Genetic characterisation of the neglected parasite species *Plasmodium malariae* and *Mansonella* sp "DEUX"

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Zusammenfassung

Parasitäre Infektionen sind eine große Bedrohung für die globale Gesundheit. Die Forschung konzentriert sich jedoch hauptsächlich auf Parasitenarten mit hoher Mortalität und Morbidität, während andere Arten oft übergangen werden. Die kontinuierliche Entwicklung molekularbiologischer Technologien hat jedoch dazu beigetragen, das Bewusstsein für die tatsächliche Belastung und Verbreitung dieser vernachlässigten Parasiten zu schärfen. In diesem Kontext fokussiert sich diese Dissertation auf die weitere Untersuchung und Charakterisierung zweier solcher Parasiten: *Plasmodium malariae* und *Mansonella* sp “DEUX“.

P. malariae ist der zweithäufigste Malariaparasit, der Menschen in Sub-Sahara Afrika infiziert. Die tatsächliche Prävalenz wurde lange Zeit unterschätzt. Zur besseren Charakterisierung der genetischen Vielfalt der verschiedenen Genotypen innerhalb der *P. malariae* Parasitenpopulationen, haben wir ein neues Genotypisierungs-Panel entwickelt, basierend auf den Markerregionen *pmmsp1* F2 und *pmtrap*. Es ist einfach anzuwenden und kann daher gut in endemischen Gebieten eingesetzt werden. Innerhalb der untersuchten *P. malariae* Parasitenproben aus Gabun haben wir mit Hilfe der Marker eine hohe genetische Vielfalt detektiert. Außerdem zeigte sich, dass *P. malariae* Infektionen meist aus mehreren Genotypen zusammengesetzt sind und somit eine hohe Komplexität aufweisen.

Ähnlich wie die unterschätzte Genotypen-Vielfalt von *P. malariae,* ist auch die tatsächliche Artenvielfalt und Prävalenz von *Mansonella* Parasiten bisher nur unzureichend beschrieben. In Zentralafrika, einschließlich Gabun, Kamerun und der Republik Kongo, sind verschiedene *Mansonella* Arten bei Primaten und auch Menschen bekannt. Jedoch beruhen diese Beschreibungen hauptsächlich auf deren Morphologie, da bisher nur wenige, bis keine Nukleotidsequenzen verfügbar sind. Kürzlich wurde bei Kindern in Gabun erstmals *Mansonella* sp “DEUX“ identifiziert. Es war jedoch unklar, ob es sich dabei um eine neue Art oder einen Genotypen von *M. perstans* handelt, welche ebenfalls in dieser Region vorkommt. Morphologisch-mikroskopische Untersuchungen zum Vergleich mit anderen *Mansonella* Arten wurden hierzu bisher nicht durchgeführt. Eine Querschnittsstudie in Gabun ergab eine hohe Prävalenz (35%) von *Mansonella* sp “DEUX“ im Vergleich zu anderen Filarien. Zur weiteren Klärung der Systematik innerhalb der Gattung *Mansonella* haben wir das

erste bisher veröffentlichte Referenzgenom für *Mansonella* sp “DEUX“ erstellt. Der Vergleich mit dem Genom von *M. perstans* zeigt, dass es sich hierbei um zwei getrennte Arten handelt, die in Sympatrie vorkommen. Weitere evolutionäre Analysen ergaben, dass die beiden Arten sich vor ca. 778.000 Jahren voneinander abgespaltet haben.

Diese Arbeit trägt durch die Verwendung verschiedener genetischer Ansätze zu einer besseren Beschreibung der beiden vernachlässigten Arten *P. malariae* und *Mansonella* sp „DEUX“ bei. Unsere Entwicklung eines Genotypisierungsmarker-Panels verbessert zum einen das Verständnis der genetischen Vielfalt von *P. malariae* Parasitenpopulation. Außerdem konnten wir nachweisen, dass *Mansonella* sp “DEUX“ eine separate Art ist. Die Verfügbarkeit eines Referenzgenoms wird die weitere Erforschung dieser vernachlässigten Parasitenart weiter voranbringen. Beide hier vorgestellten Methoden legen die Grundlage für die weitere Erforschung der Parasiten, um die Vernachlässigung der beiden Arten zu beenden.

Summary

Despite the major threat that parasitic infections pose to global health, they often remain overlooked, particularly those species that do not account for the highest mortality and morbidity rates. However, the continuous development of molecular technologies has contributed to a growing awareness of the true burden and distribution of those neglected parasites. This thesis focuses on further investigating and characterizing two such parasites, namely *Plasmodium malariae* and *Mansonella* sp “DEUX”.

P. malariae is the second most abundant human malaria parasite in sub-Saharan Africa. The true prevalence has long been underestimated. To better characterize the genetic diversity of different genotypes within the *P. malariae* parasite populations we have developed a novel genotyping marker panel based on the two marker regions *pmmsp1* F2 and *pmtrap.* The panel is easily applicable and can be implemented in endemic settings. Using our novel approach, we found a high genetic diversity among *P. malariae* parasites from Gabon. Additionally, we observed that genotype dynamics are very high, indicating that daily fluctuations in different genotypes is very common for *P. malariae.*

Similar to the underestimated genotype diversity of *P. malariae,* the true species diversity and prevalence of *Mansonella* parasites remains unknown. In the Central African Region, including Gabon, Cameroon and the Republic of Congo, different *Mansonella* species are known to infect humans and great apes. However, these descriptions are mainly based on morphology, as few nucleotide sequences are currently available. Recently, *Mansonella* sp “DEUX” was identified in Gabonese children for the first time, not knowing whether it represents a new species or a genotype of the sympatric species *Mansonella perstans.* Morphological studies have not yet been conducted to compare it to other known *Mansonella* species. Furthermore, a high prevalence of *Mansonella* sp “DEUX” (35%) was reported among a cross-sectional study population in Gabon, in comparison to other filariae. To clarify the systematics, we have generated the first reference genome for *Mansonella* sp “DEUX”. A comparison to the whole genome of *M. perstans*, that we also generated, confirmed that they are two separate species that occur in sympatry. Evolutionary analysis revealed that they separated approximately 778,000 years ago.

Using different genetic approaches this thesis has provided a better description of the two neglected parasites. With the development of a genotyping marker panel, which is based on two marker regions only (*pmmsp1* F2 and *pmtrap)*, our results contribute to a better understanding of the true diversity of the *P. malariae* parasite populations. Additionally, we could provide evidence that *Mansonella* sp “DEUX” is a distinct species and the generation of a reference genome will support further research on the neglected parasite. Both methods presented here can be applied to further samples so that the neglect of the two species can be put to an end.

List of publications

This dissertation is based on the following publications listed below:

1. **Rodi M**, Kawecka K, Stephan L, Berner L, Medina MS, Lalremruata A, Woldearegai TG, Matsiegui PB, Groger M, Manego RZ, Ekoka Mbassi D, Mombo-Ngoma G, Agnandji ST, Ramharter M, Mordmüller B, Inoue J and Held J. *Genetic diversity of Plasmodium malariae in sub-Saharan Africa: a two-marker genotyping approach for molecular epidemiological studies*. Front. Cell. Infect. Microbiol. 2024 Jul 19; 14:1405198. doi: 10.3389/fcimb.2024.1405198
2. **Rodi M***, Gross C*, Sandri TL, Berner L, Marcet-Houben M, Kocak E, Pogoda M, Casadei N, Köhler C, Kreidenweiss A, Agnandji ST, Gabaldón T, Ossowski S, Held J. *Whole genome analysis of two sympatric human Mansonella: Mansonella perstans and Mansonella sp "DEUX".* Front. Cell. Infect. Microbiol. 2023 Apr 14; 13:1159814. doi: 10.3389/fcimb.2023.1159814
   *shared first authorship

Additional publications that were published during the doctoral thesis but are not summarized in this dissertation:

1. Inoue J, Galys A, **Rodi M**, Mbassi DE, Mombo-Ngoma G, Adegnika AA, Ramharter M, Zoleko-Manego R, Kremsner PG, Mordmüller B, Held J. *Dynamics of Plasmodium species and genotype diversity in adults with asymptomatic infection in Gabon*. Int J Infect Dis. 2024 Mar 16:107013. doi: 10.1016/j.ijid.2024.107013
2. Sandri TL, Kreidenweiss A, Cavallo S, Weber D, Juhas S, **Rodi M**, Woldearegai TG, Gmeiner M, Veletzky L, Ramharter M, Tazemda-Kuitsouc GB, Matsiegui PB, Mordmüller B, Held J. *Molecular Epidemiology of Mansonella Species in Gabon*. J Infect Dis. 2021 Feb 3; 223(2):287-296. Erratum in: J Infect Dis. 2022 Mar 2; 225(5):929. doi: 10.1093/infdis/jiab439

3. Koehne E, Zander N, **Rodi M**, Held J, Hoffmann W, Zoleko-Manego R, Ramharter M, Mombo-Ngoma G, Kremsner PG, Kreidenweiss A. *Evidence for in vitro and in vivo activity of the antimalarial pyronaridine against Schistosoma.* PLoS Negl Trop Dis. 2021 Jun 24; 15(6):e0009511. Erratum in: PLoS Negl Trop Dis. 2022 Jun 2; 16(6):e0010512. doi: 10.1371/journal.pntd.0010512
4. Sulyok Z, Fendel R, Eder B, Lorenz FR, Kc N, Karnahl M, Lalremruata A, Nguyen TT, Held J, Adjadi FAC, Klockenbring T, Flügge J, Woldearegai TG, Lamsfus Calle C, Ibáñez J, **Rodi M**, Egger-Adam D, Kreidenweiss A, Köhler C, Esen M, Sulyok M, Manoj A, Richie TL, Sim BKL, Hoffman SL, Mordmüller B, Kremsner PG. *Heterologous protection against malaria by a simple chemoattenuated PfSPZ vaccine regimen in a randomized trial.* Nat Commun. 2021 May 4; 12(1):2518. doi: 10.1038/s41467-021-22740-w
5. Kreidenweiss A, Trauner F, **Rodi M**, Koehne E, Held J, Wyndorps L, Manouana GP, McCall M, Adegnika AA, Lalremruata A, Kremsner PG, Fendel R, Sandri TL. *Monitoring the threatened utility of malaria rapid diagnostic tests by novel high-throughput detection of Plasmodium falciparum hrp2 and hrp3 deletions: A cross-sectional, diagnostic accuracy study.* EBioMedicine. 2019 Dec; 50:14-22. doi: 10.1016/j.ebiom.2019.10.048

Personal contributions

My contributions to the publications summarized in this dissertation are as follows:

Publication 1:

Genetic diversity of *Plasmodium malariae* in Sub-Saharan Africa: A Two-Marker Genotyping Approach for Molecular Epidemiological Studies.

- Performance of *P. malariae* genotyping marker PCRs
- *In silico* genotype analysis
- Interpretation of results
- Drafting of the manuscript
- Revising and approving the manuscript for publication.

Publication 2:

Whole genome analysis of two sympatric human *Mansonella*: *Mansonella perstans* and *Mansonella* sp "DEUX".

- DNA extraction from whole blood
- *Mansonella* species confirmation via qPCR
- Evaluation of the data set
- Drafting of the manuscript
- Revising and approving the manuscript for publication.

Introduction

1 Neglected parasite species

Parasitic infections pose a major challenge to global health, affecting humans in endemic regions worldwide. Among the numerous parasite species, research and intervention strategies consequently focus on those responsible for the highest mortality and morbidity. In contrast, less attention is paid to those parasites whose prevalence, symptoms and burden have so far been little or not at all described. However, the rapid development of molecular technologies in the last decades provided a clearer picture of the true diversity and distribution of many of those neglected parasites. Given that the actual burden of many species has been underestimated so far, a better characterization of their biology and prevalence is extremely crucial. Many questions regarding their interaction with the host's immunity and other co-infecting parasite species needs to be clarified. This dissertation focuses on two of these neglected parasites, *Plasmodium malariae* and *Mansonella* sp "DEUX", investigating and characterizing them on a genetic level in more detail.

1.1 *Plasmodium malariae*

Malaria remains to be a global health threat, with an estimated 249 million cases and 608 000 deaths in 2022 worldwide [1]. Until recently, the following *Plasmodium* species were known to cause malaria in humans: *P. falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi.* Additional data have now confirmed *P. ovale* as being two different species: *P. ovalecurtisi* and *P. ovalewallikeri* [2]. Among those six species, *P. falciparum* accounts for the majority of cases and deaths in sub-Saharan Africa [1]. Therefore, intervention strategies and treatment are mainly directed towards this species. More recently *P. vivax*, which is highly prevalent in Asia and South America, has gained more attention. However, most people living in endemic regions are co-infected with other *Plasmodium* species. The second most abundant malaria parasite in sub-Saharan Africa is *P. malariae* [3,4]. Although a decline in the overall burden of malaria has been achieved over the last decades, less attention has been paid to *P. malariae* in these efforts. Continuous improvement and increased accessibility of molecular

diagnostics have shown that the prevalence of non-*falciparum* species and especially of *P. malariae* has been underestimated worldwide [5-8].

1.1.1 Biology of *Plasmodium malariae*

Infections with *P. malariae* occur worldwide in tropical and subtropical regions, with varying prevalences up to 40% in endemic regions [9-12].

The life cycle of *P. malariae* parasites is similar to those of the other human *Plasmodium* species. The sporozoites are transmitted through the bite of female *Anopheles* spp. mosquitoes. The parasites migrate to the liver where they develop into merozoites which eventually reach the blood stream and invade red blood cells (RBCs). These blood-stages massively replicate asexually and lead to a high parasite load in the blood stream, causing the clinical malaria symptoms. A small proportion of merozoites differentiates into gametocytes, which are ingested by the mosquito upon the next blood meal. Sexual reproduction takes place, producing new sporozoites that migrate to the mosquitos' salivary glands and can now be transmitted to the human host. Unique characteristics in the *P. malariae* life cycle include a prolonged pre-erythrocytic development in the liver, a preference for mature RBCs, a slower growth rate in general as well as a prolonged prepatent stage in the human host [13,14].

P. malariae infections are considered benign and mainly asymptomatic. Low levels of parasitaemia allow the parasite to remain undetected in the host for a longer time, in comparison to other *Plasmodium* species that infect humans. Rarely occurring serious complications mainly include severe anaemia and renal impairment [15,16]. Infections are described as rather chronic. However, case reports show that parasites can persist in the human host for a long time and recur decades after exposure [17,18]. The underlying mechanism remains unclear, as hypnozoites, the dormant liver stages described for species causing malaria tertiana, are not reported for *P. malariae*. Overall, the described characteristics indicate that the species shows several signs of successful adaptation to its host.

1.1.2 Diagnosis and treatment options of *P. malariae* infections

There are three main diagnostic tools, that can be applied to determine the underlying species of malaria infections. They include microscopy of thin and thick blood smears, detection of circulating malaria antigens in the blood via rapid diagnostic tests (RDTs), as well as the detection of nucleic acids from the parasite through molecular methods.

Microscopy of thick blood smears determines the underlying parasitaemia of infected individuals, whereas the different *Plasmodium* species can be distinguished via thin blood smears. However, the correct identification of *P. malariae* and other non-*falciparum* species requires a certain level of expertise and is less sensitive. RDTs are a fast and easy to use diagnostic tool. The targeted parasite antigens are either lactate dehydrogenase (LDH), aldolase or *P. falciparum* histidine-rich protein 2 (HRP2). Most RDTs specifically identify *P. falciparum (*HRP2, PfLDH) and *P. vivax* (PvLDH). In combination with antibodies against pan *Plasmodium* aldolase, RDTs can also be used to distinguish *P. falciparum* and *P. vivax* infections from other species, such as *P. malariae.* However, the sensitivity is comparably low and highly heterogenous, ranging from no detection to 100% in *P. malariae* mono-infections depending on the applied RDT kit [19,20]. The high variation in results is mainly due to the small number of samples the results were obtained from. The studies on the detection of *P. malariae* using RDTs have in common, that whenever reported, high parasite densities (>5001 parasites/µl) increase the probability of a positive test result. There are no RDTs targeting *P. malariae* specific antigens till date. A more reliable method to diagnose *P. malariae* in terms of specificity and sensitivity is via molecular assays, such as conventional polymerase chain reaction (PCR) or quantitative PCR (qPCR). The target which is most often amplified in molecular diagnostic assays is the18S ribosomal DNA (18S rDNA) gene. It is highly conserved among each species and provides a sensitive detection as parasites carry multiple copies of the gene [21]. Less often, other loci such as cytochrome b oxidase gene (*cytb*) or cytochrome c oxidase subunit 1 (*cox1)* are used [22-25].

According to the World Health Organization (WHO), *P. malariae* infections in endemic regions should be treated with either artemisinin combination therapies or chloroquine in areas with chloroquine-susceptible infections, similar to *P. falciparum* infections [26]. However, data on treatment success and efficacy of different drug combinations on *P. malariae* is scarce. Several studies and case reports have described the existence of persisting *P. malariae* parasites after treatment or chemoprophylaxis [27-29], while other studies indicate a high cure rate [30,31]. More recent data from *in vitro* studies suggest a different susceptibility profile against some of the tested antimalarial agents among the different species [32]. Research in this direction is also complicated by the fact that *P. malariae* has not yet been successfully adapted to *in vitro* culture, underscoring the lack of research on this species.

1.1.3 Whole genome sequencing of *P. malariae* and its relatives infecting other primates

The neglect of *P. malariae* is also reflected in the comparably sparse number of available sequencing data. Within the last decades, *P. malariae* has gained more attention, and the publication of a reference genome represents a significant step towards a better understanding of the species [33]. Subsequently, the publication of a selective whole genome amplification approach paved the way for large scale population studies on the parasite [34]. This method increases the relative amount of *P. malariae* DNA in blood samples. It can be applied directly to unprocessed blood from infected individuals, which simplifies the acquisition of further sequencing data.
Furthermore, the availability of a reference genome helped to clarify the origin of *P. malariae* which according to recent whole genome analysis lays in African apes [35]. Similar to *P. falciparum,* the high level of genetic polymorphisms in *P. malariae* indicates that it likely originated from zoonotic transmission of malaria parasite from African apes. The authors found two *P. malariae*-related lineages among gorillas and chimpanzees, that represent two separate *Plasmodium* species. Additionally, they could show that *P. brasilianum* which is infecting New World monkeys falls within the radiation of human *P. malariae* and does not represent a separate species. It is derived from a human *P. malariae* strain, that was imported from Africa to South America.
These results indicate the importance of further genetic investigations on *P. malariae,* to help answering basic questions on the parasites biology and potential further zoonotic transmission.

1.2 *Mansonella* spp.

Vector-borne infections with nematodes of the genus *Mansonella* affect many people in Africa and South America. However, there are still no reliable records on the prevalence, only outdated projections based on data from 1947 show that more than 100 million people in Africa might be infected with *Mansonella* species [36]. This indicates that the management of the disease and the causative parasites from the genus *Mansonella* has been neglected by health authorities and research [37]. The lack of comprehensive data hinders our understanding of the true burden of disease on the affected population and the public health impact of these parasites. No official large-

scale vector control programs or treatment campaigns have been implemented, further highlighting the need for immediate attention.

1.2.1 Biology and epidemiology of *Mansonella* spp.

Filarial nematodes from the genus *Mansonella* belong to the *Onchocercidae* family within the superfamily *Filarioidae.* Phylogenetically *Mansonella* spp. are closely related to other parasitic filariae from the same family, including the genera *Brugia, Loa, Onchocerca* and *Wuchereria* which cause various forms of filariasis in humans and animals. Three *Mansonella* species are known to infect humans, which vary in their geographic distribution. While *M. perstans* is endemic in Africa [38-40] as well as Central and South America [41], *M.* ozzardi is prevalent only in Central and South America [42,43], and lastly *M. streptocerca* is limited to West and Central Africa [44-46]. Infections are transmitted through the bite of blood sucking female biting midges from the genus *Culicoides* (*M. perstans* [47,48]*, M. ozzardi* [49] and *M. streptocerca* [50]) or blackflies from the genus *Simuliidae (M. ozzardi)* [51]. Thereby, infective larvae are introduced into the human host, where they develop into adult nematodes which produce unsheathed microfilariae. Depending on the species, the adults and their offspring can be found in different areas of the body. Adult worms of *M. perstans* reside in the body cavities, such as peritoneal or pleural cavity and release the microfilariae into the peripheral circulation. Whereas *M. ozzardi* adult worms are found in subcutaneous tissues and their offspring can be found in the blood stream as well. Other than these two so-called blood-dwelling species, the adult worms of the skin-dwelling species *M. streptocerca* are found in the dermis and the microfilariae reside in the skin.

Infections are usually diagnosed via microscopy or molecular assays through the detection of circulating microfilariae in the blood or skin, depending on the species. Molecular diagnosis through conventional PCR, qPCR or loop-mediated isothermal amplification (LAMP) is mainly used to differentiate the species from each other and to determine the parasitic load [52]. The main target regions for amplification include the conserved nuclear internal transcribed spacer 1 (ITS1) region, 18S rRNA and several mitochondrial genes, such as 12S, *cox1* or 5.8 rRNA [38,39,53].

Although *Mansonella* infections are highly prevalent and widely distributed, the clinical picture as well as the burden of disease are poorly defined. Infections are usually described as either mild or mainly asymptomatic. Nonspecific symptoms that have

been associated with an infection of *Mansonella* spp. include eosinophilia, pruritus, urticaria, and abdominal pain [46,54]. Standard anthelmintic treatments showing efficacy against *Mansonella* spp. either in combination or alone include mebendazole, diethylcarbamazine (DEC), and ivermectin [55-57]. However, clear treatment recommendations or guidelines for mansonellosis are still lacking. Therefore, it is of great importance to further investigate the severity of *Mansonella* infections and determine if they have a potential impact on other co-infections or the host's immune response.

1.2.2 *Mansonella* species diversity

In addition to the three species that infect humans, a potential new *Mansonella* species was detected in 2015 in the blood of febrile children in Gabon and provisionally called *Mansonella* sp "DEUX" [58]. Based on sequencing data from the ITS1 region, a highly conserved region in nematodes, it differs from *M. perstans*. Further investigations on the epidemiology of *Mansonella* spp. in Gabon revealed that it is highly prevalent. A cross-sectional study with 834 individuals from rural areas in Lambaréné and surrounding villages revealed that *Mansonella* sp "DEUX" was the most abundant blood-dwelling filaria species with a prevalence of 35%, next to *Loa loa* (32%) and *M. perstans* (9%) [38]. A detailed morphological description of the potential new species is lacking, as is prevalence data from other countries or regions. Based on *in silico* analysis, it was also shown that *Mansonella* sp "DEUX" could not be detected with any *M. perstans* specific molecular assays that were published by 29th of February 2020 [38].

1.2.3 *Mansonella* spp. and their endosymbiont *Wolbachia*

Most filarial species, such as *Onchocerca volvulus, Wuchereria bancrofti* and *Brugia malayi,* are known to carry the intracellular bacteria *Wolbachia* [59-61]. The endosymbiont seems to be essential for the survival and reproduction of the nematodes [62-64]. Therefore, it poses a potential treatment strategy. Whether or not *Wolbachia* are present in *M. perstans* was unclear for a long time, however the improved sensitivity of molecular assays helped to confirm their presence [65,66]. Also, the successful treatment of infections with antibiotics further indicated that *M. perstans* belongs to the nematodes carrying the endosymbiont [67-69]. *M. ozzardi* is also known to carry *Wolbachia* and only recently, *Wolbachia* were also found in *Mansonella* sp "DEUX" mono-infections [38,70]. In general, *Wolbachia* are classified into different supergroups,

which differ depending on the host. Interestingly, the three *Mansonella* species carry *Wolbachia* from the supergroup F, which they share with no other nematode but only arthropod host [38,71]. Other filarial *Wolbachia* fall into supergroups C, D or J, which include only nematode symbionts [72,73].

2 Different molecular techniques provide answers to parasite species and genotype diversity

According to the biological species concept, a species is a group of interbreeding individuals that produce fertile offspring [74]. Different species share a certain percentage of their genomes. Depending on the time that has passed since the speciation event, the genetic distance between the two newly evolved species differs. Genetic distance can either be related to different loci, genes or the whole genome. A genotype on the other hand can be attributed to one individual from the same species that is genetically distinct. The genetic similarity between different genotypes is much higher than between species. By genotyping a genetic fingerprint can be created and individual genotypes can be distinguished from one another.

In endemic regions, individuals are often co-infected with several species from the same genus, this is the case for infections with *Plasmodium* spp. as well as *Mansonella* spp [5,38,75]. Additionally, they can carry multiple genotypes from the same species at the same time. There are various molecular techniques and assays to investigate genetic diversity at both species and genotype level. The different *Plasmodium* species are often diagnosed based on the 18S rRNA locus, which is well known to be highly polymorphic between the different species but highly conserved among each species [21,76,77]. The same applies to the ITS1 region in filaria, which is why this locus is commonly used to differentiate *Mansonella* species [38,53].

In the following, a brief overview of the molecular techniques is given to better understand which genetic approaches were used for the genetic characterisation of *P. malariae* and *Mansonella* sp “DEUX” within this dissertation and why they were chosen.

2.1 Size polymorphic regions

The usage of size polymorphic markers is a well-established tool in epidemiological studies to differentiate single genotypes. Marker regions typically include microsatellites or size polymorphic regions with insertion and deletion sites (indels) of various lengths. Microsatellites are short repetitive sequences that are widely distributed across the genome and comprise tandem repeats of 2-6 bp. The different number of repeat units leads to genetic diversity. Regions are amplified via conventional PCR, followed by size determination using agarose gel or capillary electrophoresis. The approaches are easy to implement in different settings and have a high throughput.
As of the high level of polymorphism within species, markers amplifying regions that vary in size are not used to discriminate between species.

2.2 Single nucleotide polymorphisms (SNPs)

Differences among genotypes or species can also be present in the form of sequence polymorphisms. Single nucleotide polymorphisms (SNPs) represent a change of one single nucleotide at a specific position. They appear highly concentrated at polymorphic loci of up to 500 bp. At the same time, they occur separately distributed throughout the genome. Depending on the target different approaches for SNP typing are available. SNPs can either be targeted separately using a SNP-barcode [78,79] or amplicons with a high abundance of SNPs can be targeted using amplicon sequencing [80,81]. Both approached will be further addressed in the following.

2.2.1 SNP barcodes

SNPs that occur at different loci throughout the genome can be targeted via qPCR using a SNP-barcode approach [78,79]. Each targeted locus comprises one SNP [82]. Large-scale SNP-typing allows a higher throughput by using SNP panels. Although DNA input is much lower, advanced equipment and expertise in analysis are needed. Another disadvantage is that in the case of infections with multiple genotypes, it is not possible to identify separate genotypes from the SNP pattern. Therefore, SNP barcoding approaches are more suited to genotype samples that are infected with one

single genotype. However, especially for infectious diseases as malaria this is often not the case.

2.2.2 Amplicon sequencing of SNP-rich loci

Amplicon sequencing (AmpSeq) is a more suitable method for the detection of multiple genotypes within one infection. In this approach specific regions with a high abundance of SNPs within a certain locus of up to 500 bp are amplified via conventional PCR, followed by deep sequencing. The deep sequencing technique refers to the sequencing of a genomic region at high coverages. Other than the classic Sanger sequencing approaches, this next-generation sequencing approach allows for the detection of multiple genotypes, including those at low frequencies [81]. At the same time, the sensitivity advantage of AmpSeq over other genotyping methods comes with the challenge to distinguish true minor genotypes from PCR or sequencing errors. Therefore, quality checks based on replicates and appropriate analysis pipelines need to be included and considered.

2.3 Whole genome sequencing

The most detailed characterization of parasite species and genotypes can be obtained from whole genome sequencing data. While amplicon sequencing targets a specific region and allows for a more in-depth investigation of the region (deep sequencing), whole genome sequencing (WGS) is covering the entire genome of an organism. Comparison of whole genomes is typically being used to determine and differentiate between distinct species. WGS is also widely in use to investigate population genetics in general and drug resistance. More recently, pipelines have been developed to estimate the presence and number of multiple *P. falciparum* genotypes within the same host [83,84]. Other *Plasmodium* species as well as other parasites will benefit from such developments in the future.

When investigating human blood samples from infected individuals, WGS is always affected by the high abundance of human DNA in the samples. This can be overcome by the respective treatment of the samples prior to sequencing, such as leucocyte depletion or selective whole genome amplification [85,86].

To conclude, it is very important to choose the appropriate genotyping approach for the underlying research question. Therefore, the latest methodologies and the resulting findings on genotype and species diversity of the two parasites of interest, *P. malariae* and *Mansonella* sp "DEUX", will be described and evaluated in the following chapter.

3 Current knowledge on *P. malariae* genotype diversity and *Mansonella* species diversity

3.1 Genotyping in *Plasmodium* spp.

Genotyping is a powerful tool in malaria research, helping to understand parasite diversity and dynamics. *Plasmodium* blood stages are haploid, therefore each distinct allele that is detected at a certain timepoint can be attributed to a distinct genotype.

The application of polymorphic genotyping markers allows to identify the number of co-infecting genotypes at the same time, referring to the multiplicity of infection (MOI), and the genetic diversity of the parasite population can be investigated as well. Genotyping markers can be applied to follow a single genotype throughout the course of an infection or to investigate recurrent parasitaemia in malaria patients after antimalarial treatment. Parasites recurring after treatment can be attributed to a reinfection, relapse, or recrudescence [87]. A reinfection is caused by a new genotype, transmitted by a new mosquito bite. Relapses originate from so-called hypnozoites, reactivated dormant liver forms, that are only known to occur in *P. vivax,* and potentially also in *P. ovalecurtisi* and *P. ovalewallikeri* [88-90]. Whereas a recrudescent infection arises from previously undetectably persisting blood stages that replicate again and thus exceed the threshold of detection. The main explanation for recrudescence is due to unsuccessful or incomplete antimalarial treatment, allowing the survival of a small number of parasites. Clinically there is no possibility to distinguish between recurring parasites. Therefore, in clinical trials, conducted in endemic areas where natural infections can occur during the study period, genotyping markers are used to determine drug efficacy, by comparing genetic markers before and after drug administration.

The firstly described genotyping markers for *P. falciparum* are based on size polymorphisms in the *P. falciparum* merozoite surface proteins (*pfmsp*) 1 and 2 and *P. falciparum* glutamine rich protein (*pfglurp*) genes [91]. Since then, many other protocols have been reported, targeting microsatellites and other size polymorphic regions. However, *pfmsp1*, *pfmsp2* and *pfglurp* typing continues to be the most commonly used method, as until recently all three markers were recommended by the WHO to identify recrudescence in clinical trials. In 2021 the WHO changed their recommendations to *pfglurp* being replaced by a microsatellite, as it has a higher discriminatory power [87]. Relapse in *P. vivax* is currently assessed using the polymorphic markers *P. vivax* circumsporozoite protein (*pvcsp)* and *pvmsp1* [92]. Regarding other non-*falciparum* species few approaches have been described so far. For *P. ovalewallikeri,* a set of 8 microsatellite markers has been developed to investigate relapse patterns for the species [89]. The size polymorphic locus tryptophan-rich antigen (*potra*) has been found to be a suitable target for both, *P. ovalewallikeri* and *P. ovalecurtisi* genotyping [93]. Two *P. knowlesi* genotyping approaches have been published, one is based on the *msp1* gene, while the other is based on a microsatellite approach [94,95].

Similar to other non-*falciparum* species, the genotyping of *P. malariae* has made little progress to date. Therefore, findings from genotyping approaches that are already in use for other *Plasmodium* species can serve as a valuable reference point.

3.1.1 *P. malariae* genotype diversity

Two genotyping approaches have been described previously for *P. malariae* [96,97]. Both are based on microsatellite markers and indicate a high level of genetic diversity in the investigated *P. malariae* populations. Other genes have been investigated additionally regarding their variability. Based on the highly polymorphic character of the *msp1* gene in *P. falciparum* and *P. vivax*, the gene was further investigated in *P. malariae* [98-100]. A high level of similarity in the structural organization of the gene was found across *Plasmodium* species. The *pmmsp1* locus contains different blocks that vary in their level of genetic diversity. Conserved and variable blocks are organized alternately within the gene, whereby *P. malariae msp1* block II towards the 5'-end is of specific note, as it is characterized by short indels [100]. Other genes include *P. malariae* thrombospondin-related anonymous protein (*pmtrap),* 18S rRNA and *P. malariae* apical membrane antigen (*pmama1)* [101,102]. 18S and *pmama1* display a high level of

nucleotide diversity among the respective *P. malariae* populations. On the other hand, *pmtrap* varies in fragment size due to differences in the number of repeat units, which is similar to *pmmsp1* block II [100,102].

As more recent data is showing, the true prevalence of *P. malariae* has been clearly underestimated so far [6,7]. Therefore, it is of great importance to have further molecular tools that can be implemented easily in many different settings, providing further urgently needed data on the genetic diversity of *P. malariae*.

3.2 *Mansonella* species diversity

The genus *Mansonella* is part of the *Onchocercidae* family. Only recently the phylogeny of the family was divided into five strongly supported clades, based on seven mitochondrial and nuclear loci. *Mansonella* was grouped into one clade with other human filariae from the genus *Loa*, *Wuchereria* and *Brugia* [103]. Other phylogenetic investigations with *Mansonella* samples from humans and great apes from Gabon and Cameroon are based on the mitochondrial genes 12S rDNA and cytochrome c oxidase subunit 1 (*cox1*) [104]. These results suggest that the genus is more diverse than previously thought and that *M. perstans* has the potential for zoonotic transmission. Similar findings come from skin biopsies from Gabonese individuals, that were infected with microfilariae from the great ape infecting species *M. rodhaini* [105]. Based on morphology the following additional species have been described to infect great apes: *M. vanhoofi, M. gorilla, M. leopoldi* and *M. loopensis* [106-109]. However, till date there are no sequences available for any of the here listed species. More insights on the ape-infecting species will help clarifying the risk of further zoonotic transmission.

Whether or not *Mansonella* sp “DEUX” is truly a distinct species or only a genotype of *M. perstans* needs to be proven. Also, the question remains if it corresponds to any ape-related *Mansonella* species. Although more attention has recently been drawn to the neglected filarial species and the database is growing [110], the lack of sequencing data continues to complicate research on *Mansonella*.

Objectives

Neglected parasite species lack a proper description of their biology. In the era of elimination of infectious diseases, these so far understudied parasites will play a more important role. Therefore, further characterisation of their prevalence, population structure and diversity is urgently needed. This work focuses on the two parasites *Plasmodium malariae* and *Mansonella* sp “DEUX”. For the first one, easily applicable genotyping tools are needed to investigate the species heterogeneity and longevity. *Mansonella* sp “DEUX”, although being recently reported at high prevalence in Gabon, still needs to be confirmed as distinct species from other endemic *Mansonella* species.

To conclude this dissertation investigates *P. malariae* on a genotype level, while in contrast *Mansonella* sp “DEUX” is investigated on a species level.

The objectives presented in this dissertation are as follows:

1. Establishment of a *P. malariae* genotyping panel with the smallest possible number of markers involved, based on size polymorphic regions.
2. Application of the *P. malariae* genotyping panel on a large sample set from Gabon, to investigate *P. malariae* parasite populations and to analyse the dynamics and complexity of *P. malariae* infections.
3. Generation and description of whole genomes for *Mansonella* sp “DEUX and *M. perstans,* to confirm that *Mansonella* sp “DEUX” is a separate and sympatric species from *M. perstans.*
4. *In silico* analysis of *Mansonella* sp “DEUX” sequences regarding different non-human primates as potential zoonotic hosts.

Results

The results of the published work included in this doctoral thesis are summarized separately in the following.

Publication 1

Genetic Diversity of *Plasmodium malariae* in Sub-Saharan Africa: A Two-Marker Genotyping Approach for Molecular Epidemiological Studies

Rodi M, Kawecka K, Stephan L, Berner L, Medina MS, Lalremruata A, Woldearegai TG, Matsiegui PB, Groger M, Manego RZ, Ekoka Mbassi D, Mombo-Ngoma G, Agnandji ST, Ramharter M, Mordmüller B, Inoue J and Held J. *Genetic diversity of Plasmodium malariae in sub-Saharan Africa: a two-marker genotyping approach for molecular epidemiological studies*. Front. Cell. Infect. Microbiol. 2024 Jul 19; 14:1405198. doi: 10.3389/fcimb.2024.1405198

Here, we established a genotyping panel for *P. malariae* using size-polymorphic genotyping markers that can be easily used in epidemiological studies.

We assessed the following four polymorphic loci, two microsatellites (Pm02 and Pm09), as well as *P. malariae* thrombospondin-related anonymous protein (*pmtrap*), and *P. malariae* merozoite surface protein 1 fragment 2 (*pmmsp1* F2). We optimized the workflow for the four regions by using a nested PCR approach for the amplification and automated capillary gel electrophoresis for the analysis of the amplicons. Diversity was confirmed on a set of 95 *P. malariae* samples from Gabon, a malaria endemic country in sub-Saharan Africa. Dynamics and complexity of infections were analysed on samples from 21 asymptomatic individuals that were followed up for one week on a tight schedule.

We observed the best performance in terms of allelic diversity for *pmtrap*, with a multiplicity of infection of 1.61 and *pmmsp1* F2, with an expected heterozygosity (H_e) of 0.81. The performance of the microsatellite Pm02 was comparably well, with a mean MOI of 1.55 and a H_e of 0.68. The other microsatellite Pm09 on the other hand showed

the lowest genetic diversity, with the lowest mean MOI of 1.19 and the lowest H_e of 0.56. The agreement between the amplicon size analysed using automated capillary gel electrophoresis and the results from Sanger sequencing was very high, with maximum deviations of 21 bp for *pmmsp1* F2. Comparing the generated sequences to the database, we found three new *P. malariae* genotypes for *pmtrap* and *pmmsp1* F2 each among our central African samples. The combination of the markers *pmtrap* and *pmmsp1* F2 resulted in the best discriminative power, which is why we chose these two markers as a genotyping marker panel for *P. malariae.*

When applying the two markers to another set of 21 *P. malariae* positive individuals monitored over one week, we found a high level of consistency in their performance. These results highlight the complex nature and fluctuations of *P. malariae* infections in the asymptomatic study population from Gabon.

Publication 2

Whole genome analysis of two sympatric human *Mansonella*: *Mansonella perstans* and *Mansonella* sp "DEUX"

Rodi M*, Gross C*, Sandri TL, Berner L, Marcet-Houben M, Kocak E, Pogoda M, Casadei N, Köhler C, Kreidenweiss A, Agnandji ST, Gabaldón T, Ossowski S, Held J. *Whole genome analysis of two sympatric human Mansonella: Mansonella perstans and Mansonella sp "DEUX".* Front. Cell. Infect. Microbiol. 2023 Apr 14; 13:1159814. doi: 10.3389/fcimb.2023.1159814

*shared first authorship

In this work we generated the first whole genome of *Mansonella* sp "DEUX" and compared it to the whole genome of *M. perstans*. Based on this we investigated whether these are two sympatric species or if *Mansonella* sp "DEUX" is a genotype of *M. perstans.*

For this purpose, we screened individuals in the area of Fougamou, Gabon to identify *Mansonella* mono-infections and generated *de novo* assemblies on the corresponding samples. We reconstructed a phylogenetic tree from the concatenated alignments of the newly generated alignments, as well as 12 other nematode species. Also, divergence times of the two potential sympatric species *Mansonella* sp “DEUX” and *M. perstans* were calculated. Phylogenies based on *cox1* and 12S rDNA were generated from the respective mitogenomes and compared to published sequences originating from humans and great apes that were collected in Gabon and the neighbouring country Cameroon.

We successfully generated two whole genomes for *Mansonella* sp “DEUX” and one for *M. perstans* from mono-infected individuals, respectively. All three assemblies are similar in GC content and genome size to closely related filaria species. Phylogenetic analysis using annotated protein sequences support the classification of *Mansonella* sp “DEUX” and *M. perstans* into two different species. The genetic divergence we found is comparable to that of other established filaria species. The calculated divergence time for the two species is estimated at 778,000 years. To be able to better categorize the time span, it is useful to know that the closest *Brugia* species separated around 1.6 MyA ago and *Onchocerca* species separated around 1.8 MyA ago.

The results obtained from the mitochondrial marker genes *cox1* and 12S rDNA confirm the finding of two sympatric *Mansonella* species. Additionally, the analysis showed that published sequences obtained from great apes align to our *Mansonella* sp “DEUX” sequences. From this finding we conclude that *Mansonella* sp “DEUX” infections are not limited to the human host and that non-human primates serve as a zoonotic reservoir for this species.

Discussion

Infections with *Plasmodium malariae* and *Mansonella* sp “DEUX” are widely spread yet neglected. Prevalences are underestimated worldwide as infections are often not detected due to their asymptomatic course but also because symptoms are not properly described.

In recent years, both parasite species have benefited considerably from advances in molecular technologies. More sensitive and accurate approaches have shown that the true burden of *P. malariae* as well as *Mansonella* sp “DEUX” infections has been greatly underestimated [5,6,38]. While the focus for *P. malariae* within this thesis is set on genotyping within the species, the research on *Mansonella* infections is still at an earlier stage of systematic classification. It must first be determined whether *Mansonella* sp “DEUX” is a distinct species or a genotype of *Mansonella perstans*.

In order to address this, different molecular approaches were applied within the scope of this doctoral thesis. The results will be further discussed in this last part and the suitability of the two approaches will be evaluated.

Novel *P. malariae* genotyping marker panel

Plasmodium parasite population structures appear to vary among the different species. Several genotyping approaches have already been applied to samples from malaria endemic regions worldwide to better understand the epidemiology of the different *Plasmodium* spp. Insights into genetic diversity suggest that *P. vivax* populations are more diverse and less structured than those of *P. falciparum,* while *P. malariae* populations exhibit lower diversity compared to *P. falciparum* in the investigated populations [111,112]. These findings suggest that *P. vivax* is a more ancient species than *P. falciparum,* which might be less susceptible to evolutionary bottleneck events. It is further hypothesized that the differences in genetic diversity between *P. malariae* and *P. falciparum* are related to variations in their transmission efficiencies. To shed more light on the genetic diversity of *P. malariae* parasites, we established a *P. malariae* genotyping panel, comprising of only two highly discriminatory markers.

We assessed four marker regions regarding their potential to display genetic diversity: *P. malariae* merozoite surface protein 1 gene fragment 2 (*pmmsp1* F2), *P. malariae*

thrombospondin-related anonymous protein (*pmtrap*), as well as the two microsatellite regions Pm02 and Pm09. The genetic diversity of the four potential markers was investigated in 95 samples from *P. malariae* infected individuals from Gabon. The highest diversity in terms of highest mean multiplicity of infection (MOI) was displayed by *pmtrap* (1.61), and by *pmmsp1* F2 in terms of expected heterozygosity (0.81). These results are in line with published data on *P. malariae* genotyping of other loci [96,97]. While *pmtrap* can very well reflect the multiplicity of *P. malariae* infections, *pmmsp1* F2 can detect a large range of different *P. malariae* genotypes, which in combination results in a powerful marker panel.

When using size polymorphic molecular markers in general, the determination of the exact amplicon length is crucial. Traditionally agarose gel electrophoresis is used in many genotyping approaches. However, the low resolution of the method can lead to an underestimation of the true multiplicity of an infection. The two previously published marker panels for *P. malariae* are based on microsatellites containing repeat units between 3 – 17 bp [96,97]. An underestimation of the genotype diversity is particularly pronounced for microsatellites with a small repeat unit of maximum 6 bp. The low resolution can be overcome by using more precise methods, which include automated capillary gel electrophoresis, that provides a resolution of up to 3 bp according to the manufacturer. Therefore, we selected this method for our genotyping approach and validated it by additionally sequencing 10 samples infected with one genotype only. The consistency between the amplicon sizes measured using automated capillary gel electrophoresis and sequencing varied between the four markers. It ranged from a maximum deviation of 6 bp using *pmtrap* to 21 bp using *pmmsp1* F2. The higher discrepancy can be explained by the lower resolution (20 bp) of the analysis method that had to be used for *pmmsp1* F2, since the allele size range was bigger (92 – 694 bp) than for the other three marker regions. Findings from genotyping of other pathogens like *Mycobacterium tuberculosis* strains showed an increasing inaccuracy when sizing longer amplicons which is in line with our findings [113]. Yet, the mean deviation for the other three markers ranged between 3.6 – 4.3 bp only, which lies within the resolution of the analysis method (2-5 bp). Our results are in line with findings from genotyping of *P. vivax* microsatellites, where small deviations between automated capillary gel electrophoresis and Sanger sequencing were seen as well [114].

Overall, sequencing confirmed that variations in amplicon lengths resulted from differences in the number of repeat units for Pm02, Pm09 and *pmtrap.* The repeat units correlate to the motifs that were already reported [96,102], yet two new motifs of 17 bp and 8 bp were found for Pm09 respectively, we well as another 6 bp motif for Pm02. For *pmmsp1* F2 the differences originate from sequences of differing sizes without a clear repeat unit motif, as reported previously [97].

Another limitation of size polymorphic markers is the preferential amplification of smaller amplicons in mixed genotype infections. This is a general PCR effect and has also been described in *P. falciparum* genotyping of the *msp1* locus before, resulting in the underestimation of the multiplicity [115]. Indeed, our initial results suggest a similar bias for the *pmmsp1* F2 marker, as we observed a higher frequency of small amplicons and an overall lower MOI in comparison to the other three markers. However, a more detailed analysis, did not reveal any evidence of preferential amplification for the *pmmsp1* F2 locus. We tested the hypothesis using only two mock samples, that we prepared from original samples that were mono-infected with a single genotype only, either small or big. This might not be sufficient to find the effect in the *pmmsp1* F2 marker and further investigation is required.

Overall, the genetic diversity was high in our investigated *P. malariae* parasite population from Gabon. Samples were derived from a cross-sectional study, showing that individuals living in the study area of Lambaréné, Gabon, are exposed to a large variety of *P. malariae* genotypes. A comparison of our dataset to other *P. malariae* studies is challenging, as information on the genetic diversity of *P. malariae* is generally scarce. In a study from 2007, a first *P. malariae* genotyping marker set based on microsatellites was published. They reported a lower number of *P. malariae* genotypes in samples from symptomatic individuals from Thailand, a country with low endemicity, than in samples from asymptomatic individuals from Malawi, which in contrast is known to be a high malaria transmission area [96]. The same genotyping markers found no change in the MOI with increased transmission, when comparing two different sample sets from Malawi with seasonal and perennial transmission, respectively [111]. A second *P. malariae* marker set, based on microsatellites as well as the *pmmsp1* gene, screening symptomatic individuals from Myanmar, a country with low transmission, resulted in comparable diversity [97].

When comparing the diversity of these previously published sample sets with our data, it must be considered that the differing parameters between the sample sets might bias the results and lead to false interpretations of the influence of MOI on transmission dynamics in *P. malariae*. Both dataset that we investigated are from the same geographical region (Lambaréné and surrounding villages, Gabon). The first dataset is from a cross-sectional study, consisting of asymptomatic as well as mildly symptomatic individuals, while the second sample set consists of a highly preselected population with microscopically detectable *P. falciparum* infections, but no reported symptoms. The high number of circulating genotypes in the second sample set is surprisingly high and in line with the findings from Bruce *et al.* from his dataset from Malawi [96]. Other influencing factors, such as co-infecting *Plasmodium* species, the sampling period or the sample size must also be considered. However, a larger database, as available for *P. falciparum* and *P. vivax*, could help to identify potential trends and correlations. Therefore, it is important to generate further *P. malariae* genotyping data from different regions.

Studies on *P. falciparum* and *P. vivax* suggest that the MOI can serve as an indicator for the transmission intensity, whereby the MOI is reported to be higher in areas with high malaria transmission compared to those with low transmission [116-118]. However, the influence of the MOI on the clinical status of infected *P. falciparum* individuals remains inconsistent. While some studies report a higher MOI in severe malaria cases [119-121], others suggest that a high MOI might have a protective effect and is associated with asymptomatic cases [122-124]. Yet, these observations regarding the relationship between MOI and parameters such as transmission intensity and clinical status in *P. falciparum* might not apply directly to *P. malariae* because of differences in their biology and epidemiology.

We further evaluated to what extent the genetic diversity, that we detected in the investigated *P. malariae* population, can consistently be observed, using a sample set where participants were followed up tightly over one week. *P. malariae* genotypes were identified with high consistency throughout the follow-up period. From a methodological point of view the results indicate a very high effectiveness of our chosen marker panel. In addition, we can derive various biological aspects of *P. malariae* from these results.

Firstly, we see a high level of genotype dynamics overall. While some show many fluctuations throughout the sampling period, others are constantly detected at all

timepoints. So far, no other data is available on the dynamics of *P. malariae* genotypes in the human host. The investigated individuals are parasitaemic but asymptomatic. Comparable studies have already been performed on *P. falciparum* using the markers *pfmsp1*, *pfmsp2* and *pfglurp*, where asymptomatic populations showed similar hourly and daily dynamics of certain genotypes [125-127].

Due to the tight follow-up period, our sample set is well suited to potentially detect the 72 h periodicity of *P. malariae* as described in literature [13,128]. This periodicity is the reason for the clinical term malaria quartana. However, data on the 72 h paroxysms remain inconclusive. In our study, we did not observe any pattern in the appearance of genotypes. Our results are in line with daily records of *P. malariae* parasitaemia, which also showed no peaks in parasite density every third day – corresponding to the 72 h cycle [129]. Additional data is needed to further clarify our understanding of the this periodicity; for example, whether it only exists in new infections that are synchronous, and is absent in chronic infections.

Finally, our results show that relying on a single sampling timepoint for genotyping *P. malariae* may be insufficient. Though we detected different genotypes with a high consistency of both markers over time, we also observed fluctuations in minor genotypes within the same individuals. This may also partially reflect methodological factors, such as amplicon competition in PCR or sensitivity issues. Yet, this can lead to misinterpretation of genotype numbers and could impact especially drug efficacy studies. Our results reinforce the importance of serial sampling, as highlighted already in several studies on *P. falciparum* genotyping [130-132].

Two distinct and sympatric *Mansonella* species

The diversity of different *Mansonella* species has not yet been well investigated, though the parasites are highly prevalent throughout sub-Saharan Africa and South America. Therefore, we have generated whole genomes from *Mansonella perstans* and *Mansonella* sp “DEUX” from microfilariae from infected individuals from Gabon. Our findings will support further epidemiological studies on filariae since our data supports the hypothesis of *Mansonella* sp “DEUX” being a distinct species, providing reference genomes for both species.

The *de novo* assemblies for both species are of high quality and nearly complete, which is indicated by the high coverage, and N50 values and BUSCO scores above 90%. Both metrics are indicators of the quality and completeness of whole genome assemblies. Also, our assemblies are comparable in size and GC (guanine-cytosine) content to other human filarial species. Phylogenetic analysis which is based on the concatenated protein alignments and includes 12 other nematode species, suggests that *Mansonella s*p "DEUX" and *M. perstans* are two distinct species that occur in sympatry. Further evolutionary analysis indicate that the species have diverged 778,000 years ago. Additionally, our results are in line with the current classification of filarial nematodes into five strongly supported clades [103]. This classification groups the different *Mansonella* species together with the families of *Loa, Wuchereria* and *Brugia* into the so-called ONC5 clade. As expected, the whole genomes of *M. perstans* and *Mansonella* sp "DEUX" that we generated in our study also fall within the ONC5 clade.

In general, speciation is an evolutionary process whereby new species evolve from or within existing populations [133] . Different mechanisms can lead to such events, yet usually resulting in the creation of genetic barriers between organisms, leading to the inability to undergo genetic recombination. When comparing the whole genomes from *M. perstans* and *Mansonella* sp "DEUX" we still see sites of recombination. A potential biological explanation involves that adult worms from the two species can still occasionally recombine, meaning that the speciation event is still ongoing. Another more plausible explanation for our findings lies within our methodology. The recombination sites might result from co-infecting *Mansonella* species in our samples that we detected at low coverage only after sequencing. The initial diagnose was based on qPCR which is less sensitive. Our findings highlight the fact that further samples and data are needed, to get a better understanding of the true *Mansonella* species diversity.

We additionally investigated the mitogenomes from *M. perstans* and *Mansonella* sp "DEUX", which cluster as expected within the recently published mitogenomes from *M. perstans* and *M. ozzardi* [134,135]. In general, particular loci within the mitogenome are popular targets for systematic and population genomic studies. The conserved nature of the mitogenome among different filarial species has supported the differentiation of filarial species as well as the determination of the phylogeny of the *Onchocercidae* family [103,135-137]. We compared the12S rRNA and *cox1* loci within our generated

mitogenomes to sequences in the database from other members of the *Onchocercidae* family. We observe two *Mansonella* species clusters for both loci, they either correspond to *M. perstans* or *Mansonella* sp “DEUX”. The same two clusters based on *cox1* were also found by Gaillard et al., yet not based on 12S rRNA [104]. The two clusters reveal further information regarding the host specificity of *Mansonella* species. Our data suggests that apart from humans, *Mansonella* sp “DEUX” is also infecting great apes. Our *Mansonella* sp “DEUX” sequences align to unspecified *Mansonella* sequences that were obtained from non-human primates (NHPs) originating also from Gabon or from the neighbouring country Cameroon [104]. NHPs are known to act as a zoonotic reservoir for the other three human *Mansonella* species, namely *M. perstans, M. streptocerca* and *M. ozzardi* [104,138-142]. Given this, it is not surprising that the same applies for *Mansonella* sp “DEUX”.

Additionally, *M. rodhaini* a filarial species from chimpanzees and bonobos has been described based on morphology in skin biopsies from Gabonese individuals [105]. However, in contrast to *M. rodhaini* as well as *M. streptocerca, Mansonella* sp “DEUX” is a blood-dwelling species. Our phylogenetic comparisons do not indicate that *Mansonella* sp “DEUX” maps to other skin-dwelling *M. streptocerca.*

Other *Mansonella* species that are known to infect NHPs in Africa include *M. vanhoofi, M. gorilla, M. leopoldi* and *M. loopensis* [106-109]. So far, these species have been described based on morphology only. However, for *Mansonella* sp “DEUX” only sequencing data is available and no in-depth morphological description has been made so far. Therefore, it still needs to be determined, if *Mansonella* sp “DEUX” resembles one of the NHPs infecting species.

All blood-dwelling *Mansonella* species that infect humans were shown to carry endosymbiotic *Wolbachia* from the supergroup F [38,66,70,71]. We can further support these findings, as we could extract *Wolbachia* reads among all sequenced samples. These result point to the fact that infections with *Mansonella* sp “DEUX” can probably also be treated with antibiotics. One potential agent is doxycycline, which has already been shown to be active in different clinical trials against *M. perstans* [67,68]. Similar to *Mansonella* sp “DEUX”, no clinical trials have yet been conducted for *M. ozzardi*. Such data are needed now in order to further push the development of standardized control strategies for mansonellosis.

We could not assemble high quality whole genomes for *Wolbachia* from our reads, because the coverage was too low. A comparison of *Wolbachia* from the two species

could provide further information on the separation of the two species. Together with the recently published *Wolbachia* genomes from *M. perstans* and *M. ozzardi* this will add valuable insights into the evolution of the *Mansonella* family [71].

We detected an additional mitogenome from a *Mansonella* species that is so far not described. It does not map to any published sequences from the database. Therefore, it might present a new species that we named *"Mansonella cermeli".* Read coverage was low, which is why we could only retrieve the mitogenome but no separate whole genome. Further screening of samples from the same geographical region for "*M. cermeli"* is necessary to determine whether it is a common species that infects humans.

Our results demonstrate that *Mansonella* sp "DEUX" is a distinct species within the genus *Mansonella*, coexisting in sympatry with *M. perstans.* The species infects both humans and great apes, indicating a broad host range. The true species diversity may be higher than currently assumed, underscoring the need for further research to address the many unresolved questions regarding this genus. Thorough knowledge of the different species is essential for effective control and containment. Even though the clinical manifestation and the burden of disease associated with *Mansonella* infections remain poorly characterized, infections may have an impact on the host beyond that. It is known that filaria can modulate the host's immune system [143-145] and there is evidence suggesting that both *M. perstans* and *M. ozzardi* do so as well [146-148]. Consequently, infections with these parasites may influence the host's immune response to non-filarial infections or antigens, including vaccines. The availability of our whole genome data will facilitate further investigations, advancing our understanding of *Mansonella* species.

Suitability of the applied molecular techniques to explore the underlying research questions

To achieve a better genetic characterization of the two neglected parasites *P. malariae* and *Mansonella* sp "DEUX", we applied different molecular techniques. For analysing the genotype diversity in *P. malariae* we used a genotyping approach based on two length polymorphic marker genes (*pmmsp1* F2 and *pmtrap).* In contrast, to investigate

the species diversity in *Mansonella* we applied a whole genome sequencing approach. Both techniques proved to be effective in answering the respective research questions.

Our straightforward *P. malariae* genotyping panel remains highly valuable. The panel's simplicity makes it particularly suitable for the application in endemic settings, where technical capacities are often still limited. However, it is essential to develop methodologies that can be implemented directly on-site in the affected countries, as processing samples elsewhere delays data availability. By using a minimal number of two markers, our approach reduces the workload, costs and time required to generate results, making it an efficient option for the application in endemic settings. Moreover, the implementation of the panels on-site will also help to build local capacity and strengthen expertise in these regions. It is necessary to collect data on genotype diversity from other regions across endemic countries. Also, when further applied to longitudinal samples collected over several months or years, the markers can provide important insights into the longevity of *P. malariae* infections. This will add further knowledge on the infection dynamics and epidemiology of this neglected parasite species.

Currently, amplicon sequencing is a highly discussed methodology in the field of *Plasmodium* species genotyping, offering an increased sensitivity in detecting minor clones. Species with low parasite densities, such as *P. malariae,* could greatly benefit from such approaches [80,81]. The development towards amplicon sequencing is promising and represents a potential next step in the progression of further *P. malariae* genotyping tools.

Our results on *Mansonella* sp "DEUX" highlight the power of whole genome sequencing in phylogenetic studies. This approach proved to be a suitable tool to clarify the taxonomic status of *Mansonella* sp "DEUX" within the *Mansonella* genus. Yet, our findings also demonstrate the challenges of sequencing samples from human whole blood as well as co-infections of closely related species. In our study we used 5 µM filter to enrich the microfilariae relative to human blood cells. Nevertheless, for further blood sampling, alternative techniques such as leucocyte depletion might offer a more effective strategy to reduce the abundance of human DNA in the samples. The challenge of co-infected samples with closely related species can be resolved by cleaning raw sequencing data prior to genome assembly. The reference genomes we

have generated for *M. perstans* and *Mansonella* sp “DEUX”, along with those from other colleagues, will significantly facilitate this process [110].

The conclusions drawn here open up new directions for further investigating *Mansonella* sp “DEUX”. A next step would be to explore the prevalence of this species in other regions, as it has so far only been confirmed in humans in Gabon and in great apes in the neighbouring country Cameroon. It is very likely that the parasite is not restricted to those regions. Additionally, employing the technique used in this thesis for *P. malariae* - the development of genotyping markers based on polymorphic regions – could provide deeper insights into *Mansonella* sp “DEUX” and the diversity of genotypes within the species.

Conclusion

The improvements in the development of molecular techniques have contributed to a better understanding of the true burden of many neglected parasite species. Among those are the malaria parasite *P. malariae* as well as the filarial nematode *Mansonella sp "DEUX".* The results presented within this dissertation contribute to the current understanding of *P. malariae* genotype diversity as well as the true species diversity of *Mansonella.*

Consequently, the *P. malariae* genotyping panel should now be further applied to samples from other countries and to samples from longitudinal studies collected over several months. This may add important information on genotype distribution, as well as infection dynamics and longevity of infections. A better understanding of these factors will contribute to the management of this species in terms of transmission intensities or treatment schedules.

The generation of the whole genomes from the two *Mansonella* species and their phylogenetic analysis opens an important path on further investigations of *Mansonella* parasites. In addition to the evidence that *Mansonella* sp "DEUX" is a distinct species, we could show that the species is also infecting great apes and carries the endosymbiont *Wolbachia.* This further helps to understand transmission chains and raise awareness of zoonotic reservoirs, also it paves the way for possible treatment options for this parasite.

Using different molecular techniques, we have made a major step towards an end of the neglect on parasitic infections with *P. malariae* and *Mansonella* sp "DEUX".

References

1 World Health Organization. World Malaria Report 2023. (2023).

2 Snounou, G., Sharp, P. M. & Culleton, R. The two parasite species formerly known as Plasmodium ovale. *Trends Parasitol* **40**, 21-27 (2024). https://doi.org/10.1016/j.pt.2023.11.004

3 Miezan, A. J. S. *et al.* Molecular epidemiology of non-falciparum Plasmodium infections in three different areas of the Ivory Coast. *Malar J* **22**, 211 (2023). https://doi.org/10.1186/s12936-023-04639-7

4 Nundu, S. S. *et al.* Malaria parasite species composition of Plasmodium infections among asymptomatic and symptomatic school-age children in rural and urban areas of Kinshasa, Democratic Republic of Congo. *Malar J* **20**, 389 (2021). https://doi.org/10.1186/s12936-021-03919-4

5 Woldearegai, T. G. *et al.* Characterization of Plasmodium infections among inhabitants of rural areas in Gabon. *Sci Rep* **9**, 9784 (2019). https://doi.org/10.1038/s41598-019-46194-9

6 Yman, V. *et al.* Persistent transmission of Plasmodium malariae and Plasmodium ovale species in an area of declining Plasmodium falciparum transmission in eastern Tanzania. *PLOS Neglected Tropical Diseases* **13**, e0007414 (2019). https://doi.org/10.1371/journal.pntd.0007414

7 Nguiffo-Nguete, D. *et al.* Plasmodium malariae contributes to high levels of malaria transmission in a forest–savannah transition area in Cameroon. *Parasites & Vectors* **16** (2023). https://doi.org/10.1186/s13071-022-05635-7

8 Nainggolan, I. R. A. *et al.* The presence of Plasmodium malariae and Plasmodium knowlesi in near malaria elimination setting in western Indonesia. *Malaria Journal* **21** (2022). https://doi.org/10.1186/s12936-022-04335-y

9 Zhou, M. *et al.* High prevalence of Plasmodium malariae and Plasmodium ovale in malaria patients along the Thai-Myanmar border, as revealed by acridine orange staining and PCR-based diagnoses. *Trop Med Int Health* **3**, 304-312 (1998). https://doi.org/10.1046/j.1365-3156.1998.00223.x

10 Mbama Ntabi, J. D. *et al.* Prevalence of non-Plasmodium falciparum species in southern districts of Brazzaville in The Republic of the Congo. *Parasites & Vectors* **15** (2022). https://doi.org/10.1186/s13071-022-05312-9

11 Agonhossou, R. *et al.* Surveillance of Plasmodium malariae infection among inhabitants of rural areas in Ouidah–Kpomasse–Tori Bossito health district, Benin. *Parasitology Research* **121**, 275-286 (2022). https://doi.org/10.1007/s00436-021-07398-z

12 Camargo-Ayala, P. A. *et al.* High Plasmodium malariae Prevalence in an Endemic Area of the Colombian Amazon Region. *PLoS One* **11**, e0159968 (2016). https://doi.org/10.1371/journal.pone.0159968

13 Garnham, P. C. C. *Malaria Parasites and other Haemosporidia*. (1966).

14 Gilles, H. M. & Warrell, D. A. *Bruce-Chwatt's essential malariology*. (1993).

15 Ehrich, J. H. H. & Eke, F. U. Malaria-induced renal damage: facts and myths. *Pediatric Nephrology* **22**, 626-637 (2007). https://doi.org/10.1007/s00467-006-0332-y

16 Langford, S. *et al.* Plasmodium malariae Infection Associated with a High Burden of Anemia: A Hospital-Based Surveillance Study. *PLOS Neglected Tropical Diseases* **9**, e0004195 (2015). https://doi.org/10.1371/journal.pntd.0004195

17 Hedelius, R., Fletcher, J. J., Glass, W. F., Susanti, A. I. & Maguire, J. D. Nephrotic syndrome and unrecognized Plasmodium malariae infection in a US Navy sailor 14 years after departing Nigeria. *J Travel Med* **18**, 288-291 (2011). https://doi.org/10.1111/j.1708-8305.2011.00526.x

18 Vinetz, J. M., Li, J., McCutchan, T. F. & Kaslow, D. C. Plasmodium malariae infection in an asymptomatic 74-year-old Greek woman with splenomegaly. *N Engl J Med* **338**, 367-371 (1998). https://doi.org/10.1056/NEJM199802053380605

19 Wu, J. *et al.* Poor performance of malaria rapid diagnostic tests for the detection of Plasmodium malariae among returned international travellers in China. *Malar J* **22**, 163 (2023). https://doi.org/10.1186/s12936-023-04596-1

20 Yerlikaya, S., Campillo, A. & Gonzalez, I. J. A Systematic Review: Performance of Rapid Diagnostic Tests for the Detection of Plasmodium knowlesi, Plasmodium malariae, and Plasmodium ovale Monoinfections in Human Blood. *J Infect Dis* **218**, 265-276 (2018). https://doi.org/10.1093/infdis/jiy150

21 Groger, M. *et al.* Prospective Clinical Trial Assessing Species-Specific Efficacy of Artemether-Lumefantrine for the Treatment of Plasmodium malariae, Plasmodium ovale, and Mixed Plasmodium Malaria in Gabon. *Antimicrobial Agents and Chemotherapy* **62**, AAC.01758-01717 (2018). https://doi.org/10.1128/aac.01758-17

22 Farrugia, C. *et al.* Cytochrome b gene quantitative PCR for diagnosing Plasmodium falciparum infection in travelers. *J Clin Microbiol* **49**, 2191-2195 (2011). https://doi.org/10.1128/JCM.02156-10

23 Bourgeois, N. *et al.* Comparison of three real-time PCR methods with blood smears and rapid diagnostic test in Plasmodium sp. infection. *Clin Microbiol Infect* **16**, 1305-1311 (2010). https://doi.org/10.1111/j.1469-0691.2009.02933.x

24 Dos Santos, E. H. *et al.* A new Real Time PCR with species-specific primers from Plasmodium malariae/P. brasilianum mitochondrial cytochrome b gene. *Parasitol Int* **76**, 102069 (2020). https://doi.org/10.1016/j.parint.2020.102069

25 Xu, W. *et al.* SYBR Green real-time PCR-RFLP assay targeting the plasmodium cytochrome B gene--a highly sensitive molecular tool for malaria parasite detection and species determination. *PLoS One* **10**, e0120210 (2015). https://doi.org/10.1371/journal.pone.0120210

26 World Health Organization. WHO guidelines for malaria. (2023).

27 Betson, M., Clifford, S., Stanton, M., Kabatereine, N. B. & Stothard, J. R. Emergence of Nonfalciparum Plasmodium Infection Despite Regular Artemisinin Combination Therapy in an 18-Month Longitudinal Study of Ugandan Children and Their Mothers. *J Infect Dis* **217**, 1099-1109 (2018). https://doi.org/10.1093/infdis/jix686

28 Dinko, B., Oguike, M. C., Larbi, J. A., Bousema, T. & Sutherland, C. J. Persistent detection of Plasmodium falciparum, P. malariae, P. ovale curtisi and P. ovale wallikeri after ACT treatment of asymptomatic Ghanaian school-children. *Int J Parasitol Drugs Drug Resist* **3**, 45-50 (2013). https://doi.org/10.1016/j.ijpddr.2013.01.001

29 Teo, B. H. *et al.* Delayed Onset of Symptoms and Atovaquone-Proguanil Chemoprophylaxis Breakthrough by Plasmodium malariae in the Absence of Mutation at Codon 268 of pmcytb. *PLoS Negl Trop Dis* **9**, e0004068 (2015). https://doi.org/10.1371/journal.pntd.0004068

30 Groger, M. *et al.* Effectiveness of pyronaridine-artesunate against Plasmodium malariae, Plasmodium ovale spp, and mixed-Plasmodium infections: a post-

hoc analysis of the CANTAM-Pyramax trial. *Lancet Microbe* **3**, e598-e605 (2022). https://doi.org/10.1016/S2666-5247(22)00092-1

31 Mombo-Ngoma, G. *et al.* Prospective evaluation of artemether-lumefantrine for the treatment of non-falciparum and mixed-species malaria in Gabon. *Malar J* **11**, 120 (2012). https://doi.org/10.1186/1475-2875-11-120

32 van Schalkwyk, D. A., Moon, R. W., Duffey, M., Leroy, D. & Sutherland, C. J. Ex vivo susceptibility to new antimalarial agents differs among human-infecting Plasmodium species. *Int J Parasitol Drugs Drug Resist* **17**, 5-11 (2021). https://doi.org/10.1016/j.ijpddr.2021.07.002

33 Rutledge, G. G. *et al.* Plasmodium malariae and P. ovale genomes provide insights into malaria parasite evolution. *Nature* **542**, 101-104 (2017). https://doi.org/10.1038/nature21038

34 Ibrahim, A. *et al.* Selective whole genome amplification of Plasmodium malariae DNA from clinical samples reveals insights into population structure. *Sci Rep* **10**, 10832 (2020). https://doi.org/10.1038/s41598-020-67568-4

35 Plenderleith, L. J. *et al.* Zoonotic origin of the human malaria parasite Plasmodium malariae from African apes. *Nature Communications* **13** (2022). https://doi.org/10.1038/s41467-022-29306-4

36 Simonsen, P. E., Onapa, A. W. & Asio, S. M. Mansonella perstans filariasis in Africa. *Acta Trop* **120 Suppl 1**, S109-120 (2011). https://doi.org/10.1016/j.actatropica.2010.01.014

37 Tamarozzi, F. *et al.* A large case series of travel-related Mansonella perstans (vector-borne filarial nematode): a TropNet study in Europe. *J Travel Med* (2022). https://doi.org/10.1093/jtm/taac048

38 Sandri, T. L. *et al.* Molecular Epidemiology of Mansonella Species in Gabon. *J Infect Dis* **223**, 287-296 (2021). https://doi.org/10.1093/infdis/jiaa670

39 Bassene, H. *et al.* High Prevalence of Mansonella perstans Filariasis in Rural Senegal. *Am J Trop Med Hyg* **93**, 601-606 (2015). https://doi.org/10.4269/ajtmh.15-0051

40 Debrah, L. B. *et al.* Epidemiology of Mansonella perstans in the middle belt of Ghana. *Parasit Vectors* **10**, 15 (2017). https://doi.org/10.1186/s13071-016-1960-0

41 Tavares da Silva, L. B. *et al.* Molecular Verification of New World Mansonella perstans Parasitemias. *Emerg Infect Dis* **23**, 545-547 (2017). https://doi.org/10.3201/eid2303.161159

42 Calvopina, M., Chiluisa-Guacho, C., Toapanta, A., Fonseca, D. & Villacres, I. High Prevalence of Mansonella ozzardi Infection in the Amazon Region, Ecuador. *Emerg Infect Dis* **25**, 2081-2083 (2019). https://doi.org/10.3201/eid2511.181964

43 Kozek, W. J. & Raccurt, C. Ultrastructure of Mansonella ozzardi microfilaria, with a comparison of the South American (simuliid-transmitted) and the Caribbean (culicoid-transmitted) forms. *Tropenmed Parasitol* **34**, 38-53 (1983).

44 Fischer, P., Bamuhiiga, J. & Büttner, D. W. Occurrence and diagnosis of Mansonella streptocerca in Uganda. *Acta Trop* **63**, 43-55 (1997). https://doi.org/10.1016/s0001-706x(96)00607-9

45 Ta, T. H. *et al.* Geographical distribution and species identification of human filariasis and onchocerciasis in Bioko Island, Equatorial Guinea. *Acta Trop* **180**, 12-17 (2018). https://doi.org/10.1016/j.actatropica.2017.12.030

46 Anosike, J. C. Studies on filariasis in Bauchi State, Nigeria. V. The distribution and prevalence of mansonellosis with special reference to clinical signs. *Appl Parasitol* **35**, 189-192 (1994).

47 Ebai, R. *et al.* Species of the Rain Forest Belt of the Littoral Region of Cameroon: Their Incrimination in the Transmission of. *Pathogens* **13** (2024). https://doi.org/10.3390/pathogens13020146
48 Debrah, L. B. *et al.* Diversity of Culicoides in the middle belt of Ghana with Implications on the transmission of Mansonella perstans; a molecular approach. *Parasit Vectors* **17**, 123 (2024). https://doi.org/10.1186/s13071-024-06179-8
49 Linley, J. R., Hoch, A. L. & Pinheiro, F. P. Biting midges (Diptera: Ceratopogonidae) and human health. *J Med Entomol* **20**, 347-364 (1983). https://doi.org/10.1093/jmedent/20.4.347
50 DUKE, B. O. The intake of the microfilariae of Acanthocheilonema perstans by Culicoides grahamii and C. inornatipennis, and their subsequent development. *Ann Trop Med Parasitol* **50**, 32-38 (1956). https://doi.org/10.1080/00034983.1956.11685736
51 Shelley, A. J. & Coscarón, S. Simuliid blackflies (Diptera: Simuliidae) and ceratopogonid midges (Diptera: Ceratopogonidae) as vectors of Mansonella ozzardi (Nematoda: Onchocercidae) in northern Argentina. *Mem Inst Oswaldo Cruz* **96**, 451-458 (2001). https://doi.org/10.1590/s0074-02762001000400003
52 Amambo, G. N. *et al.* Application of loop mediated isothermal amplification (LAMP) assays for the detection of. *Front Trop Dis* **3**, 1016176 (2023). https://doi.org/10.3389/fitd.2022.1016176
53 Tang, T. H. *et al.* Nested PCR to detect and distinguish the sympatric filarial species Onchocerca volvulus, Mansonella ozzardi and Mansonella perstans in the Amazon Region. *Mem Inst Oswaldo Cruz* **105**, 823-828 (2010). https://doi.org/10.1590/s0074-02762010000600016
54 Ta-Tang, T. H., Crainey, J. L., Post, R. J., Luz, S. L. & Rubio, J. M. Mansonellosis: current perspectives. *Res Rep Trop Med* **9**, 9-24 (2018). https://doi.org/10.2147/RRTM.S125750
55 Fischer, P., Tukesiga, E. & Büttner, D. W. Long-term suppression of Mansonella streptocerca microfilariae after treatment with ivermectin. *J Infect Dis* **180**, 1403-1405 (1999). https://doi.org/10.1086/315014
56 Bregani, E. R., Rovellini, A., Mbaïdoum, N. & Magnini, M. G. Comparison of different anthelminthic drug regimens against Mansonella perstans filariasis. *Trans R Soc Trop Med Hyg* **100**, 458-463 (2006). https://doi.org/10.1016/j.trstmh.2005.07.009
57 Basano, S. e. A. *et al.* Sustained clearance of Mansonella ozzardi infection after treatment with ivermectin in the Brazilian Amazon. *Am J Trop Med Hyg* **90**, 1170-1175 (2014). https://doi.org/10.4269/ajtmh.13-0410
58 Mourembou, G. *et al.* Mansonella, including a Potential New Species, as Common Parasites in Children in Gabon. *PLoS Negl Trop Dis* **9**, e0004155 (2015). https://doi.org/10.1371/journal.pntd.0004155
59 Kozek, W. J. & Marroquin, H. F. Intracytoplasmic bacteria in Onchocerca volvulus. *Am J Trop Med Hyg* **26**, 663-678 (1977). https://doi.org/10.4269/ajtmh.1977.26.663
60 Chung, M., Small, S. T., Serre, D., Zimmerman, P. A. & Dunning Hotopp, J. C. Draft genome sequence of the Wolbachia endosymbiont of Wuchereria bancrofti wWb. *Pathog Dis* **75** (2017). https://doi.org/10.1093/femspd/ftx115
61 Foster, J. *et al.* The Wolbachia genome of Brugia malayi: endosymbiont evolution within a human pathogenic nematode. *PLoS Biol* **3**, e121 (2005). https://doi.org/10.1371/journal.pbio.0030121

62 Hoerauf, A. *et al.* Tetracycline therapy targets intracellular bacteria in the filarial nematode Litomosoides sigmodontis and results in filarial infertility. *J Clin Invest* **103**, 11-18 (1999). https://doi.org/10.1172/JCI4768
63 Hoerauf, A. *et al.* Doxycycline as a novel strategy against bancroftian filariasis-depletion of Wolbachia endosymbionts from Wuchereria bancrofti and stop of microfilaria production. *Med Microbiol Immunol* **192**, 211-216 (2003). https://doi.org/10.1007/s00430-002-0174-6
64 Hoerauf, A. *et al.* Doxycycline in the treatment of human onchocerciasis: Kinetics of Wolbachia endobacteria reduction and of inhibition of embryogenesis in female Onchocerca worms. *Microbes Infect* **5**, 261-273 (2003). https://doi.org/10.1016/s1286-4579(03)00026-1
65 Gehringer, C. *et al.* Molecular evidence of Wolbachia endosymbiosis in Mansonella perstans in Gabon, Central Africa. *J Infect Dis* **210**, 1633-1638 (2014). https://doi.org/10.1093/infdis/jiu320
66 Keiser, P. B. *et al.* Molecular identification of Wolbachia from the filarial nematode Mansonella perstans. *Mol Biochem Parasitol* **160**, 123-128 (2008). https://doi.org/10.1016/j.molbiopara.2008.04.012
67 Batsa Debrah, L. *et al.* The Efficacy of Doxycycline Treatment on. *Am J Trop Med Hyg* **101**, 84-92 (2019). https://doi.org/10.4269/ajtmh.18-0491
68 Coulibaly, Y. I. *et al.* A randomized trial of doxycycline for Mansonella perstans infection. *N Engl J Med* **361**, 1448-1458 (2009). https://doi.org/10.1056/NEJMoa0900863
69 Raoult, D. Doxycycline for Mansonella perstans infection. *N Engl J Med* **362**, 272; author reply 272-273 (2010). https://doi.org/10.1056/NEJMc0910675
70 Casiraghi, M., Favia, G., Cancrini, G., Bartoloni, A. & Bandi, C. Molecular identification of Wolbachia from the filarial nematode Mansonella ozzardi. *Parasitol Res* **87**, 417-420 (2001). https://doi.org/10.1007/s004360000368
71 Sinha, A. *et al.* Multiple lineages of nematode-Wolbachia symbiosis in supergroup F and convergent loss of bacterioferritin in filarial Wolbachia. *Genome Biol Evol* **15** (2023). https://doi.org/10.1093/gbe/evad073
72 Bandi, C., Anderson, T. J., Genchi, C. & Blaxter, M. L. Phylogeny of Wolbachia in filarial nematodes. *Proc Biol Sci* **265**, 2407-2413 (1998). https://doi.org/10.1098/rspb.1998.0591
73 Lefoulon, E. *et al.* Breakdown of coevolution between symbiotic bacteria Wolbachia and their filarial hosts. *PeerJ* **4**, e1840 (2016). https://doi.org/10.7717/peerj.1840
74 Mayr, E. Populations Species and Evolution. (Harvard University Press, 1970).
75 Crainey, J. L. *et al.* Deep Sequencing Reveals Occult Mansonellosis Coinfections in Residents From the Brazilian Amazon Village of São Gabriel da Cachoeira. *Clin Infect Dis* **71**, 1990-1993 (2020). https://doi.org/10.1093/cid/ciaa082
76 Snounou, G. *et al.* High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol Biochem Parasitol* **61**, 315-320 (1993). https://doi.org/10.1016/0166-6851(93)90077-b
77 Mordmüller, B. *et al.* Sterile protection against human malaria by chemoattenuated PfSPZ vaccine. *Nature* **542**, 445-449 (2017). https://doi.org/10.1038/nature21060
78 Baniecki, M. L. *et al.* Development of a single nucleotide polymorphism barcode to genotype Plasmodium vivax infections. *PLoS Negl Trop Dis* **9**, e0003539 (2015). https://doi.org/10.1371/journal.pntd.0003539

79 Daniels, R. *et al.* A general SNP-based molecular barcode for Plasmodium falciparum identification and tracking. *Malar J* **7**, 223 (2008). https://doi.org/10.1186/1475-2875-7-223
80 Lerch, A. *et al.* Development of amplicon deep sequencing markers and data analysis pipeline for genotyping multi-clonal malaria infections. *BMC Genomics* **18**, 864 (2017). https://doi.org/10.1186/s12864-017-4260-y
81 Early, A. M. *et al.* Detection of low-density Plasmodium falciparum infections using amplicon deep sequencing. *Malar J* **18**, 219 (2019). https://doi.org/10.1186/s12936-019-2856-1
82 Fola, A. A. *et al.* SNP barcodes provide higher resolution than microsatellite markers to measure Plasmodium vivax population genetics. *Malar J* **19**, 375 (2020). https://doi.org/10.1186/s12936-020-03440-0
83 Assefa, S. A. *et al.* estMOI: estimating multiplicity of infection using parasite deep sequencing data. *Bioinformatics* **30**, 1292-1294 (2014). https://doi.org/10.1093/bioinformatics/btu005
84 Zhu, S. J., Almagro-Garcia, J. & McVean, G. Deconvolution of multiple infections in Plasmodium falciparum from high throughput sequencing data. *Bioinformatics* **34**, 9-15 (2018). https://doi.org/10.1093/bioinformatics/btx530
85 Oyola, S. O. *et al.* Whole genome sequencing of Plasmodium falciparum from dried blood spots using selective whole genome amplification. *Malar J* **15**, 597 (2016). https://doi.org/10.1186/s12936-016-1641-7
86 Auburn, S. *et al.* An effective method to purify Plasmodium falciparum DNA directly from clinical blood samples for whole genome high-throughput sequencing. *PLoS One* **6**, e22213 (2011). https://doi.org/10.1371/journal.pone.0022213
87 World Health Organization. Informal consultation on methodology to distinguish reinfection from recrudescence in high malaria transmission areas. (2021).
88 Imwong, M. *et al.* Relapses of Plasmodium vivax infection usually result from activation of heterologous hypnozoites. *J Infect Dis* **195**, 927-933 (2007). https://doi.org/10.1086/512241
89 Joste, V. *et al.* Genetic Profiling of Plasmodium ovale wallikeri Relapses With Microsatellite Markers and Whole-Genome Sequencing. *J Infect Dis* **228**, 1089-1098 (2023). https://doi.org/10.1093/infdis/jiad216
90 Richter, J., Franken, G., Mehlhorn, H., Labisch, A. & Häussinger, D. What is the evidence for the existence of Plasmodium ovale hypnozoites? *Parasitol Res* **107**, 1285-1290 (2010). https://doi.org/10.1007/s00436-010-2071-z
91 Viriyakosol, S. *et al.* Genotyping of Plasmodium falciparum isolates by the polymerase chain reaction and potential uses in epidemiological studies. *Bull World Health Organ* **73**, 85-95 (1995).
92 Imwong, M. *et al.* Practical PCR genotyping protocols for Plasmodium vivax using Pvcs and Pvmsp1. *Malar J* **4**, 20 (2005). https://doi.org/10.1186/1475-2875-4-20
93 Tanomsing, N. *et al.* Genetic marker suitable for identification and genotyping of Plasmodium ovale curtisi and Plasmodium ovale wallikeri. *J Clin Microbiol* **51**, 4213-4216 (2013). https://doi.org/10.1128/JCM.01527-13
94 Noordin, N. R., Azhar, A., Lau, Y. L., Cheong, F. W. & Fong, M. Y. Multiplicity of infection of Plasmodium knowlesi in Malaysia: an application of Pkmsp-1 block IV. *Trop Biomed* **40**, 295-300 (2023). https://doi.org/10.47665/tb.40.3.004

95 Divis, P. C. *et al.* Admixture in Humans of Two Divergent Plasmodium knowlesi Populations Associated with Different Macaque Host Species. *PLoS Pathog* **11**, e1004888 (2015). https://doi.org/10.1371/journal.ppat.1004888
96 Bruce, C., M., Macheso, A., Galinski, R., M. & Barnwell, W., J. Characterization and application of multiple genetic markers forPlasmodium malariae. *Parasitology* **134**, 637-650 (2007). https://doi.org/10.1017/s0031182006001958
97 Mathema, V. B. *et al.* Polymorphic markers for identification of parasite population in Plasmodium malariae. *Malaria Journal* **19** (2020). https://doi.org/10.1186/s12936-020-3122-2
98 Birkenmeyer, L., Dawson, G. J., Desai, S. M. & Muerhoff, A. S. Isolation and Characterization of the MSP1 Genes from Plasmodium malariae and Plasmodium ovale. *The American Journal of Tropical Medicine and Hygiene* **82**, 996-1003 (2010). https://doi.org/10.4269/ajtmh.2010.09-0022
99 Guimarães, L. O. *et al.* Merozoite surface protein-1 genetic diversity in Plasmodium malariae and Plasmodium brasilianum from Brazil. *BMC Infectious Diseases* **15** (2015). https://doi.org/10.1186/s12879-015-1238-8
100 Putaporntip, C., Kuamsab, N., Rojrung, R., Seethamchai, S. & Jongwutiwes, S. Structural organization and sequence diversity of the complete nucleotide sequence encoding the Plasmodium malariae merozoite surface protein-1. *Scientific Reports* **12** (2022). https://doi.org/10.1038/s41598-022-19049-z
101 Lalremruata, A. *et al.* Species and genotype diversity of Plasmodium in malaria patients from Gabon analysed by next generation sequencing. *Malaria Journal* **16** (2017). https://doi.org/10.1186/s12936-017-2044-0
102 Srisutham, S. *et al.* Genetic diversity of three surface protein genes in Plasmodium malariae from three Asian countries. *Malaria Journal* **17** (2018). https://doi.org/10.1186/s12936-018-2176-x
103 Lefoulon, E. *et al.* Shaking the Tree: Multi-locus Sequence Typing Usurps Current Onchocercid (Filarial Nematode) Phylogeny. *PLoS Negl Trop Dis* **9**, e0004233 (2015). https://doi.org/10.1371/journal.pntd.0004233
104 Gaillard, C. M. *et al.* Detection of DNA of filariae closely related to Mansonella perstans in faecal samples from wild non-human primates from Cameroon and Gabon. *Parasit Vectors* **13**, 313 (2020). https://doi.org/10.1186/s13071-020-04184-1
105 Richard-Lenoble, D., Kombila, M., Bain, O., Chandenier, J. & Mariotte, O. Filariasis in Gabon: human infections with Microfilaria rodhaini. *Am J Trop Med Hyg* **39**, 91-92 (1988). https://doi.org/10.4269/ajtmh.1988.39.91
106 Peel, E. C., M. Sur des Filarides de Chimpanzés *Pan piscus* et *Pan satyrus* au Congo Belge. *Annales de la Société Belge de Médecine tropicale*, 117-150 (1946).
107 Berghe, L., van den & Chardome, M. Une microfilaire du gorille. *Microfilaria gorillae*. *Annales de la Société Belge de Médecine tropicale* **29**, 495-499 (1949).
108 Berghe, L., van den, Peel, E. & Chardome, M. M*icrofilaria leopoldi* n. sp. chez G*orilla gorilla* au Congo Belge. *ibidem* **3**, 87 (1957).
109 Bain, O., Moisson, P., Huerre, M., Landsoud-Soukate, J. & Tutin, C. Filariae from a wild gorilla in Gabon with description of a new species of Mansonella. *Parasite* **2**, 315-322 (1995). https://doi.org/10.1051/parasite/1995023315
110 Sinha, A. *et al.* Genomes of the human filarial parasites Mansonella perstans and Mansonella ozzardi. *Frontiers in Tropical Diseases* **4** (2023). https://doi.org/10.3389/fitd.2023.1139343

111 Bruce, M. C., Macheso, A., Mcconnachie, A. & Molyneux, M. E. Comparative population structure of Plasmodium malariae and Plasmodium falciparum under different transmission settings in Malawi. *Malaria Journal* **10**, 38 (2011). https://doi.org/10.1186/1475-2875-10-38
112 Barry, A. E., Waltmann, A., Koepfli, C., Barnadas, C. & Mueller, I. Uncovering the transmission dynamics of Plasmodium vivax using population genetics. *Pathog Glob Health* **109**, 142-152 (2015). https://doi.org/10.1179/2047773215Y.0000000012
113 Nikolayevskyy, V. *et al.* MIRU-VNTR Genotyping of Mycobacterium tuberculosis Strains Using QIAxcel Technology: A Multicentre Evaluation Study. *PLoS One* **11**, e0149435 (2016). https://doi.org/10.1371/journal.pone.0149435
114 Manrique, P. *et al.* Assessment of an automated capillary system for Plasmodium vivax microsatellite genotyping. *Malar J* **14**, 326 (2015). https://doi.org/10.1186/s12936-015-0842-9
115 Messerli, C., Hofmann, N. E., Beck, H. P. & Felger, I. Critical Evaluation of Molecular Monitoring in Malaria Drug Efficacy Trials and Pitfalls of Length-Polymorphic Markers. *Antimicrob Agents Chemother* **61** (2017). https://doi.org/10.1128/AAC.01500-16
116 Getachew, S. *et al.* Variation in Complexity of Infection and Transmission Stability between Neighbouring Populations of Plasmodium vivax in Southern Ethiopia. *PLoS One* **10**, e0140780 (2015). https://doi.org/10.1371/journal.pone.0140780
117 Akoniyon, O. P., Akiibinu, M., Adeleke, M. A., Maharaj, R. & Okpeku, M. A Comparative Study of Genetic Diversity and Multiplicity of Infection in Uncomplicated. *Pathogens* **13** (2024). https://doi.org/10.3390/pathogens13020172
118 Abukari, Z. *et al.* The Diversity, Multiplicity of Infection and Population Structure of. *Genes (Basel)* **10** (2019). https://doi.org/10.3390/genes10060434
119 Kiwuwa, M. S. *et al.* Genetic diversity of Plasmodium falciparum infections in mild and severe malaria of children from Kampala, Uganda. *Parasitol Res* **112**, 1691-1700 (2013). https://doi.org/10.1007/s00436-013-3325-3
120 Rout, R., Mohapatra, B. N., Kar, S. K. & Ranjit, M. Genetic complexity and transmissibility of Plasmodium falciparum parasites causing severe malaria in central-east coast India. *Trop Biomed* **26**, 165-172 (2009).
121 Ranjit, M. R. *et al.* Distribution of Plasmodium falciparum genotypes in clinically mild and severe malaria cases in Orissa, India. *Trans R Soc Trop Med Hyg* **99**, 389-395 (2005). https://doi.org/10.1016/j.trstmh.2004.09.010
122 Magesa, S. M. *et al.* Diversity of Plasmodium falciparum clones infecting children living in a holoendemic area in north-eastern Tanzania. *Acta Trop* **84**, 83-92 (2002). https://doi.org/10.1016/s0001-706x(02)00179-1
123 Durand, R. *et al.* Analysis of circulating populations of Plasmodium falciparum in mild and severe malaria in two different epidemiological patterns in Madagascar. *Trop Med Int Health* **13**, 1392-1399 (2008). https://doi.org/10.1111/j.1365-3156.2008.02156.x
124 al-Yaman, F. *et al.* Reduced risk of clinical malaria in children infected with multiple clones of Plasmodium falciparum in a highly endemic area: a prospective community study. *Trans R Soc Trop Med Hyg* **91**, 602-605 (1997). https://doi.org/10.1016/s0035-9203(97)90046-8
125 Farnert, A., Snounou, G., Rooth, I. & Bjorkman, A. Daily dynamics of Plasmodium falciparum subpopulations in asymptomatic children in a

holoendemic area. *Am J Trop Med Hyg* **56**, 538-547 (1997). https://doi.org/10.4269/ajtmh.1997.56.538
126 Jafari, S., Le Bras, J., Bouchaud, O. & Durand, R. Plasmodium falciparum clonal population dynamics during malaria treatment. *J Infect Dis* **189**, 195-203 (2004). https://doi.org/10.1086/380910
127 Bruce, M. C. *et al.* Genetic diversity and dynamics of plasmodium falciparum and P. vivax populations in multiply infected children with asymptomatic malaria infections in Papua New Guinea. *Parasitology* **121 (Pt 3)**, 257-272 (2000). https://doi.org/10.1017/s0031182099006356
128 Coatney, G. R. *Primate Malarias*. 209 (1971).
129 McKenzie, F. E., Jeffery, G. M. & Collins, W. E. Plasmodium malariae blood-stage dynamics. *J Parasitol* **87**, 626-637 (2001). https://doi.org/10.1645/0022-3395(2001)087[0626:PMBSD]2.0.CO;2
130 Inoue, J. *et al.* Dynamics of Plasmodium species and genotype diversity in adults with asymptomatic infection in Gabon. *Int J Infect Dis* **143**, 107013 (2024). https://doi.org/10.1016/j.ijid.2024.107013
131 Färnert, A., Lebbad, M., Faraja, L. & Rooth, I. Extensive dynamics of Plasmodium falciparum densities, stages and genotyping profiles. *Malar J* **7**, 241 (2008). https://doi.org/10.1186/1475-2875-7-241
132 Snounou, G. & Beck, H. P. The use of PCR genotyping in the assessment of recrudescence or reinfection after antimalarial drug treatment. *Parasitol Today* **14**, 462-467 (1998). https://doi.org/10.1016/s0169-4758(98)01340-4
133 Darwin, C. On the origin of species by means of natural selection, or preservation of favoured rases in the struggle for life. *London: John Murray* (1859).
134 Chung, M. *et al.* Complete Mitochondrial Genome Sequence of Mansonella perstans. *Microbiol Resour Announc* **9** (2020). https://doi.org/10.1128/MRA.00490-20
135 Crainey, J. L. *et al.* Mansonella ozzardi mitogenome and pseudogene characterisation provides new perspectives on filarial parasite systematics and CO-1 barcoding. *Sci Rep* **8**, 6158 (2018). https://doi.org/10.1038/s41598-018-24382-3
136 Xie, H., Bain, O. & Williams, S. A. Molecular phylogenetic studies on filarial parasites based on 5S ribosomal spacer sequences. *Parasite* **1**, 141-151 (1994). https://doi.org/10.1051/parasite/1994012141
137 Yilmaz, E. *et al.* The Mitochondrial Genomes of the Zoonotic Canine Filarial Parasites Dirofilaria (Nochtiella) repens and Candidatus Dirofilaria (Nochtiella) hongkongensis Provide Evidence for Presence of Cryptic Species. *PLoS Negl Trop Dis* **10**, e0005028 (2016). https://doi.org/10.1371/journal.pntd.0005028
138 Orihel, T. C. & Eberhard, M. L. Mansonella ozzardi: a redescription with comments on its taxonomic relationships. *Am J Trop Med Hyg* **31**, 1142-1147 (1982). https://doi.org/10.4269/ajtmh.1982.31.1142
139 Bain, O. *et al.* Review of the genus Mansonella Faust, 1929 sensu lato (Nematoda: Onchocercidae), with descriptions of a new subgenus and a new subspecies. *Zootaxa* **3918**, 151-193 (2015). https://doi.org/10.11646/zootaxa.3918.2.1
140 Medkour, H. *et al.* Parasitic Infections in African Humans and Non-Human Primates. *Pathogens* **9** (2020). https://doi.org/10.3390/pathogens9070561
141 Orihel, T. C. *et al.* Susceptibility of laboratory primates to infection with Mansonella ozzardi from man. *Am J Trop Med Hyg* **30**, 790-794 (1981). https://doi.org/10.4269/ajtmh.1981.30.790

142 de Souza, V. K. *et al.* Emergence of Mansonella sp. in free-ranging primates in southern Brazil. *Primates* **64**, 153-159 (2023). https://doi.org/10.1007/s10329-022-01038-5
143 Ritter, M. *et al.* Wuchereria bancrofti-infected individuals harbor distinct IL-10-producing regulatory B and T cell subsets which are affected by anti-filarial treatment. *PLoS Negl Trop Dis* **13**, e0007436 (2019). https://doi.org/10.1371/journal.pntd.0007436
144 Metenou, S. *et al.* Patent filarial infection modulates malaria-specific type 1 cytokine responses in an IL-10-dependent manner in a filaria/malaria-coinfected population. *J Immunol* **183**, 916-924 (2009). https://doi.org/10.4049/jimmunol.0900257
145 Semnani, R. T., Law, M., Kubofcik, J. & Nutman, T. B. Filaria-induced immune evasion: suppression by the infective stage of Brugia malayi at the earliest host-parasite interface. *J Immunol* **172**, 6229-6238 (2004). https://doi.org/10.4049/jimmunol.172.10.6229
146 Ritter, M. *et al.* Mansonella perstans microfilaremic individuals are characterized by enhanced type 2 helper T and regulatory T and B cell subsets and dampened systemic innate and adaptive immune responses. *PLoS Negl Trop Dis* **12**, e0006184 (2018). https://doi.org/10.1371/journal.pntd.0006184
147 Costa, A. G. *et al.* Immune response in Mansonella ozzardi infection modulated by IL-6/IL-10 axis in Amazon region of Brazil. *Cytokine* **104**, 98-103 (2018). https://doi.org/10.1016/j.cyto.2017.09.033
148 Bouyou-Akotet, M. K., Moussavou Boussougou, M. N., Ovono-Abessolo, F., Owono-Medang, M. & Kombila, M. Influence of Mansonella perstans microfilaraemia on total IgE levels in Gabonese patients co-infected with Loa loa. *Acta Trop* **131**, 11-15 (2014). https://doi.org/10.1016/j.actatropica.2013.11.012

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Appendix

- **Publication 1:**
  Genetic diversity of *Plasmodium malariae* in sub-Saharan Africa: a two-marker genotyping approach for molecular epidemiological studies.
- **Publication 2:**
  Whole genome analysis of two sympatric human *Mansonella*: *Mansonella perstans* and *Mansonella* sp "DEUX".

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Genetic diversity of *Plasmodium malariae* in sub-Saharan Africa: a two-marker genotyping approach for molecular epidemiological studies

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Introduction: *Plasmodium malariae* is the most common non-falciparum species in sub-Saharan Africa. Despite this, data on its genetic diversity is scarce. Therefore, we aimed to establish a *P. malariae* genotyping approach based on size polymorphic regions that can be easily applied in molecular epidemiological studies.

Methods: Four potential genotyping markers, Pm02, Pm09, *P. malariae* thrombospondin-related anonymous protein (pmtrap), and *P. malariae* merozoite surface protein fragment 2 (pmmsp1 F2) were amplified via nested PCR and analysed using automated capillary gel electrophoresis.

Results: We observed the highest allelic diversity for pmtrap (MOI = 1.61) and pmmsp1 F2 (He = 0.81). Further applying the two markers pmtrap and pmmsp1 F2 on a different sample set of 21 *P. malariae* positive individuals followed up over one week, we saw a high consistency in their performance. The results show a large complexity and high dynamics of *P. malariae* infections in the asymptomatic Gabonese study population.

Discussion: We successfully implemented a new genotyping panel for *P. malariae* consisting of only two markers: pmtrap and pmmsp1 F2. It can be easily applied in other settings to investigate the genotype diversity of *P. malariae* populations, providing further important data on the molecular epidemiology of this parasite species.

KEYWORDS

***Plasmodium malariae*, genotyping, size polymorphism, *pmtrap*, *pmmsp1* F2**

1 Introduction

Plasmodium malariae is the second most abundant *Plasmodium* species in sub-Saharan Africa causing malaria in humans (Garnham, 1966; Coatney, 1971; Sutherland, 2016; Nundu et al., 2021; Culleton et al., 2023; Miezan et al., 2023). It is detected worldwide in many tropical and subtropical regions and is often found in co-infections with other malaria parasites, mostly *P. falciparum* (Zhou et al., 1998; Herman et al., 2023; Nguiffo-Nguete et al., 2023). Over the past decades a worldwide decline in malaria cases and deaths has been reported by the World Health Organization (WHO) (Organization, 2023). However, this decrease in case numbers has not been observed for *P. malariae*. On the contrary, there are many endemic countries reporting an increase in *P. malariae* prevalence (Cao et al., 2016; Yman et al., 2019; Agonhossou et al., 2022; Nainggolan et al., 2022). Due to improvements and increased usage of molecular diagnostics, the number of *P. malariae* infections has been shown to be substantially higher than previously described (Woldearegai et al., 2019; Mbama Ntabi et al., 2022; Nguiffo-Nguete et al., 2023). However, treatment as well as epidemiological studies and vaccine development are mainly directed towards *P. falciparum* and more recently also *P. vivax*, while other species are almost entirely neglected (Groger et al., 2022). A deeper understanding of the biology and epidemiology of the non-*falciparum* species would contribute to our understanding of transmission dynamics which is a prerequisite for achieving malaria elimination.

P. malariae causes a more benign form of malaria characterized by comparably mild symptoms and low parasitemia. However, serious complications have also been reported, mainly including severe anemia (Langford et al., 2015) and acute kidney injury (Ehrich and Eke, 2007). Infections are described as more chronic, persisting in the human host for a long time, indicating that *P. malariae* is successfully adapted to its human host.

A powerful tool to further characterize a parasite population and its transmission dynamics is the usage of genotyping markers. With this approach, diverse genetic regions are used to distinguish parasite genotypes. For *P. malariae* two genotyping marker panels have been identified previously (Bruce et al., 2007; Mathema et al., 2020). Both approaches focus on microsatellite markers, that represent genomic repetitive regions displaying a high genetic diversity. Few other *P. malariae* genes have been investigated additionally regarding their variability (Guimarães et al., 2015; Lalremruata et al., 2017; Srisutham et al., 2018; Putaporntip et al., 2022). In the present study, we aimed to establish a panel with a minimum number of genotyping markers for *P. malariae* yet with a high discriminative power. We assessed four markers from previous studies - two microsatellites, Pm02 and Pm09, as well as *P. malariae* thrombospondin-related anonymous protein (*pmtrap*), and *P. malariae* merozoite surface protein 1 fragment 2 (*pmmsp1* F2) - the most promising candidate markers based on published diversity and successful amplification (Bruce et al., 2007; Srisutham et al., 2018; Mathema et al., 2020). We optimized the workflow, confirmed their diversity in a large set of *P. malariae* samples from Gabon, a malaria-endemic country in sub-Saharan Africa, and analysed dynamics and complexity of infections, by following-up asymptomatically infected individuals from Gabon in a tight schedule over one week.

2 Methods

2.1 Study populations

A total of 410 *P. malariae* samples from two different study populations were investigated in this study. Study 1 was a cross-sectional study, aiming to characterize *Plasmodium* infections in rural areas in Fougamou and surrounding villages in Gabon in February/March 2016 (Manego et al., 2017; Groger et al., 2018; Woldearegai et al., 2019). A total of 840 individuals aged from 1-96 years were included in the original study, from which 193 participants were positive by 18S rRNA qPCR for *P. malariae*. Of these, 95 samples were used here, selected according to low Cq values.

Study 2 was conducted in 2019/2020 in Lambaréné, Gabon, assessing safety and efficacy of ivermectin against *P. falciparum* infections in asymptomatic adults (Ekoka Mbassi et al., 2023); samples were collected throughout the year. As ivermectin at the given dose showed similar activity as placebo against Plasmodia, individuals are regarded as not treated for this study. Out of 49 participants, 21 were positive for *P. malariae* at inclusion. The following 15 timepoints were investigated: Screening (SCR), after 8

(H8), 16 (H16), 24 (H24), 32 (H32), 40 (H40), 48 (H48), 56 (H56), 64 (H64), 72 (H72), 96 (H96), 120 (H120) hours, day 6 (D6), day 7 (D7). On day 7 a complete treatment of artemether-lumefantrine was given, and additional sampling was done on day 14 (D14).

Both studies and the corresponding experimental protocols were approved by the Institutional Ethics Committee of CERMEL (CEI-007/2014, CEI/CERMEL 006/2019). Study 2 was registered with the Pan-African Clinical Trials Registry (PACTR201908520097051). All methods were carried out in accordance with relevant guidelines and regulations. Informed consent was obtained from all adult participants or legal guardians.

2.2 DNA extraction and *Plasmodium* species determination

Blood samples were stabilized in RNAlater prior to extraction. DNA was extracted either manually using the QIAamp DNA mini blood kit (QIAGEN) or automated in the QIAsymphony (QIAGEN) or in the KingFisher™ Flex Purification System (Thermo Fisher Scientific) with the sbeadex blood kit (LGC), according to the manufacturer's protocols. *Plasmodium* species were determined by qPCR as described previously targeting the 18S rRNA of the different *Plasmodium* species (Groger et al., 2018; Woldearegai et al., 2019; Ekoka Mbassi et al., 2023).

2.3 Identification of *P. malariae* genotyping markers

We chose four loci distributed on different regions of the *P. malariae* genome, namely Pm02, Pm09, *pmtrap*, and *pmmsp1* F2, based on high genetic diversity published in other *P. malariae* populations (Bruce et al., 2007; Guimarães et al., 2015; Srisutham et al., 2018). Pm09 is located on chromosome 1 and *pmmsp1* F2 on chromosome 7. Pm02 and *pmtrap* are both located on chromosome 12. The two microsatellite markers show the following repeat sizes: Pm02 has a repeat unit of 4 bp (CATA) and Pm09 of 17 bp (GCAAAATAACAAAAAGA). *Pmtrap* shows different patterns of 12 bp repeat units (CCAGAGGATAGA; CCAGAGAATAGA; CCAGAGAATAGT).

Each of the four length-polymorphic markers was amplified by nested PCR. In all PCR runs the following controls were used: non-template control (H_2O), negative control (human whole blood from a malaria naïve person), positive control (*P. malariae* mono infection).

Optimized cycling conditions and primer sequences for Pm02, Pm09, *pmtrap* and *pmmsp1* F2 are shown in Supplementary Table S1. New outer (Pm02 and Pm09 forward) and inner (*pmmsp1* F2 and pmtrap) primers were designed according to the amplicon length needed for the analysis method used, which is described below. Primer design was based on the *P. malariae* reference genome PmUG01 sequences (LT594622 – LT594635). Samples that were negative in two consecutive runs, were repeated with 5 more cycles, to exclude low DNA content as a source of error.

2.4 Analysis and verification of *P. malariae* marker

Before further analysis, the amplicon size and concentration were measured using automated capillary gel electrophoresis (QIAxcel, QIAGEN). Specific conditions and descriptions are listed in Supplementary Table S2 and Supplementary File S1.

Verification of automated capillary gel electrophoresis is described in Supplementary File S1.

2.5 Analysis of genetic diversity

We assessed the performance and diversity of the *P. malariae* genotyping markers along 95 *P. malariae* positive samples from study 1. Whereas samples from study 2 were used to analyze the performance of the markers over time. The maximum multiplicity of infection (MOI) was counted based on the highest number of alleles per marker in one sample. The mean MOI for each marker was calculated as followed: $\frac{total\ number\ of\ alleles}{number\ of\ positive\ samples}$. To estimate the allele frequency for each detected allele size, we divided the number of alleles detected by the total number of PCR positive samples. The expected heterozygosity (H_e) was calculated using the following formula:

$H = \frac{N}{(N-1)}(1 - \sum_{i=1}^{k} p_i^2)$ where p corresponds to the i^{th} of k alleles and N to the number of positive samples.

3 Results

3.1 Comparative performance and validation of four potential *P. malariae* genotyping markers

Based on literature and *in silico* analysis, we chose four different loci Pm02, Pm09, *pmtrap*, and *pmmsp1* F2.

3.1.1 High diversity of *P. malariae* genotypes in study population from Gabon

The allelic diversity of the markers was assessed on a set of 95 samples from study 1. The number of allele groups detected in the 95 samples was highest for *pmmsp1* F2 and Pm02, with 15 distinct groups detected each (Figure 1). While 11 allele groups were detected for *pmtrap*, Pm09 resulted in 5 distinct allele groups, which was the lowest number detected. Overall, the allele frequency of groups was unevenly distributed for all four markers, with mostly one major allele group. For *pmmsp1* F2 seven minor allele groups were identified, each appearing only once.

An overall comparison of allele characteristics showed that *pmtrap* and *pmmsp1* F2 performed best among the four markers when aiming at displaying diversity (Table 1). The highest expected heterozygosity (H_e) of 0.81 was calculated for *pmmsp1* F2 while *pmtrap* had the highest mean MOI of 1.61. Pm02 performed comparably well, with a mean MOI of 1.55 and an H_e of 0.68. Despite the high resolution of the chosen QIAxcel method, the

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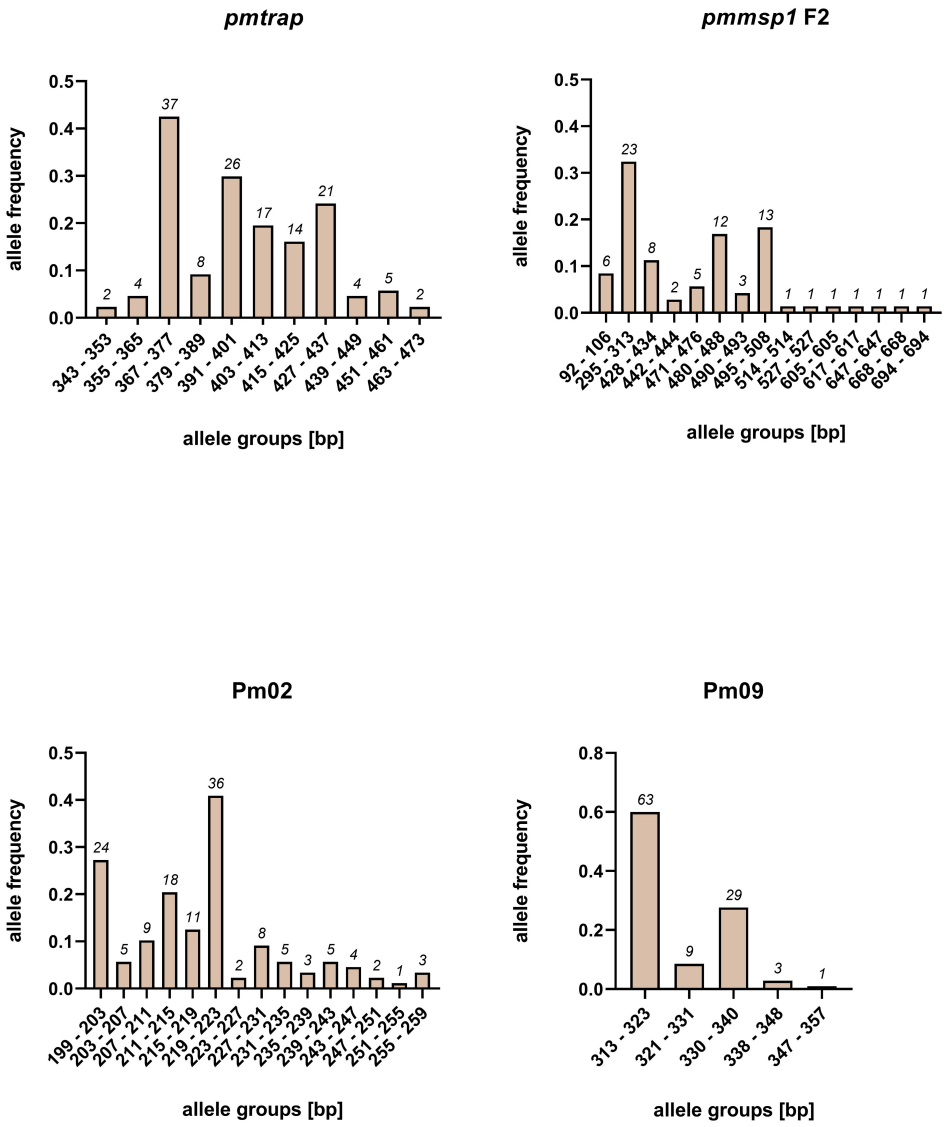

FIGURE 1
Allele frequencies of four *P. malariae* markers *pmtrap*, *pmmsp1* F2, Pm02 and Pm09, arranged in different allele groups according to the fragment size. Frequencies and sizes include alleles from multiclonal infections. In total 95 samples were screened.

differentiation of the allele groups was challenging for the marker Pm02, as it has a repeat size unit of only 4 bp, which falls within the maximum resolution of 3-5 bp that can be achieved with the underlying method. Pm09 was the least diverse locus, with the lowest H_e (0.56) as well as a low MOI (1.19). Amplification success exceeded 90% for three out of the four markers, while *pmmsp1* F2 exhibited a lower performance, with a PCR positivity rate of only 76%. The gene is known for its polymorphisms; therefore, it is likely that for some of the samples the primer regions were too diverse to anneal without mismatches. Indeed, we found one mismatch in the

TABLE 1 Allele characteristics of four *P. malariae* markers based on size polymorphisms.

Marker	*P. malariae* positivity by PCR	Number of alleles		MOI		H_e	Allele size range [bp]
		distinct	total	max.	mean		
pmtrap	87/95 [92%]	11	140	6	1.61	0.60	349 – 469
pmmsp1 F2	72/95 [76%]	15	80	3	1.11	0.81	92 – 694
Pm02	88/95 [93%]	15	136	7	1.55	0.66	200 – 257
Pm09	88/95 [93%]	5	105	4	1.19	0.56	314 - 346

MOI, Multiplicity of infection. H_e, expected Heterozygosity.

reverse primer region of 1/12 generated *pmmsp1* F2 sequences. We do not see any effect of the Cq value on the amplification success of the samples.

3.1.2 *In silico* analysis reveals new *P. malariae* genotypes from African samples

In addition to the *P. malariae* reference genome obtained from Uganda, we identified sequences from regions outside of Africa within the NCBI database that align to the four markers explored with samples from study 1. We also considered *P. brasilianum* sequences. 32 sequences were retrieved for *pmtrap* and 50 sequences for *pmmsp1*. All 32 *pmtrap* sequences are derived from samples collected in Southeast Asia. 35/50 *pmmsp1* sequences originate from Southeast Asia as well and the remaining 15/50 from South America. For Pm02 and Pm09, one and two published sequences respectively aligned to our sequences, all three originating from South America.

When comparing the sequences from *pmtrap* and *pmmsp1* F2 to the above-mentioned sequences, we found four new genotypes using *pmtrap* (accession numbers: OR829576-OR829579) and five new genotypes using *pmmsp1* F2 (accession numbers: OR829569-OR829571, OR829572, OR829573), among our samples that were not published before. For Pm09 two new genotypes (accession numbers: OR829566, OR829568) were found, each displaying a new repeat unit motif of 17 bp (GAAGAGCAAAATAACAA) and 8 bp (AACAAACA). For Pm02 seven new genotypes were found (accession numbers: OR829554, OR829557, OR829558, OR829560-OR829564), one with a new repeat unit of 6 bp (CCACAC). New genotypes are defined by a new number of repeat units for *pmtrap*, Pm02 and Pm09 or an amplicon size not reported before for *pmmsp1* F2. The genotype derived from the reference genome was found among our sequences in all four markers.

3.1.3 Combination of *P. malariae* genotyping marker reveals high number of samples with one genotype

We investigated which combination of the four markers gives the highest diversity compared to using a single marker (Supplementary Figure S1). This was assessed on the 95 samples from study 1. Hereby the number of samples with either 1 (i.e., MOI=1) or more than 1 genotype (i.e., MOI>1), was used as a benchmark to evaluate the different combinations. The more samples with an MOI>1, the better the marker combination reflects the allelic diversity of the sample set. In addition, the PCR failure rate, i.e., number of samples that were not amplified, was used for comparison. The number of negative samples was highest (at least 7/95 samples) when the markers were used separately than in any other combination (less than 6/95 samples). All combinations of two markers enhanced diversity by either reducing the number of negative samples or increasing the number of samples with multiple genotypes.

For any combination of 3 markers, the multiplicity is even higher, with at least 33/95 samples showing a MOI>1. Combining all four markers decreased the number of negative samples to 3/95 and increased samples with an MOI>1 to 40/95. Although this combination reflects diversity best, we did not select it for further investigation. We excluded Pm09 from the combinations because of its low heterozygosity. Pm02 on the other hand was diverse enough, however the analysis of the generated amplicons was challenging and time consuming as of the small repeat size of 4 bp. Instead, we chose the combination of *pmtrap* and *pmmsp1* F2, identifying 61/95 samples with one genotype only (Figure 2). While 5/95 samples

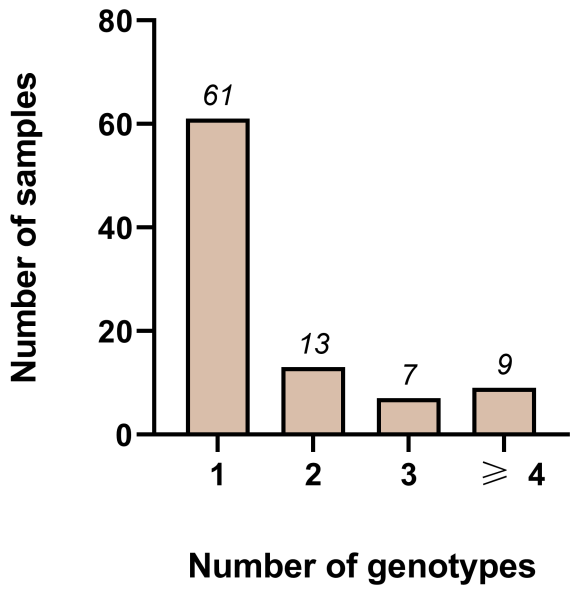

FIGURE 2
Distribution of different genotypes measured using the two markers *pmmsp1* F2 and *pmtrap* in combination among 95 samples from Gabon. Out of 95 samples, 61 were infected with one genotype only. Whereas 13, 7 and 9 samples were infected with two, three or more than three genotypes respectively. In total 5/95 samples failed to amplify.

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were not amplified by any of the two marker, 29/95 samples were carrying two or more genotypes simultaneously.

3.2 Indel-based markers *pmmsp1* F2 and *pmtrap* show high consistency in their performance and reveal a large complexity of infection in asymptomatic Gabonese individuals

We further applied the two markers (*pmmsp1* F2 and *pmtrap)* on a unique sample set from study 2, consisting of 21 P. *malariae* positive individuals that were followed for 14 days. In total, 15/21 individuals were positive for both markers by PCR while 6/21 were positive for only one marker respectively (two for *pmtrap* and four for *pmmsp1* F2).

Overall individuals showed different patterns throughout the course of the infection (for an overview of all 21 individuals see Supplementary Table S3): 2/15 individuals showed only one genotype for both markers over all sampling timepoints (individual i.01 in Figure 3) and 11/15 individuals were infected with multiple genotypes according to both markers, one was even detected with 8 genotypes using *pmtrap* (individual i.02 in Figure 3). In all 11 mentioned individuals the maximum MOI was higher for *pmtrap* than *pmmsp1* F2. The maximum MOI measured with *pmtrap* and *pmmsp1* F2 is depicted in Figure 3 for individuals i.01 – i.03 as an example for different genotype patterns detected. The remaining individuals can be seen in Supplementary Figure S2 (individuals i.04 – i.15). In 2/15 cases the maximum MOI was higher for *pmmsp1* F2 and in two other cases it was similar. Overall, the different genotypes were detected very stable along the 15 timepoints. Some genotypes were fluctuating over time, detected at some timepoints and undetected in others (individual i.03 in Figure 3). This shows the high dynamics and complexity of *P. malariae* infections. 17/21 individuals were negative on day 14 (D14), as per protocol participants were treated with artemether-lumefantrine latest on day 7.

4 Discussion

The number of *P. malariae* infections in endemic countries has been shown to be substantially higher than previously acknowledged. Nevertheless, there are many open questions regarding the genetic epidemiology of this species. In the present study, we assessed four genotyping markers and selected two of them for establishment of a genotyping panel for *P. malariae* with a minimum number of markers and a high discriminative power.

First, we analysed the individual performance of the four markers using a sample set of 95 *P. malariae* infected individuals from Gabon. Our analysis showed that *pmtrap*, *pmmsp1* F2 and Pm02 are the most diverse among the chosen loci in the population studied. They showed the highest number of distinct alleles - 11, 15 and 15, respectively -, whereas only 5 distinct alleles were found

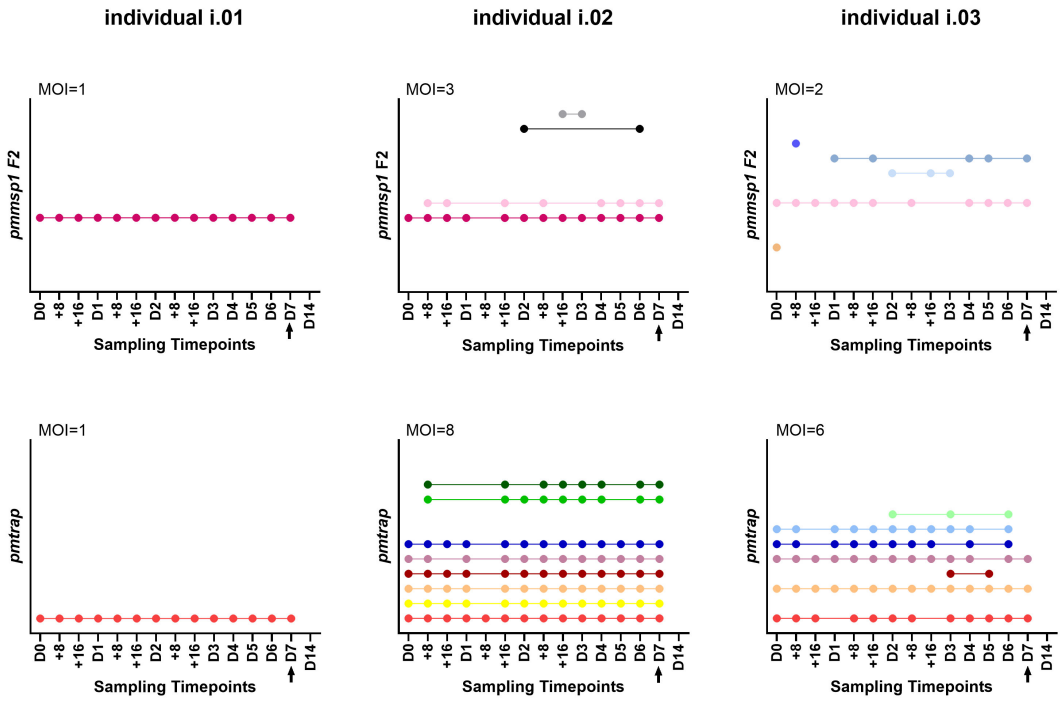

FIGURE 3
Three individuals i.01, i.02 and i.03 were genotyped at 15 timepoints using the markers *pmmsp1* F2 (upper row) and *pmtrap* (lower row). Each color represents a different genotype for the respective marker. The arrow indicates treatment with artemether-lumefantrine on day 7. The remaining 18 individuals are shown in Supplementary Figure S2. MOI, multiplicity of infection, referring to the maximum MOI measured among the 15 timepoints.

with Pm09 in the 95 samples. The mean MOI was highest for *pmtrap* (1.61), showing that this marker can very well reflect the multiplicity of *P. malariae* infections. The expected heterozygosity was highest for *pmmsp1* F2 (0.81), demonstrating that this marker can detect a wide variety of different *P. malariae* genotypes. In terms of theses parameters, Pm02 performs comparatively well. However, this marker seems unsuitable for the fragment analysis using automated capillary gel electrophoresis with a resolution of 3-5 bp. The repeat size of only 4 bp is too small to reliably distinguish single alleles in infections with multiple genotypes, a limitation of this method. Similar results were found in another study on *P. vivax* genotyping, where automated capillary gel electrophoresis also reached its limit in distinguishing alleles that are close to each other in size (Manrique et al., 2015). The observed differences in the respective PCR efficiency of each marker can be attributed to various influencing factors. Low DNA concentrations in the sample are most likely the major reason why some samples were not amplified at all. We found a mismatch in the primer region in one of the *pmmsp1* F2 sequences. This further underlines the high rate of polymorphisms in this region and might have led to the low amplification success for this marker. If primer binding is not efficient, less DNA is amplified. PCR conditions were thoroughly optimized to increase PCR efficiency to a maximum.

Overall, the range of heterozygosity (0.56 – 0.80) is comparable to other published marker panels by Bruce et al. (0.331 – 0.839) and Mathema et al. (0.530 – 0.922); the mean MOI for the four markers (1.11 – 1.61) is even slightly higher (Bruce et al., 2007; Mathema et al., 2020). However, both parameters depend on the characteristics of the investigated population, as geographical origin, clinical status, participants age, or seasonality. The samples used here for the validation of genetic diversity are from one geographical region (Lambaréné and surrounding villages, Gabon) and were collected within two months (study 1). The same is true for the *P. malariae* sample set from Myanmar of Mathema et al. In contrast Bruce et al. tested samples from diverse geographical origin (Thailand, Malawi, and Gambia), different clinical status and transmission seasons. Therefore, a direct comparison of the different marker panels using different settings can be misleading.

A notable observation for the *pmmsp1* F2 marker was that comparatively more amplicons with a small size were detected and that the MOI was lower than for the other three markers. This effect of preferential amplification of shorter over longer amplicons has been seen in *P. falciparum* genotyping before, targeting the *msp1* locus as well (Messerli et al., 2017). It can lead to an underestimation of the MOI. However, we saw no evidence of preferential amplification of smaller fragments for the *pmmsp1* F2 locus (Supplementary File S2). Also, it is unlikely that a low parasite density of the minor genotype leads to these findings in infections with multiple genotypes, as the other three markers have a higher MOI. Another explanation for the seen effect could be mutations in the primer region of the bigger alleles.

We optimized our genotyping approach based on automated capillary gel electrophoresis. To validate the method, we sequenced amplicons from samples that were infected with one genotype only and compared the amplicon lengths from both methods. The high concordance observed for all four markers shows that our chosen analysis method is very reliable (Supplementary File S3). This approach has been proven efficient in previous *Plasmodium* genotyping approaches as well (Manrique et al., 2015; Nguyen et al., 2019; Tadele et al., 2022). General advantages of the method include the fast and high throughput, as well as the straightforward analysis. The high deviation of up to a maximum of 21 bp seen for *pmmsp1* F2 can be explained by the lower resolution of 20 bp given by the used method. As the allele size range was bigger for this marker (92 – 694 bp), a method with a lower resolution (20 bp) had to be used.

Previous studies on *P. malariae* genotyping have been carried out in samples from South America and/or Southeast Asia (Guimarães et al., 2015; Srisutham et al., 2018; Putaporntip et al., 2022). The *pmtrap* and *pmmsp1* sequences generated here originate from Gabon and therefore represent a valuable set of genotypes from Africa. A comparison of spatial dynamics of *P. malariae* genotypes reveals that some genotypes appear in all three continents mentioned: for *pmtrap* 3/10 identified genotypes have been found in Southeast Asia as well as Africa; for *pmmsp1* F2 4/25 genotypes have been identified either in Southeast Asia and South America (2 genotypes), in South America and Africa (1 genotype) or in all three continents (1 genotype). However, most genotypes are shared among samples from the same region, forming spatial genotype clusters. As most of the available sequences are from Southeast Asia, the biggest cluster was identified from this region for both markers (4/10 using *pmtrap* and 14/25 using *pmmsp1* F2). Another genotype cluster exclusively seen in samples from South America, consists of three genotypes and was identified using *pmmsp1* F2. We found three new genotypes using both markers in our samples from Gabon, Africa, that were not seen in the sequencing data from any other continent before.

In the end, a combination of the two markers *pmmsp1* F2 and *pmtrap* resulted in the panel with the best discriminative power. While *pmmsp1* F2 can detect a high variety of diverse genotypes, *pmtrap* is able to best cover the multiplicity of *P. malariae* infections. In combination the two markers provide a powerful tool for studies on genetic diversity of *P. malariae*.

Based on the results from the sample set of the two-weekly follow-up study, both markers demonstrated a very consistent performance over time. Genotypes were detected consistently throughout the different sampling timepoints, which shows the method`s effectiveness. We detected up to 8 genotypes in one individual at the same timepoint. This surprisingly high number of circulating *P. malariae* genotypes in an asymptomatic population is particularly interesting. In a previous study from Gabon including symptomatic individuals, *P. malariae* was the species with the highest number of genotypes (Lalremruata et al., 2017). To which extend these findings are related to the clinical status needs further investigation.

Throughout the follow-up period, fluctuations of genotypes were evident, indicating that not all genotypes are present at every sampling occasion. This observation is likely because the amount of DNA from these minor genotypes approaches the limit of detection, so that they are detectable at one time point but not at the other. However, it is also possible that some of the appearing

genotypes are new infections. The 72-hour cycle of *P. malariae* could not be seen in the pattern of detected genotypes from our data.

We followed the *P. malariae* infections for a limited time, providing valuable insights into their dynamics. As *P. malariae* infections are known for their longevity and to persist in the human host for even decades (Ciusa et al., 2022), studying infections over longer periods would provide even more information on the biology of this species. However, the high complexity of asymptomatic *P. malariae* infections with multiple genotypes and variable detection, shown by our findings, makes longitudinal investigations challenging to interpret since a genotype may not be detected on one sampling timepoint but be present in another. This could be eventually addressed with next generation sequencing (NGS) approaches that can increase sensitivity and the probability to detect minor clones, potentially adding further insights into *P. malariae* genotypes, infection dynamics and biology (Gruenberg et al., 2019; Wamae et al., 2022).

Some individuals in our study were still PCR-positive on day 14, seven days after treatment with artemether-lumefantrine. We do not think that these are treatment failures, but rather residual DNA that can still be detected with our highly sensitive PCR. Similar results were also found for *P. falciparum* (Inoue et al., 2024). In addition, no parasites were detected microscopically at those timepoints. However, in order to be certain that these are no treatment failures and that the individuals turn PCR negative later, follow-up samples from later timepoints would have been required.

5 Conclusion

We were able to successfully implement a new *P. malariae* genotyping panel consisting of only two markers, *pmtrap* and *pmmsp1* F2. This set of markers will add a valuable tool to characterize the genetic diversity of *P. malariae* infections from different regions and to better elucidate the transmission dynamics of this neglected malarial parasite species. It can be implemented in many settings as its usage is straightforward, having a short turnaround time as only two markers are required.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, OR829554-OR829580.

Ethics statement

The studies involving humans were approved by Institutional Ethics Committee of CERMEL. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

MRo: Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. KK: Investigation, Methodology, Validation, Writing – review & editing. LS: Investigation, Writing – review & editing. LB: Investigation, Writing – review & editing. MS: Investigation, Methodology, Writing – review & editing. AL: Methodology, Writing – review & editing. TW: Resources, Writing – review & editing. PB: Resources, Writing – review & editing. MG: Resources, Writing – review & editing. RZ: Resources, Writing – review & editing. DE: Resources, Writing – review & editing. GM-N: Resources, Writing – review & editing. SA: Resources, Writing – review & editing. MRa: Resources, Writing – review & editing. BM: Resources, Writing – review & editing. JI: Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. JH: Conceptualization, Project Administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor KM declared a past co-authorship with the author JH.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2024.1405198/full#supplementary-material

References

Agonhossou, R., Akoton, R., Dossou, Y. A., Avokpaho, E., Mbama, D. N. J., Boussougou-Sambe, T. S., et al. (2022). Surveillance of Plasmodium malariae infection among inhabitants of rural areas in Ouidah–Kpomasse–Tori Bossito health district, Benin. *Parasitol. Res.* 121, 275–286. doi: 10.1007/s00436-021-07398-z

Bruce, C. M., Macheso, A., Galinski, R. M., and Barnwell, W. J. (2007). Characterization and application of multiple genetic markers for*Plasmodium malariae*. *Parasitology* 134, 637–650. doi: 10.1017/S0031182006001958

Cao, Y., Wang, W., Liu, Y., Cotter, C., Zhou, H., Zhu, G., et al. (2016). The increasing importance of Plasmodium ovale and Plasmodium malariae in a malaria elimination setting: an observational study of imported cases in Jiangsu Province, China 2011–2014. *Malaria J.* 15. doi: 10.1186/s12936-016-1504-2

Ciusa, G., Guida Marascia, F., Virruso, R., Angheben, A., Guaraldi, G., and Cascio, A. (2022). Clinical recrudescence of chronic untreated. *IDCases* 27, e01456. doi: 10.1016/j.idcr.2022.e01456

Coatney, G. R., Collins, W. E., Warren, M., and Contacos, P. G. (1971). The primate malarias. (National Institute of Allergy and Infectious Diseases (U.S.)).

Culleton, R., Pain, A., and Snounou, G. (2023). Plasmodium malariae: the persisting mysteries of a persistent parasite. *Trends Parasitol.* 39, 113–125. doi: 10.1016/j.pt.2022.11.008

Ehrich, J. H. H., and Eke, F. U. (2007). Malaria-induced renal damage: facts and myths. *Pediatr. Nephrol.* 22, 626–637. doi: 10.1007/s00467-006-0332-y

Ekoka Mbassi, D., Mombo-Ngoma, G., Held, J., Okwu, D. G., Ndzebe-Ndoumba, W., Kalkman, L. C., et al. (2023). Efficacy and safety of ivermectin for the treatment of Plasmodium falciparum infections in asymptomatic male and female Gabonese adults - a pilot randomized, double-blind, placebo-controlled single-centre phase Ib/IIa clinical trial. *EBioMedicine* 97, 104814. doi: 10.1016/j.ebiom.2023.104814

Garnham, P. C. C. (1966). *Malaria parasites and other haemosporidia*.

Groger, M., Tona Lutete, G., Mombo-Ngoma, G., Ntamabyaliro, N. Y., Kahunu Mesia, G., Muena Mujobu, T. B., et al. (2022). Effectiveness of pyronaridine-artesunate against Plasmodium malariae, Plasmodium ovale spp, and mixed-Plasmodium infections: a *post-hoc* analysis of the CANTAM-Pyramax trial. *Lancet Microbe* 3, e598–e605. doi: 10.1016/S2666-5247(22)00092-1

Groger, M., Veletzky, L., Lalremruata, A., Cattaneo, C., Mischlinger, J., Zoleko-Manego, R., et al. (2018). Prospective Clinical Trial Assessing Species-Specific Efficacy of Artemether-Lumefantrine for the Treatment of Plasmodium malariae, Plasmodium ovale, and Mixed Plasmodium Malaria in Gabon. *Antimicrobial Agents Chemotherapy* 62, AAC.01758–17. doi: 10.1128/AAC.01758-17

Gruenberg, M., LERCH, A., BECK, H. P., and FELGER, I. (2019). Amplicon deep sequencing improves Plasmodium falciparum genotyping in clinical trials of antimalarial drugs. *Sci. Rep.* 9, 17790. doi: 10.1038/s41598-019-54203-0

Guimarães, L. O., Wunderlich, G., Alves, J. M. P., Bueno, M. G., Röhe, F., Catão-Dias, J. L., et al. (2015). Merozoite surface protein-1 genetic diversity in Plasmodium malariae and Plasmodium brasilianum from Brazil. *BMC Infect. Dis.* 15. doi: 10.1186/s12879-015-1238-8

Herman, C., Leonard, C. M., Uhomoibhi, P., Maire, M., Moss, D., Inyang, U., et al. (2023). Non-falciparum malaria infection and IgG seroprevalence among children under 15 years in Nigeri. *Nat. Commun.* 14, 1360. doi: 10.1038/s41467-023-37010-0

Inoue, J., Galys, A., Rodi, M., Ekoka Mbassi, D., Mombo-Ngoma, G., Adegnika, A. A., et al. (2024). Dynamics of Plasmodium species and genotype diversity in adults with asymptomatic infection in Gabon. *Int. J. Infect. Dis.* 143, 107013. doi: 10.1016/j.ijid.2024.107013

Lalremruata, A., Jeyaraj, S., Engleitner, T., Joanny, F., Lang, A., Bélard, S., et al. (2017). Species and genotype diversity of Plasmodium in malaria patients from Gabon analysed by next generation sequencing. *Malaria J.* 16. doi: 10.1186/s12936-017-2044-0

Langford, S., Douglas, N. M., Lampah, D. A., Simpson, J. A., Kenangalem, E., Sugiarto, P., et al. (2015). Plasmodium malariae infection associated with a high burden of anemia: A hospital-based surveillance study. *PloS Negl. Trop. Dis.* 9, e0004195. doi: 10.1371/journal.pntd.0004195

Manego, R. Z., Mombo-Ngoma, G., Witte, M., Held, J., Gmeiner, M., Gebru, T., et al. (2017). Demography, maternal health and the epidemiology of malaria and other major infectious diseases in the rural department Tsamba-Magotsi, Ngounie Province, in central African Gabon. *BMC Public Health* 17, 130. doi: 10.1186/s12889-017-4045-x

Manrique, P., Hoshi, M., Fasabi, M., Nolasco, O., Yori, P., Calderón, M., et al. (2015). Assessment of an automated capillary system for Plasmodium vivax microsatellite genotyping. *Malar J.* 14, 326. doi: 10.1186/s12936-015-0842-9

Mathema, V. B., Nakeesathit, S., Pagornrat, W., Smithuis, F., White, N. J., Dondorp, A. M., et al. (2020). Polymorphic markers for identification of parasite population in Plasmodium malariae. *Malaria J.* 19. doi: 10.1186/s12936-020-3122-2

Mbama Ntabi, J. D., Lissom, A., Djontu, J. C., Diafouka-Kietela, S., Vouvoungui, C., Boumpoutou, R. K., et al. (2022). Prevalence of non-Plasmodium falciparum species in southern districts of Brazzaville in The Republic of the Congo. *Parasites Vectors* 15. doi: 10.1186/s13071-022-05312-9

Messerli, C., Hofmann, N. E., Beck, H. P., and Felger, I. (2017). Critical evaluation of molecular monitoring in malaria drug efficacy trials and pitfalls of length-polymorphic markers. *Antimicrob. Agents Chemother.* 61. doi: 10.1128/AAC.01500-16

Miezan, A. J. S., Gnagne, A. P., Bedia-Tanoh, A. V., Kone, E. G. M., Konate-Toure, A. A., Angora, K. E., et al. (2023). Molecular epidemiology of non-falciparum Plasmodium infections in three different areas of the Ivory Coast. *Malar J.* 22, 211. doi: 10.1186/s12936-023-04639-7

Nainggolan, I. R. A., Syafutri, R. D., Sinambela, M. N., Devina, C., Hasibuan, B. S., Chuangchaiya, S., et al. (2022). The presence of Plasmodium malariae and Plasmodium knowlesi in near malaria elimination setting in western Indonesia. *Malaria J.* 21. doi: 10.1186/s12936-022-04335-y

Nguiffo-Nguete, D., Nongley Nkemngo, F., Ndo, C., Agbor, J.-P., Boussougou-Sambe, S. T., Salako Djogbénou, L., et al. (2023). Plasmodium malariae contributes to high levels of malaria transmission in a forest–savannah transition area in Cameroon. *Parasites Vectors* 16. doi: 10.1186/s13071-022-05635-7

Nguyen, T. T., Nzigou Mombo, B., Lalremruata, A., Koehne, E., Zoleko Manego, R., Dimessa Mbadinga, L. B., et al. (2019). DNA recovery from archived RDTs for genetic characterization of Plasmodium falciparum in a routine setting in Lambaréné, Gabon. *Malar J.* 18, 336. doi: 10.1186/s12936-019-2972-y

Nundu, S. S., Culleton, R., Simpson, S. V., Arima, H., Muyembe, J. J., Mita, T., et al. (2021). Malaria parasite species composition of Plasmodium infections among asymptomatic and symptomatic school-age children in rural and urban areas of Kinshasa, Democratic Republic of Congo. *Malar J.* 20, 389. doi: 10.1186/s12936-021-03919-4

Organization, W. H. (2023). *World malaria report 2023*. (World Health Organization).

Putaporntip, C., Kuamsab, N., Rojrung, R., Seethamchai, S., and Jongwutiwes, S. (2022). Structural organization and sequence diversity of the complete nucleotide sequence encoding the Plasmodium malariae merozoite surface protein-1. *Sci. Rep.* 12. doi: 10.1038/s41598-022-19049-z

Srisutham, S., Saralamba, N., Sriprawat, K., Mayxay, M., Smithuis, F., Nosten, F., et al. (2018). Genetic diversity of three surface protein genes in Plasmodium malariae from three Asian countries. *Malaria J.* 17. doi: 10.1186/s12936-018-2176-x

Sutherland, C. J. (2016). Persistent parasitism: the adaptive biology of malariae and ovale malaria. *Trends Parasitol.* 32, 808–819. doi: 10.1016/j.pt.2016.07.001

Tadele, G., Jaiteh, F. K., Oboh, M., Oriero, E., Dugassa, S., Amambua-Ngwa, A., et al. (2022). Low genetic diversity of Plasmodium falciparum merozoite surface protein 1 and 2 and multiplicity of infections in western Ethiopia following effective malaria interventions. *Malar J.* 21, 383. doi: 10.1186/s12936-022-04394-1

Wamae, K., Ndwiga, L., Kharabora, O., Kimenyi, K., Osoti, V., De Laurent, Z., et al. (2022). Targeted amplicon deep sequencing of ama1 and mdr1 to track within-host. *Wellcome Open Res.* 7, 95. doi: 10.12688/wellcomeopenres

Woldearegai, T. G., Lalremruata, A., Nguyen, T. T., Gmeiner, M., Veletzky, L., Tazemda-Kuitsouc, G. B., et al. (2019). Characterization of Plasmodium infections among inhabitants of rural areas in Gabon. *Sci. Rep.* 9. doi: 10.1038/s41598-019-46194-9

Yman, V., Wandell, G., Mutemi, D. D., Miglar, A., Asghar, M., Hammar, U., et al. (2019). Persistent transmission of Plasmodium malariae and Plasmodium ovale species in an area of declining Plasmodium falciparum transmission in eastern Tanzania. *PloS Negl. Trop. Dis.* 13, e0007414. doi: 10.1371/journal.pntd.0007414

Zhou, M., Liu, Q., Wongsrichanalai, C., Suwonkerd, W., Panart, K., Prajakwong, S., et al. (1998). High prevalence of Plasmodium malariae and Plasmodium ovale in malaria patients along the Thai-Myanmar border, as revealed by acridine orange staining and PCR-based diagnoses. *Trop. Med. Int. Health* 3, 304–312. doi: 10.1046/j.1365-3156.1998.00223.x

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Whole genome analysis of two sympatric human *Mansonella*: *Mansonella perstans* and *Mansonella* sp "DEUX"

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Introduction: *Mansonella* species are filarial parasites that infect humans worldwide. Although these infections are common, knowledge of the pathology and diversity of the causative species is limited. Furthermore, the lack of sequencing data for *Mansonella* species, shows that their research is neglected. Apart from Mansonella perstans, a potential new species called *Mansonella* sp "DEUX" has been identified in Gabon, which is prevalent at high frequencies. We aimed to further determine if *Mansonella* sp "DEUX" is a genotype of M. *perstans*, or if these are two sympatric species.

Methods: We screened individuals in the area of Fougamou, Gabon for Mansonella mono-infections and generated de novo assemblies from the respective samples. For evolutionary analysis, a phylogenetic tree was reconstructed, and the differences and divergence times are presented. In addition, mitogenomes were generated and phylogenies based on 12S rDNA and cox1 were created.

Results: We successfully generated whole genomes for M. perstans and *Mansonella* sp "DEUX". Phylogenetic analysis based on annotated protein sequences, support the hypothesis of two distinct species. The inferred evolutionary analysis suggested, that M. perstans and *Mansonella* sp "DEUX" separated around 778,000 years ago. Analysis based on mitochondrial marker genes support our hypothesis of two sympatric human Mansonella species.

Discussion: The results presented indicate that *Mansonella* sp "DEUX" is a new *Mansonella* species. These findings reflect the neglect of this research topic. And the availability of whole genome data will allow further investigations of these species

KEYWORDS

Mansonella* sp "DEUX", *Mansonella perstans*, whole genome sequencing, phylogeny, *Wolbachia

1 Introduction

Infections with nematodes of the genus *Mansonella* are among the most neglected diseases, even though these parasites belong to the most widespread human filaria species. It has been estimated that more than 100 million people are infected with *Mansonella* species in Africa only and around 600 million people are at risk of infection (Simonsen et al., 2011). Three species are known to be prevalent in humans, *Mansonella perstans*, *M. ozzardi*, and *M. streptocerca* with varying geographic distribution. While *M. perstans* is prevalent in most parts of Sub-Saharan Africa and some regions in South and Central America, *M. streptocerca* is restricted to the African continent and *M. ozzardi* to Latin America. In 2015 a potential new species provisionally called *Mansonella* sp "DEUX" was described in febrile children in a hospital-based study in Gabon (Mourembou et al., 2015). Further investigations showed that this was the most frequent filarial species, as during a cross-sectional study in rural Fougamou, Gabon, 35% of individuals carried this parasite (Sandri et al., 2021).

In addition to the nuclear and the mitochondrial genomes, most filarial species comprise an additional genome - the genome of their bacterial endosymbiont *Wolbachia*. These intracellular bacteria can be found in most filarial parasites (Ferri et al., 2011). For *M. perstans* the obligate presence of *Wolbachia* was under debate for a long time (Grobusch et al., 2003; Gehringer et al., 2014; Batsa Debrah et al., 2019). However, improved molecular diagnostics, as well as results from treatment studies demonstrating the efficacy of antibiotics, indicate that *M. perstans* contains these bacteria (Keiser et al., 2008; Coulibaly et al., 2009; Gehringer et al., 2014; Batsa Debrah et al., 2019). *Wolbachia* were also detected in individuals mono infected with *Mansonella* sp "DEUX", confirming carriage of endosymbionts also by this new species/genotype (Sandri et al., 2021). As *Wolbachia* are usually transferred maternally and because of the known genetic variability of this endosymbiont among nematodes, *Wolbachia* genomes could give a further hint on the genetic relatedness of their host species (Bordenstein et al., 2009).

Until recently, no whole genome for any *Mansonella* species infecting humans was available, reflecting the neglect of these infections. Fortunately, this has been recognized and sequences of *M. perstans* and *M. ozzardi* have been generated in the last years (Crainey et al., 2018; Chung et al., 2020; Sinha et al., 2023). A recently published study from Tamarozzi et al. has confirmed the lack of consensus in the management of *Mansonella* infections (Tamarozzi et al., 2022). There are no clear treatment guidelines and also information on specific symptoms and burden of disease is not well described. To contribute to a better evolutionary understanding of *Mansonella* species, we generated and compared the whole genomes as well as the mitogenomes of *M. perstans* and *Mansonella* sp "DEUX" isolated from circulating microfilariae from infected individuals. We aimed to assess the differences between the two sympatric variants to identify if they are two separate species. In addition, we also obtained and analyzed the genomes of the *Wolbachia* symbionts.

2 Materials and methods

2.1 Ethical statement

The study protocol was approved by the Institutional Ethics Committee at the Centre de Recherches Médicales de Lambaréné, Gabon (CEI-CERMEL 002/2018). Adolescents were asked to give written assent, written informed consent was obtained from their legal guardian and of all adult individuals taking part in this study.

2.2 *Mansonella perstans* and *Mansonella* sp "DEUX" sample collection

Individuals ≥ 16 years living in the area of Fougamou, Gabon, with a confirmed *Mansonella* species microfilaria count ≥ 50 parasites/ml and no microscopical detectable *Loa loa* infection were invited to take part in the study. Diagnosis was based on the microscopic reading of two Giemsa-stained thick blood smears (2x 10 µl blood). 18 ml of venous blood was collected in EDTA tubes from participating individuals. The sample was diluted 1:2 with phosphate buffered saline (PBS; Merck) and filtered through a 5 µm filter paper (Whatman) with a syringe to enrich the microfilariae. Filtration was repeated with 30 ml of PBS to remove remaining blood cells. Subsequently the Whatman filter was removed, the sticking microfilariae were washed off with 10 ml PBS, and the mixture was centrifuged (10 minutes, 1800 rpm) to remove the supernatant. The pellet was transferred to a cryotube with RNAlater and frozen at -80°C.

2.3 DNA extraction and *Mansonella* species confirmation

DNA was extracted from a 25 µl pellet using NEB Monarch Genomic Purification Kit (NEB). Sample lysis was performed according to the animal tissue protocol provided by the manufacturer. Incubation was increased to 3 h at 56°C to improve the yield. DNA was eluted with 50 µl elution buffer. *Loa loa* infection as well as coinfection with either *M. perstans* or *Mansonella* sp "DEUX" based on the internal transcribed spacer region (ITS1 region) was excluded by qPCR according to previously published protocols (Sandri et al., 2021). These procedures resulted in three eligible samples, named mperst1, mdeux2 and mdeux3.

2.4 Genome sequencing, *de novo* assemblies and genome annotation

2.4.1 Library preparation for whole genome sequencing

Genomic DNA was quantified using Qubit dsDNA HS assay kit with a Qubit fluorometer (ThermoFisher). DNA library preparation was performed using the Nextera DNA Flex kit (Illumina) following the manufacturers instruction. Briefly, 4 to 20 ng of genomic DNA was diluted in a total volume of 30 µl of nuclease-free water and fragmented at 55°C for 15 minutes using tagmentation. The enzymatic reaction was stopped, and the adapter-tagged DNA was purified using magnetic-bead clean-up. The resulting DNA was amplified using 8 to 12 cycles of PCR, allowing the required sequencing adapters to anneal to the DNA. The resulting libraries were purified using a double-sided beads purification. Library molarity was determined by measuring the library size (approximately 600 bp) using the Fragment Analyzer with the High NGS Fragment 1-6000bp assay (Agilent) and the library concentration (approximately 10 ng/µl) using the Infinite 200Pro (Tecan) and the Quant-iT HS Assay Kit (Thermo Fisher Scientific). The libraries were denaturated, diluted to 162 pM and sequenced as paired-end 250 bp reads on an Illumina NovaSeq6000 (Illumina, San Diego, CA, USA) with a sequencing depth of approximately 200 million clusters per sample.

2.4.2 *De novo* assembly and genome annotation

The first step in the *de novo* assembly of the *Mansonella* species genomes was to remove the reads from the human host from all generated reads. Therefore, Kraken2 (Wood et al., 2019) was used to classify the reads taxonomically since unmapped reads may also indicate the presence of *Wolbachia* sequences. The reads reported as unclassified by Kraken were assumed to be *Mansonella* species reads and used for the *de novo* assembly. Information about the pre-built Kraken database used can be found in the Supplementary File 1. The assembly was performed using the SPAdes *De Novo* Assembler (Bankevich et al., 2012) without error correction, using the default parameters. The quality of the scaffolds assembled by SPAdes were assessed using the genome assembly evaluation tool QUAST (Gurevich et al., 2013) and the genome completeness was assessed using BUSCO (Simao et al., 2015). Also, the N50 values and coverage values of the scaffolds were calculated in this way. BLAST (Altschul et al., 1990) was used to investigate the similarity of the potential *Mansonella* species scaffolds to closely related species. If at least one of the 39 listed species (Supplementary Table 1) occurred in the top 10 BLAST hits of a scaffold, the scaffold was counted as a fragment of the *Mansonella* species genome. Estimated genome length was calculated by summing the lengths of these filtered scaffolds. Intron hints for the gene annotation were generated using the protein sequences of *B. malayi* with the freely available sequence alignment program, Exonerate (Slater and Birney, 2005). Subsequently, AUGUSTUS (Stanke and Morgenstern, 2005) gene prediction tool was used to predict the genes on the scaffolds using the hints and to annotate the predicted genes Supplementary File 1. The predicted genes were aligned to the NCBI protein sequence database using DIAMOND (Buchfink et al., 2021) to find similar genes.

2.5 Evolutionary analysis of *Mansonella* species genomes

2.5.1 Phylome reconstruction

The evolutionary analysis was performed using genome assemblies from 10 nematodes, that were downloaded from NCBI and Uniprot (Supplementary Table 2), in addition to mperst1 and mdeux3. Genome completeness was assessed using BUSCO v4.0.2 (Supplementary Table 2) (Simao et al., 2015). The phylome of *Mansonella* sp "DEUX" was reconstructed from mdeux3 using a set of 11 additional Nematode proteomes, including mperst1. The genomes of *Brugia timori* and *Onchocerca flexuosa* were not used in the phylome reconstruction due to their poor completeness, as well as mdeux2. An automated pipeline was used that applies the same process to reconstruct gene trees as one would do manually (Fuentes et al., 2022). First the proteome database was reconstructed using the 12 species and formatting the codes to phylomeDB format. Then a blastp search was performed between each gene in the genome of *Mansonella* sp "DEUX" and this proteome database. Blast results were filtered using an e-value threshold of 1e-05 and an overlap threshold of 50%. The number of hits was limited to the 150 best hits for each protein. Next, six different multiple sequence alignments were reconstructed using three programs [Muscle v3.8.1551 (Edgar, 2004), mafft v7.407 (Katoh et al., 2005) and kalign v2.04 (Lassmann and Sonnhammer, 2005)] and aligning the sequences in forward and in reverse direction. From this group of alignments, a consensus alignment was obtained using M-coffee from the T-coffee package v12.0 (Wallace et al., 2006). Alignments were then trimmed using trimAl v1.4.rev15 (consistency-score cut-off 0.1667, gap-score cut-off 0.9) (Capella-Gutierrez et al., 2009). IQTREE v1.6.9 (Nguyen et al., 2015) was used to reconstruct a maximum likelihood phylogenetic tree. Model selection was limited to 5 models (DCmut, JTTDCMut, LG, WAG, VT) with freerate categories set

to vary between 4 and 10. The best model according to the BIC criterion was used. 1000 rapid bootstraps were calculated. All trees and alignments were stored in phylomedb (Fuentes et al., 2022) with phylomeID 97 (http://phylomedb.org).

2.5.2 Species tree reconstruction

A species tree was reconstructed using a supertree approach as implemented in duptree v1.48 (Wehe et al., 2008) using all the trees reconstructed in the phylome as input. A second species tree was reconstructed using a gene concatenation approach, adding the three genome assemblies from the species that were not initially included in the phylome reconstruction process (*B. timori* and *O. flexuosa*, as well as the second *Mansonella* sp "DEUX" sample mdeux2). In order to do that, genes in the phylome were selected that were found in single copy in all species. 1068 such genes were selected, and a blast search was performed between these genes and the proteomes of the three newly added assemblies. Only those genes were kept that had only one hit in each of the three new assemblies, reducing the set to 562 genes. The alignment was redone for each selected protein family, adding the three new sequences, and using the same pipeline applied during phylome reconstruction. These alignments were subsequently concatenated into a single multiple sequence alignment which was trimmed using trimAl v1.4.rev15 (Capella-Gutierrez et al., 2009), producing an alignment of 260.126 amino acid positions. IQTREE v1.6.9 (Nguyen et al., 2015) was used to reconstruct the species tree using the model selection option without restrictions. The best model according to BIC was JTT+F+R4.

2.5.3 Nucleotide divergences and SNP calling

Nucleotide divergence was calculated by performing a best bidirectional hit analysis between all pairs of *Brugia*, *Onchocerca* and *Mansonella* species. For pairs of orthologs, the percent of identity was calculated by aligning 100 random pairs of nucleotide sequences using Muscle (Edgar, 2004) and using trimAl (Capella-Gutierrez et al., 2009) to calculate the pairwise identity without considering gaps.

2.5.4 Divergence time calculation

Fossil records for Nematodes were obtained from Paleobiology Database (https://paleobiodb.org/). Four fossils fell within the species tree used in this paper: *Cascofilaria baltica* (33.9 - 38.0 MyA) and *Cascofilaria dominicana* (13.82 - 20.44 MyA) which belong to the Filariidae family, as well as *Ascarites gerus* (129.4 - 122.46 MyA) and *Ascarites rufferi* (237 - 242 MyA), which belong to the Ascarididae family. SortaDate (Smith et al., 2018) was used to select the 20 best genes to use for dating based on their clock-likeness, tree length and topological similarity to the species tree. Trees were used from the phylome, despite having three genome assemblies not included. Alignments were then concatenated, a species tree was reconstructed as detailed above, and phylobayes was used to calculate divergence times using the two calibration points based on the fossil records. The rooting point was set to have happened at least 242 MyA without detailing an upper limit. The second calibration point was set at the base of Filarioidea and was set to have happened between 13 and 38 MyA. Phylobayes 4.1 (Lartillot et al., 2009) was run on the species tree and with those calibration points using two chains, the CAT model and the Birth and Death model. It was run until both chains converged as seen with tracecomp from the Phylobayes package. Readdiv was run to obtain the chronogram using a burn-in of 10.000 and a subsampling frequency of 10.

2.6 Extraction of mitogenome assemblies

The mitogenomes were extracted from the whole genome assembly of the individual samples. Using the visual tool Bandage (Wick et al., 2015) the *M. perstans* mitogenome was blasted against the assembly graph to identify contigs with mitochondrial genomic sequences. In case of multiple matching contigs these were linked based on coverage and sequence similarity to extract one or more complete mitogenomes from the assembly.

2.6.1 Phylogenetic tree reconstruction based on mitogenomic data

The analysis follows the companion repository from Chung et al. (2020). First, a multiple sequence alignment including the mitogenomes of mperst1, mdeux2 and mdeux3 as well as *Mansonella* related species from NCBI (Supplementary Table 3) was created using CLUSTAL OMEGA v.1.2.3. Then the phylogeny was calculated using IQTREE v2.1.4 (Nguyen et al., 2015) and visualized using iTOL (Letunic and Bork, 2021). For the 12S rDNA phylogenies, rRNA sequences were extracted from the genome assemblies using BARRNAP v0.9 Seemann, T (2013) and then combined with published 12S rDNA sequences from other *Mansonella* species. 12S rDNA and *cox1* phylogenies were created using the same methods described above for the metagenome data.

3 Results

3.1 *De novo* assembly and gene annotation

Genome assemblies were generated from *Mansonella* microfilaria isolated from three individuals living in the area of Fougamou, Gabon. They presented with a qPCR-confirmed mono-infections of either *M. perstans* (individual 1: mperst1) or *Mansonella* sp "DEUX" (individual 2: mdeux2 and individual 3: mdeux3) (Table 1). Reads for all three samples were obtained with a coverage of 7.4x (mperst1), 32.4x (mdeux2), and 65.5x (mdeux3), resulting in a total assembly size of 76.2 Mb, 80 Mb and 78.5 Mb, respectively (Table 2 and Supplementary Table 4). Although the generated whole genome sizes are comparable, the N50 from mdeux3 is twice and three times the size of the other two assemblies respectively. This makes mdeux3 the most contiguous assembly generated thus far. To further verify the quality of the assemblies, the completeness of the data was quantified in terms of the expected gene content, using BUSCO (Benchmarking Single-Copy Orthologs) tool. For all three samples, more than 90% of conserved eukaryotic gene orthologs were identified. GC content as

TABLE 1 Infection status, parasitemia and demographic data of individuals for mperst1, mdeux2 and mdeux3.

Sample ID	Species based on ITS1 qPCR	Ct value	Microscopic count [mf per ml]	Participant sex	Participant age [years]	Participant living area
mperst1	*Mansonella perstans*	25	350	female	71	Nzong Bang, Lambaréné, Gabon
mdeux2	*Mansonella* sp "DEUX"	26	125	male	78	Nzemba, Lambaréné, Gabon
mdeux3	*Mansonella* sp "DEUX"	24	400	male	70	Moukabou, Lambaréné, Gabon

well as the number of predicted genes are comparable among all three samples.

The respective mitochondrial and *Wolbachia* genomes were assembled independently for all three samples. *Wolbachia* reads were sequenced and assembled from all three samples called *w*Mpe1 for mperst1, *w*Mde2 for mdeux2, and *w*Mde3 for mdeux3. The *Wolbachia* assemblies are of comparable low quality (Supplementary Table 5). As a comparison, *Wolbachia* assemblies from other hosts were included. Among them are *Wolbachia* from supergroup F, as well as from supergroup D. Overall the here generated *Wolbachia* assemblies show a slightly lower GC content and a comparably high number of small contigs. In general, the assemblies are shorter than those used for comparison, especially *w*Mpe1 is four times smaller than the reference *w*Mpe (JACZHU01.1). Due to the poor quality, further analyses are not presented here.

3.2 Evolutionary analysis indicates a divergence between *Mansonella perstans* and *Mansonella* sp "DEUX"

To provide further information on the orthology and paralogy relationships across the genes of the nematode species, a phylome – i.e., a complete catalogue of gene evolutionary histories - was reconstructed for *M. perstans* from mperst1 and *Mansonella* sp "DEUX" from mdeux3, including 10 other sequenced nematode species. This allowed the selection of widespread genes that were present in single copy in the selected species. Subsequently, these genes were also identified in the second *Mansonella* sp "DEUX" sample mdeux2 as well as in two additional nematode species which were not included in the phylome analyses due to the fragmented nature of their assemblies (*Brugia timori* and *Onchocerca flexuosa*). This set of widespread genes was used to build a concatenated alignment and then a species tree was reconstructed (Figure 1). Using the inferred branch lengths from this tree, the phylogenetic distances within groups of species or strains from the same genus (*Mansonella, Brugia* and *Onchocerca*) were compared. As seen in Figure 1, branch lengths are very short for all three species groups, though the branches are shortest between the two *Mansonella* sp "DEUX" strains mdeux2 and mdeux3. The branch separating *Mansonella* sp "DEUX" and *M. perstans* is similar in length to the one separating *Onchocerca ochengi* and *O. volvulus* and the one separating *B. timori* and *B. malayi*, indicating that, at a genomic level, they have a similar genetic divergence. These findings are further confirmed by the calculations of nucleotide and amino acid diversity between the species in the three genera *Mansonella*, *Brugia* and *Onchocerca* (Supplementary Table 6). These analyses support the separation of *Mansonella* sp "DEUX" and *M. perstans* into two different species, as their genetic divergence is similar to that between established nematode species.

Our phylogenetic analysis supports the results of the multi-locus sequence typing analysis by Lefoulon et al. (2015), which suggests a division of *Onchocercidae* into distinct phylogenetic clades (ONC1-ONC5). Figure 1 additionally shows the phylogenetic *Onchocercidae* clades ONC3, ONC4, and ONC5. Consistent with Lefoulon et al., all marked species are placed into the same clades. *O. flexuosa*, *O. ochengi*, and *O. volvulus* into clade ONC3, *Litomosoides sigmodontis* and *Acanthocheilonema vitae* into clade ONC4. *Loa loa, B. pahangi, B. timori, B. malayi* as well as the three *Mansonella* assemblies from mperst1, mdeux2 and mdeux3 are placed into clade ONC5.

To calculate the divergence time of mperst1 from mdeux2 and mdeux3, a chronogram was generated (Figure 2). Two calibration points were set based on fossil records, one placed at the root of the tree and the other at the base of the Filarioidea super family. Using this calibration, the divergence time between *Mansonella* sp "DEUX" and *M. perstans* is estimated at 778,000 years. To better classify these time spans, we give the divergence time of the closest members of *Brugia*

TABLE 2 Comparison of genome assemblies mperst1, mdeux2 and mdeux3 to *L. loa*. A more detailed comparison to other filarial species can be found in Supplementary Table 4.

	mperst1	mdeux2	mdeux3	*L. loa*
Total length (Mb)	76.2	80.0	78.5	91.4
GC (%)	30.3	30.3	30.3	31.0
N50 (kb)	45.5	101.8	173.8	174.4
Complete BUSCO (%)	94.7	94.4	96.0	95.4

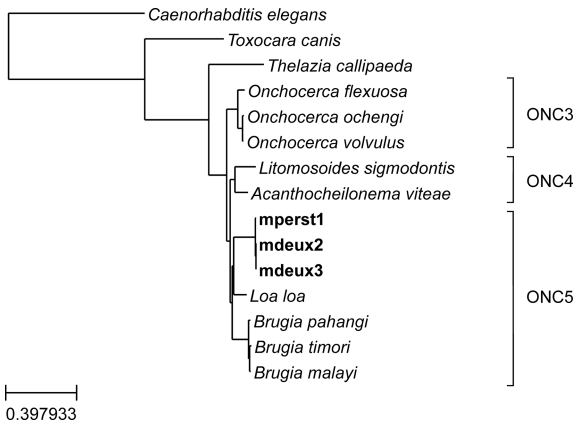

FIGURE 1
A phylogenetic tree reconstructed for *M. perstans* assembly mperst1 and the two *Mansonella* sp "DEUX" assemblies mdeux2 and mdeux3, including 12 other nematode species based on the concatenation of 562 protein alignments. *Onchocercidae* clades ONC3, ONC4 and ONC5 are shown next to the corresponding species.

(1.6 MyA) and *Onchocerca* (1.8 MyA) for comparison. The divergence time of the two strains of *Mansonella* sp "DEUX" is 33,000 years.

3.3 Phylogeny based on mitochondrial sequences reveals three distinct *Mansonella* mitogenomes

Sequence information for filaria species is rare, however a mitochondrial genome of *M. ozzardi* and *M. perstans* has been published recently (Crainey et al., 2018; Chung et al., 2020). Therefore, a phylogenetic analysis based on the mitogenomes was performed to further understand the relationship between *M. perstans* and *Mansonella* sp "DEUX".

Figure 3A shows the generated phylogeny based on the mitogenomes of 17 nematode species, including the newly

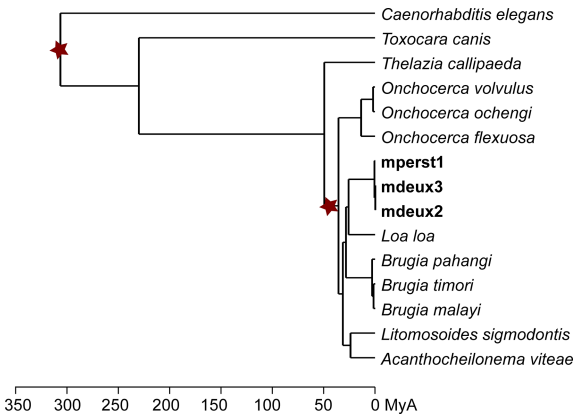

FIGURE 2
Evolutionary analysis of *Mansonella* sp "DEUX" and *M. perstans*. Chronogram detailing the divergence times of 15 nematode species. Stars indicate calibration points derived from recorded fossil evidence.

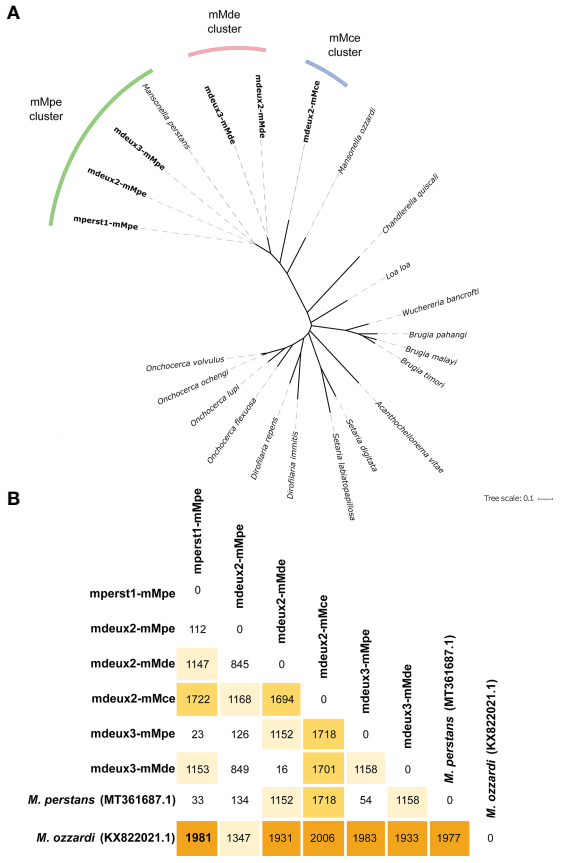

FIGURE 3
Mitogenome analysis. **(A)** Mitogenome phylogeny of different nematode species, including the newly generated mitogenomes from the three assemblies forming three *Mansonella* mitogenome clusters: Mitogenome of *M. perstans* mMpe marked in green, comprising of the mitogenomes from mperst1, and additionally two mitogenomes at low coverage of the individuals mdeux2 and mdeux3. *Mansonella* sp "DEUX" mMde marked in orange, with two mitogenomes of high coverage of mdeux2 and mdeux3. And the surprising third mitogenome of "*M. cermeli*" mMce with a single mitogenome of individual 2 (mdeux2). **(B)** SNP variant call between the three mitogenomes of each individual generated by us and the published mitogenomes of *M. perstans* (GenBank: MT361687) and *M. ozzardi* (GenBank: KX822021).

generated mitogenomes from the three assemblies. In total, three distinct mitogenome clusters from different species were identified from the assemblies. The first mitogenome cluster, designated as mMpe (mitogenome *M. perstans*), can be assigned to *M. perstans*, as it clusters with the published *M. perstans* mitogenome (GenBank: MT361687). It is found in mperst1 with a coverage of 40x. Unexpectedly, although identified as mono-infected with *Mansonella* sp "DEUX" *via* qPCR, assemblies of the individuals mdeux2 and mdeux3 were found to be mixed infected at low coverage with *M. perstans*. Both have in addition one mMpe mitogenome each, with lower coverage depths of 7x and 12x compared to the published *M. perstans* mitogenome, respectively. However, there were also two additional mitogenomes identified in each of these two assemblies that cluster together, both with a high coverage of 140x and 290x respectively. They show no similarity to any published mitogenomes and were therefore assigned to *Mansonella* sp "DEUX" and designated as mMde (mitogenome

Mansonella sp "DEUX"). Surprisingly, a third mitogenome not clustering to the other mitogenomes or any other published sequence was identified in assembly mdeux2. Though it was sequenced at low coverage of 8x, it was assembled into a single contig, therefore confidence in the sequence is high. As the phylogeny suggests that this mitogenome originates from a separate species we provisionally call it "*Mansonella cermeli*" and the respective mitogenome mMce.

To compare the similarity of the generated mitogenomes, a SNP variant call was performed, including the reference mitogenomes of *M. perstans* (MT361687) and *M. ozzardi* (KX822021) (Figure 3B). The highest number of SNPs is found between each mitogenome and *M. ozzardi*. Whereas the lowest number of SNPs is found between the mitogenomes within one cluster as well as the mitogenomes from the cluster mMpe and *M. perstans*. Both samples from the mMde cluster (mdeux2-mMde and mdeux3-mMde) as well as the mMce cluster have a high number of SNPs compared to the mMpe cluster and the two references from *M. perstans* and *M. ozzardi*. This further supports the findings from the phylogenetic tree. Overall, genome size and GC content from all generated clusters are comparable to the published mitogenomes from *M. perstans* and *M. ozzardi* for all mitogenomes that were generated (Table 3). Both *Mansonella* sp "DEUX" mitogenomes mdeux2-mMde and mdeux3-mMde have a high coverage and are very similar to each other, showing a 99.8% pairwise identity. The same applies for the three *M. perstans* mitogenomes mperst1-mMpe, mdeux2-mMpe and mdeux3-mMpe. However, mdeux2_mMpe has a lower coverage and could not be completely assembled. The other two *M. perstans* mitogenomes though show a high similarity to the published *M. perstans* mitogenome. These data further support our hypothesis of two sympatric human *Mansonella* species.

3.4 *Mansonella* sp "DEUX" infects humans as well as great apes

Parasites from the genus *Mansonella* are also known to infect non-human primates (NHPs). Only recently, filarial DNA extracted from NHPs faecal samples from Gabon and the neighbouring country Cameroon was used to investigate the *Onchocercidae* phylogeny based on two mitochondrial genes, cytochrome c oxidase subunit 1 (*cox1)* and 12S rDNA (Gaillard et al., 2020). These phylogenetic analyses showed that sequences obtained from chimpanzee cluster with samples from humans infected with *M. perstans*, concluding that chimpanzees can also be infected with *M. perstans*. In addition, they found NHPs to be infected with sequences of filaria that fall within the *Mansonella* genus, but cluster separately and are different from all published sequences.

We extended the published phylogenetic trees from Gaillard et al. with the respective *cox1* (Figure 4) and 12S rDNA (Figure 5) sequences derived from the newly generated mitogenomes. As expected, the *cox1* sequences of three mitogenomes of the mMpe cluster, map to the published sequences by Gaillard et al., that cluster to *M. perstans*. The *cox1* sequences of the *Mansonella* sp "DEUX" mitogenomes (mMde) map to the unclassified *cox1* sequences of NHPs that could not be assigned to a *Mansonella* species by Gaillard et al. Therefore, it can be assumed that *Mansonella* sp "DEUX" infects humans and great apes. *Cox1* sequences of the mMce mitogenome cluster separately. Overall, we see the same five monophyletic groups, as depicted in Gaillard et al. The phylogenetic tree (Figure 5) based on 12S rDNA clusters corresponds very well to the clusters seen for *cox1*. We see separate clusters for the potential different species, as sequences from the *M. perstans* assemblies (mMpe assemblies: mperst1-mMpe, mdeux2-mMpe and mdeux3-mMpe) cluster to the published *M. perstans* sequences. This cluster can therefore be assigned to *M. perstans*. The 12S rDNA sequence from the mMce mitogenome forms a separate cluster. A third cluster is generated from two 12S rDNA sequences potentially originating from the *Mansonella* sp "DEUX" mitogenome assemblies mMde and additional 12S rDNA sequences generated from Gaillard et al., that were formerly grouped together with *M. perstans* sequences.

4 Discussion

Infections with *Mansonella* species have been neglected in all areas, this is also reflected by the fact, that until recently no whole

TABLE 3 Comparison of different characteristics of the generated mitogenome clusters, including the published mitogenomes from *M. perstans* (MT361687) and *M. ozzardi* (KX822021).

Mitogenome	Length	GC (%)	Found in sample	Coverage
mMpe	13,616 bp	26.0	mperst1	40x
	8,978 bp	26.3	mdeux2	7x
	13,617 bp	26.0	mdeux3	12x
mMde	13,619 bp	25.5	mdeux2	140x
	13,621 bp	25.5	mdeux3	290x
mMce	12,613 bp	26.3	mdeux2	8x
***M. perstans* (MT361687)**	13,619 bp	25.9	NA	1,115x
***M. ozzardi* (KX822021)**	13,681 bp	25.7	NA	NA

NA, not applicable.

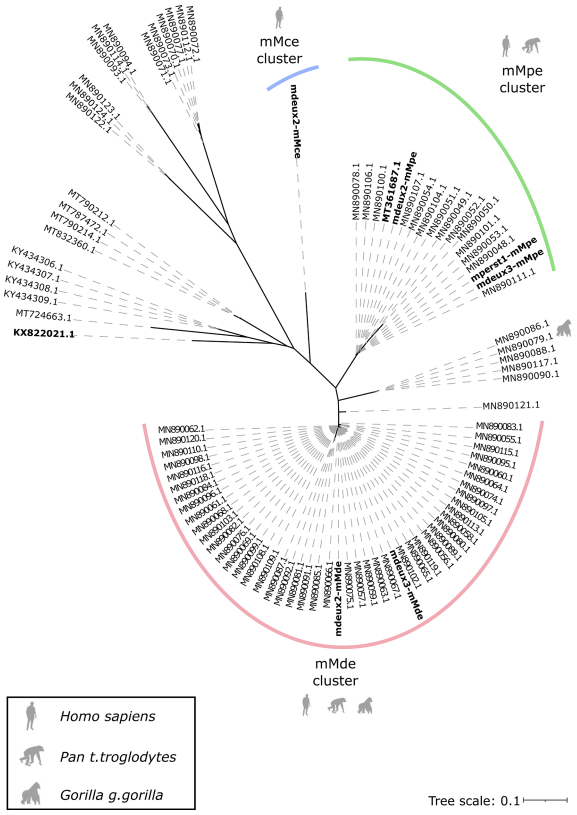

FIGURE 4
Phylogeny based on *cox1* gene sequences. The three mitogenome clusters mMpe, mMde and mMce are highlighted in green, red and blue respectively. The generated mitogenomes, as well as the published mitogenomes from *M. perstans* (GenBank: MT361687) and *M. ozzardi* (GenBank: KX822021) are highlighted in bold.

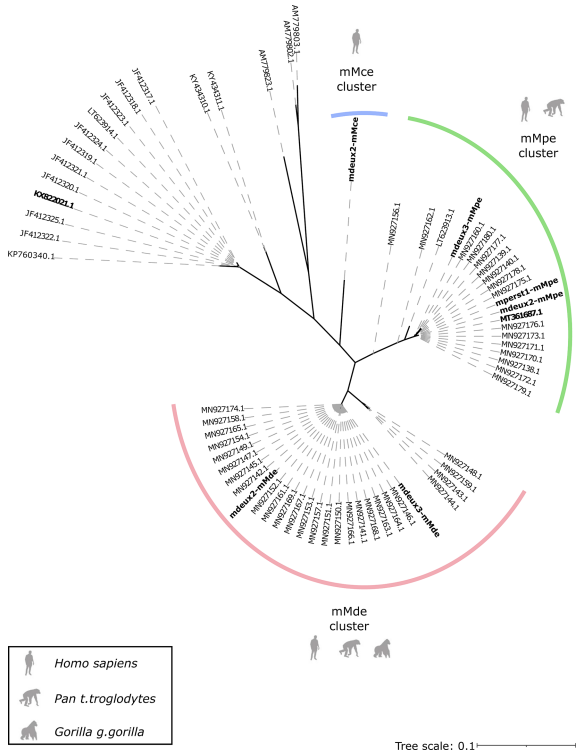

FIGURE 5
Phylogeny based on 12S rDNA gene sequences. The three mitogenome clusters mMpe, mMde and mMce are highlighted in green, red and blue respectively. The generated mitogenomes, as well as the published mitogenomes from *M. perstans* (GenBank: MT361687) and *M. ozzardi* (GenBank: KX822021) are highlighted in bold.

genome of any of the *Mansonella* species was available. We generated whole genomes of *Mansonella perstans* and the related *Mansonella* sp "DEUX" from microfilaria of infected individuals from Gabon. All assemblies show a high level of quality, as is indicated by high coverage, N50 values and BUSCO scores. The whole genomes are comparable in size, number of predicted genes and GC content to other filarial species. Phylogenetic analysis of the whole genomes as well as the mitogenomes of these two *Mansonella* suggest that these are two distinct species. Evolutionary analysis of the whole genome comparisons supported these findings as they give estimates that these sympatric species have diverged 778,000 years ago.

However, we found sites of recombination between the whole genomes of *M. perstans* and *Mansonella* sp "DEUX". This can be a true finding, indicating that these species are still able to cross in rare occasions. On the other hand, this can also be due to our whole genome assemblies. Based on mitogenomic data, we discovered that the *Mansonella* sp "DEUX" samples, which we originally assumed to be mono-infected, are in fact co-infected with *M. perstans* and/or an unknown potential third species "*M. cermeli*" at a low coverage. These co-infections have influenced our analysis, and it might be that they are responsible for the observed recombination regions that could lead to less accurate estimates. The relatively high divergence time of 33,000 years between the two *Mansonella* sp "DEUX" assemblies could also be explained by the influence of the co-infections in those samples. However, the overall results provide a good starting point for further research. With the collection of more *Mansonella* samples from different regions in the future, the genetic differences between samples of the same species can be estimated and can be included in the equations, and the divergence times can be calculated more accurately.

The first previously sequenced and published mitogenome of *M. perstans* from a sample from Cameroon (Chung et al., 2020) clusters closely to our *M. perstans* mitogenome and its inclusion in the phylogenetic analysis additionally supports the finding of two distinct species. Since sequence data on *Mansonella* species from different regions are limited, we additionally investigated the genes for which the most sequence data are available: *cox1* and 12S rDNA. Based on both genes, we have identified two *Mansonella* species clusters that correspond to either *M. perstans* or *Mansonella* sp "DEUX"; the same clustering was also seen by Gaillard et al. for *cox1* sequences (Gaillard et al., 2020). We can confirm these two separate clusters also based on the 12S rDNA sequences. Only one cluster for 12S rDNA is shown by Gaillard et al., however, when we reanalyzed Gaillard's data, we identified two clusters.

Molecular markers based on mitochondrial sequences are a commonly used tool in phylogenetic studies due to the conserved nature among species, and several markers have assisted in recent years to differentiate filarial species and determine the phylogeny of

the *Onchocercidae* family (Xie et al., 1994; Lefoulon et al., 2015; Yilmaz et al., 2016; Crainey et al., 2018). The investigated markers 12S rDNA and *cox1* overall support the evolutionary relationships and classification into different clades, as suggested by Lefoulon et al. (2015). Based on multi locus sequence typing they had identified five strongly supported clades, whereby all *Mansonella* species fall within clade ONC5. The affiliation to the different ONC clades is also reflected in our phylogeny based on the complete mitogenome and the phylogeny based on the concatenation of the 562 protein alignments as seen in Figure 1.

Our analysis of the *cox1* and 12S rDNA genes additionally showed that our *Mansonella* sp "DEUX" sequences align to *Mansonella* sequences obtained from great apes from the same country (Gabon) and the neighboring country Cameroon (Gaillard et al., 2020), suggesting that *Mansonella* sp "DEUX" is infecting humans as well as NHPs. There are a few *Mansonella* species that have been described to occur in great apes based on morphology, as *M. vanhoofi* (Peel, 1946), *M. gorillae* (van den Berghe and Chardome, 1949), *M. leopoldi* (van den Berghe et al., 1957) *and M. loopensis* (Bain et al., 1995). Most of them have been described in the form of a case report. One ape related species has been described to occasionally infect humans in Gabon – *M. rodhaini* (Richard-Lenoble et al., 1988). There is no sequence information for any of these species available to compare them on a genetic level to our assemblies. However, *M. rodhaini* is described to be a skin dwelling species like *M. streptocerca* and not a blood dwelling species like *M. perstans* or *Mansonella* sp "DEUX". We also compared our obtained sequences to the few available sequences of the skin dwelling *M. streptocerca*, but they did not map. Therefore, there is no indication that these skin dwelling species are the same as *Mansonella* sp "DEUX". To determine if one of the morphologically described great ape infecting *Mansonella* species could be *Mansonella* sp "DEUX", microscopic investigations of mono-infected samples could be done.

We additionally identified *Wolbachia* reads in all three sequenced samples. *Wolbachia* are intracellular bacteria (order *Rickettsiales*) found in a range of arthropod and nematode species (Taylor et al., 2018). In filarial nematodes *Wolbachia* are usually symbionts, beneficial for the host survival and reproduction (Bandi et al., 1999; Hoerauf et al., 1999; Volkmann et al., 2003). Antibiotic treatment of filarial infections targets the *Wolbachia* and are promising alternative treatment options, especially for *M. perstans* infections, where standard filarial treatments are not efficacious (Coulibaly et al., 2009; Raoult, 2010; Batsa Debrah et al., 2019). Currently, *Wolbachia* are taxonomically subdivided into 16 different so-called supergroups. *Wolbachia* of filaria fall in supergroups C, D, and J with the exception of *Wolbachia* of *Mansonella* species that fall in supergroup F. Interestingly, all other *Wolbachia* of supergroup F have arthropod hosts (Covacin and Barker, 2007; Ferri et al., 2011). We could confirm that *Wolbachia* reads from *Mansonella* sp "DEUX" also belong to supergroup F, similar to *Wolbachia* of *M. perstans* and *M. ozzardi* (Casiraghi et al., 2001; Keiser et al., 2008). Whole genomes as well as sequence comparisons of the *Wolbachia* of these two *Mansonella* species (*M. perstans and M. ozzardi*) are described in a preprint (Sinha et al., 2021). *Wolbachia* are usually inherited maternally and differences between *Wolbachia* of different species can give further evidence on their relatedness. The read coverage of our assembled *Wolbachia* genomes was low (<10x) leading to a high fragmentation with many small contigs. However, the finding of *Wolbachia* reads in all three samples, further supports the carriage of *Wolbachia* in *M. perstans* and *Mansonella* sp "DEUX".

Based on mitogenomic analysis, we found a third *Mansonella* mitogenome that did not cluster with any of the available sequences and might therefore even present another new *Mansonella* species, that we named "*Mansonella cermeli*". As read coverage was low, we could not construct a separate whole genome, but because of the multicopy nature of mitogenomic DNA in general, we could construct a separate mitogenome. Evidence of this separate genome is high, as the whole mitogenomes could be assembled into a single circular contig. This is the first time that sequences of "*M. cermeli*" are detected, and we do not have a morphologic proof of its existence. Also, we do not know if this is a common species/infection in that region. Further studies must be conducted to analyze if this is another common *Mansonella* species infecting humans. The mitogenomes reported here show that the species diversity within *Mansonella* might even be higher than previously thought.

Overall, the results of our study suggest that *Mansonella* sp "DEUX" represents a new *Mansonella* species. It is most probably not restricted to the human host, but also infects great apes. However, given the high prevalence of this mansonellosis in the population in this area, one can assume that it is also a true human infection. The detection of the additional "*M. cermeli*" mitogenome further illustrates the lack of knowledge about this genus. Fortunately, awareness is increasing and more data are being published, such as a preprint this year describing the entire genomes of *M. perstans* and *M. ozzardi* (Sinha et al., 2023). The authors discuss clinical differences in response to antifungal drugs in the context of molecular differences in selected genes of the different species. These publications together with the sequence data are of great value for further research on *Mansonella*. There are many open questions on *Mansonella* infections not only related to phylogeny and number of infecting species, but also on the burden of disease, adequate treatment and impact on the immune system. However, to properly address these questions, one prerequisite is to be able to identify, name, and differentiate the infecting species.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB57801 and PRJNA942613.

Author contributions

JH, TS, SO, SA, NC designed the study. LB collected samples. MR, LB, MP performed lab experiments. MR, CG, TS, MM-H, EK, TG, SO, JH analyzed data. TS, NC, AK, SA interpreted results. TG,

NC, CK, AK, JH provided resources. MR, CG, JH wrote the first draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1159814/full#supplementary-material

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215 (3), 403–410. doi: 10.1016/S0022-2836(05)80360-2

Bain, O., Moisson, P., Huerre, M., Landsoud-Soukate, J., and Tutin, C. (1995). Filariae from a wild gorilla in Gabon with description of a new species of mansonella. *Parasite.* 2 (3), 315–322. doi: 10.1051/parasite/1995023315

Bandi, C., McCall, J. W., Genchi, C., Corona, S., Venco, L., and Sacchi, L. (1999). Effects of tetracycline on the filarial worms brugia pahangi and dirofilaria immitis and their bacterial endosymbionts wolbachia. *Int. J. Parasitol.* 29 (2), 357–364. doi: 10.1016/S0020-7519(98)00200-8

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19 (5), 455–477. doi: 10.1089/cmb.2012.0021

Batsa Debrah, L., Phillips, R. O., Pfarr, K., Klarmann-Schulz, U., Opoku, V. S., Nausch, N., et al. (2019). The efficacy of doxycycline treatment on. *Am. J. Trop. Med. Hyg.* 101 (1), 84–92. doi: 10.4269/ajtmh.18-0491

Bordenstein, S. R., Paraskevopoulos, C., Dunning Hotopp, J. C., Sapountzis, P., Lo, N., Bandi, C., et al. (2009). Parasitism and mutualism in wolbachia: what the phylogenomic trees can and cannot say. *Mol. Biol. Evol.* 26 (1), 231–241. doi: 10.1093/molbev/msn243

Buchfink, B., Reuter, K., and Drost, H. G. (2021). Sensitive protein alignments at tree-of-life scale using DIAMOND. *Nat. Methods* 18 (4), 366–368. doi: 10.1038/s41592-021-01101-x

Capella-Gutierrez, S., Silla-Martinez, J. M., and Gabaldon, T. (2009). trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics.* 25 (15), 1972–1973. doi: 10.1093/bioinformatics/btp348

Casiraghi, M., Favia, G., Cancrini, G., Bartoloni, A., and Bandi, C. (2001). Molecular identification of wolbachia from the filarial nematode mansonella ozzardi. *Parasitol. Res.* 87 (5), 417–420. doi: 10.1007/s004360000368

Chung, M., Aluvathingal, J., Bromley, R. E., Nadendla, S., Fombad, F. F., Kien, C. A., et al. (2020). Complete mitochondrial genome sequence of mansonella perstans. *Microbiol. Resour Announc* 9 (30). doi: 10.1128/MRA.00490-20

Coulibaly, Y. I., Dembele, B., Diallo, A. A., Lipner, E. M., Doumbia, S. S., Coulibaly, S. Y., et al. (2009). A randomized trial of doxycycline for mansonella perstans infection. *N Engl. J. Med.* 361 (15), 1448–1458. doi: 10.1056/NEJMoa0900863

Covacin, C., and Barker, S. C. (2007). Supergroup f wolbachia bacteria parasitise lice (Insecta: Phthiraptera). *Parasitol. Res.* 100 (3), 479–485. doi: 10.1007/s00436-006-0452-0

Crainey, J. L., Marín, M. A., Silva, T. R. R. D., de Medeiros, J. F., Pessoa, F. A. C., Santos, Y. V., et al. (2018). Mansonella ozzardi mitogenome and pseudogene characterisation provides new perspectives on filarial parasite systematics and CO-1 barcoding. *Sci. Rep.* 8 (1), 6158. doi: 10.1038/s41598-018-24382-3

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32 (5), 1792–1797. doi: 10.1093/nar/gkh340

Ferri, E., Bain, O., Barbuto, M., Martin, C., Lo, N., Uni, S., et al. (2011). New insights into the evolution of wolbachia infections in filarial nematodes inferred from a large range of screened species. *PloS One* 6 (6), e20843. doi: 10.1371/journal.pone.0020843

Fuentes, D., Molina, M., Chorostecki, U., Capella-Gutiérrez, S., Marcet-Houben, M., and Gabaldón, T. (2022). PhylomeDB V5: an expanding repository for genome-wide catalogues of annotated gene phylogenies. *Nucleic Acids Res.* 50 (D1), D1062–D10D8. doi: 10.1093/nar/gkab966

Gaillard, C. M., Pion, S. D., Hamou, H., Sirima, C., Bizet, C., Lemarcis, T., et al. (2020). Detection of DNA of filariae closely related to mansonella perstans in faecal samples from wild non-human primates from Cameroon and Gabon. *Parasit Vectors.* 13 (1), 313. doi: 10.1186/s13071-020-04184-1

Gehringer, C., Kreidenweiss, A., Flamen, A., Antony, J. S., Grobusch, M. P., and Bélard, S. (2014). Molecular evidence of wolbachia endosymbiosis in mansonella perstans in Gabon, central Africa. *J. Infect. Dis.* 210 (10), 1633–1638. doi: 10.1093/infdis/jiu320

Grobusch, M. P., Kombila, M., Autenrieth, I., Mehlhorn, H., and Kremsner, P. G. (2003). No evidence of wolbachia endosymbiosis with loa loa and mansonella perstans. *Parasitol. Res.* 90 (5), 405–408. doi: 10.1007/s00436-003-0872-z

Gurevich, A., Saveliev, V., Vyahhi, N., and Tesler, G. (2013). QUAST: quality assessment tool for genome assemblies. *Bioinformatics.* 29 (8), 1072–1075. doi: 10.1093/bioinformatics/btt086

Hoerauf, A., Nissen-Pähle, K., Schmetz, C., Henkle-Dührsen, K., Blaxter, M. L., Büttner, D. W., et al. (1999). Tetracycline therapy targets intracellular bacteria in the filarial nematode litomosoides sigmodontis and results in filarial infertility. *J. Clin. Invest.* 103 (1), 11–18. doi: 10.1172/JCI4768

Katoh, K., Kuma, K., Toh, H., and Miyata, T. (2005). MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Res.* 33 (2), 511–518. doi: 10.1093/nar/gki198

Keiser, P. B., Coulibaly, Y., Kubofcik, J., Diallo, A. A., Klion, A. D., Traoré, S. F., et al. (2008). Molecular identification of wolbachia from the filarial nematode mansonella perstans. *Mol. Biochem. Parasitol.* 160 (2), 123–128. doi: 10.1016/j.molbiopara.2008.04.012

Lartillot, N., Lepage, T., and Blanquart, S. (2009). PhyloBayes 3: a Bayesian software package for phylogenetic reconstruction and molecular dating. *Bioinformatics.* 25 (17), 2286–2288. doi: 10.1093/bioinformatics/btp368

Lassmann, T., and Sonnhammer, E. L. (2005). Kalign–an accurate and fast multiple sequence alignment algorithm. *BMC Bioinf.* 6, 298. doi: 10.1186/1471-2105-6-298

Lefoulon, E., Bain, O., Bourret, J., Junker, K., Guerrero, R., Cañizales, I., et al. (2015). Shaking the tree: Multi-locus sequence typing usurps current onchocercid (Filarial nematode) phylogeny. *PloS Negl. Trop. Dis.* 9 (11), e0004233. doi: 10.1371/journal.pntd.0004233

Letunic, I., and Bork, P. (2021). Interactive tree of life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* 49 (W1), W293–W2W6. doi: 10.1093/nar/gkab301

Mourembou, G., Fenollar, F., Lekana-Douki, J. B., Ndjoyi Mbiguino, A., Maghendji Nzondo, S., Matsiegui, P. B., et al. (2015). Mansonella, including a potential new species, as common parasites in children in Gabon. *PloS Negl. Trop. Dis.* 9 (10), e0004155. doi: 10.1371/journal.pntd.0004155

Nguyen, L. T., Schmidt, H. A., von Haeseler, A., and Minh, B. Q. (2015). IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* 32 (1), 268–274. doi: 10.1093/molbev/msu300

Peel, E. C. Sur des Filarides de Chimpanzés Pan piscus et Pan satyrus au Congo Belge. *Annales de la société belge de médecine tropicale* (1946), 26, 117–150.

Raoult, D. (2010). Doxycycline for mansonella perstans infection. *N Engl. J. Med.* 362 (3), 272; author reply–3. doi: 10.1056/NEJMc0910675

Richard-Lenoble, D., Kombila, M., Bain, O., Chandenier, J., and Mariotte, O. (1988). Filariasis in Gabon: human infections with microfilaria rodhaini. *Am. J. Trop. Med. Hyg.* 39 (1), 91–92. doi: 10.4269/ajtmh.1988.39.91

Sandri, T. L., Kreidenweiss, A., Cavallo, S., Weber, D., Juhas, S., Rodi, M., et al. (2021). Molecular epidemiology of mansonella species in Gabon. *J. Infect. Dis.* 223 (2), 287–296. doi: 10.1093/infdis/jiaa670

Seemann, T. (2013). Available at: https://github.com/tseemann/barrnap.

Simao, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., and Zdobnov, E. M. (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics.* 31 (19), 3210–3212. doi: 10.1093/bioinformatics/btv351

Simonsen, P. E., Onapa, A. W., and Asio, S. M. (2011). Mansonella perstans filariasis in Africa. *Acta Trop.* 120 Suppl 1), S109–S120. doi: 10.1016/j.actatropica.2010.01.014

Sinha, A., Li, Z., Poole, C. B., Ettwiller, L., Lima, N. F., Ferreira, M. U., et al. (2021). Multiple origins of nematode-wolbachia symbiosis in supergroup f and convergent loss of bacterioferritin in filarial wolbachia. doi: 10.1101/2021.03.23.436630

Sinha, A., Li, Z., Poole, C. B., Morgan, R. D., Ettwiller, L., Lima, N. F., et al. (2023). Genomes of the human filarial parasites mansonella perstans and mansonella ozzardi. doi: 10.1101/2023.01.06.523030

Slater, GS, and Birney, E. (2018). Automated generation of heuristics for biological sequence comparison. *BMC Bioinformatics* (2005) 6, 31. doi: 10.1186/1471-2105-6-31

Smith, S. A., Brown, J. W., and Walker, J. F. (2018). So many genes, so little time: A practical approach to divergence-time estimation in the genomic era. *PloS One* 13 (5), e0197433. doi: 10.1371/journal.pone.0197433

Stanke, M., and Morgenstern, B. (2005). AUGUSTUS: a web server for gene prediction in eukaryotes that allows user-defined constraints. *Nucleic Acids Res.* 33 (Web Server issue), W465–W467. doi: 10.1093/nar/gki458

Tamarozzi, F., Rodari, P., Salas-Coronas, J., Bottieau, E., Salvador, F., Soriano-Pérez, M. J., et al. (2022). A large case series of travel-related mansonella perstans (vector-borne filarial nematode): a TropNet study in Europe. *J. Travel Med.* 29 (7). doi: 10.1093/jtm/taac048

Taylor, M. J., Bordenstein, S. R., and Slatko, B. (2018). Microbe profile: Wolbachia: a sex selector, a viral protector and a target to treat filarial nematodes. *Microbiol. (Reading).* 164 (11), 1345–1347. doi: 10.1099/mic.0.000724

van den Berghe, L., and Chardome, M. (1949). Une microfilaire du gorille. *Microfilaria gorillae*. *Annales la Société Belge Médecine tropicale. Folia Scientif. Afr. Centr.* 29, 495–499.

van den Berghe, L., Chardome, M., and Peel, E. (1957). *Microfilaria leopoldi n. sp. chez gorilla gorilla au Congo belge*. 87.

Volkmann, L., Fischer, K., Taylor, M., and Hoerauf, A. (2003). Antibiotic therapy in murine filariasis (Litomosoides sigmodontis): comparative effects of doxycycline and rifampicin on wolbachia and filarial viability. *Trop. Med. Int. Health* 8 (5), 392–401. doi: 10.1046/j.1365-3156.2003.01040.x

Wallace, I. M., O'Sullivan, O., Higgins, D. G., and Notredame, C. (2006). M-coffee: combining multiple sequence alignment methods with T-coffee. *Nucleic Acids Res.* 34 (6), 1692–1699. doi: 10.1093/nar/gkl091

Wehe, A., Bansal, M. S., Burleigh, J. G., and Eulenstein, O. (2008). DupTree: a program for large-scale phylogenetic analyses using gene tree parsimony. *Bioinformatics.* 24 (13), 1540–1541. doi: 10.1093/bioinformatics/btn230

Wick, R. R., Schultz, M. B., Zobel, J., and Holt, K. E. (2015). Bandage: interactive visualization of *de novo* genome assemblies. *Bioinformatics.* 31 (20), 3350–3352. doi: 10.1093/bioinformatics/btv383

Wood, D. E., Lu, J., and Langmead, B. (2019). Improved metagenomic analysis with kraken 2. *Genome Biol.* 20 (1), 257. doi: 10.1186/s13059-019-1891-0

Xie, H., Bain, O., and Williams, S. A. (1994). Molecular phylogenetic studies on filarial parasites based on 5S ribosomal spacer sequences. *Parasite.* 1 (2), 141–151. doi: 10.1051/parasite/1994012141

Yilmaz, E., Fritzenwanker, M., Pantchev, N., Lendner, M., Wongkamchai, S., Otranto, D., et al. (2016). The mitochondrial genomes of the zoonotic canine filarial parasites dirofilaria (Nochtiella) repens and candidatus dirofilaria (Nochtiella) hongkongensis provide evidence for presence of cryptic species. *PloS Negl. Trop. Dis.* 10 (10), e0005028. doi: 10.1371/journal.pntd.0005028