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The use of mitochondrial and nuclear sequences in prospecting for cryptic species in *Tabascotrema verai* (Digenea: Cryptogonimidae), a parasite of *Petenia splendida* (Cichlidae) in Middle America

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ABSTRACT

In this study, we used sequences of mitochondrial and nuclear markers to test the hypothesis that *Tabascotrema verai* Lamothe-Argumedo and Pineda-López, 1990, a parasite of the cichlid *Petenia splendida* Günther, 1862 in Middle America, represents a single species, following a molecular prospecting approach. One-hundred and five individuals of *T. verai* were collected from the intestine of 43 specimens of *P. splendida* in eight localities of Mexico, Belize and Guatemala. A portion (n = 53) of the collected specimens was used for molecular studies, whereas the remaining individuals were processed for morphological studies. Fifty-three partial sequences of the cytochrome *c* oxidase subunit I (*cox*1) gene were obtained. In addition, 36 sequences of the ITS (internal transcribed spacers 1, 5.8S rRNA gene, and ITS2) and of the 28S rRNA gene were generated from most of the specimens that showed nucleotide variation with the *cox*1 gene. A haplotype network obtained from *cox*1 sequences revealed three independent groups (haplogroups 1, 2, and 3). Independent phylogenetic analyses performed with maximum likelihood and Bayesian inference of *cox*1, ITS and the 28S rRNA gene recovered three genetically-distinct and reciprocally-monophyletic groups, corresponding with the 3 haplogroups obtained in the haplotype network. Values of genetic divergence between clades for *cox*1 sequences ranged between 8.3% and 11.9%, while for ITS and the 28S rRNA gene, these ranged from 0.08% to 1.2% and from 0.3% to 0.4%, respectively. Morphological observations, and measurements of 23 characters of 44 mounted individuals, showed that no morphological differences exist between individuals from the eight collecting sites, and that the ranges of most morphological traits overlap. Our results suggest that the digenean *T. verai* represents a complex of cryptic species; the haplotype network, phylogenetic analyses, and genetic differences, along with the morphological stasis recorded here support this notion. Finally, the three recovered lineages showed neither geographical association nor correlation with drainage basins.

1. Introduction

In the last 15 years, the application of molecular tools in taxonomic and phylogenetic studies of several species of digeneans (each considered to represent a single species based solely on morphology) demonstrated that some of these entities actually consist of two or more genetically distinct lineages, *i.e.*, cryptic species [1–4]. The discovery of cryptic species within a single species of digenean is often a secondary result of molecular surveys whose main goals were to reconstruct phylogenetic relationships, or to describe the population genetics and/or phylogeographic patterns of one or more parasite species [5,6]. Nevertheless, the aim of recent studies has been to use sequences from mitochondrial and nuclear markers (*e.g.*, *cox*1, ND1, ITS1 and ITS2) in order to search for evidence of cryptic species in some species of digeneans [7–10] (*i.e.*, molecular prospecting, *sensu* [11,12]). Single species of digeneans that exhibit a preference for a particular host species (specialists) distributed across a wide geographic range, or those that can infect a wide range of host species (generalists), and show paucity of morphological features, are potentially prone to show instances of cryptic diversity [13–15].

The monotypic genus *Tabascotrema* (Cryptogonimidae) was established by Lamothe-Argumedo and Pineda-López to include *T. verai* Lamothe-Argumedo and Pineda-López, 1990, as a parasite of the intestine and gall bladder of the cichlid *Petenia splendida* Günther, 1862 from Tabasco, Mexico [16]. Adults of this cryptogonimid have been recorded in four species of freshwater cichlids in eleven localities of three states of southeastern Mexico [17]. Nevertheless, *P. splendida*, the type-host, seems to be the preferential definitive host for *T. verai*,

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as it has been found harboring the parasite in nine of the 11 localities where it has been recorded [17,18].

Since cryptic species complexes have been discovered in other digenean parasites of freshwater fishes in Mexico [7,8], in this study we used sequences of the cytochrome *c* oxidase subunit 1 (*cox*1) gene, of the ribosomal RNA internal transcribed spacers (ITS1, 5.8S rRNA gene, and ITS2), and of the 28S rRNA gene, following a molecular prospecting approach to test the hypothesis that *T. verai* represents a single species [13].

2. Materials and methods

Between February 2006 and July 2009, 105 digeneans, identified *in vivo* as *T. verai* using morphology, were collected from the intestine of 43 specimens of the cichlid *P. splendida*. Hosts were obtained in eight localities of Mexico, Belize and Guatemala (Fig. 1 and Table 1). Fish were collected with gillnets. Hosts were dissected, and internal organs were examined under the stereoscope in search for parasites. Digeneans collected from the intestine were placed in a Petri dish containing 0.65% saline solution. Several specimens from each locality were relaxed in hot (near boiling) tap water and fixed in 4% formalin for morphological observations (n = 52), while others were rinsed twice with clean saline solution and, immediately placed in 100% ethanol for molecular studies (n = 53). Unflattened worms (fixed in formalin) were stained with Mayer's paracarmine or Delafield's haematoxylin, dehydrated, cleared in methyl salicylate and mounted between cover-slips with Canada balsam and held in Cobb slides. Specimens from each locality were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México (UNAM), Mexico City (Table 1).

2.1. Molecular protocols

Three to nine specimens from each sampling site were placed individually in a 1.5 ml Eppendorf tube for genomic DNA extraction (n = 53). Genomic DNA of each individual was extracted using the DNeasy® Blood & Tissue Kit (Qiagen, Valencia, California) according to the manufacturer's protocol. Amplicons of mitochondrial (*cox*1 gene) and nuclear (internal transcribed spacer 1, 5.8S rRNA gene and internal transcribed spacer 2, and 28S rRNA gene) markers were amplified by PCR with the following primer pairs: JB3: 5′-TTTTTTGGGCATCCTGAG GTTTAT-3′ [19], and CO1-R: 5′-CAACAAAATCATGATGCAAAAGG-3′ [1]; Glyp1: 5′-GCTGAGAAGACGACCAAACTTGAT-3′ [7], and BD2: 5′-TATG CTTAAATTCAGCGGGT-3′ [19]; and digl2: 5′- AAGCATATCACTAAGCGG -3′ and LO: 5′-GCTATCCTGAGRGAAACTTCG-3′ [20], respectively. With the exception of annealing temperature, 45–50 °C for *cox*1 gene or 55 °C for ITSs and 28S rRNA gene, PCR conditions of denaturation and extension were the same as those commonly reported, regardless of primer set employed. Evaluation of positive amplicons, purification and subsequent sequencing of *cox*1 gene, ITSs and 28S rRNA gene were performed according to the protocols reported by Razo-Mendivil et al. [21].

Chromatograms of mitochondrial and nuclear sequences were manually revised for accuracy using FinchTV (Geospiza Inc., Seattle, Washington). To check for the presence of mitochondrial DNA pseudogenes, *cox*1 sequences from each individual were translated to amino acids using the flatworm mitochondrial code with MEGA 5.05 [22]. Mitochondrial and nuclear sequences were aligned using MUSCLE v. 3.5 [23], implemented in the software SEAVIEW v. 4.2 [24].

2.2. Number of haplotypes and haplotype network for cox1 sequences

The number of haplotypes from *cox*1 sequences (n = 53) was identified using Collapse 1.2 [25], without considering missing data. In addition, an unrooted haplotype network was constructed with TCS v1.21 [26], in order to illustrate all connections between haplotypes with a >95% probability of being most parsimonious.

2.3. Phylogenetic analyses

Maximum likelihood (ML) and Bayesian inference (BI) analyses of *cox*1, ITS´s and 28S rRNA gene sequences were performed to reconstruct the phylogenetic relationships among the distinct isolates of *T. verai*. Because no sequences of the *cox*1 gene from other cryptogonimids have been deposited in GenBank, no outgroups were used, and trees were midpoint rooted. Two species of *Oligogonotylus* (*O. manteri* and

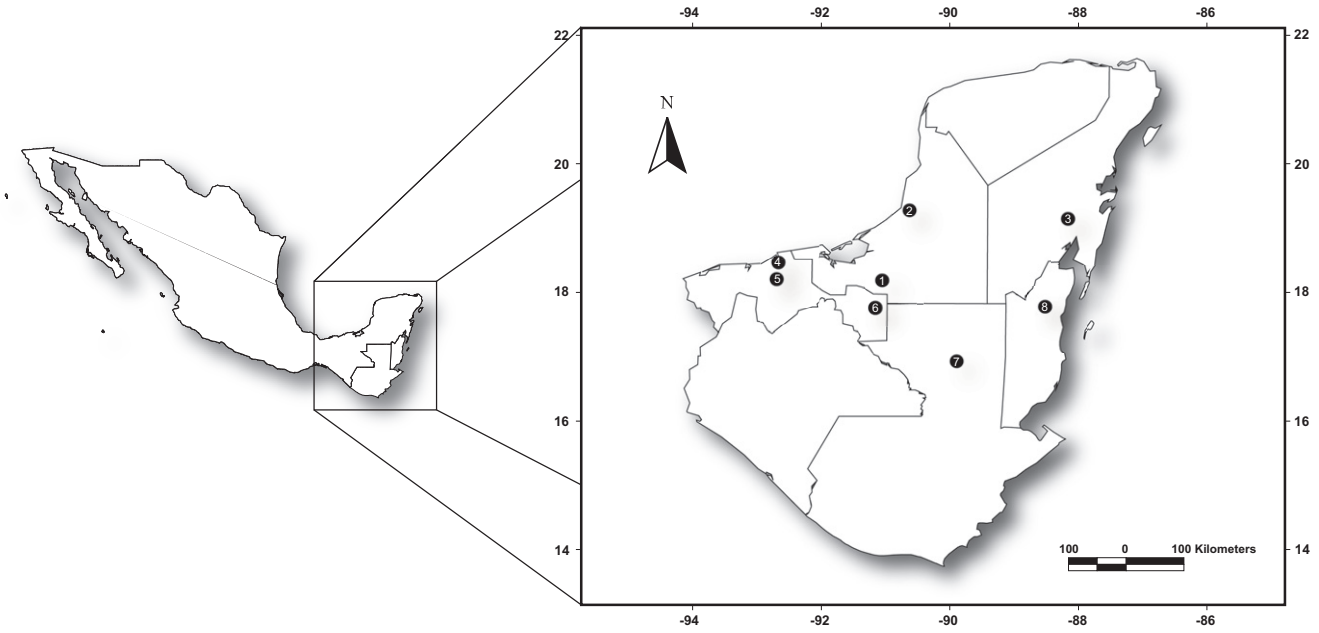

Fig. 1. Sampling localities for *Tabascotrema verai* collected from the intestine of *Petenia splendida* in Mexico, Belize and Guatemala. Numbers correspond to the following localities: 1 = Río Candelaria, Campeche; 2 = Río Champotón, Campeche; 3 = Laguna Noh Bek, Quintana Roo; 4 = Pantanos de Centla, Tabasco; 5 = Chilapa, Tabasco; 6 = San Pedro Balancán, Tabasco; 7 = Lago Petén Itzá, Guatemala; 8 = Crooked Tree Lagoon, Belize; see Table 1 for further details.