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Research note

Morphological and molecular identification of *Gnathostoma binucleatum* (Nematoda: Gnathostomatidae) advanced third stage larvae ($AdvL_3$) in the state of Colima, Mexico

Determinación morfológica y molecular de larvas del tercer estadio larvario (L_3A) de *Gnathostoma binucleatum* (Nematoda: Gnathostomatidae) del estado de Colima, México

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Abstract. As a part of an ongoing project to understand the current distribution of *Gnathostoma* species in Mexico, 22 species of vertebrates were examined for this nematode in the state of Colima. The fish species *Dormitator latifrons* ("chococo") and *Sciades guatemalensis* ("cuatete") from Cuyutlán Lagoon and the reptile *Crocodylus acutus* from the Amela Lagoon were positive for infection. Morphometric characteristics of the larvae collected in Colima were similar to those of *G. binucleatum* larvae collected from other regions and host species in Mexico. The low divergence of the ITS sequence obtained in this study from that of *G. binucleatum* (0.19%) indicates that our material belongs to this species. This is the first record of the $AdvL_3$ of *G. binucleatum* in Colima, the tenth state in Mexico in which this species has been recorded in wild vertebrates.

Key words: gnathostomiasis, morphometrics, DNA sequences, ITS2, rDNA.

Resumen. Como parte de un proyecto para definir la distribución actual de las especies de *Gnathostoma* en México, examinamos 22 especies de vertebrados en el estado de Colima en busca de este nematodo. Las especies de peces *Dormitator latifrons* ("chococo") y *Sciades guatemalensis* ("cuatete") de la laguna de Cuyutlán y el reptil *Crocodylus acutus* de la laguna de Amela resultaron positivos a la infección. Las características morfométricas de las larvas encontradas en Colima son similares a las de las larvas de *G. binucleatum* recolectadas en otras regiones y especies de hospederos en México. La escasa divergencia entre las secuencias del ITS obtenidas en este estudio con aquella de *G. binucleatum* (0.19%) indica que nuestro material pertenece a esta especie. Este es el primer registro de la L_3A de *G. binucleatum* en Colima, siendo éste el décimo estado en México en donde se ha registrado a esta especie en vertebrados silvestres.

Palabras clave: gnathostomiasis, morfometría, secuencias de ADN, ITS2, ADNr.

Three species of the genus *Gnathostoma, Gnathostoma binucleatum* Almeyda-Artigas, 1991, *G. lamothei* Bertoni-Ruiz, García-Prieto, Osorio-Sarabia and León-Règagnon, 2005, and *G. turgidum* Stossich, 1902, have been recorded in Mexico (Bertoni-Ruiz et al., 2005). However, knowledge of their geographic distribution is far from complete. To date, species of *Gnathostoma* have been observed parasitizing wild vertebrates in 82 localities in 14 states of Mexico (Pérez et al., 2008).

As a part of an ongoing project to establish the extent of the current distribution of *Gnathostoma* species in Mexico, we present the results of sampling wild vertebrates (fishes, amphibians, reptiles, and birds) in Colima state on the Pacific slope of Mexico. Likewise, we identify and describe the advanced third stage larvae (A_3L) of *G. binucleatum* through morphological and molecular studies.

Between February 2001 and October 2007, several species of wild vertebrates were collected in 2 localities

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of Colima state, Mexico: Amela Lagoon (18º50´20´´N, 103º 46´20´´W) and Cuyutlán Lagoon (18º57´00´´N, 103º57´00´´W).

Fish were caught with seine nets, amphibians and reptiles were caught manually, and birds were shot by local hunters. Amphibians and reptiles were killed by an overdose of sodium pentobarbital. Host muscle was ground individually, compressed between 2 glass plates, and examined with the aid of a magnifying glass. Some larvae were fixed in hot 70% ethanol for morphometric study, cleared in Amann's lactophenol, and measured under a compound microscope. Means are expressed in mm, ranges are shown in parentheses. Other larvae were fixed in 100% ethanol for molecular analysis. Voucher specimens were deposited in the Colección Nacional de Helmintos (CNHE: 4813, 4814), Instituto de Biología, Universidad Nacional Autónoma de México (UNAM), México City.

Standard phenol-chloroform extraction methods were used to recover DNA from the specimens. Laboratory protocols follow Palumbi (1996) and Hillis et al. (1996). The polymerase chain reaction (PCR) was used for amplifying the DNA sample; the parameters and settings followed manufacturer's recommendations and Palumbi (1996). Amplification and sequencing of the Internal Transcribed Spacer 2 (ITS2) of the ribosomal DNA was performed using the primers NEWS2 (forward) 5'TGTGTCGATGAAGAACGCAG and ITS2-RIXO (reverse) 5'TTCTATGCTTAAATTCAGGGG (Almeyda-Artigas et al., 2000). The amplification conditions consisted of 1 min denaturation at 94° C, then 35 cycles of 30 sec at 92° C, 30 sec at 50° C, and 1 min at 72° C, followed by 4 min at 72° C for the final elongation. PCR products were sequenced directly on an ABI Prism 310 automated DNA sequencer. The obtained sequence (Genbank accession number FJ497054) was aligned with available sequences from American species of *Gnathostoma*: *G. binucleatum* (AY061741), *G. lamothei* (AY818004), *G. miyazakii* (FJ497055), and *G. turgidum* (Z97175) using the computer program Clustal-W (Thompson et al., 1994), and a pair-wise distance matrix was obtained using the computer program Bioedit (Hall, 2001). *Gnathostoma spinigerum* (AB181155) was also included in this comparison due to its morphological resemblance with *G. binucleatum* in the larval stage.

Twenty-two species of vertebrates, including 15 fish species, 2 amphibian species, 2 reptile species, and 3 bird species, were examined for *Gnathostoma* (Appendix 1). Three $AdvL_3$ of *Gnathostoma* sp. were found in the skeletal muscle of 1 fish specimen, *Dormitator latifrons* ("chococo"), and 5 were found in 1 specimen of *Sciades guatemalensis* ("cuatete"); both specimens were from the Cuyutlán Lagoon. One specimen of *Crocodylus acutus* from the Amela Lagoon was infected with 4 larvae. All amphibians and birds were negative for infection (Appendix 1). Collected larvae were morphologically indistinguishable. Mean body length was 3.13 (3.12-3.14), maximum width 0.32 (0.31-0.32); cephalic bulb length 0.12 (0.11-0.13), width 0.24 (0.23-0.25); lip length 0.025 (0.024-0.026), width 0.083 (0.082-0.084); esophagus length 1.22 (1.22-1.23), width 0.20 (0.19-0.21); cervical sac length 0.7 (0.6-0.8), and width 0.087 (0.085-0.088). The number of hooklet rows on the cephalic bulb and body, as well as the location of the cervical papilla and excretory pore, are listed in Table 1. We compared the morphometric characteristics of our larvae with *G. binucleatum* from other localities, *G. procyonis* and *G. turgidum* (the other American species for which larvae have been described) and *G. spinigerum* (originally confused with *G. binucleatum* in Mexico) (Table 1).

Table 1. Morphometric comparison of advanced third stage larvae of *Gnathostoma* spp. Measurements are given in mm

Character	*G.binucleatum*[a] this study	*G.binucleatum*[b] Veracruz	*G.binucleatum*[c] Nayarit	*G. procyonis*[d] U.S.A.	*G. spinigerum*[e] Japan	*G. turgidum*[f] Guerrero
Body length	3.13 (3.12-3.14)	4.3 (2.6-5.9)	4.3 (3-5.2)	5.2	5 (4.6-5.5)	1.7 (1.5-2.0)
Hooklets number[g]						
1st row	38 (36-39)	39 (35-44)	38 (36-40)	33 (29-36)	44 (39-49)	31 (26-34)
2nd row	40 (40-41)	42 (38-47)	44 (36-48)	37 (32-40)	47 (42-54)	34 (29-38)
3rd row	45 (44-46)	45 (40-49)	44 (38-50)	41 (37-45)	50 (45-56)	37 (29-43)
4th row	46 (44-47)	48 (43-52)	45 (38-54)	45 (42-47)	52 (45-58)	39 (33-42)
4th –1st [h]	8	9	7	12	8	8
Rows of spines[i]	255	240[k]	261	---	+200	240
Position Cp/Ep[j]	14/30	12-13/28[k]	12/28	---	--/--	12/20

[a] This study; [b] Almeyda, 1991; [c] León-Règagnon et al., 2002; [d] Ash, 1962; [e] Miyazaki, 1954; [f] Mosqueda-Cabrera et al., 2009; [g] number shown as mean (range); [h] differences of hooks number between fourth and first rows; [i] number of cuticular striations covered with spines on body surface; [j] position of the cervical papillae (Cp) and excretory pore (Ep) refered to the number of body spines rows; [k] Almeyda-Artigas et al. (1994).

The morphometric characteristics of the *Gnathostoma* larvae collected in Colima are similar to those of *G. binucleatum* larvae collected from other regions and host species in Mexico, although they differ markedly from those of *G. procyonis* and *G. spinigerum* (Table 1). The minimum differences in the number of hooklets in the cephalic bulb, as well as the position of the cervical papillae, fall within the variation registered for the species (Akahane et al., 1994; Almeyda et al., 1994; Kifune et al., 2004; Martínez-Salazar and León-Règagnon, 2005).

One larva collected from *S. guatemalensis* was processed for DNA extraction. Analysis of the amplified ITS2 sequence showed 0.19% divergence with *G. binucleatum,* whereas the divergence with all other species was greater than 14.04% (Table 2). The low divergence of the obtained ITS sequence with that of *G. binucleatum* corresponds to intraspecific variation levels recorded previously for this species (Almeyda-Artigas et al., 2000; Kifune et al., 2004; León-Règagnon et al., 2002; Martínez-Salazar and León-Règagnon, 2005), providing extra support for the morphological data.

This is the first record of the $AdvL_3$ of *G. binucleatum* in Colima state, the tenth state in Mexico in which this species has been recorded in wild vertebrates (Pérez et al., 2008). Species of the fish families Eleotridae and Ariidae have been reported as etiological agents of human infections in the neighboring states of Guerrero, Nayarit, and Sinaloa (Álvarez and Alba, 2007; Díaz-Camacho et al., 2008; León-Règagnon et al., 2000, 2002).

The fish species reported as intermediate hosts in this study (*Dormitator latifrons* and *Sciades guatemalensis*) belong to the Eleotridae and Ariidae families, indicating that there is a potential zoonotic problem in Colima as well. These fish species are occasionally consumed in the form of ceviche (raw fish meat with lime juice), making this dish a potential source of infection.

The presence of Adv_3L in muscles of *C. acutus* is accidental. This host acquires the infection through the ingestion of parasitized fish; the position of crocodiles at the top of the food chain indicates that this infection represents a dead-end in the life cycle of this nematode species, especially since crocodile meat is not eaten by people locally.

Our results clearly show that *G. binucleatum* is endemic to the state of Colima. Although no human cases of gnathostomosis have ever been reported in Colima (Perez-Álvarez et al., 2008), the risk of gnathostomosis through the ingestion of raw or undercooked fish should be extensively disseminated among the residents in this region to prevent human infection.

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Literature cited

Akahane, H., R. Lamothe-Argumedo, J. M. Martínez, D. Osorio-Sarabia and L. García-Prieto. 1994. A morphological observation or the advanced third-stage larvae of Mexican *Gnathostoma*. Japanese Journal of Parasitology 43:18-22.

Almeyda-Artigas, R. J. 1991. Hallazgo de *Gnathostoma binucleatum* (Nematoda: Spirurida) en felinos silvestres y el papel de peces dulceacuículas y oligohalinos como vectores de la gnatostomiasis humana en la cuenca baja del río Papaloapan, Oaxaca-Veracruz, México. Anales del Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México 18:137-155.

Almeyda-Artigas, R. J., V. A. Acosta-Hernández, L. Alonzo-Díaz, M. Castañeda-Sánchez and V. R. Zárate-Ramírez. 1994. First record of *Gnathostoma procyonis* Chandler, 1942 in Mexico, and a note on larval *G. binucleatum* Almeyda-Artigas, 1991 (Nematoda: Spirurida). Research and Reviews in Parasitology 54:93-98.

Almeyda-Artigas R., D. M. Bargues and S. Mas-Coma. 2000. ITS-2 rDNA sequencing of *Gnathostoma* species (Nematoda) and elucidation of the species causing gnathostomiasis in the Americas. Journal of Parasitology 86:357-544.

Table 2. Pairwise distance matrix of species of *Gnathostoma* spp. percentage of divergence above diagonal; absolute distance below

		1	*2*	*3*	*4*	*5*	*6*
1	*G. binucleatum* (this study)	0.0000	0.19	23.77	16.10	14.04	52.46
2	*G. binucleatum* (AY061741)	0.0019	0.0000	23.47	15.83	13.80	51.82
3	*G. lamothei* (AY818004)	0.2377	0.2347	0.0000	21.25	31.68	59.53
4	*G. miyazaki* (XXXX)	0.1610	0.1583	0.2125	0.0000	21.09	48.67
5	*G. spinigerum* (AB181155)	0.1404	0.1380	0.3168	0.2109	0.0000	60.78
6	*G. turgidum* (Z97175)	0.5246	0.5182	0.5953	0.4867	0.6078	0.0000

Álvarez, G. C. and H. F. Alba. 2007. Estuarine fish and turtles as intermediate and paratenic hosts of *Gnathostoma binucleatum* in Nayarit, Mexico. Parasitology Research 102:117-122.

Ash, L. 1962. Development of *Gnathostoma procyonis* Chandler 1942, in the first and second intermediate host. Journal of Parasitology 48:298-305.

Bertoni-Ruiz, F., L. García-Prieto, D. Osorio-Sarabia and V. León-Règagnon. 2005. A new species of *Gnathostoma* (Nematoda:Gnathostomidae) in *Procyon lotor hernandezii* from Mexico. Journal of Parasitology 91:1143-1149.

Díaz-Camacho, S. P., M. C. Cruz-Otero, M. L. Zazueta-Ramos, A. Bojórquez-Contreras, J. Sicairos-Félix, S. Campista-León, R. Guzmán-Loreto, F. Delgado-Vargas, V. León-Règagnon and Y. Nawa. 2008. Identification of estuarine fish *Dormitator latifrons* as an intermediate host and *Eleotris picta* as a paratenic host for *Gnathostoma binucleatum* in Sinaloa, Mexico. Parasitology Research 103:1421-1425.

Hillis, D.C., C. Mortiz and B. K. Mable. 1996. Molecular Systematics. Sinauer Associates, In. Publishers. Sunderland, Massachussets, 665 p.

Hall, T. 2001. Bioedit, version 5.0.6. North Carolina State University, Department of Microbiology, Raleigh, North Carolina, 192 p.

Kifune, T., R. Lamothe-Argumedo, L. García-Prieto, A. Oceguera-Figueroa and V. León-Règagnon. 2004. *Gnathostoma binucleatum* (Spirurida: Gnathostomatidae) en peces dulceacuícolas de Tabasco, México. Revista de Biología Tropical 52: 371-376.

León-Règagnon, V., L. García-Prieto, D. Osorio-Sarabia and A. Jiménez-Ruiz. 2000. Gnatostomiasis in fish from Tres Palos Lagoon, Guerrero, Mexico. Emerging Infectious Diseases 6:429-430.

León-Règagnon, V., D. Osorio-Sarabia, L. García-Prieto, H. Akahane, R. Lamothe-Argumedo, M. Koga, M. Messina-Robles and C. Álvarez. 2002. Study of the etiological agent of gnathostomosis in Nayarit, Mexico. Parasitology International 51:201-204.

Martínez-Salazar, E. and V. León-Règagnon. 2005. Confirmation of *Gnathostoma binucleatum* Almeyda-Artigas, 1991, Advanced third-stage larvae in Tres Palos lagoon, Mexico, by morphological and molecular data. Journal Parasitology 9:962-965.

Miyazaki, I. 1954. Studies on *Gnathostoma* occurring in Japan (Nematoda: Gnathostomatidae) II Life history of *Gnathostoma* and morphological comparison of its larval forms. Kyushu Memory Medical Science 5:123-139.

Mosqueda-Cabrera, M. A., E. Sánchez-Miranda, L. Carranza-Calderón and H. E. Ortiz-Nájera. 2009. Finding of the advanced third-stage larvae of *Gnathostoma turgidum* Stossich, 1902 in Mexico from natural and experimental hosts and contributions to the life cycle description. Parasitology Research 104:1219-1225

Palumbi, S. R. 1996. Nucleic acids II: The polymerase chain reaction. *In* Molecular Systematics, D. M. Hillis, C. Moritz and B. K. Mable (eds.). Sinauer, Sunderland. Massachusetts. p. 205-247.

Pérez, A. Y., L. García-Prieto, D. Osorio-Sarabia, R. Lamothe-Argumedo and V. León-Règagnon. 2008. Present distribution of the genus *Gnathostoma* (Nematoda: Gnathostomatidae) in Mexico. Zootaxa 1930:39-55.

Thompson, J. D., D. G. Higgins and T. J. Gibson. 1994. Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequences weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22:4673-4680.

Appendix 1. Species of examined hosts from laguna de Amela and Laguna de Cuyutlán, Colima, Mexico.

Host species	*# examined / # positive/ # larvae*	*Locality (lagoons)*
Fishes		
Dormitator latifrons	245/0/0	Amela
	6/1/3	Cuyutlán
Sciades guatemalensis	173/0/0	Amela
	229/1/5	Cuyutlán
Arius seemani	181/0/0	Amela
Bagre panamensis	10/0/0	Amela
Oreochromis aureus	307/0	Amela
	80/0/0	Cuyutlán
Ciprinus carpio	100/0/0	Amela
Gobiomorus dormitor	16/0/0	Amela
Eleotris picta	29/0/0	Amela
Mugil cephalus	10/0/0	Amela
	149/0/0	Cuyutlán
Gobiidae gen. sp.	15/0/0	Amela
Astianax sp.	35/0/0	Amela
Diapterus peruvianus	193/0/0	Cuyutlán
Oligoplites altus	74/0/0	Cuyutlán
Lutjanus inermes	3/0/0	Cuyutlán
Centropomus sp.	67/0/0	Cuyutlán
Amphibians		
Lithobates sp. (Colima)	40/0/0	Amela
Bufo marinus	20/0/0	Amela
Reptiles		
Crocodylus acutus	1/1/4	Amela
Boa constrictor	3/0/0	Amela
Birds		
Ardea herodias	3/0/0	Amela
	4/0/0	Cuyutlán
Phalacrocorax olivaceus	3/0/0	Amela
	1/0/0	Cuyutlán
Pelecanus erythrorhynchus	3/0/0	Cuyutlán