New Molecular Data on Filaria and its Wolbachia from Red Howler Monkeys (Alouatta macconnelli) in French Guiana—A Preliminary Study

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Abstract: Previous studies have reported filarial parasites of the genus Dipetalonema and Mansonella from French Guiana monkeys, based on morphological taxonomy. In this study, we screened blood samples from nine howler monkeys (Alouatta macconnelli) for the presence of filaria and Wolbachia DNA. The infection rates were 88.9% for filaria and 55.6% for wolbachiae. The molecular characterization, based on the 18S gene of filariids, revealed that A. macconnelli are infected with at least three species (Mansonella sp., Brugia sp. and an unidentified Onchocercidae species.). Since the 18S and cox1 generic primers are not very effective at resolving co-infections, we developed ITS genus-specific PCRs for Mansonella and Brugia genus. The results revealed coinfections in 75% of positives. The presence of Mansonella sp. and Brugia sp. was also confirmed by the 16S phylogenetic analysis of their associated Wolbachia. Mansonella sp., which close to the species from the subgenus Tetrapetalonema encountered in New World Monkeys, while Brugia sp. was identical to the strain circulating in French Guiana dogs. We propose a novel ITS1 Brugia genus-specific qPCR. We applied it to screen for Brugia infection in howler monkeys and 66.7% were found to be positive. Our finding highlights the need for further studies to clarify the species diversity of neotropics monkeys by combining molecular and morphological features. The novel Brugia genus-specific qPCR assays could be an effective tool for the surveillance and characterization of this potential zoonosis.

Keywords: Mansonella sp.; Brugia sp.; Onchocercidae sp.; Wolbachia; neotropic monkeys; reservoir; zoonosis

1. Introduction

Filariasis unites diseases are caused by arthropod-borne filariids and nematodes belonging to the Onchocercidae family. Several species can be encountered in human and animals with some zoonotic aspects. Morphologically, the adult filariids are long, string-like, white-to-cream-colored worms [1]. They appear to be capable of living inside various tissues and cavities outside the gastrointestinal tract. Once mature, the adult females produce blood or cutaneous microfilariae, where they are available to arthropod vectors [2]. Species having a predilection for subcutaneous tissues are less or completely avirulent in comparison to those found in cavities, such as Dipetalonema species (D. gracile, D. graciliformis, D. caudispina, D. robini and D. freitasi, D. vanhoofi), Macacanema formosana where they induce serious disease manifestations such as pleuritis, fibrinopurulent peritonitis and fibrinous adhesion, resulting in the entrapment of worms [3,4]. Furthermore, species found in the circulatory system (e.g., Dirofilaria immitis and D. pongoi, Edesonfilaria malayensis), as well as those present in
the lymphatic system, such as Brugian filariids (B. malayi, B. pahangi, B. timori and B. tupaiae) and Wuchereria bancrofti, disrupt blood and lymphatic drainage, leading to serious and often irreversible vascular damage [4–9]. These filariids, along with Onchocerca volvulus, the agent of river blindness, constitute the most thread-like filarial worms and have affected up to 893 million people in 49 countries worldwide [10].

Several filariids of the subfamilies Onchocercinae and Dirofilarinae are associated with an endosymbiotic intracellular bacterium of the genus Wolbachia [11], which is present in all developmental stages of filariids that harbor Wolbachia, leading to their long-term survival [12]. The parasites’ endosymbiotic Wolbachia are implicated in severe inflammatory-mediated filarial diseases [13–16]. Anti-wolbachial therapies, based on the administration of antibiotics, are known to be effective against the most common filariasis caused by Brugia spp., i.e., W. bancrofti, Mansonella perstans and D. immitis [17–19]. The Wolbachia-filaria relationship is species-specific, wherein each filariid has a specific genotype of Wolbachia [11], thus providing an additional target suitable for the diagnosis of filarial infections [20], especially when occurring in dead-end hosts, as is the case in D. immitis in human and cats [21,22]. Recently, the simultaneous detection of both filarial and wolbachial DNAs from infected hosts is used as an improvement tool for the diagnosis of filarial infections [23–25].

Filariasis is one of the most neglected tropical diseases selected, but it is included in the Mass Drug Administration (MDA) program to achieve its elimination by 2020 [26–28]. Human filariasis was almost eliminated from Latin America [29,30]. Thanks to the MDA program, river blindness (onchocerciasis caused by O. volvulus) transmission is currently limited to the Amazon rainforest on the Venezuelan–Brazilian border, while the lymphatic filariasis caused by W. bancrofti only occurs in four countries: Brazil, the Dominican Republic, Guyana, and Haiti [31]. Another human sympatric filariasis caused by M. ozzardi and M. perstans occurs today in a small foci in South America (Amazon Basin, Yucatan, Panama and Haiti) [32–34]. In Latin America, domestic and wild animals seem to be the foci of some neglected filariasis potentially zoonotic such as Brugia guyanensis (Orihel 1964) from the lymphatic system of the coatimundi (Nasua nasua vittata) in French Guiana [35] and some unidentified Brugian filariids in dogs and ring-tailed coatis (Nasua nasua) [25,36], and the zoonotic canine filariasis (e.g., D. immitis and Acanthocheilonema reconditum) from Brazil and French Guiana [25,37].

New world monkeys are a diverse group of arboreal primates inhabiting the tropical forest environments of southern Mexico, Central and South America [38]. These primates are the natural hosts for several filariids belonging to the genus Dipetalonema and Mansonella, where they are often present as co-infected [3,39]. Howler, monkeys (Alouatta spp., Atelidae, Primata) have a wide distribution, from Mexico to northern Argentina. Only a few species of this group have been genetically characterized [40]. The red howler monkey (Alouatta macconnelli, Linnaeus 1766—Elliot 1910) is one of eight species of primates found in the French Guiana forest [41]. They are medium sized (10 kg) and about 84 cm (head and body) with a prehensile tail [38]. They live in small groups of four to eight individuals. The primary forest in the canopy high strata is often frequented by these primates who are mainly found in the north of South America and the Amazonia (Suriname, Guyana, Trinidad, French Guiana, Venezuela and Brazil). Their diet is low in energy (leaves and sometimes fruits and seeds) [40]. Population density is estimated to be 13 individuals/km$^2$ along the Approuague River, which is the location in which we conducted our investigation [42]. Nowadays, little molecular data are available on filarial parasites in howler monkeys from French Guiana. The aims of the present study are mainly to determine, at the molecular level, the presence of filarial parasites and the status of their endosymbiotic Wolbachia in red howler monkeys. To this end, we examined blood samples obtained from a game that was hunted by the natives of French Guiana [43].
2. Results

2.1. Host Identification

Folmer’s primers allowed for the amplification of DNA sequences from all blood samples, but despite several attempts, a high-quality DNA sequence of the vertebrate \textit{cox1} gene was only obtained in one from among the nine samples tested, suggesting the presence of a non-specific amplification from the latter. The partial nucleotide sequence (558 bp) of the \textit{cox1} gene obtained in this study was deposited in the GenBank under accession number MT193011. Blast analysis showed that the \textit{cox1} sequence of howler monkeys in our study had an identity of 96.06% with \textit{Alouatta seniculus} (HQ644333), 95.88% with \textit{Alouatta caraya} (KC757384) and 95.34% with \textit{Alouatta guariba} (KY202428) and a query cover of 100%. Accordingly, the phylogenetic analysis using the Maximum Likelihood (ML) method showed that the specimen of howler monkeys (\textit{Alouatta macconnelli}) is monophyletic with other \textit{Alouatta} species (Figure 1).

![Figure 1](image.png)

**Figure 1.** Phylogram generated by maximum likelihood method from 17 partial (521 bp) \textit{cox1} sequences showing the position of \textit{Alouatta macconnelli} through the neotropics monkeys. A discrete Gamma distribution was used to model evolutionary rate differences among the sites (5 categories (+G, parameter = 0.4575)). The rate variation model allowed for some sites to be evolutionarily invariable (+I, 57.2649% sites). Likelihood was –2676.5239. Numbers above and below the branches display the nod statistics and branch length, respectively. Geographical location (when available) and GenBank accession numbers are indicated in each node.

2.2. Molecular Screening for Filarial and Wolbachia DNAs in Howler Monkeys

Filarial and \textit{Wolbachia} DNAs were detected by qPCR assays in eight out of nine samples tested and six out of nine samples tested, which correspond to a frequency of infection of 88.9% and 66.7% for filaria and \textit{Wolbachia}, respectively. This is the first molecular report of filaria and its \textit{Wolbachia} from the howler monkeys of French Guiana.

2.3. Molecular Characterization of Filarial Species

To identify filaria detected by qPCR, we performed standard polymerase chain reaction (PCR) screening with primers targeting the small subunit rRNA (\textit{18S}), the internal transcribed spacer 1 (\textit{ITS1})...
and the cytochrome c oxidase subunit I (cox1) genes. A nearly full-length DNA sequence of the 18S rRNA gene was obtained from all eight samples, was positive in qPCR and was split into three isolates according to the blast results. (i) Six sequences were obtained from the monkeys B2, B3, B4, B6, B7 and B8. These amplicon sequences were identical to each other, showing an identity and query cover of 100% with Dipetalonema sp. (DQ531723) isolated from an owl monkey (Aotus nancymaae) captured in Peru and 99.6% of identification with the Mansonella species (MN432520, MN432519). (ii) One 18S sequence obtained from sample B5 was very close to the Onchocercidae members (Onchocerca cervicalis: DQ094174, and Loa loa: DQ094173), where the identification was 99.9% and 100% of the query cover. Further sequence comparisons showed that the Adenine and Thymine mutated into Cytosine at the position 304 and 879 with O. cervicalis (DQ094174) and L. loa (DQ094173), respectively (Figure S1). (iii) One sequence from sample B9 showed an identification of 100% with B. malayi (AF036588) and 99.9% with Brugia sp. (MN795087), isolated from dogs in French Guiana.

Mansonella genus-specific PCR, based on the amplification of the ITS1, allowed us to obtain ITS sequences of Mansonella sp. from seven monkeys (B2, B3, B4, B5, B6, B7 and B8). They were almost identical and displayed an identity ranging from 83.47% to 93.49% and a query cover ranging from 62% to 83% with Mansonella species (M. ozzardi: KR952332, M. perstans: MN432520, M. mariae: AB362562, M. streptocerca: KR886771, M. dunnii: KY434312 and Mansonella sp.: MN821052). Furthermore, Brugia sp. was identified in five samples (B2, 3, 4, 7 and 9) using the Brugia-specific qPCR and ITS sequences were obtained for four of them. These sequences were similar and were close to the Brugia species, wherein the identity ranged from 88.81% to 91.98% with B. malayi (JQ327147, EU419333) and from 89.10% to 91.19% with B. pahangi (EU373633, EU419348).

Primers targeting the cox1 gene amplified the expected DNA amplicon size from all the filaria-positive samples. However, only two sample (B8 and B9) sequences provided good quality electropherograms. Several overlapping peaks (double peaks) within samples B2, B3, B4, B5, B6 and B7 suggested co-infection with two or more filarial species. Blast analysis showed that the specimen amplified from monkey B8 had an identity of 88.2% with Mansonella perstans (MN890111). While the cox1 sequence amplified from monkey B9 was very close to Brugian filariids, with an identity of 99.6% with Brugia sp. (MT193074), isolated from dogs in French Guiana, 95.4% with Brugia timori (AP017686) and 94.9% with Brugia malayi (MNS64741).

Phylogenetic analysis using the maximum likelihood method of the 18S rRNA gene showed that howler monkeys from French Guiana are infected with at least three filarial species belonging to the Onchocercidae clade, namely ONC 5. The 18S sequences amplified from monkeys B2, 3, 4, 6, 7 and 8 in a separate branch with Mansonella species, while the sequence obtained from monkey B5 appeared paraphyletic with respect to L. loa (ADBU02009332) and O. volvulus (ADB01000330), suggesting an unknown onchocercid. Finally, the sequence from monkey B5 clustered with the B. pahangi strain (UZAD01013810 and JAAVKF010000006) (Figure 2). The ML tree, based on the concatenated rRNA sequences (18S and ITS1), showed that the specimens amplified from monkeys B2, 3, 4, 6, 7 and 8 clustered with other monophyletic species of the genus Mansonella, while the specimen amplified from monkey B9 clustered with the Brugia species (Figure 3). Interestingly, the cox1 phylogram replicated the same results, though with a greater degree of accuracy. The species amplified in this study belong to the clade 5 of the Onchocercidae family. More precisely, the species amplified from monkey B8 belong to the genus Mansonella and the subgenus Tetrapetalonema encountered in New World Primates [44], while the species from monkey B9 clustered with Brugia sp. (MT193074), isolated from dogs in French Guiana [45] and are monophyletic with other Brugian filariids (Figure 4). Interspecific nucleotide distances (IND) of the cox1 sequences ranged between 0.08 and 0.13 between Mansonella sp. from the monkey B8 and most species from the genus Mansonella (MN890075, MN890115, MN890111 and KY434309), while the IND ranged from 0 to 0.03 between Brugia sp. amplified from monkey B9 and Brugian filariids (Figure 5, Table S1).
Figure 2. Phylogram generated by Maximum Likelihood (ML) method based on 24 partial (941 bps) rRNA sequences showing the position of filariids from howler monkeys Onchocercidae clades (ONC). A discrete Gamma distribution was used to model evolutionary rate differences among the sites (5 categories (+G, parameter = 0.1000)). The likelihood was −1770.1752. Numbers above and below the branches display nod statistics and branch lengths, respectively. Geographical location (when available) and GenBank accession numbers are indicated in each node. (*) indicates sequences retrieved from the Worm parasites database.

Figure 3. Phylogram generated by ML method based on 24 partitioned concatenated rRNA sequences (18S ad ITS1) showing the position of Brugia sp. and Mansonella sp. through Onchocercidae clades (ONC). The total length was 1221 bp, the rate variation model allowed for some sites to be evolutionarily invariable (+I, 29.0648% sites). Likelihood was −3034.4989. Numbers above and below the branches display nod statistics and branch lengths, respectively. Geographical location (when available) and GenBank accession numbers are indicated in each node. (*) indicates sequences retrieved from Worm parasites database.
Figure 4. Phylogram generated by ML method based on 36 cox1 partial sequences (266 bp) showing the position of Brugia sp. and Mansonella sp. through Onchocercidae clades (ONC). A discrete Gamma distribution was used to model evolutionary rate differences among the sites (five categories (+G, parameter = 0.4964)). The rate variation model allowed for some sites to be evolutionarily invariable (+I, 0.000% sites). The likelihood was −2194.0587. Numbers above and below the branches display nod statistics and branch lengths, respectively. Host, geographical location (when available) and GenBank accession numbers are indicated in each node. Mansonella species are color-coded according to their subgenus.

Importantly, the cox1 DNA sequences were aligned correctly to the reference mitogenome of M. ozzardi (KX822021) [45], and when translated, there were no stop codons in the amino acid sequences, suggesting the absence of co-amplified numts. Finally, translated protein sequences of the cytochrome c oxidase subunit I (COI) showed three amino acid changes between Mansonella sp. from monkey B8 and the other Mansonella species from GenBank, namely, from threonine to alanine, threonine to isoleucine and aspartic acid to valine (Figure 6A). While Brugia sp. from monkey B9 showed a deletion of one amino acid instead of tryptophan, in comparison to Brugian filariids from GenBank (Figure 6B).
with Wolbachia were identical with each other and were 100% identical with all samples that tested positive for Wolbachia DNA through the qPCR. Three identical sequences revealed Brugia sp. (abscissa) and Mansonella sp. (ordinate) from A. macconnelli and the representative members of Onchocercidae clades. The analyses involved 112 partial (266 bp) cox1 sequences with a total of 216 positions in the final dataset. All positions containing gaps and missing data were eliminated.

Figure 5. Scatter chart showing the interspecific pairwise distance between the cox1 sequences of Brugia sp. (absissa) and Mansonella sp. (ordinate) from A. macconnelli and the representative members of Onchocercidae clades. The analyses involved 112 partial (266 bp) cox1 sequences with a total of 216 positions in the final dataset. All positions containing gaps and missing data were eliminated.

Figure 6. Cytochrome c oxidase subunit I protein sequences (COI) alignment showing the conservation of amino acid within (A) Mansonella spp., (B) Brugia spp. Protein Id and species name are indicated for each sequence. Selected boxes represent species obtained in this study.

A partial DNA sequence of the Wolbachia 16S gene (295 bps) was obtained from five out of six samples that tested positive for Wolbachia DNA through the qPCR. Three identical sequences revealed 99.32% identity with Wolbachia of M. atelensis amazone (FR827940) and 98.64% with both Wolbachia of M. perstans (AY278355) and M. ozzardi (AJ279034). These sequences were obtained from filaria-positive monkeys, including monkey B4, which was co-infected with Mansonella sp. and Brugia sp., monkey B5 co-infected with an unidentified Onchocercidae species and Mansonella sp. and monkey B8, which was mono-infected with Mansonella sp. The two remaining sequences were amplified from two filaria-positive samples, one for Mansonella sp. (B6) and the other for Brugia sp. (B9). These sequences were identical with each other and were 100% identical with all Wolbachia genotypes associated to Brugia species (CP050521, CP034333, AJ012646 and MT231956). Accordingly, the ML inference indicates that the Wolbachia genotype from monkeys B4, 5 and 8 belong to the Clade F of Wolbachia lineage infecting Mansonella species, while the genotype obtained from monkeys B6 and B9 clustered together with Wolbachia endosymbiont of Brugian filariids within Clade D of the Wolbachia lineage (Figure 7).
Finally, by combining all the molecular results for filaria and Wolbachia detection, we concluded six cases (75%) of co-infections in monkeys, including Mansonella sp.—Brugia sp. co-infection in five and Mansonella sp.—unidentified Onchocercidae species in one. Two other monkeys (25%) presented mono-infections, one with Mansonella sp. and the other with Brugia sp. (Table 1).
Table 1. Results of molecular assays used for the identification of filariids and their associated Wolbachia in the blood of red howler monkeys from French Guiana.

| Sample Code | Filariids DNA | Wolbachia DNA | Decision |
|-------------|---------------|---------------|----------|
|             | 28S qPCR      | 18S PCR       | COI PCR  | Mansonella spp. | Brugia spp. | Wolbachia 16S qPCR | Wolbachia 16S PCR | Combined Assays |
| B1          | N/A           | N/A           | N/A      | Mansonella sp.  | N/A         | Neg.               | Neg.             | N/A            | Negative.      |
| B2          | Pos.          | O/P           | Mansonella sp. | N/A         | Brugia sp.  | Pos.               | Neg.             | N/A            | Mansonella sp. + Brugia sp. |
| B3          | Pos.          | O/P           | Mansonella sp. | [MT341516]  | Brugia sp.  | Pos.               | Pos.             | O/P            | Mansonella sp. + Brugia sp. |
| B4          | Pos.          | O/P           | Mansonella sp. | [MT341517]  | Brugia sp.  | Pos.               | Pos.             | M. Mansonella sp. [MT231961] | Mansonella sp. + Brugia sp. |
| B5          | Pos.          | O/P           | Mansonella sp. | [MT341518]  | N/A         | Neg.               | Pos.             | W-Mansonella sp. [MT231962] | Mansonella sp. + unidentified Onchocercidae species |
| B6          | Pos.          | O/P           | Mansonella sp. | [MT341519]  | N/A         | Neg.               | Pos.             | W-Brugia sp. [MT231964] | Mansonella sp. + Brugia sp. |
| B7          | Pos.          | O/P           | Mansonella sp. | [MT341520]  | Brugia sp.  | Pos.               | Neg.             | N/A            | Mansonella sp. + Brugia sp. |
| B8          | Pos.          | O/P           | Mansonella sp. | [MT341521]  | Brugia sp.  | N/A                | Pos.             | W-Mansonella sp. [MT231963] | Mansonella sp. |
| B9          | Pos.          | O/P           | Mansonella sp. | [MT341522]  | Brugia sp.  | N/A                | Pos.             | W-Brugia sp. [MT231965] | Brugia sp. |

N/A: no amplification, O/P: overlapping peaks on the electropherograms, Pos: positive reaction, Neg: negative reaction, W-Mansonella sp.: Wolbachia endosymbiont of Mansonella sp., W-Brugia sp.: Wolbachia endosymbiont of Brugia sp. GenBank accession numbers are given in square brackets.
3. Discussion

This is the first molecular report of filaria and Wolbachia infection from red howler monkeys (*Alouatta macconnelli*, Linnaeus 1766—Elliot 1910) in French Guiana. These monkeys were morphologically considered as a distinct species from *A. seniculus* and they are not a subspecies [46]. Our data confirmed that, molecularly, both species can be distinguished by their *cox1* sequences. The wide distribution of howler monkeys (from Mexico to northern Argentina) constitutes a non-negligible reservoir for zoonotic disease [43] and should be monitored. Our study is limited in the number of species and samples, due to the difficulties encountered in the field. The number of monkeys tested was much lower than those tested in Reference [47], where 1353 free-ranging mammals, including 114 howler monkeys (*A. seniculus*) and 84 red handed tamarins (*Saguinus midas*) from the neotropical primary rainforest in French Guiana were studied for haemoparasites and microfilariae.

However, the prevalence of filarial infection we recorded using molecular assays is close to that reported in tamarins and howler monkeys using blood smear, where the infection rates were 80% and 92% of filaria infections (*Dipetalonema* and *Mansonella* (*Tetrapetalonema*) species), respectively [47]. Our data indicate that the prevalence of filarial infection was higher than that of sloths, anteaters and porcupines in French Guiana, where the infection rate of 40% was reported using blood smears test [47]. The higher prevalence observed in monkeys may be related to the lower host specificity of filariids [48] and/or similar biotope of potential vectors [49]. Another hypothesis is that the lifestyle of these animals increases the risk of vector-borne disease transmission between infected and non-infected individuals in the monkey colony. Therefore, the highest mixed-infection detected in our study corroborates previous reports [50], but it is still unknown whether it is geographical or host-specific. Several species of filariids are reported from a wide range of neo-tropical primates based on morphological taxonomy (Table 2). Most of them belong to the genus *Dipetalonema* and *Mansonella* (*Tetrapetalonema*). However, data in DNA barcoding of these species is lacking.

The use of two (or more) different molecular markers for species delimitation remained necessary for the accurate identification of nematode species [51]. In the present study, our molecular approach, based on generic and genus specific primers, permits the detection and characterization of filarial infections and resolved the co-infections. This is due to the ability of ITS genus-specific PCR assays to separately amplify DNA amplicons depending on their specificity. Filarial nematodes could be misclassified when the 18S gene is used alone as a barcode. This gene is often limited to the genus level and has proven to be inconclusive for the molecular taxonomy of nematodes [52], while the ITS 1 gene appears to be a satisfactory barcode in resolving taxonomic relationships among species [53–55]. Furthermore, as suggested by previous authors [56], the use of partitioned concatenated DNA sequences enables the accurate identification of filarial nematodes. We used both the 18S and the partitioned concatenated rRNA (18S and ITS1) gene, which confirmed the presence of at least three potential new species from clade 5 of the Onchocercidae family present in howler monkeys in French Guiana, including *Mansonella* sp., *Brugia* sp. and an unidentified Onchocercidae species.

The *cox1* gene enabled the accurate identification of the *Mansonella* species from wild non-human primates from Cameroon and Gabon [57], and has been proven to be a satisfactory discrimination between filarial species. This gene was described by its low nucleotide distances (from 0 to 0.02) within filarial species [58] and a larger variation between congeneric species (i.e., 0.098 to 0.2) [58,59]. In the present study, we used two different phylogenetic methods for the analysis of *cox1*, together with the alignment of COI protein sequences, which confirmed that species from monkeys B8 and B9 clustered, respectively, with *Mansonella Tetrapetalonema* subgenus and *Brugia* species, with the distance ranging between 0.02 and 0.2, suggesting unidentified or potential new species from these genera.

Wolbachia are host-specific, and each genotype is associated with a specific filarial species [11,60]. Bacterial genotype-specific identification was previously proposed for the speciation of *Brugia* parasites that infect humans [9]. Several studies showed the utility of the specific detection of Wolbachia in determining the subject as infected or not with filarial species (e.g., *D. immitis*, *D. repens*, *B. pahangi* and *B. malayi*) from domestic animals [14,21,23–25,61,62]. Accordingly, the phylogenetic analysis of the
Wolbachia 16S DNA sequences demonstrated the presence of two bacterial genotypes belonging to the supergroup F and D encountered in Mansonella and Brugia species, thus corroborating with filariae phylogenies. The inconsistency between the bacterial genotype and filaria species was observed in monkey B6. The presence of Mansonella sp. and Wolbachia of Brugia sp. DNAs highlights a co-infection with both filarial species. However, the absence of Wallachia of Mansonella sp. could be explained by a weaker infection density in this species, while the absence of Brugia sp. DNA, despite the presence of its Wolbachia, could be result to an amicrofilaraemic infection due to single sex infection, an earlier infection stage or any other causes. Such inconsistencies were previously reported between Brugia and Dirofilaria species in dogs [63]. Wolbachia-filaria interactions within co-infected hosts are not well understood. Despite the presence of both parasites in co-infected dogs with D. immitis and D. repens, the single detection of Wolbachia of D. immitis is frequent [24] and may result in an unexplained suppression effect on the production of D. immitis microfilariae induced by the presence of D. repens [64,65].

Our findings extend the presence of Brugia sp. and an unidentified Onchocercidae species to the New World Monkeys (e.g., Alouatta macconnelli). Several species of filariae have been described from these primates and they all belong to the genus Dipetalonema or Mansonella subgenus Tetrapetalonema [4] (Table 2). The genus Dipetalonema (Diesing 1861) is restricted to non-human primates (NHPs) of the neotropics, according to the phylogenetic study conducted by Lefoulon et al. [56]. Adult worms are prevalent in the serous cavities of the hosts. A high species diversity of this genus was observed in a wide range of New World monkeys. D. gracile (Rudolphi 1819), D. graciliformis (Freitas 1964) and D. caudispina (Molin 1858) are the main species found in Guiana monkeys, using a morphological taxonomy (Table 2).

The subgenus Mansonella (Tetrapetalonema) is one of the five subgenera derived from the genus Mansonella. Adult filariids are small, slender and can be found in subcutaneous tissues. The Tetrapetalonema subgenus comprises 13 species (Table 2), which have been restricted to platyrrhine (neotropical) primates [66]. Human mansonellensisiasis across South America regions are caused by M. ozzardi type species of Mansonella (Mansonella) subgen. n. [44,45] causing fever, pruritis, arthralgias, headache, rashes, lymphadenopathy, edema, and pulmonary symptoms and a common eosinophilia mainly associated with corneal lesions [67–70]. M. perstans type species of Mansonella (Esslingeria, Chabaud and Bain 1976) subgen. n. [44] is another agent of human mansonellensisiasis in some neotropical regions of Central and South America that causes the bung-eye diseases [71]. These species have been found in both humans and non-human primates [4,44]. However, the possibility that the Mansonella sp. we have detected here is one of the 13 Mansonella (Tetrapetalonema) species or a new species from this subgenus cannot be ruled out in the absence of morphological identification.

Brugia spp. are incidental filariids that parasitize non-human vertebrates [72]. The classical brugian filariids involved in lymphatic filariasis are found in Asia, while species reported from North and South America constitute the most zoonotic species of this genus [73]. In Latin America, Brugia sp. infection was reported from the ring-tailed coatis (Nasua nasua nasua) in Brazil [36], Brugia guyanensis from the lymphatic system of the coatimundi (Nasua nasua vittata) in British Guiana [35] and Brugia sp. from domestic dogs in French Guiana [25]. Our findings indicate that Brugia sp. detected from howler monkeys is the same as that recently detected in domestic dogs [25]. Unlike Asian primates in which infection with B. malayi and B. pahangi has been reported [74], Brugian filariid has not been reported in neotropical primates [75]. Cases of human infection by Brugia sp. have been reported in several localities (Amazon, Peru, Colombia) in South America, but the reservoir of the parasites is unknown [72,73]. However, the possibility that the Brugia sp. we detected from howler monkeys and dogs in our previous study [25] is of the same species circulating in humans cannot be ruled out in the absence of molecular data.
Table 2. Filarial parasites and host diversity from neotropic monkeys.

| Genera                        | Species                          | Host                                                                                           | References |
|-------------------------------|----------------------------------|-------------------------------------------------------------------------------------------------|------------|
| Mansonella (Faust, 1929),     | Mansonella (T.) marmosetae       | Saginus geoffroyi, Saimiri oerstedii oerstedii, Ateles paniscus, Saimiri boliviensis, Saimiri sciureus and |           |
| Mansonella (T.) zakii (Nagaty | Mansonella (T.) panamensis       | Leontopithecus (= Leontocebus) rosalia                                                         | [44,66,67,76]|           |
| Mannsonella (T.) panamensis   | Mansonella (T.) atelensis atelensis | Cebus capucinus, Saimiri oerstedii oerstedii, Aotus lemurinus zonalis, C. apella and A. trivirgatus |           |
| Mannsonella (T.) atelensis    | Mansonella (T.) atelensis amazonae| Ateles geoffroyi, A. fusciceps rufiventris                                                     |           |
| Mannsonella (T.) atelensis    | Mansonella (T.) atelensis         | Cebus olivaceus                                                                                 |           |
| Dipetalonema (Diesing 1861)   | Dipetalonema (Diesing 1861)      | Species in bold are occurring in French Guiana monkeys.                                          | [76–82]    |
4. Materials and Methods

4.1. Samples and Ethic Statement

In January 2016, we obtained samples from howler monkeys that were legally hunted by two Amerindian hunters for family consumption of meat. The International Union for Conservation of Nature conservation status for this species is a “least concern” \[83,84\]. The hunters applied the provisions of the prefectural decree regulating the quotas of species that can be taken by a person in the department of Guiana (No. 583/DEAL of 12 April 2011). The hunt took place in the deep forest (4°01’39.5” N 52°31’32.5” W), near the Approuague River, 50 km from the village of Regina. We were able to examine corpses of nine hunted howler monkeys (five females and four males). Blood was collected by a heart-puncture in sterile tubes containing Ethylene-Diamine-Tetra-Acetic acid (EDTA) and was kept in a cooler before being frozen at −20 °C until further analysis.

4.2. DNA Extraction

Genomic DNA was extracted from 200 µL of each blood samples. The extraction was performed using QIAGEN DNA tissues kit (QIAGEN, Hilden, Germany) following the manufacturer’s recommendations. Two lysis steps were applied before the extraction procedure: (i) mechanical lyses performed on FastPrep-24™ 5G homogenizer using high speed stirring for 40 s in the presence of glass powder, (ii) enzymatic digestion of proteins with buffer G2 and proteinase K for 12 h at 56 °C. The extracted DNA was eluted in a total volume of 100 µL and stored at −20 °C.

4.3. Host Identification

The universal \textit{cox1} DNA barcoding region of metazoans [85] was targeted using the degenerated primers of Folmer, as described elsewhere [86]. The PCR products were purified, sequenced and edited, as described below, and were then aligned against \textit{cox1} sequences of \textit{Alouatta} spp. (HQ644333, KC757384, KY202428), \textit{Ateles} spp. (AB016730, KC757386, JF459104, EF658646, EF568717), \textit{Callicebus} personatus (MH101707), \textit{Chiropotes israelita} (KC592392, KC757393), \textit{Lagothrix lagotricha} (EF568626, KC757398), \textit{Sapajus} spp. (KY703885) and \textit{Aotus trivirgatus} (HQ005481) as representative New World monkeys [46]. The sequence (MH177805) of human \textit{cox1} was used as an out-group. Finally, the Hasegawa-Kishino-Yano (+G, +I) [87] was selected as a best fit model according to the Akaike Information Criterion (AIC) option in MEGA6 [88]. The maximum likelihood (ML) phylogenetic inference was used with 1000 bootstrap replicates to generate the phylogenetic tree using the same software.

4.4. Molecular Screening for Filaria and Wolbachia

First, all blood samples were screened for the presence of filaria and \textit{Wolbachia} DNAs using, respectively, the pan-filarial [Pan-fil 28S] and pan-\textit{Wolbachia} [All-Wol 16S] qPCRs, as described elsewhere [24].

4.5. Molecular Characterization of Filaroids and their Associated \textit{Wolbachia} Using Generic Primers

Samples positive for filaria and \textit{Wolbachia} by qPCR were subjected to amplification and sequencing analysis using the pan-Nematoda-18S primers [61] and pan-filarial \textit{cox1} based PCR [Pan-fil \textit{cox1}] [24] to generate 1127–1155 bp and 509 bp from the filarial 18S and \textit{cox1} genes, respectively. The third PCR system [W16S-Spec] PCR [89] was used to amplify 438 bp from the 16S gene of \textit{Wolbachia} spp. (Table 3).
Table 3. The primers and probes used in this study.

| System Name               | Target Gene     | Primer and Probe Name         | Sequence (5′-3′)                                      | Amplicon Size (bp) | Tm/Elongation Time | Assay Specificity | Ref.   |
|---------------------------|-----------------|--------------------------------|------------------------------------------------------|--------------------|---------------------|-------------------|--------|
| Pan-fil 28S qPCR-based system | LSU rRNA (28S) | qFil-28S-F                    | TTGTGGAGATTGCAGCCCA                                   | 151                | 60 °C/30'          | Filarids          | [24]   |
|                           |                 | qFil-28S-P                    | 6FAM-CAAGTACCCTGAGGGAAAGT-TAMRA                       |                    |                     |                   |        |
|                           |                 | qFil-28S-R                    | GTTCCCACTCTACGCCAGTTTTC                               |                    |                     |                   |        |
| All-Wol 16S qPCR-based system | 16S rRNA gene | all.Wol.16S.301-F             | TGGAACGTGAGATACGCTCAGG                                 | 177                | 61 °C/30'          | Wolbachia         |        |
|                           |                 | all.Wol.16S.347-P             | 6FAM-AATAATGGACAATGGCGCAA-TAMRA                       |                    |                     |                   |        |
|                           |                 | all.Wol.16S.478-R             | GCACGGAGTTACCCAGGACT                                   |                    |                     |                   |        |
|                           | W-Spec          | W.Specf                       | CATACT TATTCGAAGGGATAG                                 | 438                | 60 °C/1'           |                   | [89]   |
| Brug-gen-spec qPCR        | Internal Transcribed Spacer 1 (ITS1) | Brug.ITS.f.260             | AGCGTAGCCTAAATTTAATTACCTATT                           | 161                | 61 °C/30'          | Brugia spp.       | This study |
|                           |                 | Brug.ITS.p.307               | 6FAM-CAATTGCTAGATATGCCTACCAAA-TAMRA                   |                    |                     |                   |        |
|                           |                 | Brug.ITS.r.421               | CCACCGCTAAGGTTAAAAAATT                                 |                    |                     |                   |        |
| Manso-gen-spec PCR        | SSU rRNA (18S)  | Fwd.18S.631                  | TCGTCATTGCGGTTAAG                                   | 1127–1155          | 54 °C/1'30'        | Mansonella spp.   | [61]   |
|                           |                 | Rwd.18S.1825r                | GGTTCAAGGATTAAG                                       |                    |                     |                   |        |
| Pan-fil cox1 PCR          | Cytochrome c oxidase subunit 1 gene (cox1) | Fwd.957                  | ATGTTATCAAGGTTATTTTTTTTTTTATGG                      | 509                | 52 °C/1'           | Filarids          | [24]   |
| dg-Folmer’s primers       |                 | dgLCO-1490                   | GGTCAACAAATCAAAAGAYAYYAG                             | 708                | 44 °C/40'          | Metazoans         | [86]   |
|                           |                 | dgHCO-2198                   | TAAAATTTCCAGGGTGACCAARAAAYCA                          |                    |                     |                   |        |
4.6. Molecular Characterization of Filariids Using Genus Specific PCR Assays

4.6.1. Design of Oligonucleotides

In order to complete the molecular characterization of filariids detected by the 18S and cox1 genes, we targeted the Internal Transcribed Spacer 1 (ITS1) gene to design genus-specific PCR assays targeting Brugia and Mansonella species. The choice for this gene was based on the following criteria: a higher divergence between filarial species especially among Brugia species [90], its tandem repeat that increases PCR sensitivity [91] and its availability in the GenBank database for these species. Three PCR assays were designed by the alignment of ITS1 sequences of Brugia sp. (HE856316), B. malayi (EU419346, JQ327149), B. timori (AF499132), B. pahangi (EU373628), M. ozzardi (MN432519, LT623912, AF228559), M. perstans (MN432520, KJ631373, EU272184) and M. mariae (KX932484) against 33 sequences (data not showed) from a representative member of Onchocercidae using the MUSCLE application within DNAstar software [92]. Three genus specific PCR systems were proposed (Table 3). This includes two PCRs: one specific for Brugia spp. [Brug-gen-spec] and the other specific for Mansonella spp. [Manso-gen-spec], and qPCR system [Brug-gen-spec qPCR] targeting Brugia spp.

Assay specificity was confirmed in silico and in vitro for each system, as described elsewhere [24]. Briefly, the in silico validation was conducted using Primer-BLAST [93]. Genomic DNA of M. perstens was used to validate the PCR for Mansonella, while the B. malayi DNA was used to validate both the qPCR and PCR for Brugia spp. Moreover, all PCR assays were challenged against the genomic DNA of filariids other than Brugia and Mansonella, as well as several nematodes, arthropods, vertebrate hosts (e.g., human, monkey, donkey, horse, cattle, mouse and dog) and laboratory-maintained colonies [24].

4.6.2. Amplification, Sequencing and Run Protocol

All blood samples from howler monkeys were screened for the presence of Mansonella and Brugia DNA using the genus specific PCR. The PCR reactions were carried out in a total volume of 50 µL, comprising 25 µL of AmpliTaq Gold master mix (Thermo Fisher Scientific, Saint Herblain, France), 18 µL of ultrapure water free of DNase-RNase, 1 µL of each primer and 5 µL of genomic DNA. PCR reactions were run under the following protocol: the incubation step at 95 °C for 15 min, 40 cycles of one minute at 95 °C, 30 s for the annealing at a different melting temperature for each PCR assays (Table 3), and 72 °C of elongation step (Table 3) with a final extension step of five minutes at 72 °C. PCR reactions were performed in a Peltier PTC-200 model thermal cycler (MJ Research Inc., Watertown, MA, USA).

DNA amplicons generated throughout each PCR reaction were purified using NucleoFast® 96 PCR DNA purification plate (Macherey Nagel EURL, Hoerdt, France). Purified DNAs were subjected to the second amplification using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA), then the BigDye PCR products were purified on the Sephadex G-50 Superfine gel filtration resin prior to sequencing on the ABI Prism 3130XL (Applied Biosystems, Courtaboeuf, France).

4.6.3. Molecular Screening for Brugia

In order to reveal the infection rate of Brugia spp., all the samples were subjected to the amplification using the genus-specific qPCR. The qPCR reaction was performed in a total volume of 20 µL including 5 µL of DNA template, 10 µL of Master Mix Roche (Eurogentec France, Angers, France), 3 µL of ultra-purified water DNase-RNase free and 0.5 µL of each primer, UDG and each probe. The TaqMan reaction of both systems was run using the same cycling conditions. This included two hold steps at 50 °C and 95 °C for 2 and 15 min, respectively, followed by 40 cycles of two steps each († 95 °C for 30 s and 60 °C for 30 s). The qPCR reaction was performed in a CFX96 Real-Time system (Bio-Rad Laboratories, Foster City, CA, USA).
4.7. Phylogenetic Analysis

First, nucleotide sequences of the filarial *cox1*, *18S* and *ITS1* genes, as well as the *16S* gene of *Wolbachia*, were assembled and edited by Chromas-Pro 2.0.0 (http://technelysium.com.au/wp/chromaspro/). The absence of co-amplification of nuclear mitochondrial genes (numts) was verified by aligning the obtained *cox1* sequences with the *Mansonella ozzardi* mitogenome (KX822021) [45]. Furthermore, ambiguities in the sequence chromatograms, stop codons and indels were visually verified, as recommended in Reference [94]. All the sequences were subjected separately to a preliminary analysis using Basic Local Alignment Search Tool (BLAST) [95].

Both the nuclear *18S* rRNA alone or concatenated with the *ITS1* (if amplified) gene from each filarial species generated through the present study were separately aligned against the previously published sequences from the complete rRNA sequences or draft/complete genomes from the Onchocercidae clade ONC2, ONC3, ONC4 and ONC5 [56]. While, the *cox1* sequences were aligned against the representative members of the clade ONC4 and ONC5 encountered in primates [56]. The *Wolbachia* *16S* DNA sequences were aligned against the representative members of *Wolbachia* lineages (C, D, F and J) infecting filarial parasites [11,16]. MAFFT alignment was performed on the concatenated nuclear (*18S* rRNA and *ITS1*) sequences using DNastar software [92], while the *18S*, the *cox1* and the *16S* DNA sequences were aligned using ClustalW application within Bioedit v.7.2.5. [96]. The Akaike Information Criterion (AIC) option in MEGA6 [88] was used to establish the best nucleotide substitution model adapted to each sequence alignment. The Kimura 2-parameter model (+G) [97] was used to generate the *18S* and the *16S* trees, while the Tamura 3-parameter model (+I) [98] and the General Time Reversible model (+G, +I) [98] were, respectively, used for the concatenated rRNA (*18S* and *ITS1*) and the *cox1* alignments. A maximum likelihood (ML) phylogenetic inference was used with 1000 bootstrap replicates to generate the phylogenetic tree in MEGA6 [88]. *Gongylonema nepalensis* (LC278392) rRNA sequence, both Filarioidea species (KP728088) and *Physaloptera amazonica* (MK309356) *cox1* sequences and the *16S* DNA sequence of *Rickettsia* sp. (AB795333) were used as out groups to root the trees.

In addition, we generated another *cox1* alignment, including the representative members of all the Onchocercidae clades (ONC1, ONC2, ONC3, ONC4 and ONC5) [56]. Two Filaridae and four Physalopteridae sequences were included as out-groups. The interspecific nucleotide pairwise distance (IND) was used to estimate the evolutionary divergence between *cox1* sequences among Onchocercidae. Standard error was obtained by a bootstrap procedure with 1000 replicates. Analyses were inferred on MEGA6 software [88], based on the Maximum Composite Likelihood model [99]. A scatter chart based on the IND between Onchocercidae members and the *cox1* sequences generated in the present study was drown using XLSTAT Addinsoft version 4.1 (XLSTAT 2019: Data Analysis and Statistical Solution for Microsoft Excel, Paris, France).

Finally, COI protein sequences of *Brugia* species (Protein Id: QIL51350, QDE55703, ALR73830, QDE55700 and ALR73832) and those of *Mansonella* species (Protein Id: CAO83087, QHA95050, AVA30206, CAO83074 and SCW25063) were retrieved from the GenBank database and aligned against the COI sequences obtained from monkeys B9 and B8, respectively. The alignment was performed using the ClustalW application within Bioedit v.7.2.5. [96]. Amino acids conservation between the COI sequences from this study comparatively to GenBank sequences was visualized on the CLC Sequence Viewer 7 (CLC Bio Qiagen, Aarhus, Denmark).

5. Conclusions

In this study, we phylogenetically describe filarial parasites belonging to three distinct genera: *Mansonella* sp., *Brugia* sp. and an unidentified Onchocercidae species. Funding extends the presence of *Brugia* sp. and the unidentified Onchocercidae species to Guiana monkeys. In addition, phylogenetic analyses highlight the necessity of completing the classification of filariosis of neo-tropical monkeys by combining morphological and molecular-based identification for an integrative taxonomical perspective. Filaria associated *Wolbachia* can be used as diagnostic markers since they are genus specific endosymbionts. Regarding the presence of *Brugia* sp. in Guiana monkeys, the same genotype
circulates in French Guiana dogs, suggesting host diversity of this filariids. We therefore developed a novel qPCR assay that could be useful for the surveillance of brugian filariasis in vectors, animals, and humans. Further studies will be needed to shed light on the life cycle, epidemiology and circulation of this potentially zoonotic parasite.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-0817/9/8/626/s1, Figure S1: 18S sequences alignment showing the nucleotide conservation of the unidentified Onchocercidae species obtained from howler monkey against the GenBank sequences of O. volvulus and L. loa, Table S1: Estimates of the evolutionary divergence between the cytochrome c oxidase subunit I (cox1) sequences of Mansonella sp. and Brugia sp. obtained in this study comparatively with Onchocercidae members from GenBank database.

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