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Molecular identification and first description of the male of *Neoechinorhynchus schmidti* (Acanthocephala: Neoechinorhynchidae), a parasite of *Trachemys scripta* (Testudines) in México

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ABSTRACT

The morphology of the males of *Neoechinorhynchus schmidti* (Acanthocephala: Neoechinorhynchidae) is unknown, because this species was described based exclusively on females. However, recently we collected 2 common slider turtles *Trachemys scripta* in Centla swamps, Tabasco, Mexico, parasitized by 27 specimens of an acanthocephalan whose females were morphologically identical to *N. schmidti*. The domains D2 and D3 of the large subunit of the nuclear ribosomal RNA (LSU) of 3 males and 2 females of this material were sequenced. The sequences of both sexes were identical, and based on this result, we described for the first time the morphology of the males of *N. schmidti*. In addition, 6 sequences of a congeneric species, also parasite of turtles (*Neoechinorhynchus emyditoides*) were generated in the current research. The 11 sequences of these 2 species were aligned with 13 sequences of another 4 species of the same genus, producing a data set of 24 taxa with 674 nucleotides. The genetic divergence between *N. schmidti* and *N. emyditoides* was 4% and intraspecific differences ranged from 0.01 to 0.02%. Pairwise differences between either of these species and 4 other congeners parasitic in fresh and brackish water fishes (*Neoechinorhynchus golvani*, *Neoechinorhynchus roseum*, *Neoechinorhynchus saginatus*, and *Neoechinorhynchus* sp.) varied from 9.5 to 33%. Maximum likelihood and maximum parsimony analyses show that *N. schmidti* and *N. emyditoides* are sister taxa. Bootstrap analysis also indicates that the sister relationship is reliably supported.

1. Introduction

Neoechinorhynchus Stiles and Hassall 1905 is one of the most speciose and widely distributed genera within Acanthocephala. Ten of the 92 described species of this genus are parasites of emydid turtles distributed in North America [1–7]. These 10 species are distinguished based on the contour of the caudal extremity of the female and on the shape of the fully formed eggs [5,6], because male worms associated with freshwater turtles are presumed to be nearly identical among species [5].

In México, 2 species of *Neoechinorhynchus* from freshwater turtles have been described. Both are parasites of *Trachemys scripta*, i.e., *N. emyditoides* Fisher, 1960 and *N. schmidti* Barger, Thatcher and Nickol, 2004 [5,8,9]. However, type specimens of *N. schmidti* were found in a vial mixed with specimens of *N. emyditoides*, and for this reason, Barger et al. [4] could not describe the males of *N. schmidti*.

During a helminthological survey, adult acanthocephalans were collected in the intestine of freshwater turtles from 4 localities of México. These acanthocephalans were determined as *N. schmidti* and *N. emyditoides* based on female morphology. The molecular identification of the males and females of both species allowed us to describe for the first time the male of *N. schmidti*, which is the main goal of the current research.

2. Materials and methods

2.1. Acanthocephalan collection and identification

Adult acanthocephalans were collected between 2007 and 2008 from the intestine of freshwater turtles *Trachemys scripta* and *Trachemys gaigeae* from 4 localities of the Gulf of Mexico (Table 1). Worms were washed 3 times in 0.65% (w/v) saline, preserved in absolute ethanol, and stored at 4 °C. Some specimens were stained with Mayer's paracarmine, cleared with methyl salicylate, and mounted in Canada balsam for morphometric study. Figures were drawn with the aid of a drawing tube. Measurements are given in millimeters (mm). In addition, eight adult acanthocephalans were studied under scanning electron microscopy (SEM) following

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Table 1
Specimens information, Sample number, host species, collection sites, locality name, geographical coordinates, GenBank accession number and catalog number (CNHE) for specimen studied in this work. Sequences marked with an (*) asterisk were obtained in this study. (Nd) Not determined.

Sample no.	Host	Locality	Coordinates		GenBank accession no.	Vouchers (CNHE)
			North	West	LSU	
N. golvani 1	*Dormitator maculatus*	Alvarado Lagoon, Veracruz	18°45′	95°45′	FJ968153	6760
N. golvani 2	*Dormitator maculatus*	Alvarado Lagoon, Veracruz	18°45′	95°45′	FJ968154	6760
N. golvani 3	*Dormitator maculatus*	Alvarado Lagoon, Veracruz	18°45′	95°45′	FJ968155	6760
N. golvani 4	*Dormitator latifrons*	Tres Palos Lagoon, Guerrero	16°47′47″	99°44′30″	FJ968156	4347–4348
N. golvani 5	*Dormitator latifrons*	Tres Palos Lagoon, Guerrero	16°47′47″	99°44′30″	FJ968157	4347–4348
N. golvani 6	*Dormitator latifrons*	Tres Palos Lagoon, Guerrero	16°47′47″	99°44′30″	FJ968158	4347–4348
N. golvani 7	*Cichlasoma urophthalmus*	Carrizal River, Tabasco	18°1′45″	92°55′00″	FJ968134	6754
N. golvani 8	*Cichlasoma urophthalmus*	Carrizal River, Tabasco	18°1′45″	92°55′00″	FJ968135	6754
N. golvani 9	*Vieja pearsei*	Chicoasen Dam, Chiapas	16°56′02″	93°05′16″	FJ968136	6755
N. roseum 1	*Achiurus mazatlanus*	El Caimanero Estuary, Sinaloa	25°36′30″	108°26′25″	FJ388999	6762
N. roseum 2	*Citharichthys gilbertei*	La Tovara Estuary, Nayarit	21°31′37″	105°14′29″	FJ389000	6763
N. schmidti 1	*Trachemys scripta*	Swamps de Centla, Tabasco	18°28′18.9″	92°39′14.9″	FJ389001	6764
N. schmidti 2	*Trachemys scripta*	Swamps de Centla, Tabasco	18°28′18.9″	92°39′14.9″	HQ634785*	6764
N. schmidti 3	*Trachemys scripta*	Swamps de Centla, Tabasco	18°28′18.9″	92°39′14.9″	HQ634786*	6764
N. schmidti 4	*Trachemys scripta*	Swamps de Centla, Tabasco	18°28′18.9″	92°39′14.9″	HQ634787*	6764
N. schmidti 5	*Trachemys scripta*	Swamps de Centla, Tabasco	18°28′18.9″	92°39′14.9″	HQ634788*	6764
N. emyditoides 1	*Trachemys scripta*	Papaloapan River, Tlacotalpan, Veracruz	18° 42′ 13.4″	95° 45′ 27.9″	HQ634779*	6695
N. emyditoides 2	*Trachemys scripta*	Papaloapan River, Tlacotalpan, Veracruz	18° 42′ 13.4″	95° 45′ 27.9″	HQ634780*	6695
N. emyditoides 3	*Trachemys scripta*	Catemaco Lake, Veracruz	18°25′	95°07′	HQ634781*	6737
N. emyditoides 4	*Trachemys scripta*	Catemaco Lake, Veracruz	18°25′	95°07′	HQ634782*	6737
N. emyditoides 5	*Trachemys gaigeae*	Herradura Pond, Monterrey Nuevo León	25° 54′ 15″	98° 52′ 06″	HQ634783*	6696
N. emyditoides 6	*Trachemys gaigeae*	Herradura Pond, Monterrey Nuevo León	25° 54′ 15″	98° 52′ 06″	HQ634784*	6696
N. saginatus	Nd	Nd	Nd	Nd	AY829091	
Neoechinorhynchus sp.	Nd	Nd	Nd	Nd	HQ634789*	

standard methods [10], using a Hitachi S2460N microscope. Voucher specimens were deposited at the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City (Table 1).

2.2. Principal component analysis of males

To test morphometric differences between males of both species a morphometric comparison was carried out using the principal component analysis (PCA). Thirteen morphometric variables (presented in Table 2) were considered from 10 males (5 *N. emyditoides* from Papaloapan River, Tlacotalpan, Veracruz and 5 *N. schmidti* from Centla swamps, Tabasco). A classificatory hypothesis of the populations was tested using a discriminant function analysis with Hotelling's test (DFA). The analysis was conducted with the statistics packages PAST v. 1.60 [12].

2.3. DNA extraction, PCR amplification and sequencing

The female acanthocephalans were identified by conventional morphological criteria [5]; then, males and females collected in the same individual host were processed to obtain DNA. Individual specimens were digested overnight at 56 °C in a solution containing 10 mM Tris–HCl (pH 7.6), 20 mM NaCl, 100 mM Na_2 EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. Following digestion, DNA was extracted from the supernatant using the DNAzol reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions.

The domains D2 + D3 (~800 bp) of the large subunit of the nuclear ribosomal DNA (LSU) were amplified using the forward primer 5′ CAAGTACCGTGAGGGAAAGTTGC 3′ and reverse primer 5′ GTCGATAGGACTCCCTTTG 3′ [11]. PCR reactions (25 μl) consisted of 10 μM of each primer, 2.5 μl of 10X buffer, 1.5 μl of 15 mM of $MgCl_2$, and 1 U of Taq DNA polymerase (Platinum Taq, Invitrogen Corporation, São Paulo, Brazil). PCR cycling parameters for rDNA amplifications included denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min, followed by a post-amplification incubation at 72 °C for 10 min.

Each PCR product was purified using Millipore columns (Amicon, Billerica, Massachusetts). Purified products were sequenced for both DNA strands. Sequencing reactions were performed using ABI Big Dye (PE Applied Biosystems, Boston, Massachusetts) terminator sequencing chemistry, and reaction products were separated and detected using an ABI 310 capillary DNA sequencer. Contigs were assembled and base-calling differences resolved using Codoncode Aligner version 3.0.1 (Codoncode Corporation, Dedham, Massachusetts). All sequences were deposited in the GenBank database (accession numbers in Table 1).

2.4. Alignment and phylogenetic analyses

The D2 and D3 domains from LSU were aligned using the software ProAlign version 0.5 [13]. A guide tree was constructed using corrected (for multiple hits) pair-wise distances; this guide tree was used to estimate the hidden Markov model parameters (δ and ε) for progressive multiple alignment. Program (Java) memory and band-width were increased as required to complete the alignment. The minimum posterior probability of sites was used as the criterion for detecting and removing unreliably aligned sequence. To reduce the likelihood of excluding correctly aligned sites, the filter threshold was set to 60% minimum posterior probability. For the LSU dataset, 164 of 838 sites were excluded based on posterior probability, constituting a LSU dataset included 674 characters.

Tree searches were conducted with the optimality criteria of maximum parsimony (MP) and maximum likelihood (ML) using PAUP* 4.0b10 software [16]. For ML analyses, the Akaike Information Criterion (AIC) was used to assess the fit nucleotide substitution models [14] as implemented on the Modeltest version 3.0 [15]. The best substitution model for LSU dataset was used for likelihood analysis. Tree searches were performed using 100 (ML) random addition heuristic searches with Tree-bisection–reconnection (TBR) branch swapping. Branch and bound searches were performed using the MP method. The support of the clades was assessed by bootstrap re-sampling, with 10,000 (MP) or 1000 (ML) bootstrap replicates with the software PAUP* 4.0b10 [16]. Trees were drawn using RETREE and DRAWGRAM from PHYLIP [17]. The genetic divergence among congeneric species was estimated using p distances. Congeneric species, i.e., *N. golvani*, Salgado-Maldonado, 1978; *N. roseum* Salgado-Maldonado, 1978; *Neoechinorhynchus* sp. and