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Efficient Surveillance of *Plasmodium knowlesi* Genetic Subpopulations, Malaysian Borneo, 2000–2018

Appendix

Appendix Table 1. Unique primer pairs designed at each locus to discriminate the 2 *Plasmodium knowlesi* subpopulation clusters

Subpopulation, set	Chromosome, location of locus*	Primer ID	Sequence, 5′→3′†
Cluster 1			
C1A	1	C101AF	GTTTGGTACGTTCAAGTGTTGC**G**CTA**TGG**
	(745,563–745,763)[a]	CX01AR	CGTCTTCCGCTTGTCGTTTTCCATGTAC
C1C	6	C106BF	GATATAACCACATGTTTGCTTCGAAGGAA
	(17,264–17,523)[c]	C106BR	GGAAAGGTACCTCTTCCTCATAGTCC**C**
C1B	6	C106AF	TCCATGTGCACCCTGGCATACATGG**T**A**C**
	(35,925–36,162)[b]	C106AR	TGTACAGAGTGTACAGGAGCTGGGAC
C1D	6	C106CF	GGATGATTTAGGTAAGGATGAGGAG**G**G**T**
	(1,014,117 – 1,014,286)[d]	CX06CR	CGTCATCCTTATCCTTTTTACCCTTATCC
C1E	6	C106DF	GATGATAATTATCTTAAAGAGCCGGA**TG**
	(1,039,470–1,039,954)[e]	C106DR	CAAGACATTATGAACATTGGACCGA**T**T**A**
Cluster 2			
C2F	1	C201AF	GTTTGGTACGTTCAAGTGTTGC**T**CTA**CAT**
	(745,563–745,763)[a]	CX01AR	CGTCTTCCGCTTGTCGTTTTCCATGTAC
C2G	6	C206AF	GATATAACCACATGTTTGCTTCGAA**A**GA**G**
	(17,264 – 17,523)[c]	C206AR	GGAAAGGTACCTCTTCCTCATAGTCC**A**
C2H	6	C206BF	TCCATGTGCACCCTGGCATACATGG**C**A**T**
	(35,925–36,162)[b]	C106AR	TGTACAGAGTGTACAGGAGCTGGGAC
C2I	6	C206CF	GGATGATTTAGGTAAGGATGAGGAG**T**G**C**
	(1,014,117–1,014,286)[d]	CX06CR	CGTCATCCTTATCCTTTTTACCCTTATCC
C2J	6	C206DF	GATGATAATTATCTTAAAGAGCCGGA**G**
	(1,039,470–1,039,954)[e]	C206DR	CAAGACATTATGAACATTGGACCGA**C**T**G**

*Superscripted letters at the end of locations indicate same loci for both subpopulation clusters.
†Single-nucleotide polymorphisms with complete fixed (FST = 1) as shown at the 3′-end sequence are in bold and underlined.

Appendix Table 2. Summary of PCR optimizations for discriminating 2 subpopulations of *Plasmodium knowlesi* infection*

Primer set	Types of PCR	Optimum annealing temperature, °C	Allele specificity	PCR summary
C1A	Conventional PCR	60	Cluster 1	Cluster 1-specific
C1B	Conventional PCR	ND	ND	Weak amplification
C1C	Conventional PCR	61	Cluster 1 and Cluster 2	Not specific
C1D	Conventional PCR	ND	ND	Weak amplification
C1E	Touchdown PCR	68, 60	Cluster 1 and Cluster 2	Not specific
C2F	Conventional PCR	60	Cluster 2	Cluster 1–specific but inconsistent
C2G	Touchdown PCR	68, 60	Cluster 1 and Cluster 2	Not specific
C2H	Conventional PCR	ND	ND	Weak amplification
C2I	Conventional PCR	ND	ND	Weak amplification
C2J	Touchdown PCR	68, 62	Cluster 2	Cluster 2–specific

*The specificity of primers tested on DNA of Cluster 1 and Cluster 2 clinical infections where cluster identity of infections was confirmed previously by multilocus microsatellite analysis or whole-genome sequencing. ND, further optimization of annealing temperature was not done due to weak amplification during gradient PCR when visualized on agarose gel.

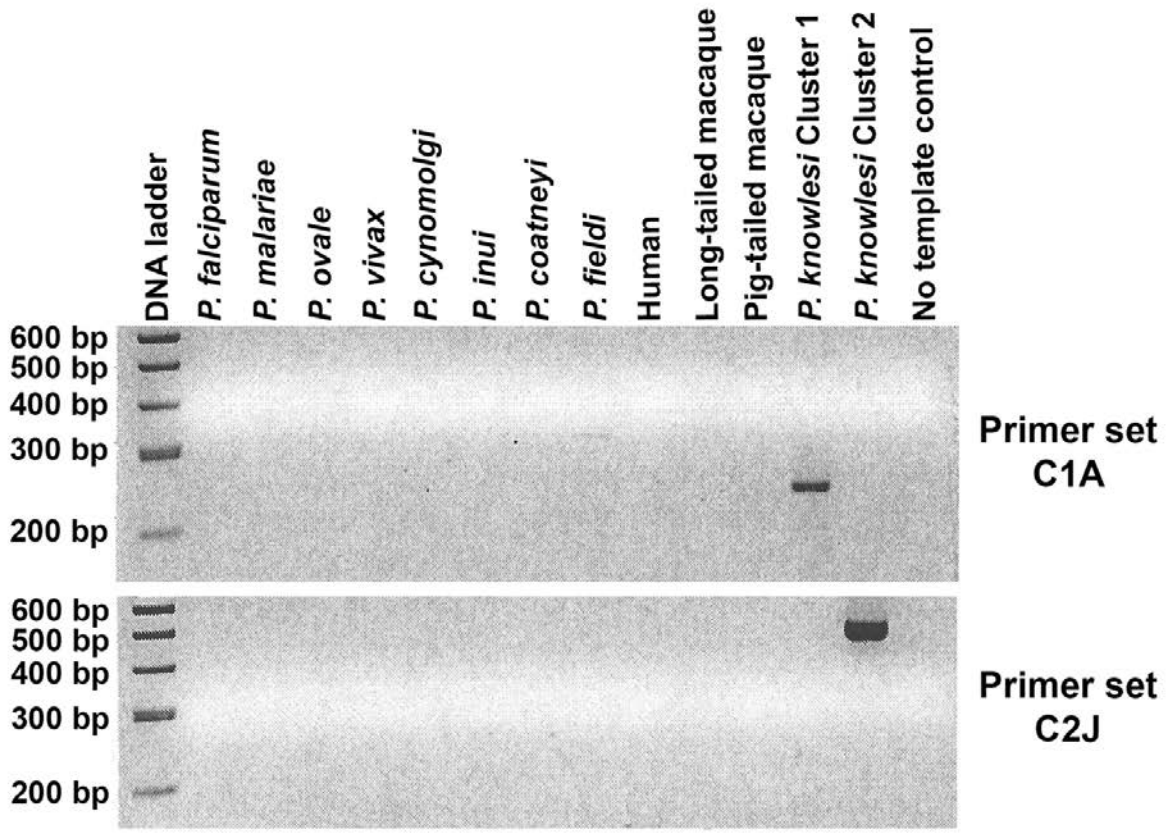
Appendix Table 3: Specificity of allele-specific PCRs for primer sets C1A and C2J for *Plasmodium knowlesi* Cluster 1 and Cluster 2 subpopulations, respectively, Malaysian Borneo*

Location	Host	Total	Infections per primer set, no. (%)		
			C1A	C2J	Both
Betong	Human	29	21 (72)	7 (24)	1 (4)
Kanowit	Human	34	14 (41)	19 (56)	1 (3)
Miri	Human	46	15 (33)	31 (67)	0
Sarikei	Human	23	14 (61)	9 (39)	0
Kapit	Human	1,204	833 (69)	342 (29)	29 (2)
Kudat	Human	46	46 (100)	0	0
Ranau	Human	62	53 (85)	9 (15)	0
Tenom	Human	48	43 (90)	4 (8)	1 (2)
Kapit	Long-tailed macaque	10	9 (90)	0	1 (10)
Kapit	Pig-tailed macaque	5	0	5 (100)	0

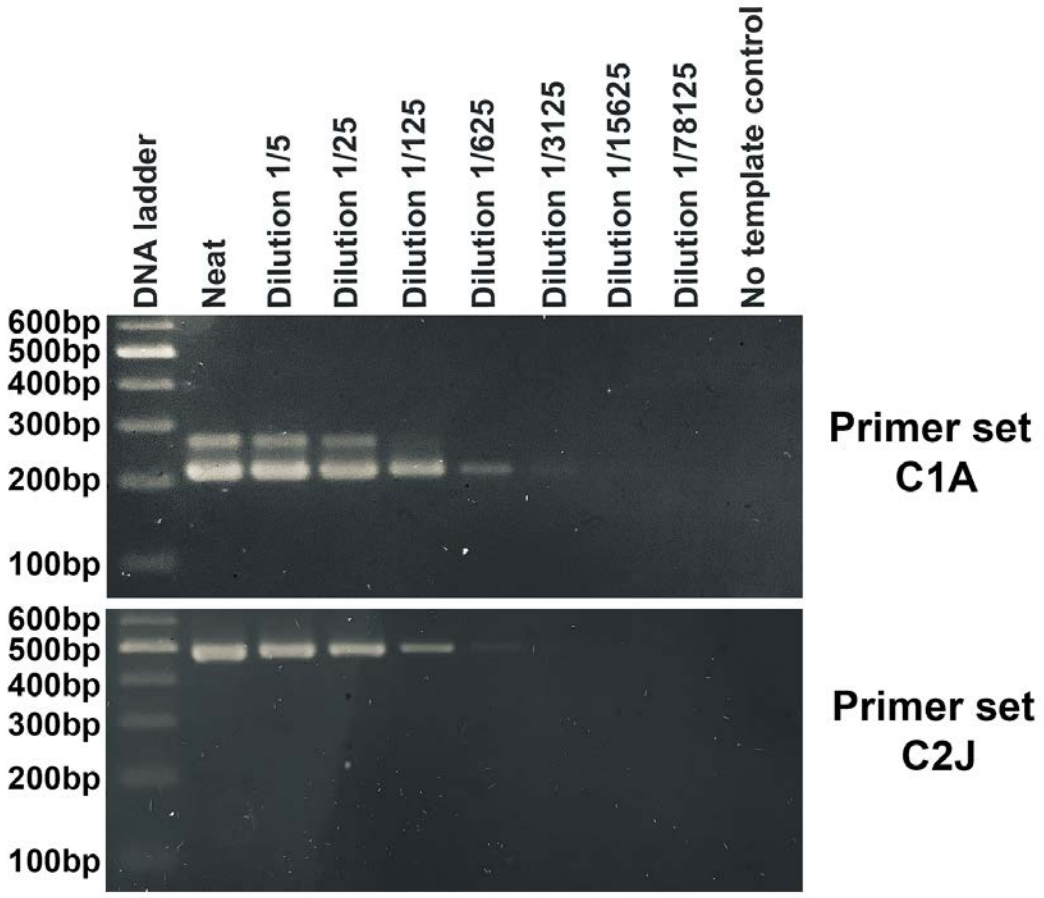
*All DNA samples were blinded with regards to their cluster assignments deduced from microsatellite (*1*) and whole genome sequencing (*2*) analyses.

Appendix Table 4. *Plasmodium knowlesi* subpopulation genotyping results for 3 study periods, Kapit division, Sarawak state, Malaysian Borneo, 2000–2018

Study period	Total no. samples	Infections, no. (%)		
		Cluster 1	Cluster 2	Mixed
2000–2002	110	74 (67)	33 (30)	3 (3)
2006–2008	176	122 (69)	51 (29)	3 (2)
2013–2018	918	637 (69)	258 (28)	23 (3)
Total	1,204	833 (69)	342 (28)	29 (3)



Appendix Figure 1. Species specificity of PCR primer sets C1A and C2J for discriminating *Plasmodium knowlesi* infections of Cluster 1 and Cluster 2 subpopulations. Gel electrophoresis on 2.7% agarose shows both primer sets did not cross-react to DNA of human *Plasmodium* species (*P. falciparum*, *P. malariae*, *P. ovale*, and *P. vivax)* and common *Plasmodium* species found in Southeast Asian macaques *(P. cynomolgi*, *P. inui*, *P. coatneyi*, and *P. fieldi*), as well as DNA of humans, long-tailed macaque, and pig-tailed macaque.



Appendix Figure 2. Analytical sensitivity of PCR primer sets C1A and C2J for discriminating *Plasmodium knowlesi* infections of Cluster 1 and Cluster 2 subpopulations. Pure DNA of Cluster 1 sample with parasitemia of 13,793 parasites/μL blood and Cluster 2 sample of 8,017 parasites/μL blood were used (denoted as 'Neat') for respective primer sets C1A and C2J. Each pure DNA sample was diluted 5-fold with nuclease free water and served as template in separate PCRs.

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