulu Kelaka, Jelebu, Negeri Sembilan | PK7       |
| MK351351              | <i>P. knowlesi</i>     | Kg Sungai Bil, Slim River, Perak       | PK8       |
| MK351352              | <i>P. knowlesi</i>     | Kg Sungai Bil, Slim River, Perak       | PK9       |
| MK351353              | <i>P. knowlesi</i>     | Kg Sungai Bil, Slim River, Perak       | PK10      |
| MK351354              | <i>P. knowlesi</i>     | Kg Sungai Bil, Slim River, Perak       | PK11      |
| MK351355              | <i>P. knowlesi</i>     | Kg Sungai Bil, Slim River, Perak       | PK12      |
| MK351356              | <i>P. knowlesi</i>     | Kg Sungai Bil, Slim River, Perak       | PK13      |
| MK351357              | <i>P. knowlesi</i>     | Kg Sungai Bil, Slim River, Perak       | PK14      |
| MK351358              | <i>P. knowlesi</i>     | Kg Sungai Bil, Slim River, Perak       | PK15      |
| MK351359              | <i>P. knowlesi</i>     | Kg Sungai Bil, Slim River, Perak       | PK16      |
| MK351360              | <i>P. knowlesi</i>     | Kg Sungai Bil, Slim River, Perak       | PK17      |
| MK351361              | <i>P. knowlesi</i>     | Kg Sungai Bil, Slim River, Perak       | PK18      |
| MK351362              | <i>P. knowlesi</i>     | Kg Sungai Bil, Slim River, Perak       | PK19      |
| MK351363              | <i>P. knowlesi</i>     | Kg Sungai Bil, Slim River, Perak       | PK20      |
| MK351364              | <i>P. knowlesi</i>     | Kg Sungai Bil, Slim River, Perak       | PK21      |
| MK351365              | <i>P. knowlesi</i>     | Kg Sungai Bil, Slim River, Perak       | PK22      |
| MK351366              | <i>P. knowlesi</i>     | Kg Batu 7 1/2, Tapah, Perak            | PK23      |
| MK351367              | <i>P. knowlesi</i>     | Kg Batu 7 1/2, Tapah, Perak            | PK24      |
| MK351368              | <i>P. knowlesi</i>     | Kg Batu 7 1/2, Tapah, Perak            | PK25      |
| MK351369              | <i>P. knowlesi</i>     | Kg Batu 7 1/2, Tapah, Perak            | PK26      |
| MK351370              | <i>P. knowlesi</i>     | Kg Batu 8, Tapah, Perak                | PK27      |
| MK351371              | <i>P. knowlesi</i>     | Kg Batu 8, Tapah, Perak                | PK28      |
| MK351372              | <i>P. knowlesi</i>     | Kg Batu 8, Tapah, Perak                | PK29      |
| MK351373              | <i>P. knowlesi</i>     | Kg Batu 8, Tapah, Perak                | PK30      |
| MK351374              | <i>P. knowlesi</i>     | Kg Batu 8, Tapah, Perak                | PK31      |
| MK351375              | <i>P. knowlesi</i>     | Kg Batu 8, Tapah, Perak                | PK32      |
| MK351376              | <i>P. knowlesi</i>     | Kg Batu 8, Tapah, Perak                | PK33      |
| MK351377              | <i>P. knowlesi</i>     | Kg Bukit Sebang, Alor Gajah, Melaka    | PK34      |
| MK351378              | <i>P. knowlesi</i>     | Kg Kuala Lah, Gua Musang, Kelantan     | PK35      |
| MK351379              | <i>P. knowlesi</i>     | Kg Kuala Lah, Gua Musang, Kelantan     | PK36      |
| MK351380              | <i>P. knowlesi</i>     | Kg Kuala Lah, Gua Musang, Kelantan     | PK37      |
| MK351381              | <i>P. knowlesi</i>     | Kg Aring 5, Gua Musang, Kelantan       | PK38      |
| MK351382              | <i>P. knowlesi</i>     | Kg Aring 5, Gua Musang, Kelantan       | PK39      |
| MK351383              | <i>P. knowlesi</i>     | Kg Aring 5, Gua Musang, Kelantan       | PK40      |
| MK351405              | <i>P. coatneyi</i>     | Kg Sungai Bil, Slim River, Perak       | UM1       |
| MK351406              | <i>P. coatneyi</i>     | Kg Batu 7 1/2, Tapah, Perak            | UM2       |
| MK351407              | <i>P. coatneyi</i>     | Kg Batu 8, Tapah, Perak                | UM3       |
| MK351409              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM9       |
| MK351410              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM11      |
| MK351411              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM15      |
| MK351412              | <i>P. cynomolgi</i>    | Kg Batu 8, Tapah, Perak                | UM17      |
| MK351413              | <i>P. cynomolgi</i>    | Kg Batu 8, Tapah, Perak                | UM12      |
| MK351414              | <i>P. cynomolgi</i>    | Kg Ulu Kelaka, Jelebu, Negeri Sembilan | UM14      |
| MK351415              | <i>Plasmodium</i> spp. | Kg Ulu Kelaka, Jelebu, Negeri Sembilan | UM16      |
| MK351416              | <i>P. cynomolgi</i>    | Kg Bukit Sebang, Alor Gajah, Melaka    | UM18      |
| MK351420              | <i>P. inui</i>         | Kg Bukit Sebang, Alor Gajah, Melaka    | UM5       |
| MK351421              | <i>P. inui</i>         | Pakan, Sarikei, Sarawak                | UM6       |
| MK351422              | <i>P. inui</i>         | 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. | <i>Plasmodium</i> spp. | Location                               | Clone identity* |
|-----------------------|------------------------|--|-----------------|
| MT992662              | <i>P. cf. inui</i>     | Pakan, Sarikei, Sarawak                | UM6A_54         |
| MT992663              | <i>P. simiovale</i>    | Pakan, Sarikei, Sarawak                | UM6D            |
| MT992664              | <i>P. cf. inui</i>     | Pakan, Sarikei, Sarawak                | UM7B            |
| MT992665              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM9A_1N         |
| MT992666              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM9A_2N         |
| MT992667              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM9A_2          |
| MT992668              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM9A_20         |
| MT992669              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM9A_21         |
| MT992670              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM9B_66         |
| MT992671              | <i>Plasmodium</i> sp.  | Kg Sungai Bil, Slim River, Perak       | UM9B_75         |
| MT992672              | <i>P. cynomolgi</i>    | Kg Aring 5, Gua Musang, Kelantan       | UM10A_5         |
| MT992673              | <i>P. cynomolgi</i>    | Kg Aring 5, Gua Musang, Kelantan       | UM10A_9         |
| MT992674              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM11A_7         |
| MT992675              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM11B_8         |
| MT992676              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM11B_10        |
| MT992677              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM11B_13        |
| MT992678              | <i>P. cynomolgi</i>    | Kg Batu 8, Tapah, Perak                | UM12A_2         |
| MT992679              | <i>P. cynomolgi</i>    | Kg Batu 8, Tapah, Perak                | UM12B_13N       |
| MT992680              | <i>P. cynomolgi</i>    | Kg Batu 8, Tapah, Perak                | UM12B_14N       |
| MT992681              | <i>P. cynomolgi</i>    | Kg Batu 8, Tapah, Perak                | UM12C_107       |
| MT992682              | <i>P. cynomolgi</i>    | Kg Ulu Kelaka, Jelebu, Negeri Sembilan | UM14A_2         |
| MT992683              | <i>P. cynomolgi</i>    | Kg Ulu Kelaka, Jelebu, Negeri Sembilan | UM14B_28        |
| MT992684              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM15A_2         |
| MT992685              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM15A_30        |
| MT992686              | <i>Plasmodium</i> sp.  | Kg Sungai Bil, Slim River, Perak       | UM15B_9         |
| MT992687              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM15B_11        |
| MT992688              | <i>Plasmodium</i> sp.  | Kg Sungai Bil, Slim River, Perak       | UM15B_12        |
| MT992689              | <i>Plasmodium</i> sp.  | Kg Sungai Bil, Slim River, Perak       | UM15B_13        |
| MT992690              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM15B_14        |
| MT992691              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM15B_24        |
| MT992692              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM15B_28        |
| MT992693              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM15B_34        |
| MT992694              | <i>P. cynomolgi</i>    | Kg Sungai Bil, Slim River, Perak       | UM15B_39        |
| MT992695              | <i>P. simiovale</i>    | Kg Ulu Kelaka, Jelebu, Negeri Sembilan | UM16A_1         |
| MT992696              | <i>P. cf. inui</i>     | Kg Ulu Kelaka, Jelebu, Negeri Sembilan | UM16A_9         |
| MT992697              | <i>P. cynomolgi</i>    | Kg Ulu Kelaka, Jelebu, Negeri Sembilan | UM16B_18        |
| MT992698              | <i>P. cynomolgi</i>    | Kg Ulu Kelaka, Jelebu, Negeri Sembilan | UM16B_21        |
| MT992699              | <i>P. cynomolgi</i>    | Kg Ulu Kelaka, Jelebu, Negeri Sembilan | UM16C_12        |
| MT992700              | <i>P. cynomolgi</i>    | Kg Batu 8, Tapah, Perak                | UM17B           |
| MT992701              | <i>P. cynomolgi</i>    | Kg Bukit Sebang, Alor Gajah, Melaka    | UM18A           |
| MT992702              | <i>P. cynomolgi</i>    | 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.

