Three Divergent Subpopulations of the Malaria Parasite *Plasmodium knowlesi*

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Multilocus microsatellite genotyping of *Plasmodium knowlesi* isolates previously indicated 2 divergent parasite subpopulations in humans on the island of Borneo, each associated with a different macaque reservoir host species. Geographic divergence was also apparent, and independent sequence data have indicated particularly deep divergence between parasites from mainland Southeast Asia and Borneo. To resolve the overall population structure, multilocus microsatellite genotyping was conducted on a new sample of 182 *P. knowlesi* infections (obtained from 134 humans and 48 wild macaques) from diverse areas of Malaysia, first analyzed separately and then in combination with previous data. All analyses confirmed 2 divergent clusters of human cases in Malaysian Borneo, associated with long-tailed macaques and pig-tailed macaques, and a third cluster in humans and most macaques in peninsular Malaysia. High levels of pairwise divergence between each of these sympatric and allopatric subpopulations have implications for the epidemiology and control of this zoonotic species.

Plasmodium knowlesi is a zoonotic malaria parasite that has only recently been recognized as a notable cause of malaria (*1*). Although cases have now been seen in most countries in Southeast Asia, the largest numbers have been reported in Malaysia (*1*–*4*). The extent to which this is a result of varying efforts in diagnosis is unclear, as specific molecular identification is required to discriminate *P. knowlesi* from other malaria parasite species. Moreover, although most reports are of cases presenting with clinical symptoms, asymptomatic infections may also occur (*5*).

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The *Plasmodium knowlesi* parasite is transmitted by mosquitoes to humans from monkey reservoir hosts, with different *Anopheles* species of the Leucosphyrus group having been incriminated as potential vectors in different areas (*1*,*6*). Two macaque species, the long-tailed macaque (*Macaca fascicularis*) and the pig-tailed macaque (*M. nemestrina*), are the major reservoirs of infection (*7*,*8*). Human infections in Malaysian Borneo, the portion of Malaysia on the island of Borneo, have divergent genetic subpopulations that are seen in the different macaque species locally, indicating that 2 independent zoonoses may be occurring sympatrically (*9*). Noticeable geographic differentiation of parasites between Malaysian Borneo and peninsular Malaysia was also evident in microsatellite analysis; separate studies have revealed divergence between the 2 regions at unlinked genes encoding the normocyte binding protein (*10*–*12*) and the Duffy binding protein (*13*,*14*), as well as the 18S rRNA and mitochondrial cytochrome oxidase subunit 1 (*15*). Whole-genome sequencing has confirmed the presence of 2 divergent subpopulations of *P. knowlesi* in Malaysian Borneo and revealed a third divergent cluster of laboratory isolates maintained in laboratories since the 1960s; most of these were recorded to have originated from peninsular Malaysia (*16*).

To resolve the population structure in relation to host species and geography, a new collection of 182 *P. knowlesi* infection samples from humans and wild macaques living in diverse areas of Malaysia was genotyped at 10 microsatellite loci. We first analyzed the new dataset separately and then analyzed a combined dataset incorporating previous multilocus microsatellite data, using several independent and complementary statistical approaches to identify genetic substructure. All analyses revealed that 2 divergent genetic subpopulations of human cases occur sympatrically in Malaysian Borneo, detected separately in long-tailed macaques and pig-tailed macaques in the same region, whereas a third divergent genetic subpopulation occurs in humans and most macaques in peninsular Malaysia. This parasite species has undergone different sympatric and allopatric processes of divergence, which will affect its future adaptation to a changing environmental landscape. Current