Molecular characterization of Dipetalonema yatesi from the black-faced spider monkey (Ateles chamek) with phylogenetic inference of relationships among Dipetalonema of Neotropical primates

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ABSTRACT

Species of the genus Dipetalonema are parasitic nematodes of the family Onchocercidae (Nematoda; Filarioidea) which infect the peritoneal cavity of Neotropical primates. Of these, six species have been taxonomically described, two of these have been reported infecting the black-faced spider monkey (Ateles chamek): Dipetalonema gracile and Dipetalonema yatesi. Description of Dipetalonema species have been based on morphological characteristics, and their phylogenetic relationships remain unresolved. A few molecular studies have been carried out in Dipetalonema spp. infecting Neotropical primates. Seven filarioid nematodes (6 females and one male) recovered from one A. chamek in the Peruvian Amazon rainforest were morphologically identified as D. yatesi and molecularly characterized. A multi-locus genetic analysis of nuclear ribosomal region (18S) and mitochondrial (cox1, 12S, and nad5) gene sequences supported D. yatesi as a distinct lineage and yielded a highly resolved phylogenetic lineage tree for this filarioid genus of Neotropical primates. Our results highlighted that Dipetalonema species are divided in two well-supported clades, one containing D. yatesi and D. caudispina, and the second containing D. robinii, D. gracile, and D. graciliformis. Due to sequence ambiguities from GenBank entries, relationships among isolates of D. gracile and D. graciliformis cannot be fully resolved, which requires further investigation. However, this suggests that these could represent a species complex. Our study confirms that D. yatesi is a valid species and constitutes the first molecular phylogenetic analysis of this parasite in black-faced spider monkeys.

1. Introduction

Non-human primates constitute a diverse group of species living in tropical and sub-tropical regions of America, Africa, and Asia, with only few species adapted to temperate climates (Dolhinow and Fuentes, 1999). Due to their close relationship to human beings, they were subjected to numerous studies for their role as reservoir for pathogens, including parasitic nematodes (Canizales and Guerrero, 2017; Solórzano-García and Pérez-Ponce de León, 2018). Primates are particularly vulnerable to the effects of parasitic infections since their tight social lifestyle (Freeland, 1983). The Atelidae comprises the largest family of monkeys across South and Central America (Rylands et al., 2012). Atelid monkeys are currently grouped into two subfamilies (i.e., Alouattinae and Atelinae) and five genera (i.e., Alouatta, Ateles, Brachyteles, Lagothrix, and Oreonax). The taxonomy within the genus Ateles has changed considerably (Morales-Jimenez et al., 2015). For instance, the black-faced spider monkey Ateles chamek was first described as Atelopus paniscus chamek (see Morales-Jimenez et al., 2015) before it was recognized as a separate species (Wallace, 2008).

Filaroid nematodes are parasites that belong to the Superfamily Onchocercidae (Nematoda; Filarioidea) which infect the peritoneal cavity of Neotropical primates. Of these, six species have been taxonomically described, two of these have been reported infecting the black-faced spider monkey (Ateles chamek): Dipetalonema gracile and Dipetalonema yatesi. Description of Dipetalonema species have been based on morphological characteristics, and their phylogenetic relationships remain unresolved. A few molecular studies have been carried out in Dipetalonema spp. infecting Neotropical primates. Seven filarioid nematodes (6 females and one male) recovered from one A. chamek in the Peruvian Amazon rainforest were morphologically identified as D. yatesi and molecularly characterized. A multi-locus genetic analysis of nuclear ribosomal region (18S) and mitochondrial (cox1, 12S, and nad5) gene sequences supported D. yatesi as a distinct lineage and yielded a highly resolved phylogenetic lineage tree for this filarioid genus of Neotropical primates. Our results highlighted that Dipetalonema species are divided in two well-supported clades, one containing D. yatesi and D. caudispina, and the second containing D. robinii, D. gracile, and D. graciliformis. Due to sequence ambiguities from GenBank entries, relationships among isolates of D. gracile and D. graciliformis cannot be fully resolved, which requires further investigation. However, this suggests that these could represent a species complex. Our study confirms that D. yatesi is a valid species and constitutes the first molecular phylogenetic analysis of this parasite in black-faced spider monkeys.
Filarioidea (Order Spirurida) and infect tissues and body cavities of vertebrate hosts (Anderson et al., 2000). All filarioids have an indirect life-cycle, requiring an arthropod intermediate host for development and transmission. In addition to the recent molecular report of an unidentified Brugia species from the red howler monkey in French Guiana (Laidoudi et al., 2020), two other genera within the Family Oncho cercidae, Mansonella and Dipetalonema, have been reported infecting nonhuman primates in the Americas (Bain et al., 1986; Laidoudi et al., 2020). Adult nematodes within the genus Dipetalonema parasitize the peritoneal cavity of their definitive hosts, while their microfilariae are found circulating in the bloodstream. Cavitary Dipetalonema infections can cause mild inflammatory reactions, including peritonitis and pleuritis with fibrinous adhesions (Strait et al., 2012). To date, biting midges of the genus Culicoides (Arthropoda: Ceratopogonidae) are the only biologically confirmed intermediate hosts and biological vectors of Dipetalonema (Eberhard et al., 1979; Travi et al., 1985; Notarnicola et al., 2007). There are six species in the genus Dipetalonema which parasitize Neotropical primates: D. gracile (Rudolphi, 1809); D. caudispina (Molin, 1858); D. graciliformis Freitas (1964); D. robbini Petit, Bain, and Roussillon, 1985; D. fretassi Bain et al. (1987); and D. yatesi Notarnicola et al. (2007) (Vanderhoeven et al., 2017). These nematodes have been isolated from over 20 species of monkeys from nine different genera of Neotropical primates of the tribe Platyrrhini. However, the true geographic distribution of many of these species is unknown since most reports of Dipetalonema infection in Neotropical primates come from animals in captivity (Conga et al., 2018). Only two Dipetalonema species have been reported in the black-faced spider monkey A. chamek: D. gracile, found in the Noel Kempff Mercado National Park, Bolivia (Karesh et al., 1998), and D. yatesi, a newly described species first isolated in north-eastern Bolivia (Notarnicola et al., 2007). The description of this latter species was solely based in morphological characteristics (i.e., structure and dimensions of the spicules and gubernaculum in males; morphology of the vulva and posterior end in females) (Notarnicola et al., 2007). As no molecular data are available for D. yatesi, much remains unclear regarding its phylogenetic relationships with other congeneric species.

Few molecular studies focusing on the genus Dipetalonema have been performed and most have been related to D. gracile. The present work integrated morphological and molecular analyses within a phylogenetic framework to confirm D. yatesi as a valid species infecting the peritoneal cavity of A. chamek in the Peruvian Amazon and highlighted new features on the evolutionary relationships among species of the genus Dipetalonema.

2. Materials and methods

2.1. Specimens collection

A total of seven specimens of filarioid nematodes were collected from the abdominal cavity of a juvenile male black-faced spider monkey at Taricaya Rescue Center in Madre de Dios, southern Peruvian Amazon (12° 31' 09" S, 68° 58' 47" W). This monkey was confiscated by Peruvian authorities from wildlife trafficking in Puerto Maldonado and sent to the program for the rehabilitation and reintroduction of spider monkeys at Taricaya Rescue Center. It arrived in good physical condition and was apparently healthy but was euthanized six months later due to a chronic herpesvirus infection detected during quarantine. Necropsy findings included multifocal pneumonia, pleural adhesions, ascites, and multiple cysts of approximately 5 mm of diameter with fibrinous adhesions in the mesenteries, peritoneum, and retroperitoneal spaces. A large number of filarioid nematodes of 10–15 cm in length were observed infecting the abdominal cavity (Fig. 1). A total of seven specimens (6 females and 1 male) were collected and stored in 96% ethanol for morphological and molecular identification as described below.

Specimens’ collection was authorized by the Peruvian government through the research permit RDG #067-2020-MINAGRI-SERFOR/DGGSPFFS and export permit CITES #21 PE003987/SP.

2.2. Laboratory analyses

For morphological identification, fragments of the anterior and posterior extremities of three specimens were cut using a sterile scalpel blade, cleared in lactophenol for 1 h, and subsequently mounted for observation under an Olympus BX53 optical microscope at 10X, 20X and 40× magnification.

One female and one male parasite were used for molecular analysis. Genomic DNA was extracted using the DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer’s instructions. DNA extracts were amplified for the partial 18S region of the nuclear ribosomal DNA (rDNA), the partial mitochondrial 12S gene sequence of the ribosomal RNA (rRNA), the partial cytochrome c oxidase subunit 1 (cox1) and NADH dehydrogenase subunit 5 (nad5) of the mitochondrial DNA (mtDNA). Polymerase chain reaction (PCR) was performed in 25 μL reactions containing 0.25 μM of each primer, 1x GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA) and 2.5 μL of DNA template. We amplified the 18S rDNA using two primer pairs based on previously published sequences (Floyd et al., 2005; Lefoulon et al., 2015). The primers F18ScF1 (5′-ACCGGCCATGTTCTCAGGTAAC-3′) and F18ScR1 (5′-CTCTGGCTTGATCCTGCT-3′) were used under the following cycling conditions: initial denaturation 95 °C for 2 min,
followed by 40 cycles of 95 °C for 30 s, 58 °C for 45 s, and 72 °C for 90 s, and final extension at 72 °C for 5 min. The primer pair Nem_18S_F (5′-CGGGAATGCTTGATCCACAGC-3′) and Nem_18S_R (5′-GGCGGTATCTGATGCC-3′) were used under the following cycling conditions: initial denaturation 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, and final extension at 72 °C for 5 min. The 12S rRNA region was amplified using primers 12SF (5′-CGCGAATGCTTGATCCACAGC-3′) and 12SR (5′-ATTGACGGAGTGTTTGTATCC-3′) under the following cycling conditions: initial denaturation 95 °C for 2 min, followed by 40 cycles of 95 °C for 40 s, 50 °C for 45 s, and 72 °C for 90 s, and final extension at 72 °C for 5 min. The cox1 mtDNA gene sequence was amplified using COIinf (5′-TGA TTG GTT GTG GTA A-3′) and COIintR (5′-ATA AGT ACG AGT ATC AAT ATC-3′) (Casiraghi et al., 2001) under the following cycling conditions: initial denaturation 95 °C for 2 min, followed by 40 cycles of 95 °C for 45 s, 52 °C for 45 s, and 72 °C for 90 s, and final extension at 72 °C for 5 min. The nads5 mtDNA gene sequence was amplified using NDS5-Ov5A-F (5′-TTT GTA CC-3′) and NDS5Ov5R (5′-GGGCGGTATCTGATGCC-3′) (Casiraghi et al., 2004) under the following cycling conditions: 95 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and final extension at 72 °C for 5 min. PCR products were purified using E.Z.N.A.® Cycle Pure Kit (Omega Bio-tek, Norcross, GA, USA), then sequenced in both directions using the original PCR primers in a 3730xl DNA Analyzer at Eurofins Genomics (Louisville, KY, USA). We assembled and edited contigs using CodonCode Aligner v9.0.1 (CodonCode Corporation, Centerville, MA, USA). These data, together with previously published sequences available in the GenBank™ database (Table 1), were aligned using MUSCLE (Edgar, 2004) as implemented in CodonCode Aligner v9.0.1 since no internal gaps were present. The 12S data were aligned using ProAlign v0.5 (Löytynoja and Milinkovitch, 2003) and 60% minimum posterior probability across the sites as the criterion for detecting and removing unreliably aligned characters.

Alignments of the 18S, 12S, and cox1 were analysed both separately and as concatenated data. We concatenated the sequences, and partitioned the datasets, using SequenceMatrix v1.8 (Vaidya et al., 2011) after executing an incongruence length difference (ILD) test (Farris et al., 1995) in PAUP* v4.0a (Sinauer Associates, Sunderland, MA, USA) to assess homogeneity between partitions. We performed the ILD test using 100,000 replicates, random addition of sequences (10 replicates), and the tree-bisection-reconnection algorithm for branch swapping. We inferred phylogenetic relationships by executing maximum likelihood (ML) in RAxML v8.2 (Stamatakis, 2014) and Bayesian inference (BI) in MrBayes v3.2.6 (Ronquist et al., 2012) as implemented in the Cyberinfrastructure for Phylogenetic Research (CIPRES) web portal (http://www.phylo.org). We used PartitionFinder v2.1.1 (Lanfear et al., 2016) to select the best-fit evolutionary models. For the ML analysis we enforced a generalized time-reversible (GTR) substitution model with rate heterogeneity across all partitions (i.e., 18S, 12S, and cox1’s first, second, and third codon positions), selected automatic arrest of bootstrap resampling to assess nodal support, and specified outgroups belonging to the family Onchocercidae (i.e., Acantocheloniella vietiae (GenBank™ accession numbers DQ994171 and HQ186249), Litomosoides sigmodontis (GenBank™ accession numbers AP227233 and AP017689), and Wuchereria bancrofti (GenBank™ accession numbers AY843436 and JQ316200)). For the BI analysis we enforced a Kimura Two-Parameter (K2P) substitution model with rate heterogeneity across the 18S, a GTR model with invariable sites across the 12S, a Hasegawa-Kishino-Yano (HKY) model with invariable sites across the cox1’s first and third codon positions, and a HKY model with rate heterogeneity across the cox1’s second codon position. The BI analysis was performed without specifying any outgroup and using two independent runs with four Markov Chain Monte Carlo (MCMC) chains and 10 million generations. MCMC chains were sampled every 10,000 generations and the first 25% of the trees was discarded as burn-in. The trees remaining after burn-in were used to create a 50% majority-rule consensus tree with posterior probabilities indicating nodal support. The resulting tree topologies were visualized using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

3. Results

The nematode specimens were identified as D. yatesi under microscopy based on published identification keys (Notarnicola et al., 2007). Specimens were deposited in the Museo de Historia Natural de la Universidad Nacional Federico Villarreal, Lima, Peru (Accession number: MUFV: ZOO-HPIA 205). Two specimens of D. yatesi were sequenced for their 18S rDNA (1642 base pairs (bp)), 12S rRNA (398 bp), cox1 mtDNA (649 bp), and nad5 mtDNA (428 bp). The 12S alignment excluded 33 of 405 sites based on posterior probability filtering. The ILD test validated the concatenation of the partitions since the null hypothesis of congruence was rejected (P = 0.36). Phylogenetic analysis of the concatenated alignment (1615 bp), which included 18S, 12S, and cox1 datasets (657 bp, 372 bp, and 586 bp, respectively), yielded a single best-scoring tree strongly supporting D. yatesi as a distinct lineage. ML bootstrap values and BI posterior probabilities supported a highly resolved topology indicating two clades, one of which was composed by parasites of spider monkeys (i.e., D. caudispina and D. yatesi) (Fig. 2). The second clade contains D. robini, D. gracile, and D. graciliformis. Relationships among isolates within the D. gracile/D. graciliformis clade are not fully resolved. Both ML and BI analyses of each aligned dataset (i.e., 18S rDNA, 12S rRNA, and cox1 mtDNA) yielded similar, although less defined, relationships among Dipetalonema species. Nevertheless, the phylogenetic tree for the cox1 dataset confirmed the revised identification of Dipetalonema evansi as Deraiothorornema evansi (Sazmand et al., 2016, 2019;
Bilegjargal et al., 2021), a filarioid nematode that infects Old World camelids, since this taxon failed to cluster within the *Dipetalonema* complex of species (Fig. 3).

We deposited molecular sequences of *D. yatesi* in GenBank™ under the accession numbers MW192232 and MW192233 (18S rDNA), MW209693 and MW209694 (12S rRNA), MW199182 and MW199183 (cox1 mtDNA), and MW194891 and MW194892 (nad5 mtDNA).

4. Discussion

Our work provides the first report of *D. yatesi* in *A. chamek* from Peru after its original description, which was based only on morphology and morphometry of specimens isolated from the same host in Bolivia (Notarnicola et al., 2007). The geographic distribution of the black-faced spider monkey ranges from north-eastern Peru and north-central Bolivia to areas of the Brazilian Amazonian rainforest in the states of Acre, Rondonia, Mato Grosso, and Amazonas (Rabelo et al., 2014, 2018) To date, there have been no reports of *D. yatesi* in *A. chamek* from Brazil and other areas of Peru and Bolivia, but this might be due to a lack of surveillance associated with the somewhat recent description of the species. In contrast, *D. gracile* was reported in *A. chamek* from Bolivia (Karesh et al., 1998) and Peru (Dunn and Lambrecht, 1963), but these specimens were identified only based on morphology. Current knowledge suggests that *D. yatesi* may be host specific, whereas *D. gracile* has been reported only in *A. chamek* but also other Atelidae species, including *A. paniscus* from Brazil, Panama, and Peru (Caballero, 1947; Freitas, 1964), *Ateles geoffroyi* from Mexico and Panama (Caballero, 1947; Lamothe-Argumedo et al., 1997), *Ateles fusciceps* from Panama (McCoy, 1936; Caballero, 1947), and *Ateles nigrlicollis* from Panama (Dunn and Lambrecht, 1963). The relatively recent advances in the knowledge of the biodiversity and taxonomy of *Dipetalonema* Neotropical primates are aiding species-specific diagnostics and are providing insight into host-parasite co-evolutionary history (Lefoulon et al., 2015, 2017; Milstein et al., 2020; Zhang et al., 2020).

Currently, there are six valid species in the *Dipetalonema* genus, with the addition of *D. robini*, *D. freitasi*, and *D. yatesi*. Our analysis supports *D. yatesi* as the sister species of *D. caudispina*, indicating that filarioid nematodes of spider monkeys form a well-supported clade. Currently, there are no genetic data available for *D. freitasi* and therefore its phylogenetic relationships with other species within the genus cannot be inferred molecularly. However, the morphological similarity between *A. chamek*, *D. yatesi*, and *D. caudispina*, as females belonging to these three species possess a sinuous vagina vera (Notarnicola et al., 2007), suggest a close phylogenetic relationship among them.

Many early reports of *Dipetalonema* in Neotropical primates were assumed to belong to *D. caudispina* and *D. gracile* since these were the only known species for decades. Therefore, historical records may have biased the rather broad host range of these filarioid species. For instance, *D. caudispina* has been reported in nine primate species belonging to nine genera across three families (i.e., Atelidae, Callithricidae, and Cebidae). Similarly, *D. gracile* has been reported in at least 16 species belonging to seven genera within four families (i.e., Atelidae, Aotidae, Cebidae, and Pithecidae) (Notarnicola et al., 2008; Conga et al., 2018, 2019a). While it is possible that both species are host generalists, and even have been found in co-infections (Conga et al., 2019b), our phylogenetic analysis suggests that *D. gracile/D. graciliformis* may represent a species complex. The type-host for *D. gracile* is the capuchin *Cebus capucinus* (Cebidae) and for *D. graciliformis* the tamarin *Saguinus midas* (Callithricidae) (Freitas, 1943, 1964; Bain et al., 1987). Recent molecular studies based on partial cox1 sequences support...
Declaration of competing interest

The authors declare no conflict of interest.

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Frelandroid et al. as a multi-host species, infecting S. midas and Saimiri sciureus (Cebidae) (Lefoulon et al., 2015; Milstein et al., 2020; Laidoudi et al., 2021).
All currently available sequences of D. caudispina come from specimens isolated from its type-host, A. paniscus (Lefoulon et al., 2015; Milstein et al., 2020), however the original description of D. caudispina by Molin listed various primate species as potential hosts (Freitas, 1943). The material used by Freitas (1943) to confirm D. caudispina as a valid species originated only from A. paniscus. Therefore, future molecular characterizations of putative D. caudispina from different hosts may also reveal cryptic diversity. In summary, the current knowledge on the host-parasite associations of D. caudispina, D. gracile, and D. graciliformis should be interpreted cautiously and should be revisited through integrated classical and molecular methods, ideally including material from the type hosts and type localities of each filarioid species.
Further investigations based on integrated morphological and molecular approaches are required to shed further light into the diversity, host associations, and geographic distribution of Dipetalonema species infecting Neotropical primates. Nevertheless, there are numerous challenges for robust and comprehensive sampling of adult nematode specimens through necropsy, including the remote locations and the conservation status of many of the host species. A potential strategy to examination of animals clearly showing clinical symptoms (e.g., our specimens through necropsy, including the remote locations and the geographic distribution of D. caudispina from different hosts may also reveal cryptic diversity. In summary, the current knowledge on the host-parasite associations of D. caudispina, D. gracile, and D. graciliformis should be interpreted cautiously and should be revisited through integrated classical and molecular methods, ideally including material from the type hosts and type localities of each filarioid species.
Further investigations based on integrated morphological and molecular approaches are required to shed further light into the diversity, host associations, and geographic distribution of Dipetalonema species infecting Neotropical primates. Nevertheless, there are numerous challenges for robust and comprehensive sampling of adult nematode specimens through necropsy, including the remote locations and the conservation status of many of the host species. A potential strategy to overcome some of these knowledge gaps is the application of less invasive and non-terminal methods for sample collection. Screening and characterizing microfilariae found in blood of animals that are captured or rescued could assist in answering some of the abovementioned questions (Laidoudi et al., 2021). Infections by filarioid nematodes in South American non-human primates is well known to local and indigenous communities despite the scant scientific reports of D. yatesi and other Dipetalonema species (Milstein et al., 2020). Furthermore, microfilariae are commonly found in blood smears of rescued A. chamek (P. Mendoza, pers. comm.) and several species of confiscated primates in Peru (Zariquiey Marcos, 2014). Filarioid infections have been described as benign in Neotropical primates (Chalifoux, 1993); however, the intensity of Dipetalonema spp. infections, observed at the post-mortem examination of animals clearly showing clinical symptoms (e.g., our current study; Karesh et al., 1998; Milstein et al., 2020), suggests that these parasitic infections may contribute as a co-morbidity factor in captive settings (such as rescue centres, wild markets, and confinement facilities) in which vectors are abundant and primate ecology has been severely disrupted (Shance et al., 2017).
Other aspects of the biology of D. yatesi remain unknown (Notarnicola et al., 2007, 2008). While Culicoides biting midges have been biologically proven to serve as intermediate hosts for other Dipetalonema species infecting Neotropical primates, there have been no studies assessing their role in the cycle of D. yatesi (Eberhard et al., 1979; Travi et al., 1985). Nevertheless, the molecular markers characterized in the present study may be useful for the xenomonitoring of Culicoides and other potential dipteran vectors, including their application to broader studies on the epidemiology of filarioid parasites.
5. Conclusions

Integrating the rapid collection of molecular data with opportunistic sampling is a vital effort to further non-invasive disease diagnostics and ecological knowledge of A. chamek and other endangered wildlife populations which continue to decline, primarily due to deforestation and hunting pressure (Salazar-García and Pérez-Ponce de León, 2018; Bogoni et al., 2020). Our study expands the known range of D. yatesi, which was previously only recorded in the black-faced spider monkey A. chamek in northern Bolivia, to southern Peru. Our phylogenetic analysis confirms that D. yatesi is a valid species that is closely related to D. caudispina, resolves phylogenetic relationships among within Dipetalonema species, and highlights the potential for hidden diversity within the genus.
