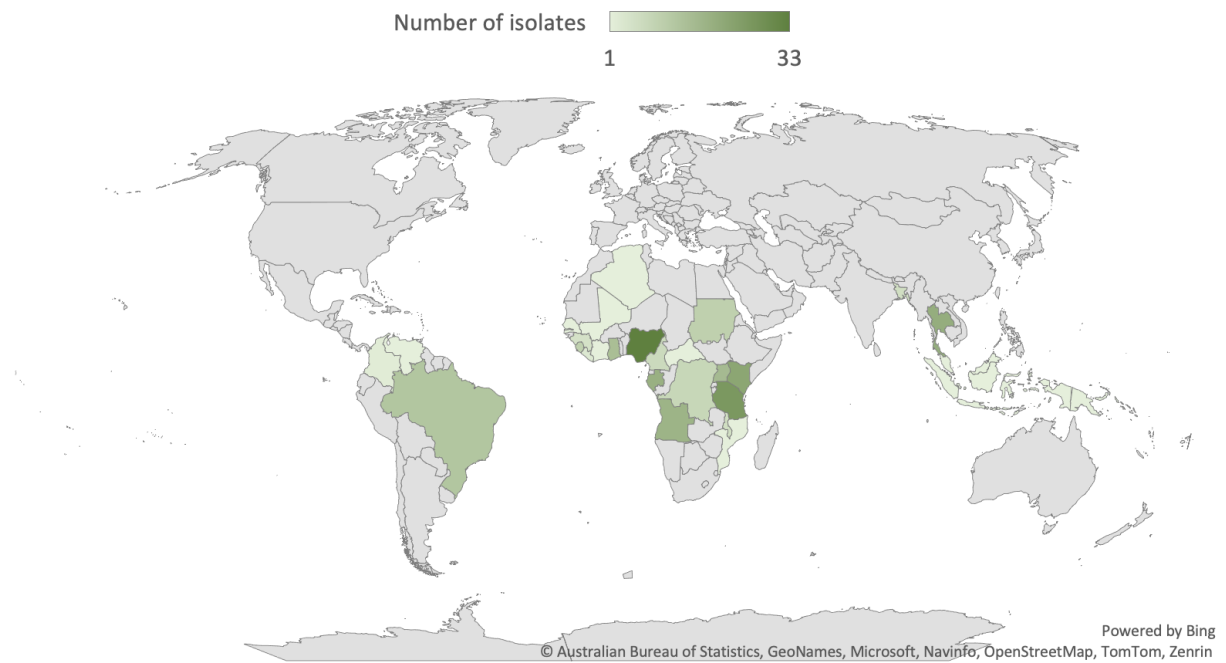
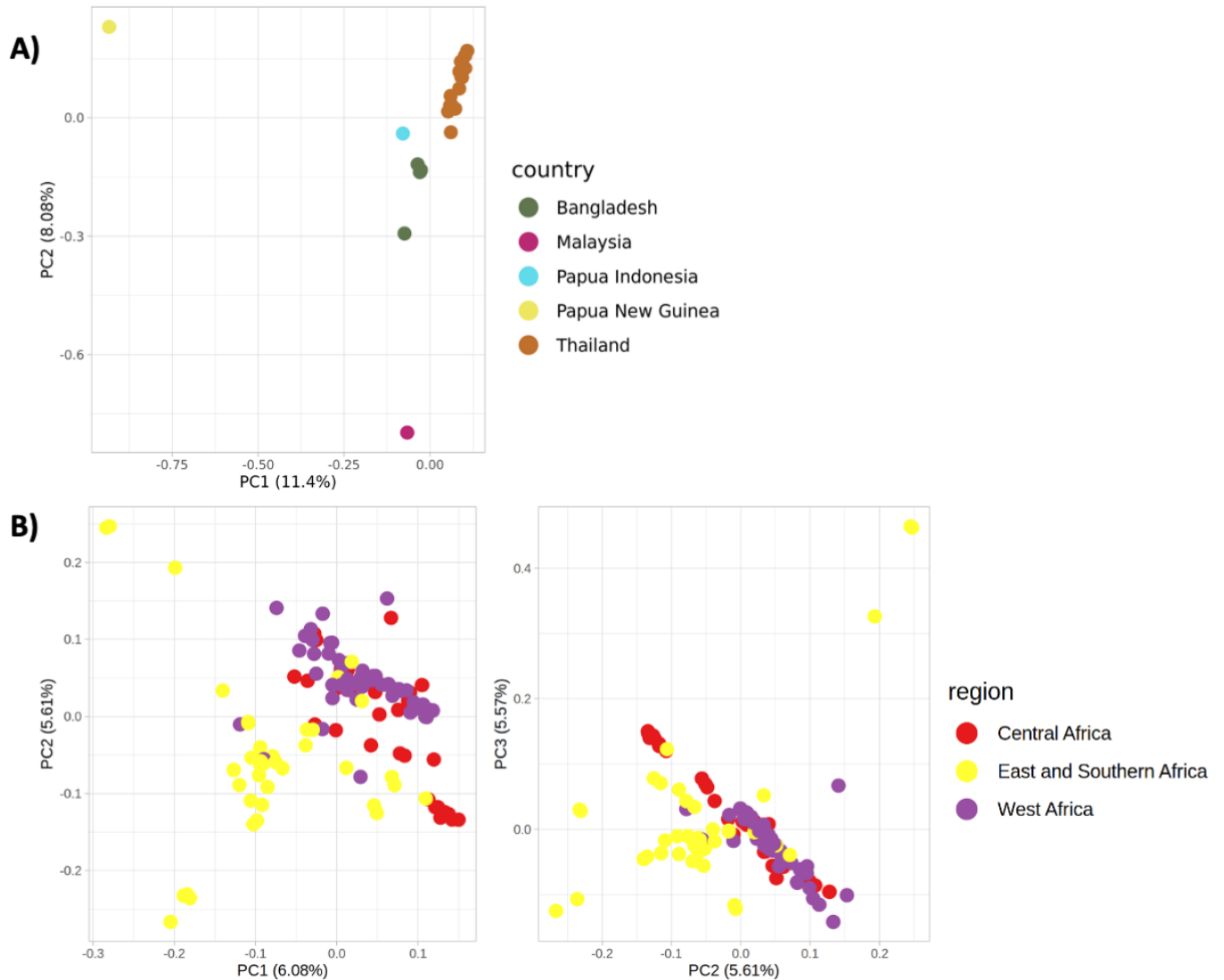


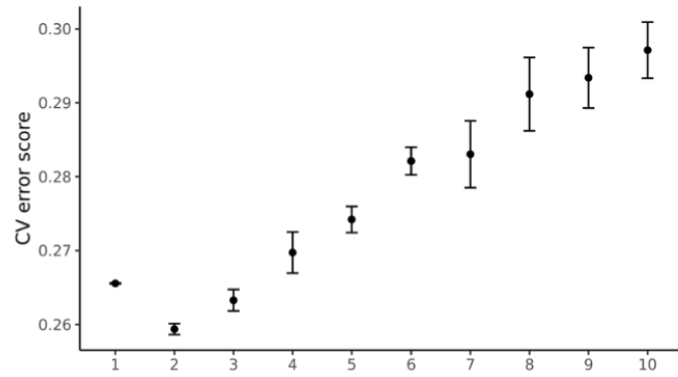
Supplementary figures



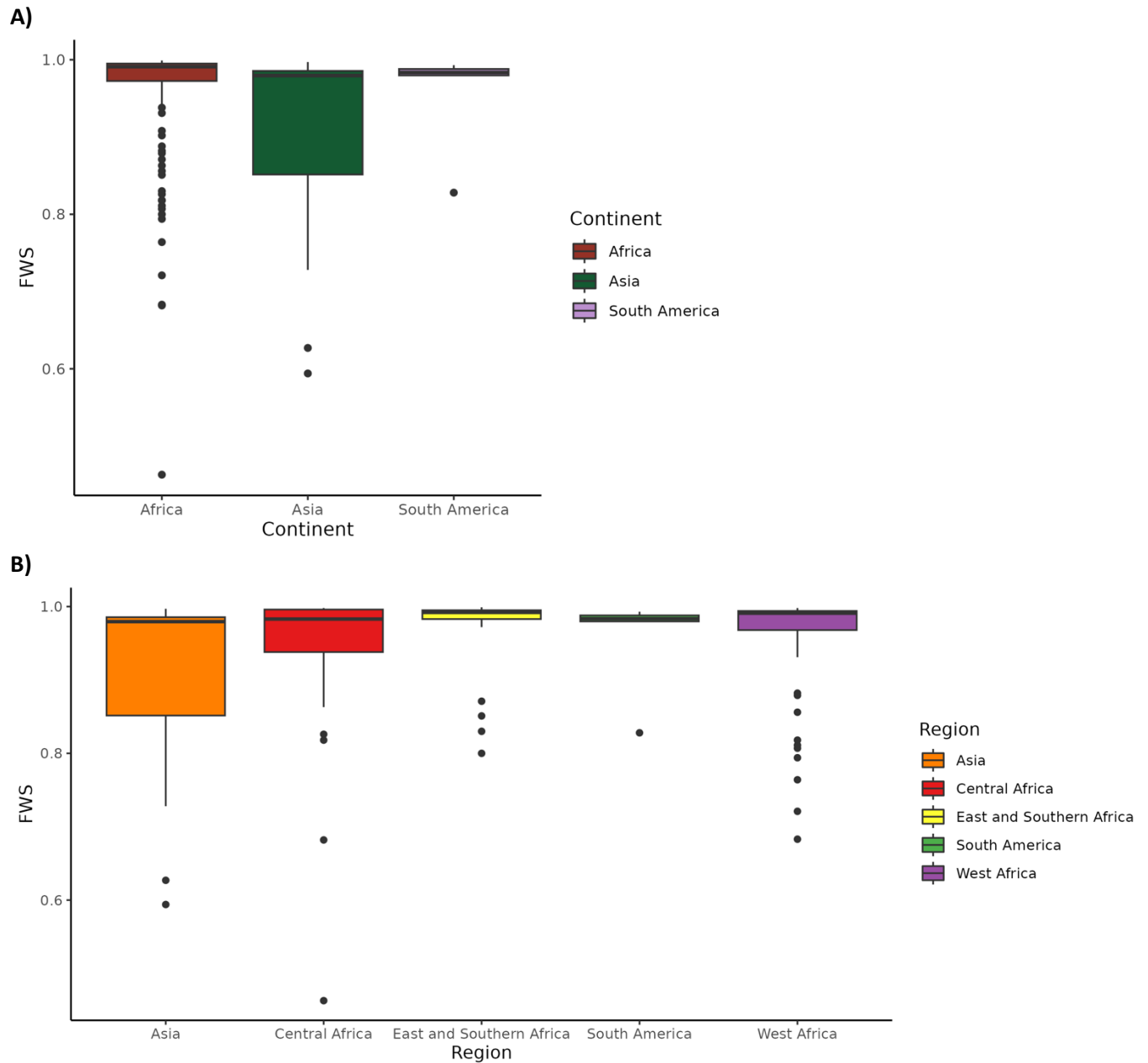
Supplementary Fig. SF1. Distribution, by country, of all *P. malariae* isolates with sequence data generated (novel and previously published) before filtering (N = 251). See **Supplementary Table ST2** for metadata of all isolates and accession numbers. Map was created using data from OpenStreetMap (openstreetmap.org) which is open data under license in the Open Data Commons Open Database License (ODbL) (opendatacommons.org) by the OpenStreetMap Foundation (OSMF) (<https://www.openstreetmap.org/copyright>).



Supplementary Fig. SF2. Principal Component analysis of population structure. A) The two major axes (PC1 and PC2) differentiate 22 Asian isolates and an individual isolate from Oceania (Papua New Guinea) coloured by their country of origin. Each data point represents an individual isolate; **B)** The three major axes (PC1, PC2 and PC3) differentiate 128 *P. malariae* isolates obtained in Africa, coloured by the region of source (Central Africa, East and Southern Africa or West Africa). Each data point represents an individual isolate.

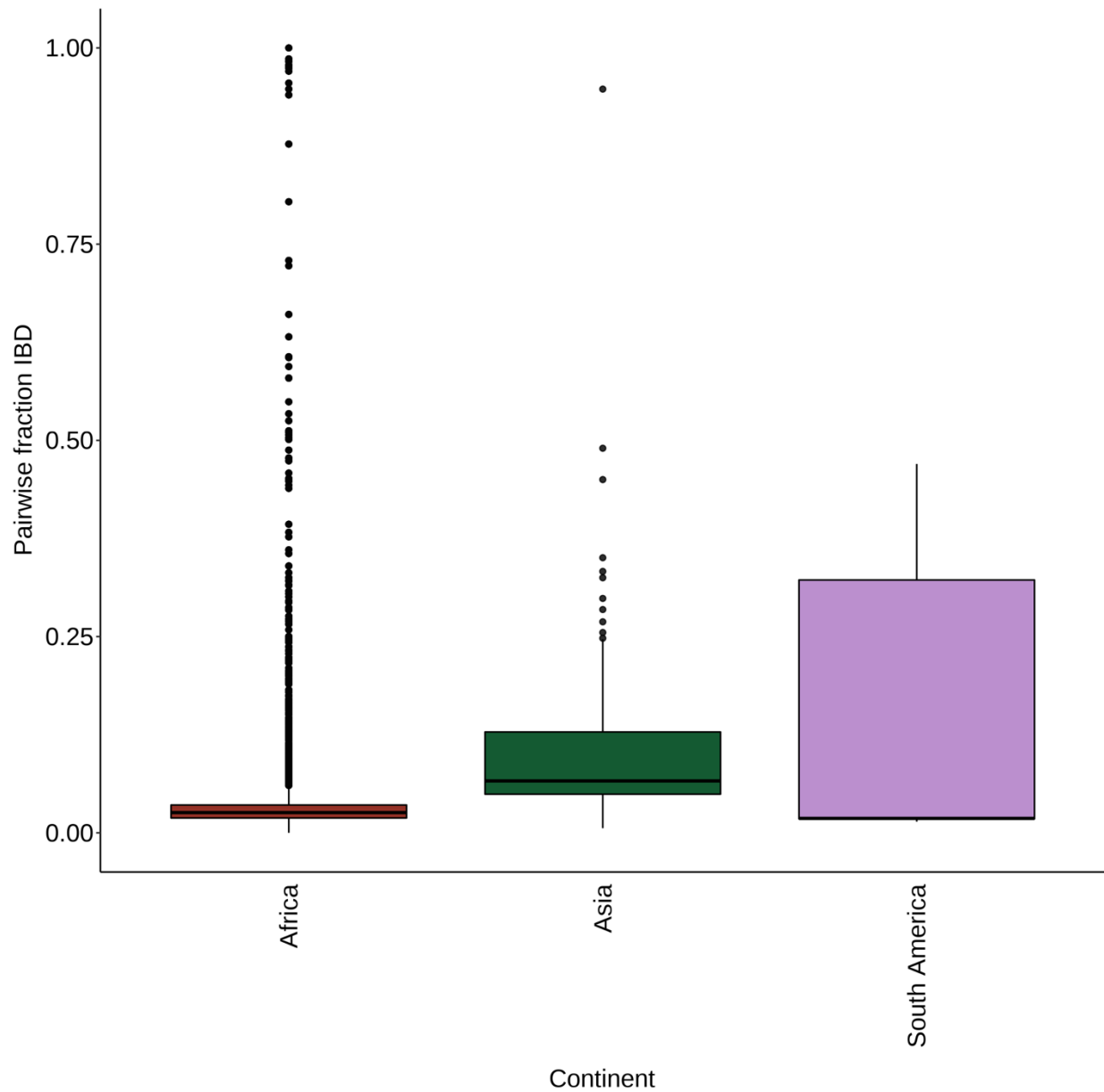


Supplementary Fig. SF3. Ancestral analysis. Estimation of number of ancestral populations amongst 141 isolates using ADMIXTURE software. ADMIXTURE was performed independently in triplicate for up to 10 populations to determine the cross-validation (CV) error score, and the average error scores were calculated. Individual data points on the graph represent the average error score, with error bars showing the range of error scores calculated over the three independent replicates. The lowest CV error score was obtained for 2 populations (average CV error = 0.259).

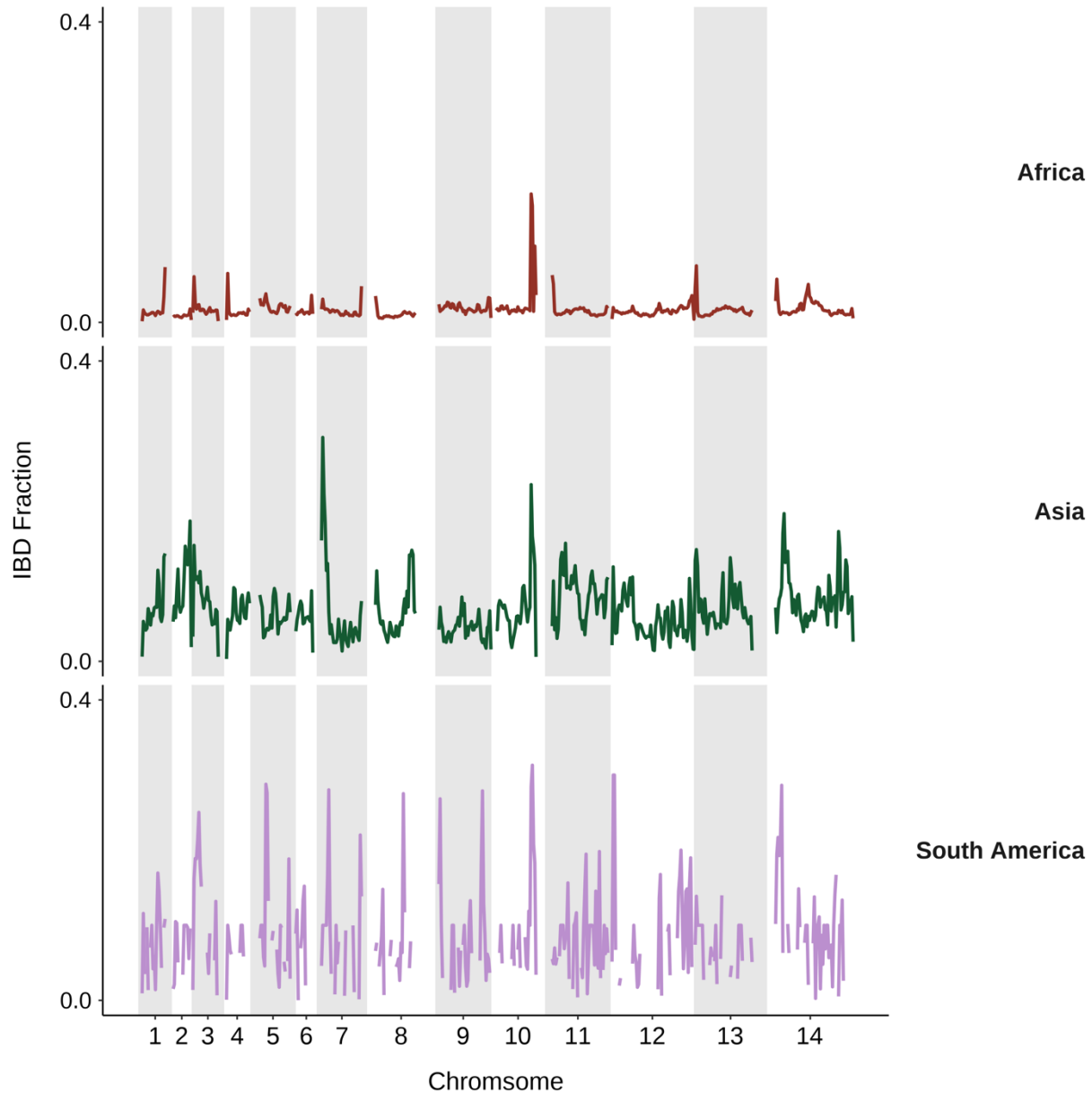


Supplementary Fig. SF4. Multiclonality in isolates in the filtered global database (n = 157).

Multiclonality is calculated using F_{WS} scores through Moimix software. Boxplots demonstrate the range of clonality in isolates in each: **A)** Continent (Africa n = 128, Asia n = 22, South America n = 6), or **B)** Region (Asia n=6, Central Africa n = 29, East and Southern Africa n = 39, South America n = 6, West Africa n = 54). Boxes are coloured according to the origin of isolates, matching all other population genetics analyses. Samples were grouped by region to calculate F_{WS} scores, and the single isolate within Oceania has been removed from F_{WS} scoring due to small sample size. The mean (SD) F_{WS} are: Africa Central (0.939 (0.110)), Africa East and Southern (0.976 (0.047)), Africa West (0.968 (0.056)), Africa Continent (0.956 (0.081)), Asia (0.903 (0.129)), and South America (0.959 (0.065)).



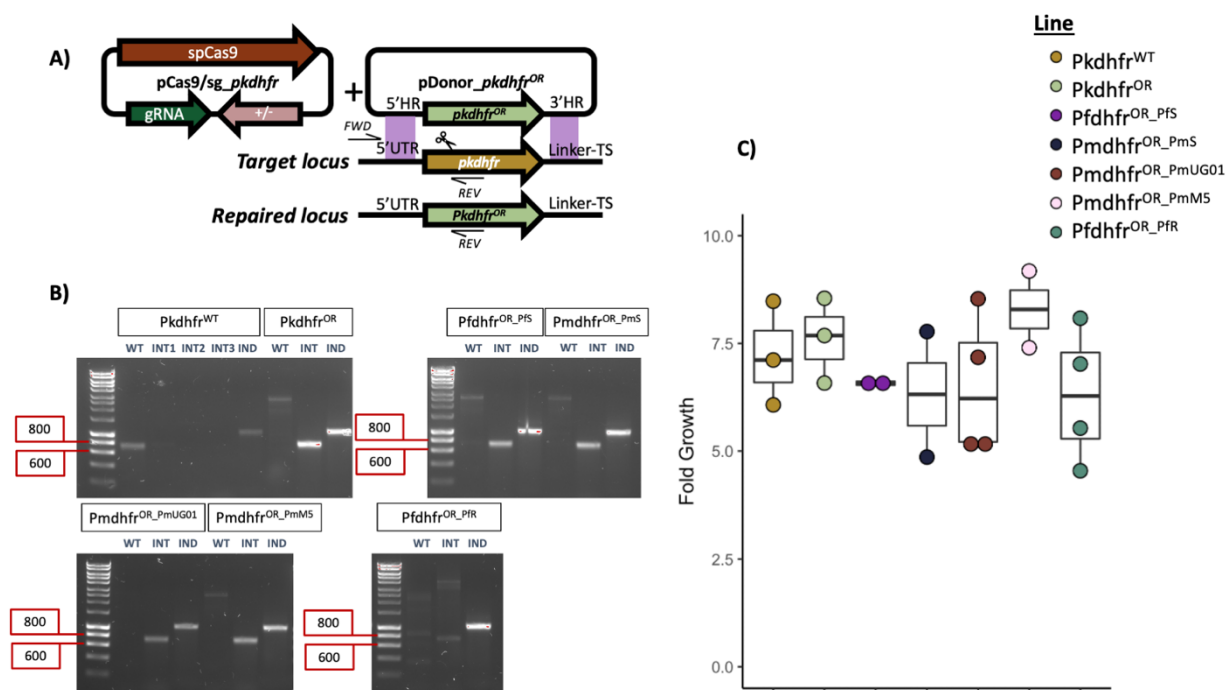
Supplementary Fig. SF5. Signals of homology within parasite populations in the global dataset determined using hmmIBD software. Identity by descent (IBD) was calculated for each continent group where there were > 5 monoclonal isolates (excluding Oceania). Monoclonality determined by F_{WS} score > 0.85, leaving a total of 136 monoclonal isolates (n: Africa 115, Asia 16, South America 5). Total IBD scores for all isolates in each continental group are visualised in boxplots.



Supplementary Fig. SF6. Genome-wide signals of high identity by descent (IBD) across the three continents. Average identity by descent (IBD) scores amongst all monoclonal isolates ($F_{WS} > 0.85$, $n = 136$) within each continent (n : Africa 115, Asia 16, South America 5) summarised across the length of the *P. malariae* genome using sliding windows of 10 kbp. IBD scores calculated using hmmlBD.

A)	pmaat1/1-662	1 MNENGSVAAYDHNKSIENEQSSYKGDHNEINNDYKNSLYVEEESNMNGYVNGYIEANESNHSKSSSVKTVPPENKKKQKQKKYKNIYEKEININGGCKINQYVNLGDN	113
	pfaat1/1-606	1 MNKKYGTSSNNHNDKKK-----NNADKN--KNNKNTTGEENKDSKSLVNDSKKN-----DSSKNKYNI-----VKANIKNIIFASDKKE-----KS	79
	pmaat1/1-662	114 DKDNENNDYNDINIGIANYEIEINTSVSYDEKQVVEAEKGGYVHQVAEVAIEIQVAEYVGHSEIEHAEYVGHSEIEHAEVEAEIEEMEVEIEERKIRKKNWKGRTFSRFTPGG	226
	pfaat1/1-606	80 DKNEKNES--SKSSKNTETVTNVND-----KKSNNLI--T-----KQSDKKKKKKDSK--KNSNNNNNTIWDISOGDYNDDEGKNPKRNRWKGRTFSRFTPGG	170
	pmaat1/1-662	127 VRSSVTLFICTAIGVGFSLPYVFSKLGITLSVLTIFENAIIESVYVTNIIQLSSLEHNTFVYGNLKKIKHKKYKTIIDIGTFPGFSSYLILLLISNLFSLIFVVFNEPAP	339
	pfaat1/1-606	221 VRSSVTLFICTAIGVGFSLPYVFSKLGITLSVLTIFENAIIESVYVTNIIQLSSLEHNTFVYGNLKKIKHKKYKTIIDIGTFPGFSSYLILLLISNLFSLIFVVFNEPAP	283
	pmaat1/1-662	340 FCNHIETIETICLLLPVTFTRDQVGLNSELVFLFSLSITVLTQWTRAYVYNLLNDKKVLEPNIIDHFFKCFNILLFSFSQDQACFITGQFNQPTHKRLTSAYSRSLVQ	452
	pfaat1/1-606	294 FTFNVFLVLLICLLILPITFRNKGSLNHLIFSLFSLSLITVLTIGLQKTSKNNLLINREVLKFMKDHFFKCFNILLFSFSQDQACFITGQFNQPTHKRLTSAYSRSLVQ	396
	pmaat1/1-662	453 IFYFTLFGFLGYLSFLNTAKDNVNLNYESNSVSIILCKFLLSVTFFFSVPLNFMGYSYSISLEYOSGRNRLFLYLYIFRRNYSLENLSALLREDTQNFQENIPDOVTENT	565
	pfaat1/1-606	397 VIFYFTLFGFLGYLSFLNTAKDNVNLNYESNSVSIILCKFLLSVTFFFSVPLNFMGYSYSISLEYOSGRNRLFLYLYIFRRNYSLENLSALLREDTQNFQENIPDOVTENT	509
	pmaat1/1-662	566 THESQTDQDKDQRMVLSICVTILCALIAFNKKLSNVIGIGGGITSTLSICLLPLNLIYKNNHNVKNRFRYLTLCMLFFSFMGFFSVVTSVLVLF	602
	pfaat1/1-606	510 VSESQTDQDKQRMVLSIVITIFCALIACKVKKLSNVIGIGGGITSTLSICLLPLNLIYKNNHNVKNRFRYLTLCMLFFSFMGFFSVVTSVLVLF	606
B)	pmcrt1-423	1 MKIKKKKKKKNHKTSDTPYRDLNLQVNGNDIQR--ISVRIKNTFISLVEHETRINTFMTLSITLYSVCVMNKLAKRTLNNKIGNYSFITSETHNFCMVVSSLYFFFT	113
	pfcrt1-424	1 MKIFA--SKKNQKNSKNDERYRDLNLVQIGNGSRGGSCGCKCAHVEKLIKKEIKDNIEIYILSIILYLSVQVNMIFAKRTLNNKIGNYSFITSETHNFCMVVSSLYFFFT	113
	pmcrt1-423	114 RSKMSAKERQDQFGLQFAFISLEDASSVILAFIQLTRTTGNIGQSFLVQLSIPINMFFCFILIRYRYHLINYLGAIVVYIAIVEMI LSFETQEENSIIFNLVLIGSLVPLCF	227
	pfcrt1-424	114 RSKMSAKERQDQFGLQFAFISLEDASSVILAFIQLTRTTGNIGQSFLVQLSIPINMFFCFILIRYRYHLINYLGAIVVYIAIVEMI LSFETQEENSIIFNLVLIGSLVPLCF	227
	pmcrt1-423	228 NMTREIVFQKHIDILRLNAVVSFFQIFITTCFILLPVYTPFLKQLHLPSEIGSNKNGFNCLIFGKNTIENVCGLMAKMDDCDGAWKTLAYSFNINCONLITSYITIEKFS	341
	pfcrt1-424	228 NMTREIVFQKHIDILRLNAVVSFFQIFITTCFILLPVYTPFLKQLHLPSEIGSNKNGFNCLIFGKNTIENVCGLMAKMDDCDGAWKTLAYSFNINCONLITSYITIEKFS	341
	pmcrt1-423	342 TMTYTTIVSICIGGPAIAIAYYKFLAGDVREPRILDFVTLVLYIGSVFYRIENIILEREKLEGNDADE--ELTNADGATA	423
	pfcrt1-424	342 TMTYTTIVSICIGGPAIAIAYYKFLAGDVREPRILDFVTLVLYIGSVFYRIENIILEREKLEGNDADE--ELTNADGATA	423
C)	pmcrt1-423	1 MEDLADIIDIYAICCKPVNQCCEGKKEIEFSTKTRGCLNGKGLPWKNSLDMKYPSVTTYVNMKYKCLKYKREKYLEKEIESNENSVTFENISLSSSKLQNVVMGR	112
	pfcrt1-424	1 MMEQVCDVDFIYAICCKPVNQCCEGKKEIEFSTKTRGCLNGKGLPWKNSLDMKYPSVTTYVNMKYKCLKYKREKYLEKEIESNENSVTFENISLSSSKLQNVVMGR	106
	pmcrt1-423	113 SSVYSIPKQYKPLNRIINVLVSRLLKKEDEVDIEIINNMDQVLLKLLKLYKCFIIGGAIYKCELRNLIKQIYFTRINNVYECDFVFFIENVFIQTSVSDVYTSKGT	225
	pfcrt1-424	107 TSVISIPKQYKPLNRIINVLVSRLLKKEDEVDIEIINNMDQVLLKLLKLYKCFIIGGAIYKCELRNLIKQIYFTRINNVYECDFVFFIENVFIQTSVSDVYTSKGT	219
	pmcrt1-423	226 SLDVFIIFSKKKKALYQESLPHOSGDSKNTSSTISNGAMSSNTIRGSTSSSGCKGCGGSEIFEREYFMGDEEDDLVYFNFNKNKE--YKNAENANDFKIYNSLKFKHHP	337
	pfcrt1-424	220 TLDVFIIFSKKKKALYQESLPHOSGDSKNTSSTISNGAMSSNTIRGSTSSSGCKGCGGSEIFEREYFMGDEEDDLVYFNFNKNKE--YKNAENANDFKIYNSLKFKHHP	324
	pmcrt1-423	338 EYQYLSIYDIIMMGNKNKSDRTGCVGLSKFGYIMKFNLDQVFPFLTTTKKFLRGIIEELLWFIETGCTNGLLNKNVRWEANGTREFLDNRKLFHREVDOLGPIYGFQWHR	437
	pfcrt1-424	325 EYQYLSIYDIIMMGNKNKSDRTGCVGLSKFGYIMKFNLDQVFPFLTTTKKFLRGIIEELLWFIETGCTNGLLNKNVRWEANGTREFLDNRKLFHREVDOLGPIYGFQWHR	437
	pmcrt1-423	451 GAETYNMNYDQKVDQLKNIILIKNDPTSRRIILCAWNVKLDQMALPCHILCQFYVFDGKLSIMYQKSCDLGLGVPIIASYSIFTHMIAQVCNQLQPAQFIHVLGNA	550
	pfcrt1-424	438 GAETYNMNYDQKVDQLKNIILIKNDPTSRRIILCAWNVKLDQMALPCHILCQFYVFDGKLSIMYQKSCDLGLGVPIIASYSIFTHMIAQVCNQLQPAQFIHVLGNA	550
	pmcrt1-423	564 HYYNNHISDLKQLNRIYPPFPTKLNPIKNIEDFTISDFTIQNYVHROKISMDMAA	621
	pfcrt1-424	551 HYYNNHISDLKQLNRIYPPFPTKLNPIKNIEDFTISDFTIQNYVHROKISMDMAA	608
D)	pmppk-dhps/1-651	1 MAVIEIEMTSRNRNMKNIAVLNIGTNDNRNRSVITIEATLEYT-----ENSYIETVPEYIAAIEQIVKSVKVEYESDIKWKEELLSTCESEKYEENSLIYEENLE	103
	pfpkp-dhps/1-706	1 METVEIILEENKTIENKNTIAVLNIGTNDNRNRSVITIEATLEYT-----ENSYIETVPEYIADKRESCEKIKDCRIYDVNYINELMQLCESEKYEENKLEIKDGEYE	111
	pmppk-dhps/1-651	104 KFMKNEKLNENMLKEIIEHEYEKTKNKLKAQDEIKNNNLEKFNKYYNYNFIETVVVATFIDDFLSMLVIEKYIEQIMKREOLKDQIEKERRIDIDITLFFNNYTFME	214
	pfpkp-dhps/1-706	112 TFLKNGKVDNSILKEVNFENGLLECNIIIVKNDIEIMKNLSYKOKYKTSKYFNTLVVVKFEVNDPLSLMLVIEKYIEQIMKREOVKEREKFNRIIDIDITLFFNNYTFME	222
	pmppk-dhps/1-651	215 NLELEKEYIYKVTITTYIETDNRNRH-----IETIERIKNKIQFLSIPHLYETKRYSLILGL	271
	pfpkp-dhps/1-706	223 NIKLEKNIMIKIIEKSYIHELEEDIKNGNDMSKVNMDKIDNLNNNNIKKKNNVIDCCDQVQNMNNHVNKNYINISFDPQIEINNMVDNIEFLSIPHLYETKRYSLILGL	333
	pmppk-dhps/1-651	272 NDIYPNYKHIVLEKDTINTLYLNFIRNFKETYNIINIKFNRMVPLVDKESVLEKKTNIIGILNVNYSFSDGGLFVFNPTKAVERMFEINEGASVIDIGGESAPYVFNPN	382
	pfpkp-dhps/1-706	334 NDMIEYKHNVLNNTIRCLYKYSVRMKEQYNIINIKENKRIYKVLDRISYLEKKTNIIGILNVNYSFSDGGLFVFNPTKAVERMFEINEGASVIDIGGESAPYVFNPN	444
	pmppk-dhps/1-651	383 SISERDLVPIVETLFLKKKWNIEIKYKLEVDIQNLEKIDRIKPIISIDITVNYVFKCEVNDLVDILNDISACTNNPEIILKLLKKNKFYVVLMMHKGPNHTMDLTNDYN	493
	pfpkp-dhps/1-706	445 KISERDLVPIVETLFLKKKWNIEIKYKLEVDIQNLEKIDRIKPIISIDITVNYVFKCEVNDLVDILNDISACTNNPEIILKLLKKNKFYVVLMMHKGPNHTMDLTNDYN	546
	pmppk-dhps/1-651	494 VYDIKTYIENRINFLVNLNGIPRYRILFDVGLGFAKKHDSIKLLQIHVVYDPLFIIGYSKRRIIAHCMODHNGVINAELNLYDD--KNDENDESKWLEKYNVMRMD	602
	pfpkp-dhps/1-706	547 LVYDIKTYIENRINFLVNLNGIPRYRILFDVGLGFAKKHDSIKLLQIHVVYDPLFIIGYSKRRIIAHCMODHNGVINAELNLYDD--KNDENDESKWLEKYNVMRMD	602
	pmppk-dhps/1-651	603 KDQLEPQKNIICGGLAIASYSYKKVDLIRVHDLVLETKAVLDVLTIKHES	651
	pfpkp-dhps/1-706	658 KDQLEPQKNIICGGLAIASYSYKKVDLIRVHDLVLETKAVLDVLTIKHES	706
E)	pmk13/1-714	1 MEGEKI--KSNISINFSYTYDRESGVNSNDSRSESSEENSNFNMNSTDKNKTEENSFALNNSFVNMDKSLLESIDSLVDSNFDTKDQFLPSNFKNFNNLKSEINIS	111
	pfx13/1-726	1 MEGEVKTKANSINFSYTYDRESGVNSNDSRSESSEENSNFNMNSTDKNKTEENSFALNNSFVNMDKSLLESIDSLVDSNFDTKDQFLPSNFKNFNNLKSEINIS	113
	pmk13/1-714	112 KYLNKFLNKSDDSMFSKSDOMLTDASNNV-----NISVKNNTKKEIIMDAATLANAEENAMNNKKFTNTNNINNDTYEKKIIELESDSDFENMVGD	210
	pfx13/1-726	114 KYLNKFLNKKKDTITNENNIHNNNNNLATANNITNLLINNMMSPISIMNTNKKENFLDAANL--INDOSGLNLLKKFST--VNNNDTYEKKIIELESDSDFENMVGD	222
	pmk13/1-714	211 RITTFINWLKKTQMFIREKDKLFKKKKELEMERILYKEIENKRIIEEQKIHDERKKLDIDISNGYKQIKKEKEHKKRFDEERLRFQIEIDKIKLVLYLEKEYFOEYKNE	323
	pfx13/1-726	223 RITTFINWLKKTQMFIREKDKLFKKKKELEMERILYKEIENKRIIEEQKIHDERKKLDIDISNGYKQIKKEKEHKKRFDEERLRFQIEIDKIKLVLYLEKEYFOEYKNE	335
	pmk13/1-714	324 NDKKKIVDANIATETMIDIVNGGAIETSRHTLTQQKDSFIEKLLSGRHVTRDKQGRIFLORDSEFRILINFLRNPLTIPKIDLSSEALLKAEFPYGIKFLPFLVFCI	436
	pfx13/1-726	336 NDKKKIVDANIATETMIDIVNGGAIETSRHTLTQQKDSFIEKLLSGRHVTRDKQGRIFLORDSEFRILINFLRNPLTIPKIDLSSEALLKAEFPYGIKFLPFLVFCI	448
	pmk13/1-714	437 GGFQDGYELNSMELLISQQQWRMTFMTSKAYFGSAVNNFLVYFGNNYDYKALFETEVYDRLDITVSSNLIIPRRNCGVTSNGRIYCYGGYDSSIIIPNVEAYDHR	549
	pfx13/1-726	449 GGFQDGYELNSMELLISQQQWRMTFMTSKAYFGSAVNNFLVYFGNNYDYKALFETEVYDRLDITVSSNLIIPRRNCGVTSNGRIYCYGGYDSSIIIPNVEAYDHR	561
	pmk13/1-714	550 MKAWVEIAPLNTPRSSMCVAFDNKIYVIGGTNGERLNSIEVYEKMMKWEQFPYALLERASSGAAFNYLNQIYVYGGIDNEHNI LDSVEQYQPFNKRQFLNGVPEKKMMFG	662
	pfx13/1-726	562 MKAWVEIAPLNTPRSSMCVAFDNKIYVIGGTNGERLNSIEVYEKMMKWEQFPYALLERASSGAAFNYLNQIYVYGGIDNEHNI LDSVEQYQPFNKRQFLNGVPEKKMMFG	674
	pmk13/1-714	663 ATLSDSYIITGGEGEVLSNCHFFSPDTEWQIGPLSLVPRFGHSVLIANI	714
	pfx13/1-726	675 ATLSDSYIITGGEGEVLSNCHFFSPDTEWQIGPLSLVPRFGHSVLIANI	726

Supplementary Fig. SF7. Amino acid alignments to investigate relevance of SNPs found within orthologs of resistance-associated genes. Both the Pf3D7 and PmUG01 reference genome predicted protein sequences for each gene of interest were downloaded from PlasmoDB and aligned using Clustal Omega for: **A)** AAT1 (PF3D7_0629500 and PmUG01_11034100), **B)** CRT (PF3D7_07090000 and PmUG01_01020700), **C)** DHFR-TS (PF3D7_04172000 and PmUG01_05034700), with the linker region annotated by a black outlining box, **D)** PPPK-DHPS (PF3D7_08108000 and PmUG01_14045500), and **E)** Kelch13 (PF3D7_13437000 and PmUG01_120212000). Alignments were visualised in JalView, with conserved amino acids highlighted in grey, residues validated to be involved in resistance in *P. falciparum* in red, and residues found to be mutated in the *P. malariae* database in blue.



Supplementary Fig. SF8. Ortholog replacement of *pkdhfr*. **A)** Diagram outlining the strategy for genome editing of *P. knowlesi* parasites using CRISPR-Cas9 with a two-plasmid approach. One plasmid encoding the Cas9 endonuclease in addition to the guide RNA targeting Cas9 to the DHFR domain, and the other plasmid containing the donor DNA flanked by two 500 bp homology regions. The same guide plasmid (pCas9/sg_pkdhfr) was used for all 6 transfections, with 6 different donor plasmids for each individual ortholog replacement. Ortholog replacement occurred through homologous recombination after Cas9 induced double strand DNA break, with 500 bp 5' and 3' homology regions flanking either side of the replacement DHFR sequence (marked 5' HR and 3' HR). The locations of the primers used to confirm successful recombination are indicated with 'WT FWD' 'OR FWD' or 'REV'. The same FWD primer is used for all lines (oIAI034), situated outside of the genetically modified locus within the *P. knowlesi* genome, and the REV primer is located within the new DHFR sequence after ortholog replacement, with a genus specific REV primer for *P. falciparum* (oIAI039), *P. malariae* (oIAI037), the recodonised *P. knowlesi* sequence (oIAI036) and the parental *P. knowlesi* line (oIAI035). **B)** Diagnostic PCRs of all parasite lines after transfection, clonal dilution, and DNA extraction to confirm parasites only harbour the modified locus. Three separate PCRs are completed for each line (WT - wild-type locus, oIAI035 and oIAI034; INT - integration locus, primers described above; IND - an independent PCR as a control targeting an unrelated locus within the *P. knowlesi* genome, primers oI75 and oI76). PCR primers and band sizes in **Supplementary Table ST11**. The parental parasite line *pkdhfr*^{WT} was tested with all three integration primer sets (INT1 = *P. falciparum*, INT2 = *P. malariae*, and INT3 = *P. knowlesi* recodonised sequence). All primer sets were additionally tested with water as a negative control and showed no amplification. **C)** Fold growth of all parasite lines in human erythrocytes (Duffy positive) using one intraerythrocytic cycle of 27 hours. Fold growth was measured using a SYBR-Green I assay and flow cytometry, with all isolates having three technical replicates.