Scientific Note

Nucleotide sequence differentiation of argentine isolates of the mosquito parasitic nematode *Strelkovimermis spiculatus* (Nematoda: Mermithidae)

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The mermithids are a large, important group of nematode parasites, principally of insects; infection is almost always lethal to their host. Their potential as biological control agents against mosquitoes has been well documented (Platzer 2007). *Strelkovimermis spiculatus* Poinar and Camino 1986 (Nematoda: Mermithidae) is a parasite isolated initially from *Aedes* (*Ochlerotatus*) *albifasciatus* (Macquart) larvae in rain-flooded ponds (Poinar and Camino 1986) and subsequently from other mosquito species (Micieli et al. 2012) and from *Culex pipiens* in house drainage ditches (Muttis et al. 2013) within the Buenos Aires, Argentina region. This nematode was morphologically characterized by a ventrally-shifted mouth opening and spicules fused at the tips (Poinar and Camino 1986). Epizootics produced by this nematode are commonly observed in natural populations of *Ae. albifasciatus,* since *S. spiculatus* is a natural enemy of the immature insect stages (Campos and Sy 2003). Infective preparasitic (second stage, J2) juveniles hatch from eggs when mosquito breeding sites become flooded and then actively seek out and penetrate host mosquito larvae. Third stage juvenile (J3) develop within the mosquito in six to eight days, at which time postparasitic juveniles (J4) emerge, killing the host. Adults mate and lay eggs in an aquatic environment where the cycle is completed (Platzer 2007). Previous studies of *Strelkovimermis spiculatus* include reports of their mass rearing (Camino and Reboredo 1996), the effects of biotic and abiotic factors on their infectivity (Micieli et al. 2012), and mosquito host range (Achinelly et al. 2004). However, little is known about the population dynamics of this nematode that could be easily addressed if robust molecular markers were available to identify populations and characterize genetic variation within and among populations.

In this study, *Strelkovimermis spiculatus* isolates from two different environments (permanent and temporary flood ponds) and three hosts (*Aedes albifasciatus*, *Culex pipiens,* and *Culex dolosus*) were discriminated using simple cloning and PCR techniques.

Nematodes were collected from two different types of mosquito breeding habitats located in the suburbs of La Plata and Ensenada, Buenos Aires province, Argentina. Permanent flood ponds were characterized by household drainage systems, a common breeding site for *Cx. pipiens*. Two sites were selected: Site 1 (34° 54 ‘29 “S, 58° 2’ 25” W) located in San Carlos, La Plata City and Site 2 (34° 52 ‘49 “S, 58° 0’ 38” W) at Gonnet, La Plata. Temporary flood ponds correspond to soil depressions where water accumulates exclusively from rainfall, the larval site of the mosquito host *Ae. albifasciatus*; depressions ranged in size from 2 to 5 m wide and 0.5 cm deep, depending on ambient precipitation amounts. Site 3 was located in a coastal suburb of Punta Lara, Partido de la Ensenada (34° 49 19.9 “S, 57° 58 7.5” 3W). Site 4, located in Los Hornos, 7 km from the La Plata city center (34° 59 16.1 “S, 57° 59 43” W); this breeding ground was a shaded and ephemeral pond, filled by rainwater, persisting from several days to a few weeks. More specifically, this site was a drainage ditch where water pooled as runoff from an adjacent field after its flooding by rain. For collection of *S. spiculatus*, water samples containing mosquito larvae were collected using a 300 ml dipper and filtered through a fine mesh net. The mosquito larvae retained in the net were placed in plastic buckets and transported to the laboratory for further analysis. Approximately 10% of the mosquito larvae in each collection were randomly sampled and both stereo and optical microscopes were used to determine the presence of the nematode. Mosquito larvae were placed in plastic trays of 38 x 28 cm with 1,000 ml of tap water and allowed to mature to stage IV. At that time, individual larvae were placed in 10 ml distilled water and post-parasitic nematodes allowed to emerge. Adults were killed in 60° C and transferred to a fixative of 50% (v/v) aqueous triethanolamine formalin for 48 h and then placed in 100% triethanolamine formalin before transfer to glycerol for slow evaporation in order to clear the parasites (Seinhorst 1959). The fixed specimens were used for taxonomic identification following the key of Poinar (1977) and description by Poinar and Camino (1986). The nematodes were frozen in distilled water at -20° C and were stored for subsequent molecular analysis. *Strelkovimermis spiculatus* DNA was purified as in Kobylinski et al. (2012). DNA preparations were used as templates in PCR amplification assays using Taq DNA polymerase (Promega). For all sites, DNA samples were obtained from 50 pooled postparasitic nematodes. Nuclear *18S* rDNA gene forward

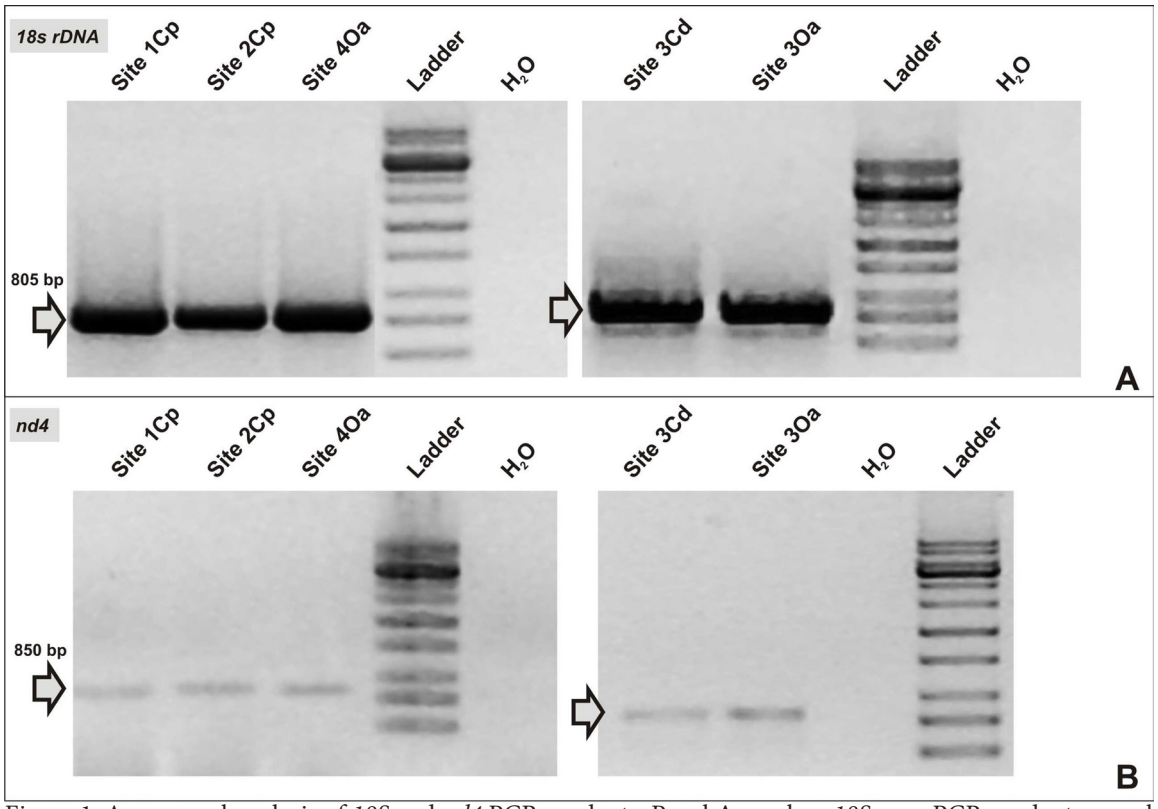

Figure 1. Agarose gel analysis of *18S* and *nd4* PCR products. Panel **A,** nuclear *18S* gene PCR products; panel **B,** mitochondrial *nd4* gene products. Abbreviations for mosquito hosts: Cp: *Culex pipiens*, Cd: *Culex dolosus*, Oa: *Ae. albifasciatus*. Ladder, 1 kb ladder size standard (Productos Biológicos, Bernal, Argentina). H_2O, no DNA template amplification reaction (negative control).

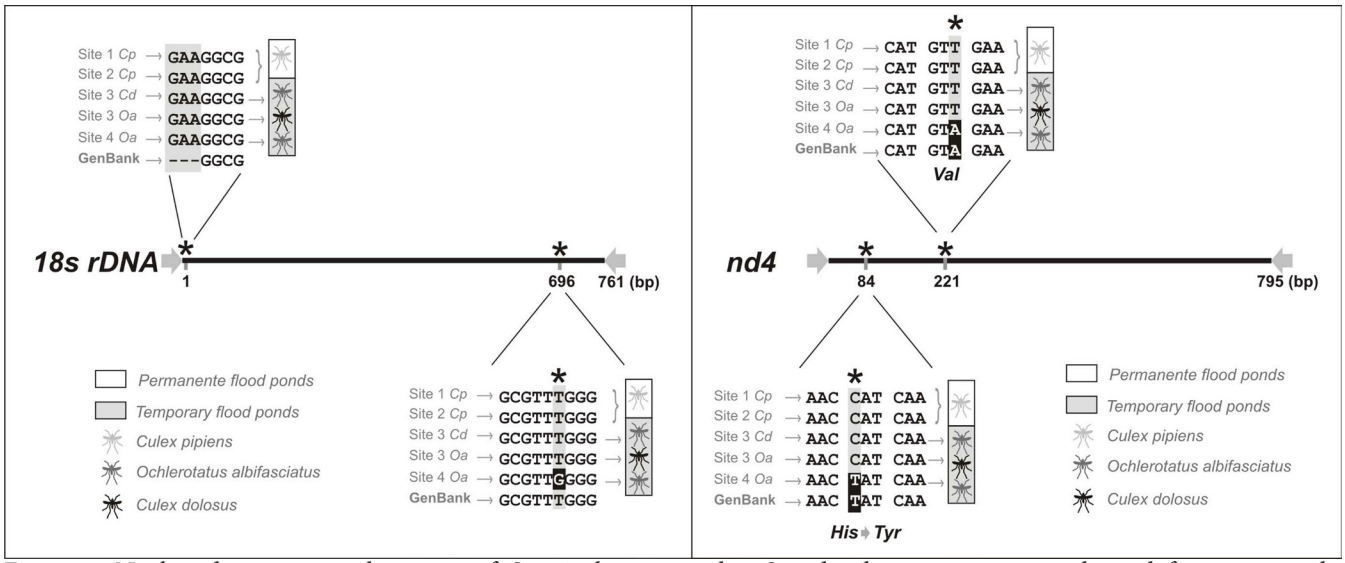

Figure 2. Nucleotide sequence alignment of *S. spiculatus* partial *18S* and *nd4* gene sequences derived from nematodes propagated in permanent and temporary ponds. Only segments containing nucleotide sequence polymorphisms are displayed. Full sequences from PCR products are available in GenBank [accession numbers: (18S_Site 1_Cp: KP270701); (18S_Site 2_Cp: KP270702); (18S_Site 3_Cd: KP270700); (18S_Site 3_Oa: KP270703); (18S_Site 4_Oa: KP270704); (nd4_Site 1_Cp: KP270706); (nd4_Site 2_Cp: KP270707); (nd4_Site 3_Cd: KP270705); (nd4_Site 3_Oa: KP270708); (nd4_Site 4_Oa: KP270709)].

and reverse primers described in Kobylinski et al. (2012) were used: Merm—5'-CAAGGACGAAAGTTAGAGGTTC-3'; Merm reverse— 5'-GGAAACCTTGTTACGACTTTTA-3′. Mitochondrial DNA (mtDNA) *nd4* gene forward and reverse primers were *ad hoc* designed using the *S. spiculatus* complete mitochondrial genome sequence (NCBI Reference Sequence: NC_008047.1): SPIC forward—5'-CTTATTGTTGGCTGAGGGCTTAATC-3'; SPIC reverse—5'-TTAAAGATGATAGCATAGGCTTGAC-3'. For PCR amplifications, $MgCl_2$ concentrations between 1.5 mM and 3 mM and annealing temperatures ranging from 50° C to 58° C were employed in the presence or absence of 10% (v/v) DMSO and were tested until a robust and unique amplification product was generated, as analyzed by 0.8% w/v agarose gel electrophoresis. DNA was detected by ethidium bromide (0.5 µg/mL) staining followed by UV illumination. Amplified fragments were recovered from agarose plugs using Zimoclean™ Gel DNA Recovery Kit (Zymo Research) according to manufacturer´s instructions. PCR products were cloned into the pGEM ®-T Easy vector (Promega) and propagated in the *Escherichia coli* strain Top 10. Plasmid DNAs were isolated by the alkaline lysis method (Sambrook et al. 1989). Cloned *S. spiculatus* DNA inserts (three recombinant plasmids for each gene and site) were sequenced using the automated method of Sanger (Macrogen Services; Seoul, Republic of Korea) with the universal primers Sp6 and T7. Confirmation that the cloned nucleotide sequences represented the *18S* and *nd4* genes was accomplished using BLASTN (www.ncbi.nlm.nih.gov).

In our data, alignment of nucleotide sequences obtained from nematodes infecting the mosquito hosts *Cx. pipiens* (site 1 and 2), *Cx. dolosus* and *Ae. albifasciatus* (site 3), and *Ae. albifasciatus* (site 4) revealed high nucleotide sequence identity to the S. *spiculatus* GenBank entry for both the partial nuclear *18S* and mitochondrial *nd4* genes sequences, with identity values ranging between 97 to 100% compared voucher sequences available in GenBank. Amplifications using *18S* primers resulted in a single product of 805 bp (Figure 1A); PCR primers targeting the *nd4* gene generated a unique product of 850 bp (Figure 1B). For the nuclear *18S* gene, the sequences obtained in this study contained an additional three contiguous nucletotides (GAA) at the beginning of the sequence not found in the GenBank *S. spiculatus* entry (Figure 2), which is also an Argentine *S. spiculatus* isolate (Platzer 2007), however, we were unable to trace with certainty the precise geographic location where the GenBank isolate was sampled. The *18S* gene sequence from site 4 nematodes presented one polymorphism, a G to T transversion at position 696, when aligned with the GenBank submission (Figure 2). Mitochondrial *nd4* sequences from *S. spiculatus* isolates collected from sites 1, 2, and 3 were identical but differed from the site 4 population and the GenBank entry at two positions. Specifically, at position 84, a T to C transition resulted in a predicted amino acid change; a TAT codon in the site 4 isolate and in GenBank entry was found to be a CAT codon in the sites 1, 2, and 3 populations. This predicted Tyr to His amino acid change resulted in a somewhat conservative substitution (from a polar to charged amino acid). A second polymorphism was an A to T transversion in a GTA codon at position 221, a silent substitution maintaining the amino acid Val at this position.

Mermithids are the only obligate nematode parasites isolated from mosquitoes. There are few studies concerning molecular taxonomy. Among them are the characterization of mitochondrial DNA size polymorphism in the mosquito parasite *Romanomermis culicivorax* (Powers et al. 1986) and a comparative mitochondrial genomics approach that revealed gene order differences among *Romanomermis* congeners, as well as large mtDNA rearrangements among *Romanomermis* and *Thaumamermis* conspecifics (Hyman et al. 2010).

The PCR primers used in this study would be useful for screening parasitism by mermithid nematodes in mosquito populations, to identify novel or cryptic species, and to study nucleotide variation and its utility in population dynamics of these parasites.

We conclude that despite a small sample size and short partial nucleotide sequence from one nuclear and one mitochondrial locus employed in the current study, isolates of *S. spiculatus* from different environments and mosquito host species could be differentiated by sequence analysis.

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