ORIGINAL ARTICLE

Ribosomal, mitochondrial and bacterial (*Wolbachia*) reference sequences for *Dipetalonema gracile* obtained from a wild pied tamarin (*Saguinus bicolor*) host in Manaus, Brazil

Carlos Henrique Aguiar COSTA[1,2], James Lee CRAINEY[1*], Ana Carolina Paulo VICENTE[3],
David Fernandez CONGA[4], Marcelo GORDO[5], Sérgio Luiz Bessa LUZ[1], Cindy Alves DIAS[1,6],
Túllio Romão Ribeiro da SILVA[1,7], Caroline Coelho FERREIRA[1,6], Alessandra Ferreira Dales NAVA[1]

[1] Instituto Leônidas e Maria Deane/ILMD/Fiocruz Amazônia, Laboratório de Ecologia de Doenças Transmissíveis na Amazônia, Manaus, AM, Brazil

[2] Instituto Oswaldo Cruz (PGBCS/IOC/Fiocruz), Programa de Pós-graduação em Biologia Computacional e Sistemas, Rio de Janeiro, RJ, Brazil

[3] Instituto Oswaldo Cruz/IOC/FIOCRUZ, Laboratório de Genética Molecular de Microrganismos, Rio de Janeiro, RJ, Brazil

[4] Universidade Federal Rural da Amazônia (UFRA), Av. Presidente Tancredo Neves 2501, Terra Firme, 66077-830 Belém, Pará, Brazil

[5] Universidade Federal do Amazonas, Instituto de Ciências Biológicas, Laboratório de Biologia da Conservação, Projeto Sauim-de-Coleira, Av. General Rodrigo Octávio Jordão Ramos 6200, Coroado, Manaus, AM, Brazil

[6] Instituto Leônidas e Maria Deane/ ILMD/Fiocruz Amazônia, Programa de Pós-Graduação em Biologia da Interação Patógeno Hospedeiro (PPGBIO-Interação), Manaus, AM, Brazil

[7] Instituto Oswaldo Cruz/IOC/FIOCRUZ, Programa de Pós-graduação em Biologia Parasitária, Rio de Janeiro, RJ, Brazil

* Corresponding author: James.lee@fiocruz.br; https://orcid.org/0000-0001-6812-9327

ABSTRACT

The primates that inhabit the rainforest surrounding the city of Manaus (Amazonas, Brazil) have long been recognised as potentially important reservoirs of emerging and re-emerging infectious diseases (ERIDs). PCR amplification of filarial sequences from wild-caught *Simulium oyapockense* has been used to incriminate potentially important Amazon-region ERID bridge vectors by showing they had previously fed on non-human primates. The broader use of filarial parasite sequences for the incrimination of biting insects as potentially important zoonotic disease vectors is limited by a paucity of primate-derived filarial parasite reference sequences which can be matched to the PCR amplified sequences obtained from insect-vector vectors. Here we have used shotgun sequencing to obtain reference data from an adult *Dipetalonema gracile* parasite which was found infecting a wild pied tamarin (*Saguinus bicolor*) in a peripheral region of Manaus. We report the parasite´s complete mitochondrial genome (which is 13,647 base pairs in length), 894,846 base pairs of its *Wolbachia* genome and 6,426 base pairs of its ribosomal DNA locus (spanning from the start of its 18S subunit to the end of its 28S subunit). Despite being critically endangered, *S. bicolor* is commonly encountered around the periphery of Manaus and in urban forest fragments. The reported sequences may be a useful reference tool for identifying ERID bridge vectors and potentially provide some insights into the amount and the nature of contact between primate pathogen reservoirs and the residents of Manaus.

KEYWORDS: zoonotic filariasis, non-human primates, Amazonia, emerging infectious disease

Sequências ribossômicas, mitocondriais e bacterianas (*Wolbachia*) de referência para *Dipetalonema gracile* obtidas de um hospedeiro sauim-de-coleira (*Saguinus bicolor*) em Manaus, Brasil

RESUMO

Os primatas que habitam a floresta tropical ao redor da cidade de Manaus (Amazonas, Brasil) há muito são reconhecidos como reservatórios potencialmente importantes de doenças infecciosas emergentes e reemergentes. Sequências de DNA de parasitas filariais detectadas por PCR em amostras de *Simulium oyapockense* foram usadas para demonstrar que eles haviam se alimentado anteriormente de primatas não humanos e, dessa maneira, incriminar vetores-ponte da região amazônica. O uso mais amplo de detecção de parasitas filariais para a incriminação de vetores-ponte é limitado por uma escassez de sequências referência de parasitas filarias obtidas de hospedeiros. Aqui nós usamos o sequenciamento tipo *shotgun* para obter dados de referência de um parasita adulto *Dipetalonema gracile* encontrado infectando um sauim-de-coleira, *Saguinus bicolor* no entorno de Manaus. Relatamos o genoma mitocondrial completo do parasita (que tem 13.647 pares de bases de comprimento), 894.846 pares de bases de seu genoma de *Wolbachia* e 6.426 pares de bases de seu locus de DNA ribossômico (desde o início de sua subunidade 18S até o final de sua subunidade 28S). Apesar de criticamente ameaçado, *S. bicolor* é comumente encontrado no entorno de Manaus e em fragmentos florestais urbanos. As sequências relatadas podem ser uma ferramenta de referência útil para identificar vetores ponte e potencialmente fornecer algumas informações sobre o contato entre reservatórios de patógenos de primatas e os moradores de Manaus.

PALAVRAS-CHAVE: filariose zoonótica, primatas não humanos, Amazônia, doença infecciosa emergente

INTRODUCTION

When the eminent British naturalist Alfred Russel Wallace visited Manaus in 1851, he estimated it had as few as 6,000 residents (Wallace 1853). More than 170 years after Wallace´s visit, the Amazon state capital has expanded its boundaries with unrelenting deforestation and the population has mushroomed to well over 2 million (IBGE 2021; Prates-Clark *et al.* 2009). As the city has expanded so too has its outer circumference and the number of forest fragments left isolated within it (Prates-Clark *et al.* 2009; Nava *et al.* 2017). An important public health consequence of this expansion is the increase in the area of urban-forest interface zones, which is likely to have increased arthropod-mediated contact between sylvatic pathogens and the city´s residents and thus the risk of a regional zoonotic disease outbreak (Prates-Clark *et al.* 2009; Nava *et al.* 2017; Lowe *et al.* 2020).

Recent surveys performed within the urban-forest interface zones of Manaus have found a diverse range of biting insects, but have not yet definitively incriminated any as a zoonotic disease bridge vector (Hendy *et al.* 2020a; Hendy *et al.* 2020b). Because filarial parasites usually cause chronic and persistent blood parasitaemias in their vertebrate hosts, they can often be detected in disease vectors, even when the vector is not a competent host for the parasite (Shelley *et al.* 1994; Czajka *et al.* 2012; Czajka *et al.* 2014; Simonsen *et al.* 2014; Manoj *et al.* 2021; Silva *et al.* 2022;). Because filarial parasites typically have limited host ranges, their detection in biting arthropods can and has been used to infer feeding preferences and, in this way, incriminate potential zoonotic disease bridge vectors (Garms and Voelker 1969; Lefoulon *et al.* 2015; Crainey *et al.* 2017a; Silva *et al.* 2022). Although the extent to which feeding preferences have been inferred from filarial parasites has historically been limited by how difficult it is to morphologically identify the filarial larvae found in arthropod vectors, the potential of such bridge-vector-incriminating approaches has been demonstrated by the recent application of these molecular tools (Garms and Voelker 1969; Czajka *et al.* 2012; Czajka *et al.* 2014; Manoj *et al.* 2021; Silva *et al.* 2022). The identification of zoonotic filarial parasites in arthropod disease vectors could, therefore, prove extremely helpful in identifying which arthropod species are the most likely to be involved in a zoonotic disease outbreak within Manaus and indeed beyond (Silva *et al.* 2022).

Filarial parasite identifying tools usually target slow-evolving DNA sequences and are thus typically of limited use for detecting cryptic species complexes or population sub-structuring (Lefoulon *et al.* 2015; Morales-Hojas *et al.* 2001; Morales-Hojas *et al.* 2006). Despite their limitations, however, molecular identification tools have already provided evidence of high-levels of intraspecific genetic diversity within *Dipetalonema gracile* (Rudolphi, 1809), suggesting it may be possible to genetically discriminate between the *D. gracile*

parasites that infect different non-human primates if the right population genetics tools are developed for the species (Lefoulon *et al.* 2015). The development and standardisation of traditional population genetics tools, like microsatellite markers, usually requires substantial resources which are not typically available for zoonotic filarial parasite research projects (Vieira *et al.* 2016). However, Yilmaz *et al.* (2016) investigated the population genetics of *Dirofilaria repens* (Railliet and Henry, 1911) by designing novel variable-region-amplifying PCR assays after they determined the species´ complete mitogenome sequence.

In this study, we report mitogenome, rDNA and *Wolbachia* genome reference sequences obtained from a single adult *D. gracile* specimen found parasitising a wild pied tamarin (*Saguinus bicolor* [Spix, 1823]) host in the periphery of Manaus. We believe these reference sequences could prove a useful resource in the design of novel population genetics tools to evaluate the zoonotic disease risk that non-human primates pose to the residents of Manaus and in the wider Amazon region. Although the critically endangered *S. bicolor* has a very limited geographical distribution, its range includes the city of Manaus, where it is among the most commonly encountered non-human primates in the forest fragments within the city (Gordo *et al.* 2013).

MATERIAL AND METHODS

Species identification

The work was approved by Conselho Nacional de Controle de Experimentação Animal (CONCEA), after analysis by the ethics commission on the use of animals from the Universidade Federal do Amazonas (approval # 017/2020 CEUA/UFAM). A *S. bicolor* killed in a road traffic accident near Manaus was identified to species level following Gordo *et al.* (2013). An adult *D. graclie* parasite, was removed from the primate's peritoneal cavity during a necropsy and was morphologically identified following Conga *et al.* (2018) and Yeh (1956).

DNA extraction and sequencing of *D. gracile*

After storage in 100% ethanol, the parasite was transferred to sterile distilled water for 4 hours before having its DNA extracted using a QIAGEN DNeasy Blood & Tissue kit and protocol. A sterile plastic pestle was used to grind the parasite in 180 μl of QIAGEN ATL buffer before it was incubated with 20 μl of proteinase K at 56 ºC overnight. The extraction procedure then followed the DNeasy Blood & Tissue kit manufacture's protocol "purification of Total DNA from Animal Tissues (Spin-Column Protocol)" from step 3 onward. Shotgun sequencing of this whole parasite DNA extract was performed using a NexteraTruSeq paired-end sequence library and an Illumina HiSeq 2500 instrument in the Oswaldo Cruz Foundation, Rio de Janeiro (Brazil). Adapter and low-quality sequence reads were removed with

Trimmomatic v.036 (Bolger *et al.* 2014) and a quality check was done using FASTQC. Initial sequence contig assembly was performed with MEGAHIT v.1.2.9 software; all the statistical metrics were assessed with metaQUAST v.5.0.2 (Mikheenko *et al.* 2016).

Dipetalonema gracile mitochondrial genome

A complete circular mitogenome from the *D. gracile* parasite was identified from our initial contig library using 48 Onchocercidae family query sequences in BLASTn searches. Sequence reads were mapped over the positive mitogenome contigs, using Bowtie2 (Langmead and Salzberg 2012) and then were used for a second assembly using SPAdes v.13.0.1. The mitogenome sequence was annotated using MITOS2 (Bernt *et al.* 2012) and Arwen (Laslett *et al.* 2008) and validated manually. Mitogenome circularity was confirmed using MAFFT v7.310 (Katoh *et al.* 2002). Genome coverage was assessed by read mapping using Bowtie2 (Langmead and Salzberg 2012), Samtools (Li *et al.* 2009a) and BEDTools v2.26.0 (Quinlan and Hall 2010). A figure showing synteny among Onchocercidae family mitogenomic architecture was prepared with the genome comparison software Easyfig (Sullivan *et al.* 2011).

Dipetalonema gracile Wolbachia (*w*Dgra) sequence contigs

Putative *Wolbachia*-origin contigs were identified from our initial shotgun sequence contig library with a *w*Dcau genome (NZ_CP046580.1) query sequence. Contigs with identity ≥ 90 were considered positive. Reads were remapped over the positive contigs and reassembled using the same approach used for the mitogenome (see above). Genome annotation was performed using PROKKA v.1.14.5 (Seemann 2014). Genome completeness was assessed using BUSCO v.5.1.3 (Seppey *et al.* 2019), with a proteobacteria_odb10 database (219 unique genes), and genome mapping over a *w*Dcau reference genome using BWA (Li *et al.* 2009b) and Samtools (Li *et al.* 2009a). Insertion (ISs) and WO (phage) sequences contained within the *w*Dgra contigs were searched for using the PHASTER and ISsaga web-server tools (Arndt *et al.* 2016; Varani *et al.* 2011).

Phylogenetic analysis

The *Wolbachia* phylogenomic analysis used for this study followed an approach described by Lefoulon *et al.* (2020). For our mitogenome phylogenetic analysis, all the translated PCGs from our mitogenome were concatenated and aligned with 27 other Onchocercidae family mitogenome sequences retrieved from GenBank. A four-gene (CO1, 12S and 18S and 28S) Multi Locus Sequence Typing (MLST) analysis was performed using sequences obtained from this study and nine reference concatemer sequences previously used for a 7-gene MLST analysis of the Onchocercidae family (Lefoulon *et al.* 2015). For our CO1 barcoding analysis, we created an alignment

using our sequence and 63 *Dipetalonema* genus CO1 sequences retrieved from GenBank. All of our alignments were created using MAFFT v7.310, and then edited with trimAl 1.2 (Capella-Gutiérrez 2009). Maximum likelihood inference was performed using IQ-TREE2 software, with 1000 bootstrap replicates.

RESULTS

Dipetalonema gracile mitogenome

A total of 10,630,646 reads (92.8% of the total generated) passed through our initial sequence quality filtering steps and were assembled into 14,815 contigs, with a N50 of 13,250 base pairs. This raw sequence data can be accessed at the NCBI´s sequence read archive (SRA) database under accession number SRR21859278. BLASTn searches using reference onchocercidae mitogenomes as queries, identified a single contig of 13,647 base pairs in length pertaining to the mitogenome of our *D. gracile* parasite. This sequence has been deposited in GenBank and ascribed the accession number MZ636522 and represents the first complete mitogenome for the genus *Dipetalonema* to be made publicly available. Our complete annotation using standard mitochondrial annotation software predicted the existence of a total of 12 protein coding genes; 22 tRNA gene sequences and two ribosomal RNA gene sequences (Figure 1). In a synteny analysis of Onchocercidae mitogenome architecture, very little evidence was found of variation in the gene order and orientation across the whole of the Onchocercidae family (Supplementary Material, Figure S1). The phylogenetic tree inferred from a whole mitogenome sequence alignment includes filarial parasite representatives from all species with complete or near complete publicly available mitogenomes (Figure 2).

Dipetalonema gracile CO1 barcode and MLST classification

Our barcoding analysis grouped the *Dipetalonema* sequences we used in our analysis into two bootstrap supported monophyletic groups: Dipgroup1 and Dipgroup2 (Figure 3). Dipgroup1 contains CO1 sequences that have been recovered from *D. caudispina* (Molin, 1858) and *D. yatesi* (Notarnicola et al. [2007]) which were found infecting at least two different species of *Ateles* spider monkeys in Guyana and Peru (Lefoulon *et al.* 2015; Laidoudi *et al.* 2021; Zárate-Rendóna *et al.* 2022; Erkenswick *et al.* 2017). Dipgroup2 contains 24 CO1 sequences that were obtained from at least three different *Dipetalonema* species (*D. robini* [Bain and Roussilhon, 1985], *D. gracile* and *D. graciliformis* [Freitas, 1964]) and which were recovered from more than seven different primate host species. Our MLST analysis produced the same Dipgroup1 and Dipgroup2 clusters that our CO1 barcoding analysis produced and also produced bootstrap support for the clustering of all *D. gracile* and *D. graciliformis*
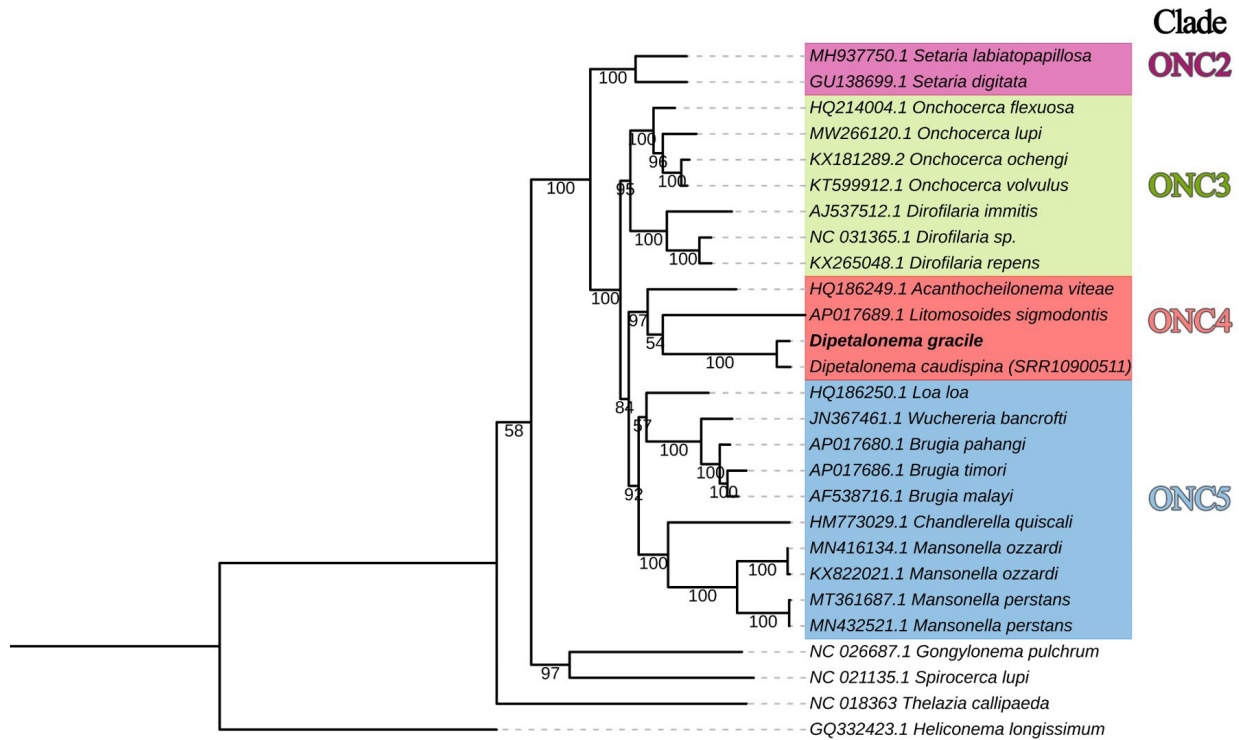
Figure 1. Genomic architecture of a *Dipetalonema gracile* mitogenome recovered from a *Saguinus bicolor*. Colours show functional gene groupings. The inner grey circle indicates the GC content. Gene names are abbreviated as follows: Cytochrome oxidase subunit 1 (COX1); NADH dehydrogenase subunit 4 gene (ND4); tryptophan transfer RNA gene (trnT); NADH dehydrogenase subunit 2 gene (ND2) tRNA serine 1 (trnS1) glutamine transfer RNA gene (trnE), NADH dehydrogenase subunit 5 (ND5); Valine transfer RNA gene (trnV); Aspartate transfer RNA gene (trnD); Proline transfer RNA gene (trnP); tRNA serine 2 (trnS2); Cysteine transfer RNA gene (trnC); NADH dehydrogenase subunit 3 gene (ND3); 16S ribosomal RNA gene (rRNL); Histidine tRNA gene (trnH) Cytochrome oxidase subunit 2 subunit (COX2); Glycine RNA transfer gene (trnG); Lysidine RNA transfer gene (trnI); ATP synthase F0 subunit 6 gene (ATP6); Phenylalanine transfer RNA (trnF); NADH dehydrogenase subunit 1 gene (ND1); Tyrosine transfer RNA gene (trnY); 12S ribosomal RNA gene (rRNS); NADH dehydrogenase subunit 4L (ND4L); Lysine transfer RNA gene (trnK); Methionine transfer RNA gene (trnM); Asparaginyl transfer RNA gene (trnN); Leucine Transfer RNA gene 2 (trnL2); Cytochrome oxidase subunit 3 gene (COX3); Alanine transfer RNA gene (trnA); Leucine transfer RNA gene 1 (trnL1); Glycine transfer RNA gene (trnQ); Cytochrome b gene (CYTB); Arginine transfer RNA gene (trnR); NADH dehydrogenase subunit 6 gene (ND6); Tryptophan transfer RNA gene (trnW). This figure was prepared with Organellar Genome DRAW (OGDRAW) version 1.3.1 (Greiner *et al.* 2019). This figure is in colour in the electronic version.

Figure 2. Phylogenetic tree inferred from an alignment of concatenated protein sequences prepared from 29 nematode mitogenome gene sequences. Nodes with greater than 50% bootstrap support have their support shown as percentages obtained from 1000 replicates. All 25 Onchocercidae sequences used are grouped into four bootstrap supported clades, highlighted with different colours (ONC2 to ONC5). These clades were first reported by Lefoulon *et al.* 2015. The four nematode outgroup sequences used in this analysis are shown without colours. This figure is in colour in the electronic version.
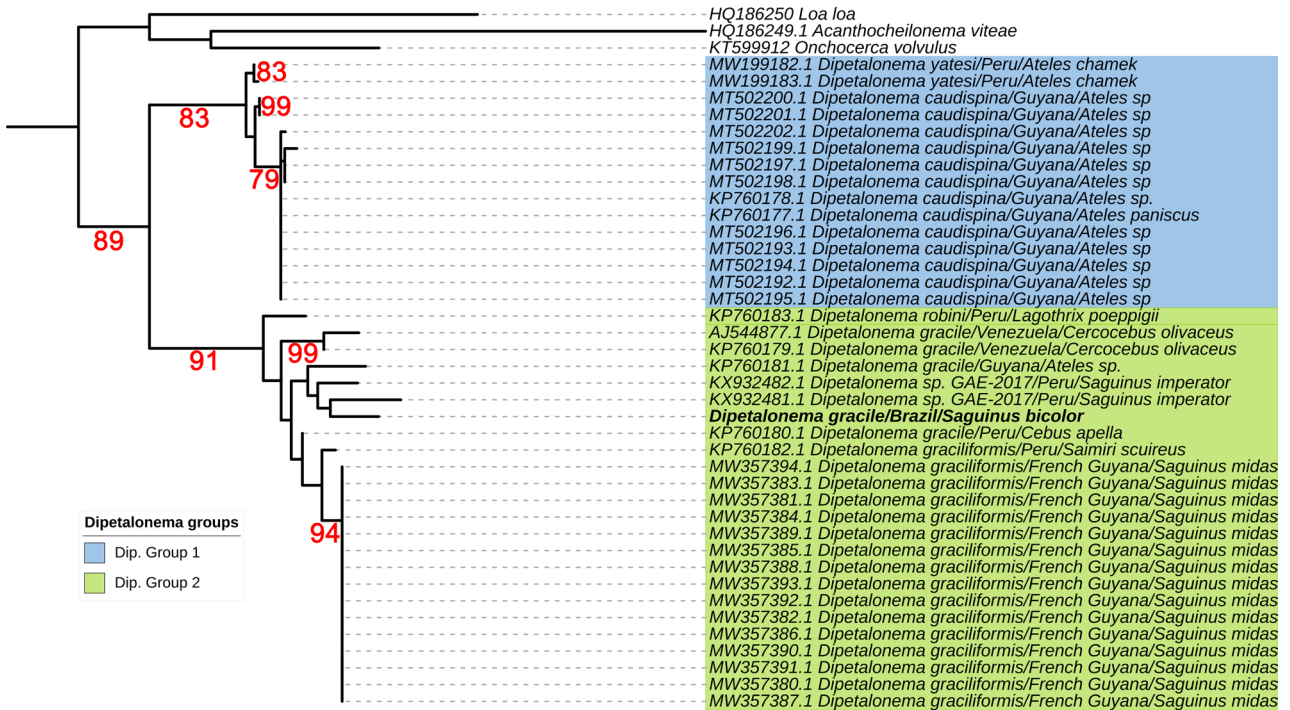
Figure 3. Phylogenetic tree inferred from a sequence alignment prepared with 39 *Dipetalonema* CO1 barcode sequences and three outgroup sequences from three distinct Onchocercidae genera. Monophyletic groups with >50% bootstrap support have their values indicated beneath their branch nodes. Two groupings referred to in the text are highlighted in colours and labelled. This figure is in colour in the electronic version.
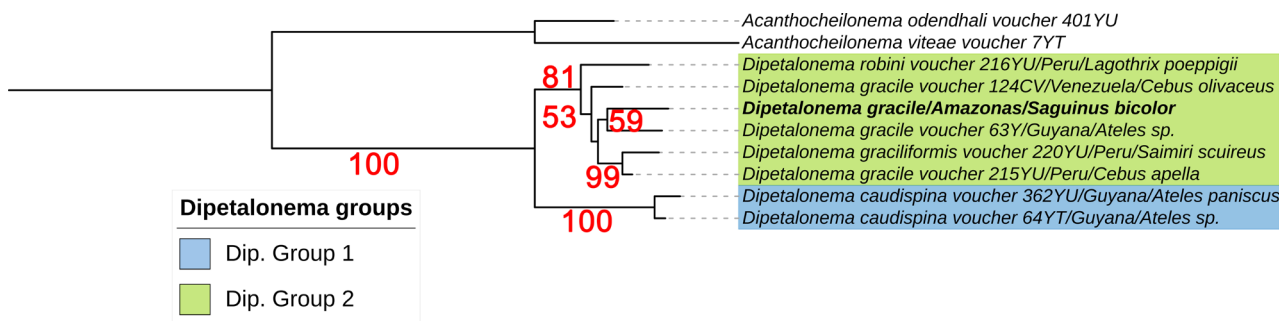
Figure 4. Phylogenetic tree inferred from a sequence alignment of 10 four-gene concatemer sequences derived from eight *Dipetalonema* parasites from the Dipgroup2 clade (see the main text), as well as two outgroup parasites from the closely related *Acanthocheilonema* genus. The *Saguinus bicolor* parasite isolated in this study can be seen to cluster within a 53% bootstrap-supported monophyletic group with five other *D. gracile* complex (*D. gracile* and *D. graciliformis*) parasites. This figure is in colour in the electronic version.

sequences in a single boostrap supported monophyletic group (Figure 4).

Ribosomal DNA locus of *Dipetalonema gracile*

Our initial assembly of shotgun sequence reads also produced a 6,426 base pair contig corresponding to a portion of our *Dipetalonema gracile* parasite´s ribosomal locus, spanning from the start of its 18S gene through to the end of its 28S gene. This ribosomal DNA sequence has been deposited in GenBank and assigned the accession number MZ727043. The ITS-1 sequence we have deposited has a length of 329 nucleotides, our ITS-2 fragment has a length of 310 nucleotides.

Wolbachia genome of *Dipetalonema gracile*

Using the *Wolbachia* genome of the *D. caudispina* endosymbiont (*wDcau)* as a reference query sequence, we identified 425 *Wolbachia* origin contigs from our shotgun sequence library. These contigs total 894,846 base pairs of unique sequence with a mean depth of coverage of 7.25 X of the genome. The contigs have been deposited in GenBank and assigned the accession number JAOWBY000000000. Our *Wolbachia* contig annotation using Prokka predicted that our contigs contain 697 PCGs as well as three rRNA genes and 33 tRNAs and that they share 525 single copy genes with the *wDcau* genome, which the same software predicted contain 619 PCGs. Our genome completeness analysis, using 219 genes from the BUSCO (proteobacteria_odb10) databases, identified 127 complete and 34 partial single-copy genes. A similar BUSCO analysis of recently published *wDcau* genome (Lefoulon *et al.*, 2020) identified a comparable number of single copy genes: 169 single (161 complete and 8 partial) and concluded that the *wDcau* genome was complete and around 863,427 base pairs in size. Our phylogenomic analysis used a set of 101 complete single copy genes that we recovered from our *wDgra* contigs (Figure 5) and generated a near identical phylogenetic tree to that produced by Lefoulon *et al.* (2020). As expected, the tree placed *wDgra* as a very close relative of *wDcau* and *wCtub* within the filarial parasite

Wolbachia super group J. Consistent with expectations and their reported absence from the *wDcau* genome, our searches did not detect any evidence of *Wolbachia* phages (WO) within the *wDgra* genome.

DISCUSSION

Dipetalonema gracile mitogenome

The *D. gracile* mitogenome sequence which we have deposited in GenBank provides publicly available *D. gracile* reference sequences for at least seven genes (12S, 16S, ND1, ND3, ND5, CO1, CO2) which have been previously targeted for species identification and/or filarial parasite molecular systematic studies (Morales-Hojas *et al.* 2006; Krueger *et al.* 2007; Liu *et al.* 2014; Fischer *et al.* 2002; Tavares da Silva *et al.* 2017). Our mitogenome phylogenomic analysis included 23 Onchocercidae family mitogenomes from 21 species, which is five more than has been used in comparable recent mitogenome phylogenetic analyses (Crainey *et al.* 2018; Yilmaz *et al.* 2019). Our mitogenome based tree produced the same ONC2 to ONC5 groupings as the MLST tree produced by Lefoulon *et al.* (2015), which was based on nuclear as well as mitochondrial gene sequences. Comparing mitogenomic analysis with MLST analysis it is thus increasingly clear that molecular phylogenetics are bringing a welcome stability to filaria parasite taxonomy, which was historically based on morphology and subject to frequent major re-organisations (Bain 2002; Lefoulon *et al.* 2015; Yilmaz *et al.* 2016; Crainey *et al.* 2018; Yilmaz *et al.* 2019).

Dipetalonema gracile CO1 barcode and MLST classification

While the mitogenomic analysis of our *Dipetalonema* parasite provides a good test of subfamily robustness, the number of filarial parasites with complete mitogenome sequences available limits its use for species level classifications. With more than 10 million CO1 reference sequences deposited in a single database, CO1 barcoding is an increasingly reliably way of molecularly identifying a species
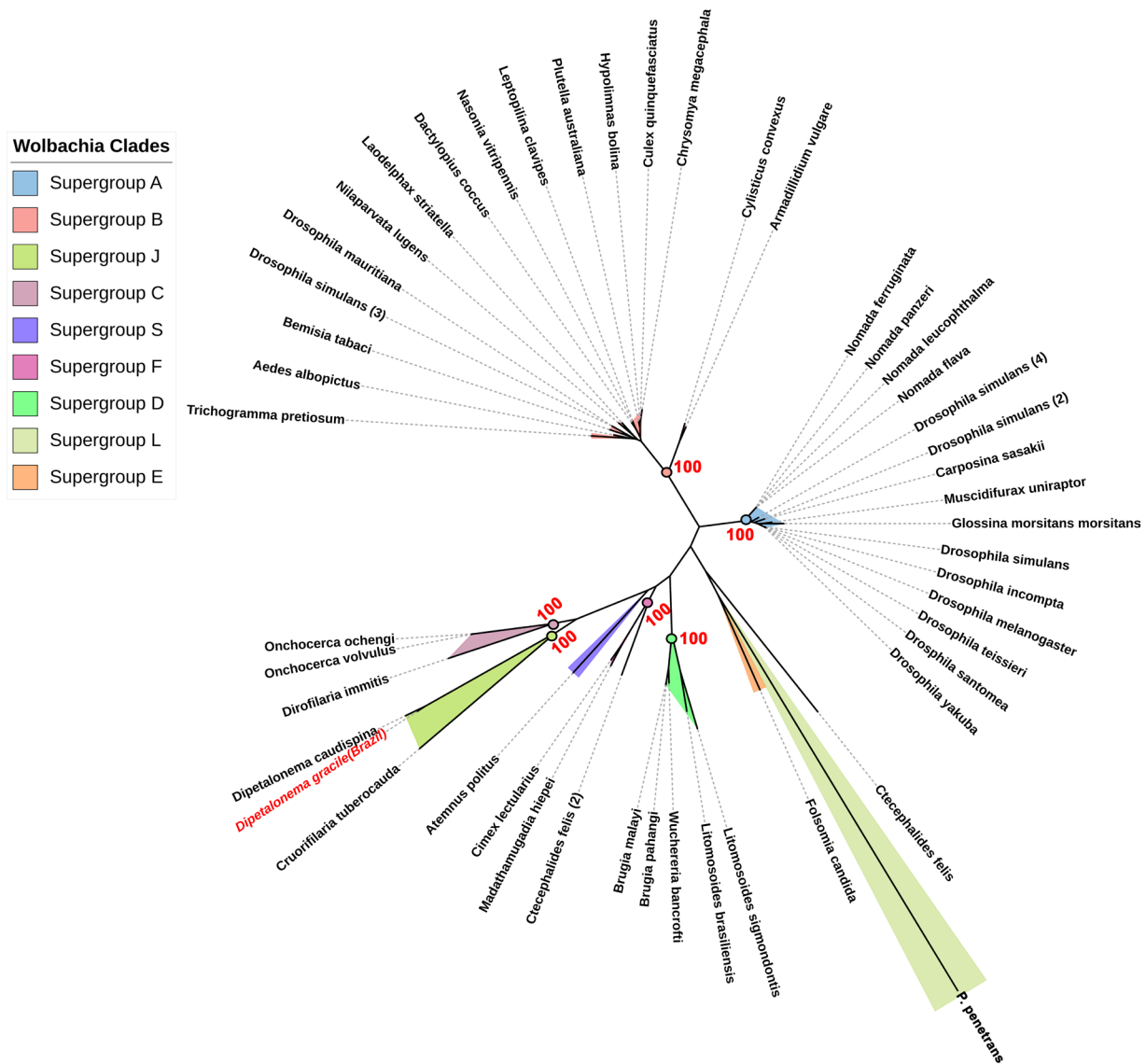
Figure 5. Phylogenetic tree inferred from a 101 gene sequence concatemer alignment prepared from 48 complete and near complete *Wolbachia* genomes available in GenBank. All major super clade groupings recovered in the analysis of Lefoulon *et al.* 2020 have been recovered in our analysis and are labelled here accordingly. The branch labels indicate both the super clades that the *Wolbachia* strains belong to and also the host organisms that the *Wolbachia* have been recovered from. This figure is in colour in the electronic version.

(Young *et al.* 2021; http://www.boldsystems.org/index.php/TaxBrowser_Home). Both the Dipgroup1 and Dipgroup2 clusters observed here can also be observed in the seven-gene MLST analysis of Lefoulon *et al.* (2015). Interestingly, though not surprisingly, given their MLST analysis used far more DNA sequence than ours, the study of Lefoulon *et al.* (2015) produced a robust sub cluster within DipGroup2, which our CO1 barcoding analysis was unable to resolve. In the Lefoulon *et al.* (2015) analysis, *D. robini* is robustly separated from the other parasite sequences in Dipgroup2, suggesting it is a distinct species separate from *D. gracile* and *D. graciliformis*. By contrast, two *D. gracile* parasites (215YU and 124CV) and a *D. graciliformis* parasite are robustly clustered

together suggesting they share a common ancestor before either does with the *D. gracile* parasite D63YT and thus that the morphological characters previously used to distinguish between *D. gracile* and *D. graciliformis* should more properly be seen as variable characters within a broader species complex (hereafter described as the *D. gracile* complex). Even though our CO1 barcoding analysis was entirely consistent with the analysis of Lefoulon *et al.* (2015), it failed to robustly separate our *D. gracile* CO1 sequence from *D. robini* and thus confirm that our sequence was not from a *D. robini* parasite. However, as the reference sequences we obtained from our *D. gracile* parasite included four (CO1, 12S, 18S and 28S) of the seven gene sequences used in the Lefoulon *et al.* (2015)

MLST analysis, it was possible for us to use a similar four-gene MLST analysis to confirm our parasite belongs to the *D. gracile* complex. The *D. gracile* parasite we recovered from a *Saguinus bicolor* host is nested in a bootstrap-supported grouping excluding *D. robini*, but containing all the available *D. graciliformis* complex members (see Figure 4).

Ribosomal DNA locus of *Dipetalonema gracile*

Like the mitochondrial sequences mentioned above, ribosomal DNA sequences from within this region of the rDNA locus (the 18S; ITS-1, 5S; ITS-2 and 28S sequences) have been used extensively as the targets of PCR and Sanger sequencing based filarial parasite molecular analyses (Morales-Hojas *et al.* 2001; Morales-Hojas *et al.* 2007; Lefoulon *et al.* 2015; Tang *et al.* 2018; Laidoudi *et al.* 2021). While rDNA 18S and 28S are commonly used in molecular taxonomic studies, rDNA ITS-1, ITS-2 and 5.8S sequences are probably the most commonly targeted sequences for filarial parasite species identification (Morales-Hojas *et al.* 2001; Krueger *et al.* 2007; Morales-Hojas *et al.* 2007; Lefoulon *et al.* 2015; Tavares da Silva *et al.* 2017; Tang *et al.* 2018; Laidoudi *et al.* 2021). To date, however, there are only 18S and 28S rDNA sequences available for species of *Diptalonema* (Lefoulon *et al.* 2015; Laidoudi *et al.* 2021). Our ITS-1, 5.8S and ITS-2 could therefore prove valuable for the identification of filarial parasites in blood and vector samples. Because the length of ITS-1 and ITS-2 sequences is highly variable among species, the size of ITS-1 PCR fragments can be useful for species-level identification and indeed fragment-size based diagnostic PCR assays have been developed on this basis (Ta-Tang *et al.* 2010).

Wolbachia genome of *Dipetalonema gracile*

Although WO are rare in filarial parasite genomes and, until recently, were thought not to occur within them, closely related WO have been found in a diverse range of *Wolbachia* genomes and gene transfer agents (GTAs), including WO genes, in all filarial parasite lineages, except for super groups J and L (Fenn and Blaxter 2006; Crainey *et al.* 2017b; Lefoulon *et al.* 2020; Bordenstein and Bordenstein 2021). In this context, the absence of WO gene sequences within our *wDgra* is interesting, as it suggests that the common ancestor of *D. gracile* and *D. caudispina* probably harboured a superclade J *Wolbachia* strain that lacked WO, and that WO sequences have failed to establish themselves within *wDcau* and *wDgra* genomes since the two species diverged. Although we have not been able to establish the exact size of the *wDgra* genome in our study, we established that it is larger than its closets relative (*wDcau*), which is the smallest reported *Wolbachia* genome to date (Lefoulon *et al.* 2020). With *Wolbachia* genomics having an increasingly important role in the development of filarial parasite therapeutics and small genomes being especially valuable for comparative genomics studies, it is also possible that the *Wolbachia* contigs reported here could be of use to studies beyond those focused on filarial parasite

identification and systematics, and be of value for comparative genomics studies assessing what genes and metabolic pathways are essential for *Wolbachia* functionality (Slatko *et al.* 2010; Bennuru *et al.* 2017; Bakowski *et al.* 2019).

Research value of novel *D. gracile* reference sequences

Because the most easily detectable filarial parasites (microfilariae found in animal blood and tissue samples, and larvae found in the parasites' arthropod vectors) are microscopic in size and embedded in host tissues, they are typically difficult to isolate without expensive, highly specialised equipment, like laser dissection microscopes (Post *et al.* 2009; Hildebrandt *et al.* 2011). Most molecular filarial parasite detection and identification in both their vertebrate and arthropod hosts is therefore typically done using PCR and Sanger sequence of filarial parasite specific DNA sequences (Simonsen *et al.* 2014; Alhassan *et al.* 2015; Ta-Tang *et al.* 2018). This means that the success in identifying filarial parasites usually depends on the existence of publicly available reference sequences to match those recovered from a disease vector or host tissue sample such as blood (Alhassan *et al.* 2015; Ta-Tang *et al.* 2018; Silva *et al.* 2022). Although these PCR assays almost always amplify ribosomal or mitochondrial DNA, a wide variety of sequences within these regions are targeted, meaning that even when mtDNA or rDNA reference sequences for a filarial parasite exist in a database, it will not be possible to use them in a PCR assay for identification of an unknown parasite if the assay has not targeted the same region as the reference sequence (Alhassan *et al.* 2015). Our shotgun sequencing of a morphologically identified adult *D. gracile* parasite has allowed us to recover a complete mitochondrial DNA genome and a near complete rDNA locus, as well as large portions of its *Wolbachia* genome from a very pure sample. This means that any filarial parasite which is detected with a standard mtDNA or rDNA PCR identification tool can now be compared to the *D. gracile* parasite we recovered.

Although our CO1 barcoding analysis was unable to confirm our morphological identification of *D. gracile*, our analysis was consistent with the MLST analysis of Lefoulon *et al.* (2015) and thus suggests that *D. gracile* is part of a species complex with high levels of cryptic diversity. Yilmaz *et al.* (2017) recovered the whole mitogenome sequence of a *D. repens* filarial parasite and used it to design primers to amplify variable regions, thus unveiling species substructuring within *D. repens*. The mitogenome recovered here could be used in a similar way to unravel any potential subspecies genetic substructuring. Even though pied tamarins are critically endangered, their occurrence in forest fragments within Manaus and peripheral forests surrounding the city represents an important potential disease reservoir for ERIDs in the Manaus region. If the mitogenome described here was used in a population genetics analysis similar to that by Yilmaz

et al. (2016) and shows that the genetic substructuring of *D. gracile* relates to its host range, it could be used to detect these parasites in arthropod vectors and evaluate the importance of *S. bicolor* as a reservoir of ERIDs in Manaus. Recently, we used molecular identification tools to identify the filarial parasite *Mansonella mariae* (Petit, Bain & Roussilhon, 1985) infecting the blackfly species *Simulum oyapockense* (Floch and Abonnenc, 1946) and, in this way, incriminate it as a potential bridge vector for emergent Amazon diseases (Silva *et al.* 2022). Our observations were made possible because reference ribosomal DNA sequences recovered from a primate host of *M. mariae* were available in GenBank (Sato *et al.* 2006).

Although it has long been known that filarial parasites usually have quite restricted host-ranges and that the larval stages of these parasites can be commonly encountered in arthropod disease vectors, their presence rarely has been used to infer past blood meals (Bain *et al.* 2002; Lefoulon *et al.* 2015; Crainey *et al.* 2017b; Silva *et al.* 2022). Before the advent of molecular techniques to identify filarial parasites, the detection of morphologically difficult to discriminate filarial larvae were typically only used to diagnose an arthropod vector as having zoonotic biting habits (Garms and Voelker 1969). The application of molecular techniques to screening of disease vectors has shown that precise identifications of filarial parasites can now be easily made from larvae found in vectors (Czajka *et al.* 2014; Manoj *et al.* 2021; Silva *et al.* 2022). Many similar studies that have identified filarial parasites in arthropod vectors have, however, recovered DNA sequences from unknown adult parasite sources (Czajka *et al.* 2012). While numerous filarial parasites have been described from wild animals of the Amazon rainforest, relatively few have had reference sequences recovered from them (Conga *et al.* 2019a; Conga *et al.* 2019b). The power of this approach will therefore be increased as more reference sequences (like those presented here) become publicly available in sequence databases.

CONCLUSIONS

The shotgun sequencing of a morphologically identified *D. gracile* from Manaus, in the central Brazilian Amazon, has provided a set of valuable reference sequences including a complete mitogenome sequence, a near complete ribosomal DNA locus, and a partial *Wolbachia* genome for the *D. gracile* endosymbiont *wDgra*. Our phylogenetic analysis of a selection of the recovered sequences, corroborates previous conclusions that molecular phylogenetics is bringing a welcome stability to Onchocercidae systematics and highlights the existence of cryptic diversity within the *D. gracile* species complex. The reference sequences reported here should facilitate future PCR-based *D. gracile* identifications and could thus prove useful for future studies investigating the ERID disease risk posed by urban primates living in and around Manaus.

REFERENCES

Alhassan, A.; Li, Z.; Poole, C.B.; Carlow, C.K.S. 2015. Expanding the MDx toolbox for filarial diagnosis and surveillance *Trends in Parasitology,* 31: 391-400.

Arndt, D.; Grant, J.R.; Marcu, A.; Sajed, T., Pon, A.; Liang, Y. 2016. PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Research,* 44: W16–21.

Bain, O. 2002. Evolutionary relationships among filarial nematodes. In: Klei, T.R.; Rajan, T.V. (Ed.). *The Filaria, World Class Parasites Series,* v.5. Springer, Boston, p.21-30.

Bakowski, M.A.; McNamara, C.W. 2019. Advances in Anti-*Wolbachia* Drug Discovery for Treatment of Parasitic Filarial Worm Infections. *Tropical Medicine and Infectious Disease,* 4: 108. doi: 10.3390/tropicalmed4030108.

Bennuru, S.; O'Connell, E.M.; Drame, P.M.; Nutman, T.B. 2018. Mining filarial genomes for diagnostic and therapeutic targets. *Trends in Parasitology,* 34: 80-90.

Bernt, M.; Donath, A.; Jühling, F.; Externbrink, F.; Florentz, C.; Fritzsch, G. 2012. Molecular phylogenetics and evolution MITOS: Improved de novo metazoan mitochondrial genome annotation. *Molecular Phylogenetics Evolution,* 69: 313-319.

Bolger, A.M.; Lohse, M.; Usadel, B. 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics,* 30: 2114–2120.

Bordenstein, S.R.; Bordenstein S.R. 2021. Widespread phages of endosymbionts: Phage WO genomics and the proposed taxonomic classification of Symbioviridae. *PLoS Genetics*, 18: e1010227.

Capella-Gutiérrez, S.; Silla-Martínez, J.M.; Gabaldón, T. 2009. trimAl: A tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics,* 25: 1972–1973.

Conga, D.F.; Mayor, P.; Furtado, A.P.; Giese, E.G.; Santos, J.N.D. 2018. Occurrence of *Dipetalonema gracile* in a wild population of woolly monkey *Lagothrix poeppiigii* in the northeastern Peruvian Amazon. *Revista Brasileira de Parasitologia Veterinária,* 27: 154-160

Conga, D.F.; Mayor, P.; Furtado, A.P.; Giese, E.G.; Santos, J.N.D. 2019. Co-infection with filarial nematodes in Sapajus macrocephalus and *Cebus albifrons* (Primates: Cebidae) from the Peruvian Amazon. *Journal of Helminthology,* 93: 375-378.

Conga, D.F.; Mayor, P.; Giese, E.G.; Santos, J.N.D. 2019. First report of filarial nematodes in free-living pitheciid primates. *Systematic Parasitology,* 96: 257-264.

Crainey, J.L.; Medeiros, J.F.; Pessoa, F.A.C.; Luz S.L.B. 2017a. Onchocerciasis. In: Marcondes C. (Ed.). *Arthropod Borne Diseases.* Springer, Cham, p.383-403.

Crainey, J.L.; Silva, T.R.; Encinas, F.; Marín, M.A.; Vicente, A.C.; Luz, S.L. 2016. The mitogenome of *Onchocerca volvulus* from the Brazilian Amazonia focus. *Memorias do Instituto Oswaldo Cruz,* 111: 79-81.

Crainey, J.L.; Marín, M.A.; Silva, T.R.R.; Medeiros, J.F.; Pessoa, F.A.C.; Santos, Y.V.; Ana Carolina Paulo Vicente, A.C.P.; Luz, S.L.B. 2018. *Mansonella ozzardi* mitogenome and pseudogene characterisation provides new perspectives on filarial parasite systematics and CO-1 barcoding. *Scientific Reports,* 8: 1–11.

Crainey, J.L.; Costa, C.H.A.; de Oliveira Leles, L.F.; Ribeiro da Silva, T.R.; de Aquino Narzetti, L.H.; Serra Dos Santos, Y.V.; *et al.* 2020. Deep sequencing reveals occult mansonellosis coinfections in residents from the Brazilian Amazon village of São Gabriel da Cachoeira. *Clinical Infectious Diseases,* 71: 1990-1993.

Crainey, J.L.; Hurst, J.; Lamberton, P.H.L.; Cheke, R.A.; Griffin, C.E.; Wilson, M.D.; Araújo, C.P.M.; Basáñez, M.G; Post, R.J. 2017b. The genomic architecture of novel *Simulium damnosum Wolbachia* prophage sequence elements and implications for onchocerciasis epidemiology. *Frontiers in Microbiology,* 8: 852. doi: 10.3389/fmicb.2017.00852

Czajka, C.; Becker, N.; Poppert, S.; Jöst, H.; Schmidt-Chanasit, J.; Krüger, A. 2012. Molecular detection of *Setaria tundra* (Nematoda: Filarioidea) and an unidentified filarial species in mosquitoes in Germany. *Parasites & Vectors,* 11: 5-14.

Czajka, C.; Becker, N.; Jöst, H.; Poppert, S.; Schmidt-Chanasit, J.; Krüger, A.; Tannich, E. 2014. Stable transmission of Dirofilaria repens nematodes, northern Germany. *Emerging Infectious Diseases,* 20: 328-331.

Erkenswick, G.A.; Watsa, M.; Gozalo, A.S.; Dmytryk, N.; Parker, P.G. 2017. Temporal and demographic blood parasite dynamics in two free-ranging neotropical primates. *International Journal for Parasitology: Parasites and Wildlife,* 6: 59-68.

Fischer, P.; Wibowo, H.; Pischke, S.; Rueckert, P.; Liebau, E.; Ismid, I.S.; Supali, T. 2002. PCR-based detection and identification of the filarial parasite *Brugia timori* from Alor island, Indonesia. *Annals of Tropical Medicine and Parasitology,* 96: 809-821.

Fenn, K.M.; Blaxter, M. 2006. *Wolbachia* genomes: revealing the biology of parasitism and mutualism. *Trends in Parasitology,* 22: 60-65.

Garms, R.; Voelker, J. 1969. Unknown filarial larvae and zoophily in *Simulium damnosum* in Liberia. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 63: 676-677.

Gordo, M.; Calleia, F.O.; Vasconcelos, S.A.; Leite, J.J.F.; Ferrari, S.F. 2013. The challenges of survival in a concrete jungle: Conservation of the pied tamarin (*Saguinus bicolor)* in the urban landscape of Manaus, Brazil. In: Marsh, L.; Chapman, C. (Ed.). *Primates in Fragments. Developments in Primatology: Progress and Prospects*, Springer, New York, p.357-370.

Greiner, S.; Lehwark, P.; Bock, R. 2019. Organellar Genome DRAW (OGDRAW) version 1.3.1: expanded toolkit for the graphical visualization of organellar genomes. *Nucleic Acids Research*, 47: W59–W64.

Hendy, A.; Hernandez-Acosta, E.; Valério, D.; Mendonça, C.; Costa, E.R.; Júnior, J.T.A.; *et al.* 2020. The vertical stratification of

potential bridge vectors of mosquito-borne viruses in a central Amazonian forest bordering Manaus, Brazil. *Scientific Reports,* 10: 18254.

Hendy, A.; Hernandez-Acosta, E.; Chaves, B.A.; Fé, N.F.; Valério, D.; Mendonça, C.; Lacerda, M.V.G.; Buenemann, M.; Vasilakis, N.; Hanley, K.A. 2020. Into the woods: Changes in mosquito community composition and presence of key vectors at increasing distances from the urban edge in urban forest parks in Manaus, Brazil. *Acta Tropica*, 206: 105441.

Hildebrandt, J.C.; Eisenbarth, A.; Renz, A; Streit, A. 2012. Single worm genotyping demonstrates that Onchocerca ochengi females simultaneously produce progeny sired by different males. *Parasitology Research,* 111: 2217–2221.

IBGE. 2021. Instituto Brasileiro de Geografia e Estatistica. (https://www.ibge.gov.br/en/cities-and-states/am/manaus.html). Accessed on 27 Oct 2022.

Katoh, K.; Misawa, K.; Kuma, K.I.; Miyata, T. 2002. MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research,* 30: 3059–66.

Krueger, A.; Fischer, P.; Morales-Hojas, R. 2007. Molecular phylogeny of the filaria genus Onchocerca with special emphasis on Afrotropical human and bovine parasites. *Acta Tropica,* 101: 1-14.

Laidoudi, Y.; Lia, R.P.; Mendoza-Roldan, J.A.; Modrý, D.; Broucker, C.A.; Mediannikov, O.; Davoust, B; Otranto, D. 2021. *Dipetalonema graciliformis* (Freitas, 1964) from the red-handed tamarins (Saguinus midas, Linnaeus, 1758) in French Guiana. *Parasitology,* 148: 1353-1359.

Langmead, B.; Salzberg, S.L. 2012. Fast gapped-read alignment with Bowtie 2. *Nature Methods,* 9: 357–359.

Laslett, D.; Canbäck, B. 2008. ARWEN: A program to detect tRNA genes in metazoan mitochondrial nucleotide sequences. *Bioinformatics,* 24: 172–175.

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 Neglected Tropical Diseases,* 9: e0004233.

Lefoulon E.; Clark, T.; Guerrero, R.; Cañizales, I.; Cardenas-Callirgos, J.M.; Junker, K.; *et al.* 2020. Diminutive, degraded but dissimilar: *Wolbachia* genomes from filarial nematodes do not conform to a single paradigm. *Microbial Genomics,* 6: 1–21.

Li, H.; Handsaker, B.; Wysoker, A.; Fennell, T.; Ruan, J.; Homer, N. 2009a. The Sequence Alignment/Map format and SAMtools. *Bioinformatics,* 25: 2078–2079.

Li, H.; Durbin, R. 2009a. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics,* 25: 1754–60.

Liu, T.; Yinan, L.; Zhong, X.; Wang, N.; Hu, D.; Zhou, X.; Xiaobin, Gu.; Xuerong, Peng.; Yang, G. 2014. Intraspecific genetic variation and phylogenetic analysis of *Dirofilaria immitis* samples from western China using complete ND1 and 16S rDNA gene sequences. *American Journal of Tropical Medicine and Hygiene,* 90: 1176-1179.

Lowe, R.; Lee, S.; Lana, R.M.; Codeço, C.T.; Castro, M.C.; Pascual, M. 2020. Emerging arboviruses in the urbanized Amazon rainforest. *British Medical Journal* 371: m4385.

Manoj, R.R.S.; Latrofa, M.S.; Cavalera, M.A.; Mendoza-Roldan, J.A.; Maia, C.; Otranto, D. 2021. Molecular detection of zoonotic filarioids in *Culex* spp. from Portugal. *Medical and Veterinary Entomology,* 35: 468-477.

Mikheenko, A.; Saveliev, V.; Gurevich, A. 2016. MetaQUAST: Evaluation of metagenome assemblies. *Bioinformatics,* 32: 1088–1090.

Morales-Hojas, R.; Post, R.J.; Shelley, A.J.; Maia-Herzog, M.; Coscarón, S.; Cheke, R.A. 2001. Characterisation of nuclear ribosomal DNA sequences from *Onchocerca volvulus* and *Mansonella ozzardi* (Nematoda: Filarioidea) and development of a PCR-based method for their detection in skin biopsies. *International Journal for Parasitology,* 31: 169-77.

Morales-Hojas, R.; Cheke, R.A.; Post, R.J. 2006. Molecular systematics of five Onchocerca species (Nematoda: Filarioidea) including the human parasite, *O. volvulus*, suggest sympatric speciation. *Journal of Helminthology,* 80: 281-290.

Morales-Hojas, R.; Cheke, R.A.; Post, R.J. 2007. A preliminary analysis of the population genetics and molecular phylogenetics of *Onchocerca volvulus* (Nematoda: Filarioidea) using nuclear ribosomal second internal transcribed spacer sequences. *Memorias do Instituto Oswaldo Cruz,* 102: 879-882.

Post, R.J.; Crainey, J.L.; Bivand, A.; Renz, A. 2009. Laser-assisted microdissection for the study of the ecology of parasites in their hosts. *Molecular Ecology Resources,* 9: 480-486.

Nava, A.; Shimabukuro, J.S.; Chmura, A.A.; Luz, S.L.B. 2017. The impact of global environmental changes on infectious disease emergence with a focus on risks for Brazil. *Institute for Laboratory Animal Research Journal,* 58: 393-400.

Prates-Clark, C.C.; Lucas, R.M.; Santos, J.R. 2009 Implications of land-use history for forest regeneration in the Brazilian Amazon. Canadian Journal of Remote Sensing, *Journal Canadien de Télédétection,* 35: 534-553.

Quinlan, A.R.; Hall, I.M. 2010. BEDTools: A flexible suite of utilities for comparing genomic features. *Bioinformatics,* 26: 841–842.

Sato, H.; Leo, N.; Katakai, Y.; Takano, J.; Akari, H.; Nakamura, S.; Une, Y. 2008. Prevalence and molecular phylogenetic characterization of *Trypanosoma* (Megatrypanum) *minasense* in the peripheral blood of small neotropical primates after a quarantine period. *Journal of Parasitology,* 94: 1128-1138.

Seemann, T. 2014. Prokka: Rapid prokaryotic genome annotation. *Bioinformatics,* 30: 2068–2069.

Seppey, M.; Manni, M.; Zdobnov, E.M. 2019. BUSCO: Assessing genome assembly and annotation completeness. *Methods in Molecular Biology,* 1962: 227–245.

Shelley, A.J., 1994. Factors Affecting Filarial Transmission by Simuliids. In: Harris K.F. (Ed.). *Advances in Disease Vector Research* v.10. Springer, New York, p.183-214.

Silva, T.R.R.D.; Narzetti, L.H.A.; Crainey, J.L.; Costa, C.H.; Santos, Y.V.S.D.; Leles, L.F.O.; Pessoa, F.A.C.; Vicente, A.C.P.; Luz, S.L.B. 2022. Molecular detection of *Mansonella mariae* incriminates *Simulium oyapockense* as a potentially important bridge vector for Amazon-region zoonoses. *Infection, Genetics and Evolution,* 3: 105200.

Simonsen, P.E.; Fischer, P.U.; Hoerauf, A.; Weil, G. 2014. The Filariases. In: Farrar, J.; Hotez, P.J.; Junghanss, T.; Kang, G.; Lalloo, D; White, N.J. (Ed.). *Manson's Tropical Diseases*. Elsevier, Amsterdam, p.37-765.

Slatko, B.E.; Taylor, M.J.; Foster, J.M. 2010. The *Wolbachia* endosymbiont as an anti-filarial nematode target. *Symbiosis,* 51: 55-65.

Sullivan, M.J.; Petty, N.K.; Beatson, S.A. 2011. Easyfig: a genome comparison visualizer. *Bioinformatics,* 27: 1009–1010.

Ta-Tang, T.H.; Crainey, J.L.; Post, R.J.; Luz, S.L.; Rubio, J.M. 2018. Mansonellosis: current perspectives. *Research and Reports in Tropical Medicine,* 9: 9-24.

Ta-Tang, T.H.; Luz, S.L.B.; Crainey, J.L.; Rubio, J.M. 2021. An Overview of the Management of Mansonellosis. *Research and Reports in Tropical Medicine,* 12: 93-105.

Ta-Tang, T.H.; Moya, L.; Nguema, J.; Aparicio, P.; Miguel-Oteo, M.; Cenzual, G.; *et al.* 2018. Geographical distribution and species identification of human filariasis and onchocerciasis in Bioko Island, Equatorial Guinea. *Acta Tropica,* 180: 12-17.

Ta-Tang, T.H.; López-Vélez, R.L.; Shelley, A.J.; Rubio, J.M.; Luz, S.B.L. 2010. Nested PCR to detect and distinguish the sympatric filarial species *Onchocerca volvulus*, *Mansonella ozzardi* and *Mansonella perstans* in the Amazon Region. *Memorias do Instituto Oswaldo Cruz,* 105: 823-828.

Tavares Silva, L.B.; Crainey, J.L.; Ribeiro da Silva, T.R.; Suwa, U.F.; Vicente, A.C.; Medeiros, J.F.; Pessoa, F.A.; Luz, S.L. 2017. Molecular Verification of New World *Mansonella perstans* parasitemias. *Emerging Infectious Diseases,* 23: 545-547.

Varani, A.M.; Siguier, P.; Gourbeyre, E.; Charneau, V.; Chandler, M. 2011. IS saga is an ensemble of web-based methods for high throughput identification and semi-automatic annotation of insertion sequences in prokaryotic genomes. *Genome Biology,* 12: R30.

Vieira, M.L.C.; Santini, L.; Diniz, A.L.; Munhoz, C.F. 2016. Microsatellite markers: what they mean and why they are so useful. *Genetics and Molecular Biology,* 39: 312–328.

Yeh, L.S. 1956. Morphological differentiation of *Dipetalonema caudispina* and *D. gracile*. *Transactions of the Royal Society of Tropical Medicine,* 50: 302-303.

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 Neglected Tropical Diseases,* 10: e0005028.

Yilmaz, E.; Wongkamchai, S.; Ramünke, S.; Koutsovoulos, G.D.; Blaxter, M.L.; Poppert, S.; Schaper, R.; von Samson-Himmelstjerna, G.; Krücken, J. 2019. High genetic diversity in the Dirofilaria repens species complex revealed by mitochondrial genomes of feline microfilaria samples from Narathiwat, Thailand. *Transbound Emerging Diseases,* 66: 389-399.

Young, M.R.; Waard, J.R.; Hebert, P.D.N. 2002. DNA barcodes enable higher taxonomic assignments in the Acari. *Scientific Reports,* 11: 15922.

Wallace, A.R. 1853. *A Narrative of Travels on the Amazon and Rio Negro: With an Account of the Native Tribes, and Observations on the Climate, Geology, and Natural History of the Amazon Valley*. Reeve and Company, London, 541p.

Zárate-Rendóna, D.A.; Salazar-Espinoza, M.N.; Catalano, S.; Sobotyk, C.; Mendozad, A.P.; Rosenbaum, M.; Verocaic, G. 2022. Molecular characterization of *Dipetalonema yatesi* from the black-faced spider monkey (*Ateles chamek*) with phylogenetic inference of relationships among *Dipetalonema* of Neotropical primates. *International Journal for Parasitology: Parasites and Wildlife,* 17: 152-157.

SUPPLEMENTARY MATERIAL (only available in the electronic version)

Costa *et al.* Ribosomal, mitochondrial and bacterial (*Wolbachia*) reference sequences for *Dipetalonema gracile* obtained from a wild pied tamarin (*Saguinus bicolor*) host in Manaus, Brazil
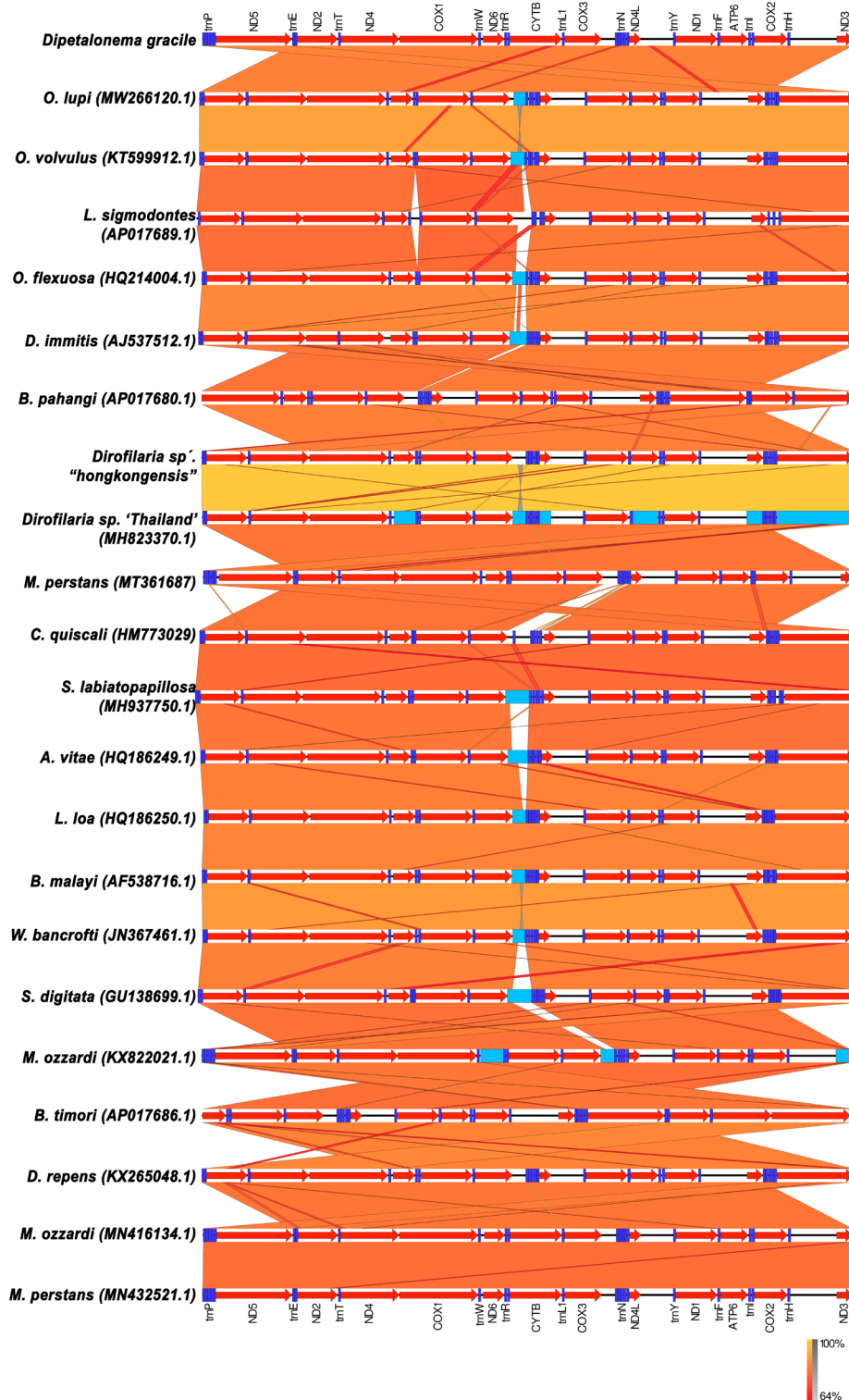

Figure S1. A graphical overview of Onchocercidae mitogenome synteny. Shared sequence identity is indicated with lines and colours. Sequence identity matches above 64% are shown in orange. Higher matches become progressively more yellow until they reach 100%. No indels or inverted sequences were detected in our analysis.