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# Mitochondrial DNA diversity in the acanthocephalan *Prosthenorchis elegans* in Colombia based on cytochrome c oxidase I (COI) gene sequence

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ABSTRACT

*Prosthenorchis elegans* is a member of the Phylum Acanthocephala and is an important parasite affecting New World Primates in the wild in South America and in captivity around the world. It is of significant management concern due to its pathogenicity and mode of transmission through intermediate hosts. Current diagnosis of *P. elegans* is based on the detection of eggs by coprological examination. However, this technique lacks both specificity and sensitivity, since eggs of most members of the genus are morphologically indistinguishable and shed intermittently, making differential diagnosis difficult, and coprological examinations are often negative in animals severely infected at death. We examined sequence variation in 633 bp of mitochondrial DNA (mtDNA) cytochrome c oxidase I (COI) sequence in 37 isolates of *P. elegans* from New World monkeys (*Saguinus leucopus* and *Cebus albifrons*) in Colombia held in rescue centers and from the wild. Intraspecific divergence ranged from 0.0 to 1.6% and was comparable with corresponding values within other species of acanthocephalans. Furthermore, comparisons of patterns of sequence divergence within the Acanthocephala suggest that *Prosthenorchis* represents a separate genus within the Oligacanthorhynchida. Six distinct haplotypes were identified within *P. elegans* which grouped into one of two well-supported mtDNA haplogroups. No association between haplogroup/haplotype, holding facility and species was found. This information will help pave the way to the development of molecular-based diagnostic tools for the detection of *P. elegans* as well as furthering research into the life cycle, intermediate hosts and epidemiological aspects of the species.


## 1. Introduction

The genus *Prosthenorchis* belongs to the Acanthocephala phylum (i.e. the thorny-headed worms), endoparasites living part of their life in arthropods and part in the digestive tract of vertebrates (Baker, 2008). Two species have been recognized for nonhuman primates on the basis of morphological differences: *Prosthenorchis elegans* and *Prosthenorchis spirula* (Pissinatti et al., 2007). Differentiation of the two species is based mainly on differences in the number and arrangement of hooks on the proboscis (Stunkard, 1965; Müller, 2007), and the presence of a collar between the proboscis and the body in *P. elegans* (Machado Filho, 1950).

The species *Prosthenorchis elegans* has been reported in the wild in New World primates and some carnivores of South America and in captivity around the world, affecting several species of primates in zoos, rescue centers and laboratories (Schoeb, 1989; Perez et al., 2007). It is an important cause of mortality in captive New World primates, especially in callitrichid species (Martin, 1978; Potkay, 1992; Garber and Kitron, 1997), where mortality in animals results from secondary bacterial infection due to lesions caused by the adult parasite in the intestinal tract of the definitive host (Takos and Thomas, 1958; Toft, 1982). Currently, the diagnosis of *P. elegans* is based on the detection of eggs (by microscopic observation in fecal samples). This technique lacks both sensitivity and specificity, since eggs of most members of the genus are morphologically indistinguishable and shed intermittently (Machado Filho, 1950).

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Where infection is identified, treatments employed have not proved completely effective (Nielsen, 1980; Jimenez, 2009; Johnson-Delaney, 2009) with surgical removal being the best option.

The application of DNA based methods has had a major impact in many areas of parasitology, where genetic information has provided important insights into taxonomic relationships and the history of evolution of parasite species and their variants (Criscione et al., 2005; Gasser, 2006; Varcasia et al., 2006; Huyse and Littlewood, 2007; Steinauer at al., 2007). Such approaches are also useful in parasite diagnosis, where they can potentially contribute to the accurate detection of species (Gasser, 2006; Al-Sabi and Kapel, 2011). Molecular based DNA approaches for the differential diagnosis of parasites have been developed for numerous parasite species (Ndao, 2009): polymerase chain reaction (PCR) based assays, using oligonucleotide primers derived from species-specific sequences, providing the greatest sensitivity (Gasser, 2006). While numerous studies have published sequence data for acanthocephalans (Westram et al., 2011; García-Varela et al., 2013; Pinacho-Pinacho et al., 2014), so far, no sequence data has been published for the acanthocephalan *P. elegans* (nor any other *Prosthenorchis* species), thus its phylogenetic relationship with other Acanthocephala or genetic variation within the species is unknown. In addition, the absence of genetic information for *Prosthenorchis* presents an obstacle to the development of molecular-based diagnostic tools for the genus.

The present study aimed to provide the first analysis of mitochondrial DNA (mtDNA) variation in *P. elegans*. The primary objectives were to: 1) obtain DNA sequence of the mitochondrial cytochrome oxidase subunit I (COI) gene of *P. elegans* and 2) provide the first description of intraspecific diversity among different isolates obtained from primate hosts in Colombia. This information will help pave the way to the development of molecular-based diagnostic tools for the detection of *P. elegans* as well as furthering research into the life cycle, intermediate hosts and epidemiological aspects of the species.

## 2. Materials and methods

### 2.1. Sample collection and morphological description

A total of 37 adult parasites (Table 1) were collected opportunistically from the intestines of primates. Twenty-two were obtained from four wild captive-held individuals (taken previously from the wild) of *Saguinus leucopus* and 13 collected from a single wild captive-held individual of *Cebus albifrons* held within wildlife rescue facilities in Colombia. In addition, two adult parasites were obtained from two wild-caught individuals of *S. leucopus* collected by the Wildlife Conservation Society – Colombia. Adult parasites were either obtained during post-mortem examination or surgical intervention carried out by qualified veterinarians following ethical practices and according to the surgical procedure described in Perez et al. (2008). All post-mortem examinations were carried out on individuals that died of natural causes. Adult parasites were stored in either 10% formaldehyde (10 samples) or absolute ethanol (27 samples). Samples received in formaldehyde were washed three times in saline solution and placed in absolute ethanol for long-term storage. The parasites were stored at −20 °C until processing. Individual parasites were identified by conventional morphological criteria following Machado Filho (1950) using a stereo and an electron microscope.

### 2.2. Amplification and sequencing of DNA

Total DNA was isolated from adult parasites using protocols of proteinase K digestion and silica/guanidinium thiocyanate extraction or standard phenol-chloroform extraction and ethanol precipitation. For silica DNA extraction, samples were digested overnight at 55 °C using 2.0 mg/ml proteinase K in lysis buffer (EDTA 0.5 M, Tris 10 mM, NaCl 100 mM, SDS buffer 2% and Triton X-100 0.5%). Binding buffer (GuSCN 5 M, NaCl 25 mM and Tris 50 mM) and silica suspension (Sigma–Aldrich, 0.5 to 10 microns particle size) were added, incubated for 3 h at room temperature and spun at 15,000 rpm for 2 min to pellet the silica. The supernatant was removed and the silica pellet was washed twice using wash buffer (ethanol 50% v/v, NaCl 125 mM, EDTA 1 mM, and Tris 10 mM). The silica pellet was dried at room temperature and DNA was recovered using ultra-pure water.

Part (703 bp) of the mitochondrial cytochrome oxidase subunit I (COI) gene was amplified using the forward primer 5′-CTAATCATAARGRTATYGG-3′ and reverse primer 5′-TAAACYTCAGGRTGACCAAARAAYCA-3′ modified from Folmer et al. (1994). M13 sequence (M13REV or M13[-21]) was added to the 5′ end of each primer in order to facilitate sequencing. Polymerase chain reactions (PCRs) were carried out in a final reaction volume of 35 μL containing 1× PCR reaction buffer [75 mM Tris–HCl, 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20; Fermentas], 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.875 U recombinant *Taq* polymerase (Fermentas) and 0.5 μM of each primer. After an initial denaturation step of 2 min at 94 °C, 34 cycles of 30 s at 94 °C, 30 s at 48 °C and 45 s at 72 °C were followed by a final extension of 72 °C for 1 min. PCR products were purified by agarose gel extraction using spin columns (MinElute Gel Extraction Kit – Qiagen; Zymoclean Gel DNA Recovery Kit – Zymo Research) and sequenced using Big-Dye (Applied Biosystems) cycle sequencing reactions with the M13REV or M13(-21) sequencing primers. Sequencing products were run on an ABI 3500 Genetic Analyzer automated sequencer (Applied Biosystems). Resulting sequence traces were checked and edited using the program CodonCode Aligner ver. 4.2. (CodonCode Corporation; www.codoncode.com). Six hundred and thirty-three bases of reliable COI gene sequence were obtained from all individuals analyzed.

**Table 1**
List of *Prosthenorchis elegans* specimens used in the present study.

| Samples | Host | Locality[a] | Source | Date of collection | Collection | Storage[b] |
|---|---|---|---|---|---|---|
| 9 | *Saguinus leucopus* 1 | URRAS-Bogotá | Rescue centre | <2011 | Unknown | Absolute ethanol |
| 13 | *Cebus albifrons* | URRAS-Bogotá | Rescue centre | 2011 | Necropsy | Absolute ethanol |
| 7 | *Saguinus leucopus* 2 | AMVA-Medellín | Rescue centre | 2011 | Surgery | 10% formaldehyde |
| 1 | *Saguinus leucopus* 3 | AMVA-Medellín | Rescue centre | 2010 | Surgery | 10% formaldehyde |
| 5 | *Saguinus leucopus* 5 | URRAS-Bogotá | Rescue centre | 2010 | Surgery | Absolute ethanol |
| 2 | *Saguinus leucopus* 6 | WCS- Puerto Berrío | Wild | 2013 | Necropsy | 10% formaldehyde |

[a] URRAS: Unidad de Rescate y Rehabilitación de Animales Silvestres, Universidad Nacional de Colombia; AMVA: Area Metropolitana del Valle de Aburrá; WCS: Wildlife Conservation Society-Colombia.
[b] Sample storage prior to receipt.