A comparative molecular survey of malaria prevalence among Eastern chimpanzee populations in the Issa valley (Tanzania) and Kalinzu (Uganda)

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Abstract

Background: Habitat types can affect vector and pathogen distribution and transmission dynamics. We investigated the prevalence and genetic diversity of *Plasmodium* spp. in two eastern chimpanzee populations - Kalinzu Forest Reserve, Uganda and Issa valley, Tanzania - inhabiting different habitat types. As a follow up study, we determined the effect of host sex and age on infections patterns in Kalinzu Forest Reserve chimpanzees.

Methods: We employed molecular methods to detect *Plasmodium* DNA from faecal samples collected from savanna-woodland (Issa valley) and forest (Kalinzu Forest Reserve) chimpanzee populations.

Results: Based on a *Cytochrome -b* PCR assay, 32 out of 160 Kalinzu chimpanzee faecal samples were positive for *Plasmodium* DNA, whilst no positive sample was detected in 171 Issa valley chimpanzee faecal samples. Sequence analysis revealed that previously known *Laverania* species (*P. reichenowi*, *P. billbrayi* and *P. billcollinsi*) are circulating in the Kalinzu chimpanzees. A significantly higher proportion of young individuals were tested positive for infections, and switching of *Plasmodium* spp. was reported in one individual. Amongst the positive individuals sampled more than once, the success of amplification of *Plasmodium* DNA from faeces varied over sampling time.

Conclusion: Our results showed marked differences in the prevalence of malaria parasites among free ranging chimpanzee populations living in different habitats. In addition, we found a clear pattern of *Plasmodium* infections with respect to host age. The results presented in this study contribute to our understanding of ecological aspects underlying the malaria infections in the wild. Nevertheless, integrative long term studies on vector abundance, *Plasmodium* diversity during different seasons between sites would provide more insight on the occurrence, distribution and ecology of these pathogens.

Keywords: Malaria, *Pan troglodytes schweinfurthii*, *Plasmodium* spp, *Laverania*, *cyt-b* gene.

Background

Parasite distribution and transmission dynamics are influenced by the ecological context of the host-parasite interactions and a variety of local environmental parameters [1-3]. In the case of vector-borne *Plasmodium* infections, the primary effect of habitat on the transmission of malaria is by affecting larvae development, abundance and distribution of competent vectors [4-7]. Numerous studies have demonstrated the relationship between specific habitats and levels of *Plasmodium* infections in humans [8-12]. However, research addressing habitat types as a source of variation in prevalence and diversity of these parasites in wild apes is lacking. In addition to habitat, host traits such as age, sex and host density may also have an influence on host parasite infection and transmission of *Plasmodium* spp. [13-15].

Chimpanzees (*Pan troglodytes*), like several other primates, harbour a multitude of malaria parasites. With the development and refinement of molecular diagnostic techniques together with non-invasive sampling, at least seven distinct *Plasmodium* species are known to infect wild chimpanzees. Four of them, *P. reichenowi*, *P. gaboni*, *P. billcollinsi* and *P. billbrayi* belong to the subgenus *Laverania* and are chimpanzee-host specific [16-22]. The remaining three species, usually referred to as *P. malariae*-like, *P. ovale*-like and *P. vivax*-like, rarely occur in chimpanzees and they are genetically related to their human counterparts. Nevertheless, the nomenclature of these rare taxa requires further investigation [19]. Given the high genetic diversity of *Plasmodium* species reported from chimpanzees and other primates including humans [19,20, 23], a better understanding of the infection dynamics and interactions between parasites, *Anopheles* mosquitoes, hosts and environmental parameters that facilitate malaria transmission in apes is required [15,18,24].

In the current study, we investigated the prevalence and genetic diversity of *Plasmodium* spp. in two populations of eastern chimpanzees (*P. t. schweinfurthii*) inhabiting

two different habitats: (i) savanna woodlands in the Issa valley, Tanzania and (ii) evergreen moist forest in Kalinzu Forest Reserve (KFR), Uganda. We compared malaria infection between these two habitats (savanna and moist evergreen forest) because of their variable environmental parameters that may influence the exposure to malaria parasites with varying degrees in chimpanzee populations. Because chimpanzees at KFR are habituated, we additionally addressed the relationship between age, sex and malaria infection patterns in this population.

Methods

Study sites

Issa valley, Tanzania: The Issa valley is located in western Tanzania (Fig. 1), about 90 km east of Mahale Mountains National Park, and approximately 70 km from Uvinza, the nearest legitimate village. Issa valley is characterised as an open area with no formal protective status, where small scale illegal human activity for hunting and logging takes place [25]. The entire region is one of the driest and most open chimpanzee habitats, with an altitudinal range of 900-1,800 m above sea level [26]. There is an extended dry season (May-September), with rains from October-April, peaking in January (unpublished data), averaging 1,095mm/year (range: 835-1395). Average daily temperature varies from 11-35°C [27]. The habitat is dominated by savanna (Miombo) woodland, characterized by *Brachystegia* and *Julbernardia* trees, with small riparian forest patches [26]. The population density of Issa chimpanzees is estimated to be ~0.25 individuals/km^2 [25]. Data on the prevalence of *Plasmodium vivax* in this population have been reported elsewhere [28]. In addition to chimpanzees, several other primate species inhabit the study site, including red colobus monkeys (*Piliocolobus tephrosceles*), yellow baboons (*Papio cynocephalus*), blue (*Cercopithecus mitis*) and red-tailed monkeys (*C. ascanius*), vervet monkeys (*Chlorocebus pygerythrus*), bushbabies (*Galago senegalensis, G. moholi*) and greater galagos (*Otolemur crassicaudatus*) [26].

KFR, Uganda: Kalinzu is one of the three largest forest blocks in Uganda. The forest reserve (~137 km^2) is located on the eastern ridge of the western Rift valley in western Uganda (Fig. 2), with an altitudinal range of 1,200-1,500 m above sea level [29]. The area is adjacent to Kashoha-Kitomi Forest Reserve and Maramagambo Forest Reserve on the north and west sides, agricultural fields to the east and tea plantations to the south [29]. Kalinzu has a bimodal distribution of rainfall with peaks between September-December and March-May, and average annual rainfall of 1,584 mm. The average daily temperature varies from 15 to 25˚C [30,31]. The vegetation is classified as medium altitude moist evergreen forest, with common species including *Musanga leo* and *Ficus* spp. [32]. The chimpanzee population density is estimated to be ~1.67 individuals/km^2 [33]. In addition to *P. t. schweinfurthii*, black and white colobus (*Colobus guereza*), olive baboons (*Papio anubis*), red tailed (*Cercopithecus ascanius*), blue (*C. mitis*), and L'hoests monkeys (*C. lhoesti*) occur in the area [32].

Sample collection

Issa valley: We collected 171 faecal samples from a single community of chimpanzees inhabiting the Issa study area between March-May 2012 and June-August 2013. It was not possible to attribute the faecal samples to specific individuals. We collected most of the faecal samples underneath fresh nests (~12 hours old) and some from chimpanzee trails. Approximately 20 g of faecal material was collected in a 50 ml tube, containing 20 ml of RNA*later*™ (Ambion Inc., Austin, TX). All faecal samples were stored in a freezer at -20°C on site, and subsequently shipped to the Czech Republic, where they were kept at -20/-80°C until DNA extraction.

KFR: Between April and July 2014, we collected faecal samples from 41 habituated chimpanzees (males, n=20; females, n=21). We collected a total of 123 fresh faecal samples, ranging from 1 to 10 faecal samples per individual. Samples were collected during direct

observations of chimpanzees. Concurrently, during tracking of chimpanzees 37 faecal samples were collected from unidentified individuals. Collection and storage protocols were the same as those at Issa, with the exception that samples were kept at 4°C in a fridge at base camp prior to shipping to the Czech Republic, where they were kept at -20/-80°C until DNA extraction.

Molecular methods

We extracted total DNA from 1.5 ml of the faecal - RNA*later*™ suspension using a QIAamp Stool DNA Mini kit (Qiagen, Valencia, CA) and PSP® Spin Stool DNA Kit (Stratec Molecular, Germany) according to the manufacturer's protocol. Bound DNA was eluted in 100 µl elution buffer. To determine the concentration of the extracted DNA, total DNA was measured by fluorometry, using a Qubit (Invitrogen, Carlsbad, CA). To screen samples for *Plasmodium*, a nested PCR was performed on each sample targeting a ~930 bp fragment of the *Plasmodium cytochrome b* (*cyt-b*) gene, as described by Prugnolle et al. [34], with modification of the second PCR reaction. A pair of short internal primers amplifying overlapping fragments (516 and 558 bp) was designed, retrieved sequences were contiged to obtain same region of *cyt-b*. First round PCRs were performed in a 25 µl reaction, containing 12.5 µl of PCR mix (Qiagen), 2.5µl of solution Q (Qiagen) and 0.2 µl of each primer (DW2 and DW4) in 10 pmol concentration and 4 µl of the DNA sample. Second nested PCR was performed using two different set of reactions, using Cytb1 (5'-CTCTATTAATTTAGTTAAAGCACA-3') and Cytb2B (5'-GCTCTATCATACCCTAAAGG-3') in the first set, and Cytb2 (5'-ACAGAATAATCTCTAGCACC-3') and Cytb1A (5'-CAAATGAGTTATTGGGGTGCAACT-3') for the second set. Two µl of first round PCR product was then used in a second round 25 µl nested PCR reaction, containing 12.5 µl common Master Mix (Top-Bio, Czech Republic) and 1 µl of each primer in 10 pmol

concentration. For details of the modified nested PCR conditions see [15]. PCR products were visualized in 2% agarose gel and stained with Gold-View. Bands of the expected size were visualized using an UV light source, excised, purified using QIAquick gel extraction kit (Qiagen, Germany) and sequenced in both directions using internal primers by Macrogen capillary sequencing services (Macrogen Europe, the Netherlands).

Sequence and phylogenetic analyses

Sequences were edited in Chromas Pro 1.5 software (Technelysium, Ltd) and alignment was prepared with ClustalW multiple alignment tool implemented in Bioedit Sequence Alignment Editor v.7.0.9.1 [35]. All suitable retrieved sequences were submitted to GenBank™ database under the Accession Numbers KT864824-KT864842.

The alignment was checked manually and the resulting sequence were (~758 bp) later used for phylogenetic analyses. To examine the phylogenetic relationship of the new dataset, we added sequences from different ape *Plasmodium* species downloaded from GenBank™ to the final alignment. For the final analyses, only haplotypes were further included (haplotypes and redundant sequences are shown in Table 1).

Phylogenetic relationships were inferred using the maximum likelihood (ML) method under the general time-reversible evolutionary model with gamma distributed substitution rates (GTR+Γ) in program PhyML 3.0 [36]. Nodal support was assessed by bootstrap using 1000 pseudoreplicates. Additionally, Bayesian methods using the program MrBayes 3.2.2 [37] was also used to reconstruct phylogenetic relationships. Setting for the evolutionary model was the same as in ML and the search was carried out in two simultaneous runs of one million generations, sampled each 100 generations, with a burn-in of 25%.

Cloning of mixed infection samples

Two samples were cloned separately with a TOPO® TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmids containing inserts were

185 isolated from positive *Escherichia coli* colonies by GenElute^{TM} plasmid mini prep kit (Sigma-
186 Aldrich, St. Louis, MO). DNA extracts from at least six randomly selected colonies were
187 sequenced in both directions.

188 **Statistical analyses**

189 We defined prevalence as the number of *Plasmodium*-positive individuals divided by
190 the total of individuals tested. Samples collected from unidentified individuals were not
191 included for the calculation of prevalence, but they were used to investigate the genetic
192 diversity of the parasites. Of the 41 habituated individuals sampled in KFR, 25 were re-
193 sampled to observe the fluctuation of the infections. In order to examine the possible effect of
194 sex and age on the occurrence of malaria in KFR chimpanzees, a general linear mixed model
195 (GLMM) with binomial distribution was fitted. Since we had a limited number of faecal
196 samples from juveniles and subadults, age classes were pooled and grouped as
197 juveniles/subadults and adults. We verified age-classes based on previously suggested
198 categorization [38]. Samples were classified according to sex (fixed factor: male, female) and
199 class of age (fixed factor: juvenile/subadult, adult). Individual identity was treated as a
200 random factor. Statistical analyses were performed in R [39].

201 **Results**

202 In total, we examined 331 chimpanzee faecal samples (Table 2) from the Issa valley.
203 All faecal samples collected from Issa chimpanzees were negative for *Plasmodium* DNA. On
204 the contrary, *Plasmodium* spp. was detected in 32 out of 160 (both identified and unidentified
205 individuals) faecal samples collected from KFR chimpanzees. In total, 22 out of 123 samples
206 collected from identified individuals were positive for *Plasmodium* DNA; 10 out of 37
207 samples from unidentified individuals were *Plasmodium*-positive. The prevalence among
208 identified individuals was 43.9% (n =18/41). The general linear mixed model showed that sex
209 had no significant effect on the susceptibility to infection (GLMM: z = -0.027, p = 0.283),

while age was a significant factor influencing *Plasmodium* infection. The total prevalence of *Plasmodium* spp. was significantly higher among juvenile/subadult individuals than adults (GLMM: z = 2.308, p = 0.020). Of the re-sampled individuals (n=25), eleven were found positive at least once. Variation on detection of *Plasmodium* DNA (negative-to-positive and *vice versa*) was common and observed in 18 identified individuals (Table 3). Switching of *Plasmodium* spp. was observed in one individual (Table 3).

Alignment and phylogenetic analysis of the obtained *cyt-b* sequences (both from identified and unidentified individuals) with reference sequences indicated the presence of *Plasmodium* strains that specifically infect only chimpanzees (see Additional files 1). Among the retrieved sequences, 12 were *P. reichenowi*, 11 *P. billbrayi* and seven *P. billcollinsi*. All sequences obtained in this study clustered with their homologous sequences retrieved from GenBank™ and form well-supported clades. Geographical sub-structuring among *P. reichenowi* was observed, whereby sequences obtained from *P. t. schweinfurthii* clustered separately from other *P. reichenowi* sequences from *P. t. troglodytes* and *P. t. ellioti*. No samples containing *cyt-b* of *P. gaboni* or non-*Laverania* species (*P. vivax*-like, *P. malariae*-like and *P. ovale*-like) were detected in our dataset. Mixed infections were detected in two samples. Sequences of two PCR amplicons showed double peaks in the chromatograms, suggesting mixed infections. These samples were further processed by cloning to identify *Plasmodium* to species level. In the first sample (from an unidentified individual), we obtained 15 sequences with two representative sequence patterns that were in agreement with BLAST-searches for the *cyt-b* sequences: 14 sequences were 99-100% similar to *P. reichenowi* (acc. number: HM235389), and one sequence was 99% similar to *P. billbrayi* (acc. GQ355468). In the second sample (from an identified individual), we obtained 12 sequences with three representative sequences patterns: four sequences were 99% similar to *P. reichenowi* (acc. number: HM235389), five sequences were 99-100% to *P. billbrayi* (acc.

number: GQ355468), and three sequences were 99% similar to *P. billcollinsi* (acc. number: HM235392).

Discussion

A number of studies have described the distribution and genetic diversity of *Plasmodium* spp. in African great apes [17,18,22,34,40,41], yet there is substantial lack of knowledge on the effect of intrinsic and extrinsic factors that govern malaria parasite transmission and frequencies of infections in free ranging chimpanzees. To our knowledge, this is the first study to investigate the prevalence and genetic diversity of *Plasmodium* spp. in KFR. Our finding from KFR is comparable to previous studies by Liu et al. [18] that were conducted at multiple field sites, as well as to the study by Kaiser et al. [41] from Budongo Forest in Uganda. While we did not detect any species of *Plasmodium* from Issa valley samples, results from a previous study [28] revealed that four out of three hundred thirteen chimpanzee samples from this population to be positive for *P. vivax*-like. Variation in the prevalence between this study and that of Liu et al. [28] is most likely to be attributable to our smaller sample set, and, possibly also to differences in sensitivity of detection methods. Looking at this discrepancy from a different perspective, *P. vivax* tends to stay dormant in the liver for many years [42]. Consequently, we can speculate that during our sampling time shedding of *Plasmodium* DNA into the intestinal lumen was minimal, leading to failure to detect *P. vivax* DNA in faecal samples.

An overall prevalence of *Plasmodium* spp. in KFR was 43.9%, while all faecal samples from Issa valley were negative. The remarkable ecological differences between KFR and Issa valley habitats represent most plausible explanation for observed differences, as they may impact on the species diversity and abundance of anopheline mosquitoes. However, also host density may have significant impact on the transmission and maintenance of infections in a given population [12]. Kalinzu chimpanzees live at a relatively high density (~1.67

individuals/km^2, [33]) compared to Issa chimpanzees (~0.25 individuals/km^2, [25]). Then, the abundance of hosts may act as an additional factor influencing the prevalence of *Plasmodium* spp.

Liu et al. [28] screened another but forest-inhabiting eastern chimpanzee population (*Pan t. schweinfurthii*) from Gombe National Park, and none of the samples was positive for *P. vivax*-like. The absence (or very low prevalence Liu et al. [28]) of *Plasmodium* infection is these eastern chimpanzee populations (Issa valley and Gombe National Park) could be also attributed to the genetic factors related to hosts as observed in human [43] rather than to their habitat. Unfortunately, it is difficult to reliably compare the results of these two studies due to the different diagnostic techniques employed (*P. vivax* species-specific assay in the Gombe study [28], and *Plasmodium* genus-specific in the present study). Nevertheless, screening of near-by forested (Mahale Mountains National Park) and other savanna-dwelling chimpanzees (e.g. Semliki, Uganda; Fongoli, Senegal), as well as re-screening of the Gombe chimpanzee population for presence of *Laverania* species would offer an insight into the factors the influence the occurrence of *Plasmodium* spp. in eastern chimpanzees.

Over the past five years, numerous *Plasmodium* species have been reported to circulate in free-ranging great apes [19]. Consistent with previous studies [18,22,34,41], sequence analyses of the *cyt-b* gene of *Plasmodium* spp. from Kalinzu chimpanzees revealed a high diversity of malaria parasites. With the exception of *P. gaboni*, which was not detected in our sample set, most of the sequences were identified as *P. billbrayi*, however, *P. reichenowi* and *P. billcollinsi* were also confirmed. Phylogenetic analysis showed that all sequences in our study cluster within the clades of subgenus *Laverania*, no sequence belonging to non-*Laverania* (*P. vivax*-like, *P. ovale*-like and *P. malariae*-like) lineage was identified. Our results agree with recent findings on ape malaria, where *Laverania* lineages were the only ones reported from central chimpanzees across multiple field sites in Gabon

[22], although, non-*Laverania* parasites are known to circulate within the same chimpanzee populations [44].

In our initial phylogenetic analysis, a geographical sub-structuring in *P. reichenowi* related to host phylogeography appeared (Fig. 3). A phylogram resulting from the extended dataset confirmed this sub-structure. All *P. reichenowi* sequences obtained from *P. t. schweinfurthii* formed a separated subclade as previously observed by Liu et al. [18]. This sub-structuring could be influenced by the geographical barriers or differences in mosquito vectors responsible for transmission of malaria parasites. Further investigation into ape-malaria from other chimpanzee populations, as well as the inclusion of environmental factors that may influence *Plasmodium* species distribution and abundance in wild great apes, will further contribute to a better understanding of *Plasmodium* species diversity and dynamics.

Of the two host traits analysed in this study, only age was found to be statistically significant, with young chimpanzees more likely to be infected with *Plasmodium* spp. than older ones. A similar trend was observed in western chimpanzees of Taï, Ivory Coast [14], western lowland gorillas inhabiting Dzanga-Sangha Protected Areas [15], as well as in humans [45,46]. The time needed to develop semi- immunity against the malaria parasite may explain why *Plasmodium* was encountered more frequently among younger individuals [47]. Also the failure to find differences in infection levels between the sexes is consistent with previous results from western lowland gorillas [15] and western chimpanzees [14]. Indeed, the scarcity of information about the biology and ecology of *Laverania* lineages and their interactions with hosts, preclude us from drawing a precise picture of the infection dynamics.

The pattern of infections (negative-to-positive and *vice versa*) was observed in 18 individuals sampled more than once over the course of the sampling period. It is worth noting that negative samples observed in this study do not necessary reflect the absence of infections. Rather, this phenomenon might be explained by fluctuation of parasitaemia level and

shedding of parasite DNA in faeces, combined with sensitivity of the *Plasmodium* detection in faecal samples expected to be lower compared to blood samples [18,48]. These findings may indicate that detection of *Plasmodium* DNA in faeces is prone to high risk of false negativity, hindering adequate assessment of actual prevalence of malaria in free ranging chimpanzee populations.

Conclusion

The findings of our study contribute to a broader understanding of malaria occurrence among wild chimpanzees. The differences observed may result from local variation in host exposure to mosquito vectors, extrinsic factors, differences in chimpanzee density, as well as host genetic related factors. Future research should focus not only on screening chimpanzees that live in a variety of habitats, but also identifying potential vectors and vector abundance, in order to provide insights on the distribution and occurrence of *Plasmodium* spp. in chimpanzees.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MIM, JB, FAS and AP collected faecal samples in the field. MIM, ES, KB, KH performed the molecular work. PV performed phylogenetic analyses. JB performed statistical analyses. HPF and MAQ supervised the laboratory work. KJP, CH and DM coordinated and designed the research project. MIM compiled the results and wrote the manuscript. KJP, DM, AKP, FAS, KH, PV, JB, HPF and MAQ edited the manuscript. All authors read and approved the final manuscript.

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Acknowledgment

We are grateful to the Tanzania Wildlife Research Institute (TAWIRI), the Tanzania Commission for Science and Technology (COSTECH), Uganda National Council for Science and Technology, Uganda Wildlife Authority and Uganda National Forest Authority for approval to conduct the field work and faecal sample collections. We would like to express our thanks to Barbora Kalousová for sample collection at the Issa valley, as well as to field assistants from both field sites. Further, we are thankful to Dr. Petr Lany from VFU for access to the P3 laboratory and Barbara Eigner and Walpurga Wille-Piazzai from the Institute of Parasitology, Department of Pathobiology, University of Veterinary Medicine Vienna, Austria for assist with laboratory work. We would like to thank also Omar Radaideh from Department of Geological Science, Masaryk University for formatting the maps. This work was funded by IGA 90/2014/FVL, grant of the Czech Academy of Sciences M200961204 and the EurNegVec COST Action TD1303. This research was carried out under the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and

Sports of the Czech Republic under the National Sustainability Programme II and further co-financed from European Social Fund and state budget of the Czech Republic (project OPVK CZ.1.07/2.3.00/20.0300). The study was further supported by the Institute of Vertebrate Biology Academy of Science of the Czech Republic (RVO: 68081766) and by project LO1218 under the NPU I program. We also acknowledge a grant for the development of research organization (RVO: RO0516). Research at Issa (and for the Ugalla Primate Project) is supported by the UCSD/Salk Center for Academic Research and Training in Anthropogeny (CARTA).

References

1. Craig MH, Snow RW, le Sueur D. A climate-based distribution model of malaria transmission in sub-Saharan Africa. Parasitol Today. 1999; 15: 105–111.

2. Grover-Kopec E, Kawamo M, Klaver RW, Blumenthal B, Ceccato P, Connor SJ. An online operational rainfall-monitoring resource for epidemic malaria early warning systems in Africa. Malaria J. 2005; 4:6.

3. Blanford JI, Blanford S, Crane RG, Mann ME, Paaijmans KP, Schreiber KV, Thomas MB. Implications of temperature variation for malaria parasite development across Africa. Sci Rep. 2013; 3:1300.

4. Kovats RS, Campbell-Lendrum DH, McMichael AJ, Woodward A, Cox JS. Early effects of climate change: do they include vector-borne disease? Philos Trans R Soc Lond B Biol Sci. 2001; 356(1411): 1057-1068.

5. Hunter PR. Climate change and waterborne and vector-borne disease. J Appl Microbiol. 2003; 94: 37S-46S.

6. McMichael AJ, Campbell-Lendrum DH, Corvalán CF, Ebi KL, Githeko AK, Scheraga JD et al.. Climate Change and Human Health: Risks and Responses. Geneva 2003: WHO.

7. Patz JA, Campbell-Lendrum D, Holloway T, Foley JA. Impact of regional climate change on human health. Nature. 2005; 438: 310-317.

8. Dery DB, Brown C, Asante KP, Adams M, Dosoo D, Amenga-Etego et al.. Patterns and seasonality of malaria transmission in the forest-savannah transitional zones of Ghana. Malaria J. 2010; 9:314.

9. Abonuusum A, Owusu-Daako K, Tannich E, May J, Garms R, Kruppa T. Malaria transmission in two rural communities in the forest zone of Ghana. Parasitol. Res. 2011; 108(6): 1465-1471.

10. Badu K, Brenya RC, Timmann C, Garms R, Kruppa TF. Malaria transmission intensity and dynamics of clinical malaria incidence in a mountainous forest region of Ghana. WMJ. 2013; 4:14

11. Songue E, Tagne C, Mbouyap P, Essomba P, Moyou-Somo R. Epidemiology of Malaria in three Geo-Ecological Zones along the Chad-Cameroon Pipeline. AJEID. 2013; 1(4):27-33.

12. Kar NP, Kumar A, Singh OP, Carlton JM, Nanda N. A review of malaria transmission dynamics in forest ecosystems. Parasit Vectors 2014; 7: 265.

13. Doolan DL, Dobano C, Baird JK. Acquired immunity to malaria. Clin Microbiol Rev. 2009; 22(1): 13-36.

14. De Nys HM, Calvignac-Spencer S, Thiesen U, Boesch C, Wittig RM, Mundry R et al.. Age-related effects on malaria parasite infection in wild chimpanzees. Biol lett. 2013; 9(4): 20121160.

15. Mapua MI, Qablan MA, Pomajbíková K, Petrželková KJ, Huzová Z, Rádrová J et al.. Ecology of malaria infections in western lowland gorillas inhabiting Dzanga Sangha Protected Areas, Central African Republic. Parasitology. 2015; 142(7): 890-900.

16. Ollomo B, Durand P, Prugnolle F, Douzery E, Arnathau C, Nkoghe D, et al.. A new malaria agent in African hominids. PLoS Pathog. 2009;5:e1000446.

17. Krief S, Escalante AA, Pacheco MA, Mugisha L, André C, Halbwax M et al.. On the diversity of malaria parasites in African apes and the origin of *Plasmodium falciparum* from Bonobos. PLoS Pathog. 2010; 6(2): e1000765.

18. Liu W, Li Y, Learn GH, Rudicell RS, Robertson JD, Keele BF et al.. Origin of the human parasite Plasmodium falciparum in gorillas. Nature. 2010; 467(7314): 420-425.

19. Rayner JC, Liu W, Peeters M, Sharp PM, Hahn BH. A plethora of Plasmodium species in wild apes: a source of human infection? Trends Parasitol. 2011;27:222–9.

20. Duval L, Ariey F. Ape Plasmodium parasites as a source of human outbreaks.

419 Clin Microbiol Infect. 2012; 18(6): 528-532.
420 21. Pacheco MA, Cranfield M, Cameron K, Escalante AA. Malarial parasite diversity in
421 chimpanzees: the value of comparative approaches to ascertain the evolution of Plasmodium
422 falciparum antigens. Malar J. 2013;12:328.
423 22. Boundenga L, Ollomo B, Rougeron V, Mouele LY, Mve-Ondo B, Delicat-Loembet LM et
424 al.. Diversity of malaria parasites in great apes in Gabon. Malar J. 2015; 14: 111.
425 23. Singh B, Daneshvar C. Human infections and detection of *Plasmodium knowlesi*. Clin
426 Microbiol Rev. 2013; 26(2): 165-84.
427 24. Verhulst NO, Smallegange RC, Takken W. Mosquitoes as potential bridge vectors of
428 malaria parasites from non-human primates to humans. Front Physiol. 2012; 3: 197.
429 25. Piel AK, Lenoel A, Johnson C, Stewart FA. Deterring poaching in western Tanzania: The
430 presence of wildlife researchers. Glob Ecol Conserv. 2015; 3: 188-199.
431 26. Hernandez-Aguilar RA. Chimpanzee nest distribution and site reuse in a dry habitat:
432 Implications for early hominin ranging. J Hum Evol. 2009; 57(4): 350-364.
433 27. Stewart FA. The evolution of shelter: Ecology and ethology of chimpanzee nest building.
434 Ph.D thesis, University of Cambridge 2011.
435 28. Liu W, Li Y, Shaw KS, Learn GH, Plenderleith LJ, Malenke JA et al.. African origin of
436 the malaria parasite *Plasmodium vivax*. Nat Commun. 2014; 5: 3346.
437 29. Yasuoka H, Kimura D, Hashimoto C, Furuichi T. Quantitative assessment of livelihoods
438 around great ape reserves: cases in luo scientific reserve, dr congo, and kalinzu forest reserve,
439 uganda. Afr Study Monogr 2012; 43: 137-159.
440 30. Hashimoto C. Population census of the chimpanzees in the Kalinzu Forest, Uganda:
441 Comparison between methods with nest counts. Primates. 1995; 36: 477-488.

442 31. Furuichi T, Hashimoto C, Tashiro Y. Extended application of a marked-nest census
443 method to examine seasonal changes in habitat use by chimpanzees. Inter J Primatol. 2001;
444 22: 913-928.
445 32. Howard PC. Nature Conservation in Uganda's Tropical Forest Reserves. IUCN, Gland,
446 Switzerland 1991.
447 33. Plumptre AJ, Cox D. Counting primates for conservation: primate surveys in Uganda.
448 *Primates*. 2006; 47(1): 65-73.
449 34. Prugnolle F, Durand P, Neel C, Ollomo B, Ayala FJ, Arnathau C et al.. African great
450 apes are natural hosts of multiple related malaria species, including *Plasmodium*
451 *falciparum*. Proc Natl Acad Sci U S A. 2010; 107(4): 1458-1463.
452 35. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis
453 program for Windows 95/98/NT. Nucl Acids Symp Ser. 1999; 41: 95- 98.
454 36. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms
455 and methods to estimate maximum-likelihood phylogenies: assessing the performance of
456 PhyML 3.0. Syst Biol. 2010; 59: 307-321.
457 37. Ronquist F, Huelsenbeck JP. MRBAYES 3: Bayesian phylogenetics inference under
458 mixed models. Bionformatics. 2003; 19: 1572-1574.
459 38. Nishida T, Corp N, Hamai M, Hasegawa T, Hiraiwa-Hasegawa M, Hosaka K et
460 al..Demography, female life history, and reproductive profiles among the chimpanzees of
461 Mahale. Am J Primatol. 2003; 59(3): 99-121.
462 39. R Development Core Team. R: A Language and Environment for Statistical
463 Computing. Vienna, Austria: the R Foundation for Statistical Computing. 2011; ISBN: 3-
464 900051-07-0.

40. Duval L, Fourment M, Nerrienet E, Rousset D, Sadeuh SA, Goodman SM et al.. African apes as reservoirs of Plasmodium falciparum and the origin and diversification of the Laverania subgenus. Proc Natl Acad Sci U S A. 2010; 107:10561–10566.

41. Kaiser M, Löwa A, Ulrich M, Ellerbrok H, Goffe AS, Blasse A et al..Wild chimpanzees infected with 5 *Plasmodium* species. Emerg Infect Dis. 2010; 16(12): 1956-1959.

42. Galinski MR, Meyer EV, Barnwell JW. *Plasmodium vivax*: modern strategies to study a persistent parasite's life cycle. Adv Parasitol. 2013; 81: 1-26.

43. Kwiatkowski DP. How malaria has affected the human genome and what human genetics can teach us about malaria. Am J Hum Genet. 2005; 77: 171-192.

44. Prugnolle F, Rougeron V, Becquart P, Berry A, Makanga B, Rahola N et al.. Diversity, host switching and evolution of *Plasmodium vivax* infecting African great apes. Proc Natl Acad Sci U S A. 2013; 110: 8123-81238.

45. Doolan DL, Dobano C, Baird JK. Acquired immunity to malaria. Clin Microbiol Rev. 2009; 22(1): 13-36.

46. De Beaudrap P, Nabasumba C, Grandesso F, Turyakira E, Schramm B, Boum Yet al.. Heterogeneous decrease in malaria prevalence in children over a six-year period in south-western Uganda. Malar J. 2011;10: 132.

47. Pouniotis DS, Proudfoot O, Minigo G, Hanley JL, Plebanski M. Malaria parasite interactions with the human host. J Postgrad Med. 2004; 50(1): 30-34.

48. Nwakanma DC, Gomez-Escobar N, Walther M, Crozier S, Dubovsky F, Malkin E, et al.. Quantitative detection of Plasmodium falciparum DNA in saliva, blood, and urine. J Infect Dis. 2009;199:1567-1574.

488

489 Table 1. List of haplotypes used in phylogenetic analyses

Haplotype	Isolate	Reference
KFR144	KFR 144, KFR177, KFR5A, KFR9A, KFR 21, KFR45,	This study
	HM235389_Pts,	Liu et al., 2010
	HM235389_Pts	Liu et al., 2010
HM235394	HM235394_Pts	Liu et al., 2010
HM235048	HM235048_Pts	Liu et al., 2010
HM235391	HM235391_Pts, HM235388 _Pts	Liu et al., 2010
HM235029	HM235029_Ptt	Liu et al., 2010
HM235028	HM235028_Ptt	Liu et al., 2010
HM235328	HM235364_Pte, HM235328_Ptt, HM235359_Ptt, HM235299_Ptt	Liu et al., 2010
HM235362	HM235362_Pte, HM235097_Pte, HM235096_Pte, HM235089_Pte	Liu et al., 2010
KFR3A	KFR3A	This study
KFR150	KFR150, KFR167,	This study
	HM235402_Pts, HM235401_Pts	Liu et al., 2010
KFR72	KFR72	This study
HM235341	KFR149	This study
	HM235341_Ptt, HM235339_Ptt, HM235108_Pts, HM235340_Ptt, HM235392_Pts, HM235342_Ptt, HM235395_Pts	Liu et al., 2010
HM235351	HM235351_Ptt	Liu et al., 2010
HM235380	HM235380_Ggg	Liu et al., 2010
HM235367	HM235367_Ggg	Liu et al., 2010
KC175316	KC175316	Sundararaman et al., 2013
AY282929	AY282929	Joy et al., 2003
HM235382	HM23538_Ggg, HM235294 Ggg, HM235304 Ggg	Liu et al., 2010
HM235400	HM235400_Pts, HM235076_Pts, HM235399_Pts	Liu et al., 2010
KFR178	KFR178	This study
HM235320	HM235320	Liu et al., 2010
HM235052	HM235052	Liu et al., 2010
GQ355470	GQ355470_Pts	Krief et al., 2010
GQ355471	GQ355471_Pts	Krief et al., 2010
KFR90	KFR90	This study
KFR36	KFR36	This study
KFR105	KFR105	This study
KFR32A	KFR32A, KFR93, KFR188, KFR7A	This study
FJ895308	FJ895308_Ptt	Ollomo et al., 2009
JX893151	JX893151_Ptt	Pacheco et al., 2013
HM235102	HM235102_Pte	Liu et al., 2010
HM234997Ptt	HM234997_Ptt, HM235315_Ptt, HM235348_Ptt, HM235309_Ptt, HM235280_Ptt	
	HM235114_Pte, HM235113_Ptt, HM235112_Ptt, HM235088_Pte, HM235086_Pte HM235083_Pte	Liu et al., 2010
HM235100	HM235100_Pte	Liu et al., 2010
HM235077	HM235077_Ptt	Liu et al., 2010
HM235375	HM235375_Ggg, HM235284_Ggg	Liu et al., 2010
HM235313	HM235313_Ggg	Liu et al., 2010
JQ240419	JQ240419	Miao et al., 2012
KC175307	KC175307	Sundararaman et al., 2013
AB489194	AB489194	Hayakawa et l., 2009

490 KFR stand for Kalinzu forest reserve

491 Acronyms under accession number represent chimpanzee and gorilla sub-species

492 Ptt; *Pan troglodytes troglodytes*, Pte; *Pan troglodytes ellioti*, Pts; *Pan troglodytes*
493 *schweinfurthii*

494 Ggg; *Gorilla gorilla gorilla*

495

496 Table 2. Results of PCR detection of *Plasmodium* DNA in feces of chimpanzees from Ugalla
497 and Kalinzu study sites and determination of *Plasmodium* spp. by subsequent sequencing.

	Plasmodium spp.			
Field site	*P. reichenowi*	*P. gaboni*	*P. billcollinsi*	Mixed infection
Ugalla (n= 171)	-	-	-	-
Kalinzu (n= 160)	12	11	7	2

498

499 Table 3. Pattern of *Plasmodium* spp. infection among identified chimpanzees' individuals.

500

			Sampling time and *Plasmodium* spp. identified			
Individuals	Sex	Age Category	April	May	June	July
Buru	M	2	-	-		-
Ross	M	1	-/-	-/-	-	-
Ota	M	1	-		*P. reichenowi*	
Tange	M	2	-/-	-/-	-/-	-
Yawara	M	2	*P. billbrayi*	*P. billcollinsi*/- /-	-/*P. billbrayi*/-/-	
Ichiro	M	2	*P. reichenowi*/-	-/-/-	-	-
Goku	M	2	-	-	-/-/-/-/-	-
Black	M	1	-/-	-/-/*P. billbrayi*	-/-/-/-	-
Gure	M	2	-/-/-	-/-		*P. reichenowi*
Ponta	M	2	-	/-/-/-	*P. billcollinsi*	
Deo	M	2	-	-		-
Pieten	M	1	-/*P. reichowi*, *P. billbrayi*, *P .billcollinsi*		-	-
Kanta	M	1	*P. billcollinsi*	*P. billcollinsi*		
Marute	M		-	-		-
Ricky	M	1	*P. billcollinsi*			
JO	M	1				*P. billbrayi* / *P. billbrayi*
Taike	M	1		*P. billbrayi*	-/-	
Iso	M	1		-	-	
Prince	M	1			-/-/-	
Max	M	1	*P. billbrayi*			
Pinka	F	2	-/-	-		
Kakumu	F	2	-/-			
Tae's daughter	F	1	-			
Nono	F	2	-	-	-	
Haro	F	2	-	-		
Haruka	F	1	*P.reichenowi*			
Shoko	F	2	-/-		-	-
Tae	F	2	-			
Gai	F	2	*P. billcollinsi*	-		
Migi	F	2		-	-	
Ida	F	2	-			
Iku	F	1	*P. billbrayi*			
Nakko	F	2		*P. reichenowi*		
Kanna	F	2	*P.reichenowi*			
Minny	F	2		-		
Umuoge	F	1		-		
Ume	F	2		-		
Miki	F	1			-	
Rina	F	2			-	
Michio	F	2			-	
Mami	F	2				*P. reichenowi*

501 (-) = negative for *Plasmodium*; 1, juvenile/ sub-adult; 2, adult.

502

FIGURE

Fig. 1: Ugalla Map
Map of the study site Issa valley, Western Tanzania.
Alex Piel

Fig. 2: Kalinzu Map
Map of the study site in Kalinzu Forest Reserve, Western Uganda.
Chie Hashimoto

Fig. 3: Phylogenetic tree of *Plasmodium* mitochondrial *cytochrome b* sequences (758bp). Nodal support from 1000 bootstrap pseudoreplicates under ML and Bayesian methods are indicated above and below branches, respectively.

Additional file 1. *Plasmodium* partial cytochrome b gene sequences obtained from GenBank and this study