Parasitology International 66 (2017) 126–133

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Morphological and molecular characterization of the metacercaria of *Paragonimus caliensis*, as a separate species from *P. mexicanus* in Costa Rica

Roderico Hernández-Chea [a,*], Ana Eugenia Jiménez-Rocha [b], Ruth Castro [a], David Blair [c], Gaby Dolz [a,b]

[a] Maestría en Enfermedades Tropicales, Posgrado Regional en Ciencias Veterinarias Tropicales, Universidad Nacional, Campus Benjamín Nuñez, Barreal de Heredia, P.O. Box 86, 3000 Heredia, Costa Rica
[b] Escuela de Medicina Veterinaria, Universidad Nacional, Campus Benjamín Núñez, Barreal de Heredia, Costa Rica
[c] College of Marine and Environmental Sciences, James Cook University, Townsville, QLD, 4811, Australia

ARTICLE INFO

Article history:
Received 13 September 2016
Received in revised form 6 December 2016
Accepted 7 December 2016
Available online 24 December 2016

Keywords:
Paragonimus mexicanus
Paragonimus caliensis
Molecular characterization
Food-borne trematodiasis
Costa Rica

ABSTRACT

The trematode *Paragonimus mexicanus* is the etiological agent of paragonimiasis, a food-borne zoonotic disease in Latin America. This species, as well as *Paragonimus caliensis*, have been reported from Costa Rica, but it is not known if the two are synonymous. Two types of *Paragonimus* metacercariae from freshwater pseudothelphusid crabs from several localities in Costa Rica were recognized by light microscopy. Morphologically, these corresponded to descriptions of *P. mexicanus* and *P. caliensis*. Metacercariae of the former species lacked a membrane or cyst and their bodies were yellow in color. Those of *P. caliensis* were contained in a transparent thin cyst and were pink in color. Morphotypes of metacercariae were determined using scanning electron microscopy (SEM). Based on the number and distribution of papillae in the ventral sucker, three morphotypes were found for *P. mexicanus* and two for *P. caliensis*. Analysis of DNA sequences (nuclear ribosomal 28S and ITS2 genes, and partial mitochondrial *cox*1 gene) confirmed the presence of *P. mexicanus* and provided the first molecular data for *P. caliensis*. The two species are phylogenetically distinct from each other and distant from the Asian species. The confirmation of *P. caliensis* as a separate species from *P. mexicanus* raises several questions about the ecology, biological diversity, and epidemiology of the genus *Paragonimus* in Costa Rica.

1. Introduction

Paragonimiasis is a food-borne zoonotic disease, which is listed among the most important trematodiases in Asia, Africa and Latin America. It is caused by several species of the genus *Paragonimus*. Around 50 species have been described since this genus was erected by Braun in 1899 [1]. Of these, about ten are pathogenic for human beings. The best-known human pathogen is *Paragonimus westermani*, widely distributed in eastern and southern Asia, but others occur in Asia (*P. skrjabini* and *P. heterotremus*), Africa (*P. africanus* and *P. uterobilateralis*) and in the Americas (*P. kellicotti* and *P. mexicanus*) [2]. Eight species of *Paragonimus* have been named from the Americas [3]. The earliest of these was *P. rudis* (Diesing, 1850), adult worms of which were found in the lungs of the Brazilian otter, *Ptenourura brasiliensis*, on a single occasion in 1828 [4]. In recent years, *P. rudis* has been considered a *nomen nudum*, because the original description was inadequate and the type specimens are poorly preserved and do not allow a clear morphological identification [4,7]. The first report of a lung fluke from Costa Rica was by Caballero [5], who described adult worms he regarded as representing *P. rudis* in a gray fox, *Urocyon cinereoargenteus*. Brenes et al. [6] later described a non-encysted metacercaria of a "*Paragonimus* sp". The first report of *P. mexicanus* in Costa Rica was in 1968 [7]. It is most likely that the worms Caballero described as *P. rudis* from Costa Rica and Guatemala in fact belonged to *P. mexicanus* [6]. Rojas et al. [8] reported a morphologically different type of metacercaria that matched with the description of the metacercaria of *P. caliensis*, first discovered in Colombia by Little [9]. *Paragonimus caliensis* is often considered a morphological variant of *P. mexicanus* [10,11].

Given the relative paucity of morphological characters available for distinguishing among *Paragonimus* species, scanning electron microscopy has often been used to reveal details of subtle morphological features, such as tegumentary papillae, that might assist in taxonomy [12]. This approach has been used only rarely for metacercariae of *Paragonimus* species in Latin America. Hernandez and Monge [13] reported two different morphotypes of metacercariae in an ultrastructural study of the papillae on the ventral sucker. These morphotypes were indistinguishable using light microscopy. Given this background, it is important to discern whether or not different morphotypes of

* Corresponding author.
E-mail address: roderik123animal@gmail.com (R. Hernández-Chea).

http://dx.doi.org/10.1016/j.parint.2016.12.006

metacercariae represent different species, a task well suited to molecular studies.

To date, molecular studies have identified four complexes of *Paragonimus* species in Asia [1]. Among these are the *P. westermani* complex, including *P. westermani* and *P. siamensis* and the *P. ohirai* complex, including a number of nominal species such as *P. ohirai* and *P. harinasutai* [14]. It is not known whether *P. mexicanus* constitutes a complex of species in Latin America, or if it is the only species as suggested by Tongu [10].

The first reported human cases of paragonimiasis in Costa Rica were in two young girls with pleural effusion [15]. Since then 28 cases have been described, primarily in children from rural areas. The most recent case occurred in an eight-year-old boy with cerebral paragonimiasis in 2013, who fortunately recovered after neurosurgery [Adriana Yock, Hospital Nacional de Niños, personal communication, July 5, 2014]. Despite the long history and morphological descriptions of lung flukes in Costa Rica, there is no molecular evidence of the presence of this trematode in the country.

The objective of this study was to determine the species of *Paragonimus* present in freshwater crabs in Costa Rica through morphological, molecular, and phylogenetic analyses.

2. Materials and methods

2.1. Crab collection and dissection

Three hundred and forty-seven pseudothelphusid crabs were collected between March and November of 2015, in streams of the Atlantic and Pacific slopes of Costa Rica, in the provinces of Limón (Veragua: 9.9258 N, −83.1909 W, Guácimo: 10.1252 N, −83.64838 W, Talamanca: 9.5586 N, −82.8555 W), Heredia (Sarapiquí: 10.2442 N, −83.9693 W), Alajuela (Upala: 10.7262 N, −85.0562 W), and Puntarenas (Buenos Aires: 9.2821 N, −83.37071 W, Coto Brus 8.8229 N, −83.0193 W; Parrita 9.6048 N, −84.2323 W). Crabs were collected by lifting rocks and organic material on the edges of the streams. The cephalothorax of each crab was separated from the body, leaving soft tissues exposed. The organs (false lung, gills, heart and hepatopancreas) and muscle tissue were placed individually on Petri plates with 0.9% saline solution, while the chelae and limbs were crushed and then placed on Petri plates with 0.9% saline solution. Metacercariae were isolated from four genera of crabs: *Ptychophallus* spp., *Achlidon* spp., *Potamocarcinus* spp., and *Allacanthos* spp. Genera of crabs were identified by the morphological appearance of the gonopods by Dr. Ingo S. Wehrtmann and Fresia Villalobos, Centro de Investigación en Ciencias del Mar y Limnología (CIMAR), Universidad de Costa Rica.

2.2. Morphological characterization of metacercariae

All the Petri plates with the organs and tissues were directly examined using a stereoscope. Visible metacercariae were collected with a dropper and stored in tubes with 0.9% saline solution until processed. Some were fixed with 70% ethanol for observation using a light microscope (Nikon Eclipse E200, objectives 10× and 40×). Various measurements were taken; length and width of the body, length and width of the oral sucker, and length and width of the ventral sucker. For scanning electron microscopy, metacercariae were fixed in 80% glutaraldehyde, 10% paraformaldehyde, and 10% phosphate buffer, pH 7.2 for 2 min, then left to dry at room temperature for 5 min. The scanning electron microscope used was a Hitachi TM 3000. The ventral sucker was located, and the metacercariae morphotype was determined based on the number and distribution of papillae on this structure.

2.3. DNA extraction, PCR amplification, and sequencing

Total DNA was extracted from each metacercaria using the Dneasy® Blood & Tissue kit (QIAGEN 2006), following the manufacturer's recommendations. A portion of the nuclear 28S ribosomal DNA (rDNA) gene was amplified using the primers TSD2 (5′ GTACCGTGAGGGAAAGTTG-3′) and D4AR (5′-GTCCGTGTTTCAAGAC-GGG-3′) [16]. The nuclear ribosomal ITS2 region was amplified using the primers 3S (5′-CGG TGG ATC ACT CGG CTC GT-3′) and A28 (5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′) [17], and a portion of the mitochondrial *cox*1 gene was amplified using primers FH5 (5-TTT TTT GGG CAT CCT GAG GTT TA-3′) and FH3 (5-TAA AGA AAG AAC ATA ATG AAA ATG-3′) [17]. Each PCR reaction contained 12.5 μl of Dream Taq™ PCR Master Mix (Fermentas®), 2.25 μl (10 pmol/μl) of each primer, 3 μl of genomic DNA and 5 μl of nuclease-free water, for a final volume of 25 μl. The DNA of an adult *Paragonimus heterotremus* was used as a positive control (kindly donated by Dr. Paron Dekumyoy, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand). A reaction lacking only genomic DNA was used as a negative control. For the 28S rDNA gene, cycling conditions were an initial denaturation at 94 °C for 5 min, then 30 cycles (94 °C for 30 s, annealing of the primers at t 50 °C for 30 s, extension at 72 °C for 45 s), and a final extension at 72 °C for 5 min. For the ITS2 and *cox*1 regions, amplification conditions consisted of 35 cycles of denaturation at 94 °C for 1 min, annealing of the primers at 50 °C (*cox*1) or 52 °C (ITS2) for 1 min, extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis in 1% agarose gel, in TBE 1× buffer (Tris base, boric acid, EDTA, pH 8, 0.5 M), and stained with GelRed™ Nucleic Acid Gel Strain. The GeneRuler 100 pb DNA Ladder Plus (Fermentas®, Ontario, Canada) was used as a molecular weight marker. The expected products of 560, 285 and 335 bp for 28S rDNA, ITS2 and *cox*1, respectively, were sent to Macrogen Inc. (Seoul, Korea) for sequencing.

2.4. Sequence analyses and construction of phylogenetic trees

The similarity of each sequence to those available in the data base (GenBank) of the U.S. National Center for Biotechnology Information was assessed using the BLASTn algorithm [18]. Sequence editing and alignment were performed using BioEdit 7.2 sequence editor program [19] and ClustalW [20] multiple alignment, respectively. The best nucleotide substitution model was estimated independently for each dataset using jModelTest 2.1.3 [21]. The best-fitting model for the 28S rDNA sequence alignment was the General Time Reversible (GTR + G), and for the ITS2 and *cox*1 datasets it was the Hasegawa-Kishino-Yano plus Gamma (HKY + G) model. Phylogenetic reconstruction was performed using maximum likelihood (ML) analysis with MEGA 6 [22], and Bayesian inference (BI) was conducted using MrBayes [23,24]. In MrBayes, two parallel runs, each of four chains, were run for 1000,000 generations and sampled every 1000 generations, the first 25% of the sampled trees were discarded as "burn-in" for each dataset. Convergence of runs was considered complete when the average standard deviation of split frequencies was <0.01. The consensus trees were drawn and labeled using the program Figtree v.1.4 [25]. Percentage similarities between sequences were calculated using the sequence identity matrix in BioEdit. A pairwise distance matrix of the 28S rDNA sequences was calculated with MEGA 6 [22]: the analysis involved 25 nucleotide sequences and all ambiguous positions were removed for each sequence pair.

3. Results

3.1. Morphology of metacercariae by light microscopy

Five hundred and ninety-nine metacercariae were collected from a total of 347 crabs examined. Two types of metacercariae of the genus *Paragonimus* could be distinguished using light microscopy (Fig. 1A). The first type of metacercaria (588 specimens found) was observed freely moving in the crab tissues (without membrane or cyst) and was yellow in body color but contained reddish granules, and yellow caeca (Fig. 1B). The morphometric details of a total of 20 metacercariae of

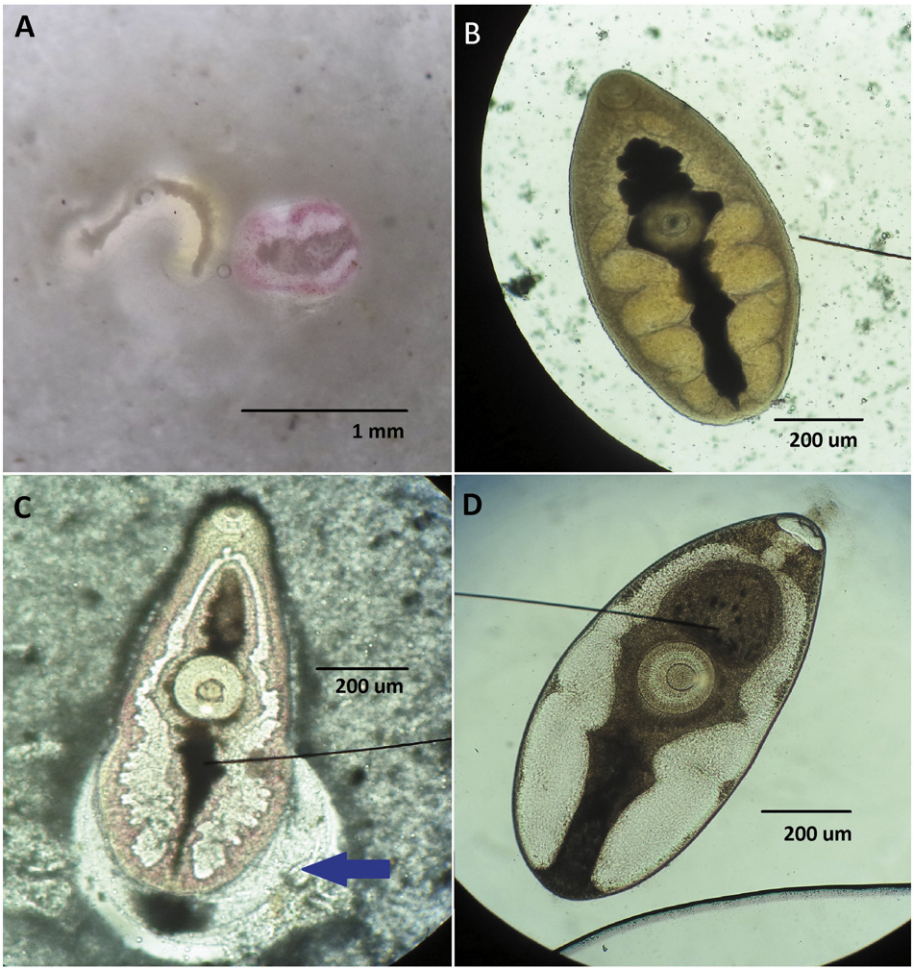

Fig. 1. Morphological characteristics of *P. mexicanus* and *P. caliensis* metacercariae in Costa Rica. (A) *P. mexicanus* metacercaria (yellow), left, and *P. caliensis* metacercaria (pink) right, seen through a stereoscope. (B) *P. mexicanus* metacercaria, light microscopy, objective 10×. (C) *P. caliensis* metacercaria emerging from the cyst; the arrow indicates the cyst. (D) *P. caliensis* metacercaria, light microscopy, objective 10×.

the first type are provided in Table 1. The second type of metacercaria (11 specimens found) possessed a more elongated body than did those of the first type, and was enclosed by a single cyst wall, very thin and flexible, which made the metacercaria difficult to manipulate. The body was pink with reddish granules. Once free of the cyst, these metacercariae were observed to have transparent caeca and were actively moving (Fig. 1C and D). Average measurements of a total of four metacercariae of the second type are specified in Table 1. The first type of metacercaria matched with the description of *P. mexicanus* [7]. The second type matched with the description of *P. caliensis* [9].

3.2. Morphology of metacercariae of P. mexicanus and P. caliensis by SEM

Based on the number and distribution of papillae on the ventral sucker, three morphotypes of *P. mexicanus* metacercariae were recognized using SEM: morphotype 1, with 19 to 30 outer papillae (Fig. 2A); morphotype 2, with 6 larger inner papillae on the ventral sucker (Fig. 2B), and morphotype 3, with 6 inner papillae, and 36–38 outer papillae (Fig. 2C). Two morphotypes were noted for *P. caliensis*: morphotype 1 with 6 inner papillae and 7 papillae on the inner rim of the ventral sucker (Fig. 2D), and morphotype 2, with 6 inner papillae (Fig. 2E). Tegumentary spines were observed covering the whole body of both types of metacercaria (Fig. 3, Table 2).

Table 1
Morphometric measurements average (AVG) and standard deviation (STD) of *P. mexicanus* and *P. caliensis* metacercariae.

Measurements (μm)	*P. mexicanus* (n = 20)		*P. caliensis* (n = 4)	
	AVG	STD	AVG	STD
Body length/width	1202/631	493.1–48.3	1300/683	173.2–125.8
Oral sucker length/width	105/112	8.8–12	154/158	6.92–6.92
Ventral sucker length/width	222/215	22.8–13.3	263/263	32.14–32.14

3.3. Molecular phylogenetic analyses

Sequences were obtained from 17 metacercariae (11 *P. mexicanus* and 6 *P. caliensis*) (Table 3). Available sequences of 28S rDNA, ITS2, and *cox*1 of *P. mexicanus*, *P. kellicotti*, and some Asian *Paragonimus* species were downloaded from GenBank and included in the analyses for comparative purposes (Table 4). The 28S rDNA sequence alignment was 561 bp in length. All but one 28S rDNA sequences of *P. mexicanus* were identical, including *P. mexicanus* from Ecuador (GenBank HM172619). The only exception was a sequence from Veragua morphotype 1 (GenBank KX289337), that differed at 4 (4/561, 0.71%) sites relative to the others. All sequences from *P. caliensis* were completely identical (100%): no differences per locality or morphotype were observed. Identity between *P. mexicanus* and *P. caliensis* was 96% (19/561 sites differed between the species; 3.38%). Table 5 provides pairwise differences among 28S rDNA nucleotide sequences. Many of the sites (16) at which *P. mexicanus* and *P. caliensis* differed were also variable among Asian species. However, the overall number of differences among Asian species was greater than among American species (Table 5). The single sequence of *P. kellicotti* differed from *P. mexicanus*

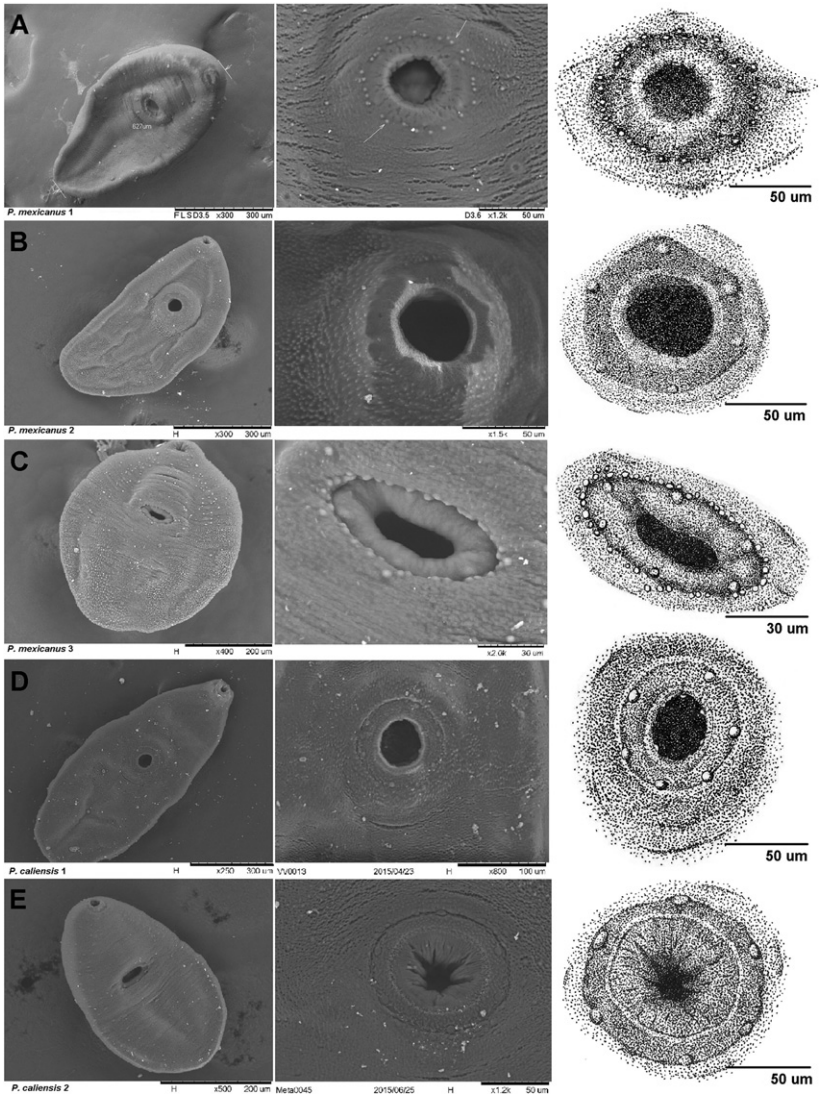

Fig. 2. Morphological characteristics of morphotypes of *P. mexicanus* and *P. caliensis* metacercariae seen using SEM (left); characteristics of the ventral sucker papillae as seen through SEM (center), and drawings (right). (A) Morphotype 1 *P. mexicanus*; (B) Morphotype 2 *P. mexicanus*; (C) Morphotype 3 *P. mexicanus*; (D) Morphotype 1 *P. caliensis*; (E) Morphotype 2 *P. caliensis*.

at 16 sites, and from *P. caliensis* also at 16 sites; seven of these sites were common to both.

In the ITS2 sequence alignment (285 bp); sequences of *P. mexicanus* from Costa Rica were near-identical (99–100%). Sequences of *P. mexicanus* from Mexico, Ecuador and Guatemala (GenBank KC562256, KC562269, KC562271, AF538945 and AF538946) were almost identical to those from Costa Rica (98–100%). In the case of *P. caliensis*, ITS2 sequences shared 99–100% identity. Differences between ITS2 sequences of these two species were observed at 37 (37/285; 13%) sites. Sequences

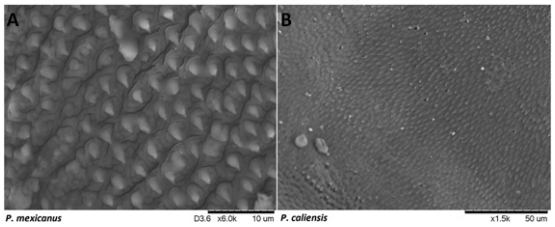

Fig. 3. Morphological characteristics of spines in the teguments of metacercariae. *Paragonimus mexicanus* (A) and *P. caliensis* (B) seen using SEM; spines of *P. mexicanus* were small with a pointed tip. Spines of *P. caliensis* were single, thinner and flatter than those of *P. mexicanus*.

of *P. mexicanus* for the *cox*1 gene showed an identity of 98%–100%. *Paragonimus caliensis* sequences were almost identical (99–100%). Differences between *cox*1 sequences of *P. mexicanus* and *P. caliensis* were noted at 52 (52/335; 15.5%) sites.

Of the nuclear gene regions (28S and ITS2), only ITS2 provided unequivocal support for a distinct American clade of *Paragonimus* species (Fig. 4, Fig. 5). The least conserved of the three regions used, mitochondrial *cox*1, demonstrated considerable variation among *P. mexicanus* individuals, but little variation in *P. caliensis* (Fig. 6). A monophyletic clade of American species was not inferred from the *cox*1 data. In all phylogenetic trees, *P. mexicanus* and *P. caliensis* were clearly very distinct from one another.

4. Discussion

The morphologies of metacercariae we found in Costa Rica are consistent (on the basis of light microscopy) with those reported by Little [9] for *P. caliensis*, and by Miyazaki and Ishii [7] and Brenes et al. [6] for *P. mexicanus*.

Based on the arrangement of the papillae around the ventral sucker, three morphotypes of *P. mexicanus* metacercariae and 2 morphotypes of *P. caliensis* metacercariae were characterized using SEM. Morphotype 3

Table 2
Morphological features of metacercariae through SEM by number of papillae in the acetabulum and host collection site.

Province/locality	Species	Morphotype	No. of sensory papillae present in the acetabulum or ventral sucker			Spines
			Outer papillae	Inner papillae	Papillae on the inner rim	
Limón-Veragua	*P. mexicanus*	1	19–30	0	0	Single-tip
Puntarenas-Buenos Aires	*P. mexicanus*	2	0	6	0	Single-tip
Limón-Talamanca Limón-Guácimo	*P. mexicanus*	3	36–38	6	0	Single-tip
Limón-Veragua	*P. caliensis*	1	0	6	7	Single-flatter
Alajuela-Upala Puntarenas-Parrita	*P. caliensis*	2	0	6	0	Single-flatter

of *P. mexicanus*, possessing 6 inner papillae and 28–38 outer papillae, was exactly the same as that reported by Tongu et al. [26] and Hernandez and Monge [13] from Costa Rica. Morphotype 2 of *P. mexicanus*, possessing the 6 inner papillae but lacking the outer ring, is of a type not reported previously. Morphotype 1 of *P. mexicanus* exhibited an outer ring of 19–30 papillae but lacked the inner ring of 6 papillae and was the most frequently seen (in 23 specimens from Veragua). This contradicts the common opinion that an inner ring of 6 papillae is always present in Latin American *Paragonimus* species [26,27,28], and indeed in Asian species [28].

The variation we noted in two American *Paragonimus* species indicates that, contrary to Aji et al. [27], arrangement of papillae on the ventral sucker is of little or no use in taxonomy of the genus. Similarly, Komalamisra et al. [12] summarized different studies noting variation in numbers of papillae in five species of *Paragonimus* present in Thailand (*P. westermani, P. bangkokensis, P. harinasutai, P. heterotremus* and *P. siamensis*). In addition to variation within each of the American species, near-identical morphotypes were shared by *P. mexicanus* and *P. caliensis* (Fig. 2).

Tegumentary spines of *P. mexicanus* found in all morphotypes were identical to those reported by Hernandez and Monge [13] on *P. mexicanus* metacercariae from Atenas and Tabarcia de Mora, Costa Rica. Spines found on the metacercariae of *P. caliensis* matched with the holotype (his Fig. 22) reported by Little [9] of *P. caliensis* from Colombia. Ours is the first ultrastructural study by SEM of *P. caliensis* metacercariae.

The molecular and phylogenetic analysis confirmed that *P. mexicanus* and *P. caliensis* are two separate species. The nucleotide sequences of morphotypes 1, 2 and 3 of *P. mexicanus* for all gene regions were 99% identical and different morphotypes intermingled in phylogenetic trees, which indicate that they belong to the same species. Likewise, the sequences of morphotypes 1 and 2 of *P. caliensis* were 100% identical for the 3 regions analyzed. Interestingly, some geographical structure is implied by the partial *cox*1 sequences of *P. mexicanus* of the three morphotypes (1-Veragua, 2-Puntarenas and 3-Talamanca/Guácimo). The potential of *cox*1 sequences to separate morphotypes of *P. mexicanus* should be explored in future studies.

The taxonomy of the genus *Paragonimus* in Latin America has been controversial, due to the morphological variation observed in specimens from Central and South America in both metacercariae and adult worms [10]. Nevertheless, the results of the morphology of light microscopy in this study agree with the phylogenetic analysis in regard to *P. mexicanus* and *P. caliensis*. The trees inferred from 28S rDNA, ITS2 and *cox*1 genes show additional evidence of the existence of three different species in the Americas.

The proposed evolutionary history of the genus *Paragonimus* [29] assumes a monophyletic clade of American species. According to this view, it is probable that *Paragonimus* originated and diversified approximately 200 million years ago in Gondwana during the Mesozoic, before the separation of India, Africa and South America [29], followed by evolution *in situ* in Asia, Africa and the Americas. This hypothesis would explain the diversity of gastropod intermediate hosts and of *Paragonimus* species in these continents. Our analysis has not unequivocally supported monophyly of species from the Americas: only the ITS2 tree demonstrated this. Relevant nodes in the 28S tree were separated by very short internodes and received low levels of support from the ML analysis, suggesting poor resolution. The basal position of the *P. westermani* group in the genus was well supported, in agreement with other studies, and suggesting an Asian origin [30]. It is possible that the history of the genus is more complicated than previously thought, requiring much further investigation.

We found many more metacercariae of *P. mexicanus* (588) than of *P. caliensis* (11). It might be that *P. mexicanus* infects a greater range of mammal species than does *P. caliensis*. Adults of *P. mexicanus* have

Table 3
Taxa, morphotype of metacercaria by species, geographic origin, and GenBank accession numbers of DNA sequences used in this analysis.

Species	Morphotype	Province/locality	28S rDNA	ITS2 rDNA	*cox*1	Source
P. mexicanus	1(19-30 Op[a])	Limón/Veragua	KX289332	KX379696	KX344899	Present study
P. mexicanus	1(19-30 Op[a])	Limón/Veragua	KX289333	KX379697	KX344900	Present study
P. mexicanus	1(19-30 Op[a])	Limón/Veragua	KX289334	KX379698	KX344901	Present study
P. mexicanus	1(19-30 Op[a])	Limón/Veragua	KX289335	KX379699	KX344902	Present study
P. mexicanus	1(19-30 Op[a])	Limón/Veragua	KX289336	KX379700	KX344903	Present study
P. mexicanus	1(19-30 Op[a])	Limón/Veragua	KX289337	KX379701	KX344904	Present study
P. mexicanus	2 (6 Ip[b])	Puntarenas/Buenos Aires	KX289345	KX379709	KX379692	Present study
P. mexicanus	2 (6 Ip[b])	Puntarenas/Buenos Aires	KX289346	KX379710	KX379693	Present study
P. mexicanus	2 (6 Ip[b])	Puntarenas/Buenos Aires	KX289347	KX379711	KX379694	Present study
P. mexicanus	3 (36 Op[a]-6 Ip[b])	Limón/Guácimo	KX289343	KX379707	KX379690	Present study
P. mexicanus	3 (36 Op[a]-6 Ip[b])	Limón/Talamanca	KX289344	KX379708	KX379691	Present study
P. caliensis	1 (6 Ip[b]-7 Pir[c])	Limón/Veragua	KX289338	KX379702	KX344905	Present study
P. caliensis	2(6 Ip[b])	Alajuela/Upala	KX289339	KX379703	KX354390	Present study
P. caliensis	2(6 Ip[b])	Alajuela/Upala	KX289340	KX379704	KX354391	Present study
P. caliensis	2(6 Ip[b])	Alajuela/Upala	KX289341	KX379705	KX354392	Present study
P. caliensis	2(6 Ip[b])	Alajuela/Upala	KX289342	KX379706	KX379689	Present study
P. caliensis	2(6 Ip[b])	Puntarenas/Parrita	KX289348	KX379712	KX379695	Present study

[a] Outer papillae.
[b] Inner papillae
[c] Papillae on the inner rim.

Table 4
Taxa, geographic origins, and GenBank accession numbers of previously published DNA sequences used in this analysis.

Species	Country	28S rDNA	Source
P. mexicanus	Ecuador/La Concordia	HM172619	Devi et al. (2010)
P. kellicotti	United States/Missouri	HQ900670	Fischer et al. (2011)
P. siamensis	Sri Lanka	HM172624	Devi et al. (2010)
P. heterotremus	India/Arunachal	HM172615	Devi et al. (2010)
P. harinasutai	Thailand/Nakorn Nayok	HM172616	Devi et al. (2010)
P. ohirai	Japan/Kinosaki	HM172621	Devi et al. (2010)
P. westermani	China/Beiguho	HM172628	Devi et al. (2010)
P. miyazakii	Japan/Kochi	HM172620	Devi et al. (2010)
Skrjabinophyetus neomidis	–	AF184252	Tkach et al. (2001)
Nephrotrema truncatum	–	AF151936	Tkach et al. (2000)
Nanophyetus salminicola	–	AY116873	Olson et al. (2002)
Species	Country	ITS2 rDNA	Source
P. mexicanus	Mexico/Colima	KC562256	López-Caballero et al. (2013)
P. mexicanus	Mexico/Colima	KC562271	López-Caballero et al. (2013)
P. mexicanus	Mexico/Colima	KC562269	López-Caballero et al. (2013)
P. mexicanus	Ecuador	AF538945	Iwagami et al. (2002)
P. mexicanus	Guatemala	AF538946	Iwagami et al. (2002)
P. kellicotti	United States/Missouri	HQ900670	Fischer et al. (2011)
P. ohirai	Japan	U96911	Blair et al. (1997)
P. harinasutai	Thailand/Nakorn Nayok	AF159609	Blair et al. (1999)
P. siamensis	Sri Lanka	AY240943	Iwagami et al. (2003)
P. westermani	India: Meghalaya	DQ836243	Narain et al. (2006)
P. miyazakii	Japan	AY618757	Blair et al. (2005)
Species	Country	*cox*1	Source
P. mexicanus	Mexico/Chiapas	KC562285	Blair et al. (1999)
P. mexicanus	Ecuador	AF538937	Iwagami et al. (2002)
P. mexicanus	Guatemala	AF538943	Iwagami et al. (2002)
P. kellicotti	United States/Missouri	HQ900671	Fischer et al. (2011)
P. ohirai	Japan	U97214	Blair et al. (1997)
P. harinasutai	Thailand/Nakorn Nayok	AF159600	Blair et al. (1999)
P. siamensis	Sri Lanka	AY240941	Iwagami et al. (2003)
P. westermani	India/Meghalaya	HM172633	Blair et al. (2005)
P. heterotremus	Vietnam/Yenbai	AB270675	Doanh et al. (2007)
P. miyazakii	Japan	AY618809	Blair et al. (2005)

been reported in opossums (*Didelphis marsupialis*, *Philander opossum*), procyonids and mephitids, whereas *P. caliensis* has only been reported in raccoons (*Procyon lotor*) and water opossums (*Chironectes minimus*) [8]. Nevertheless, the ecological niche of both species seems to be very similar. We found a second intermediate host in common for the two species of lung fluke: a single individual male crab of the genus *Ptychophallus* from Veragua was infected with one metacercaria of *P. caliensis* and several of *P. mexicanus*. This finding showed that co-infection of second intermediate hosts can occur, as noted in other species of freshwater crabs from Asia, infected with *P. westermani* and *P. heterotremus* [31]. Further studies are necessary to unveil which first and second intermediate hosts harbor the two species of *Paragonimus* in Central and South America.

Table 5
Estimates of evolutionary divergence over 28S rDNA sequence pairs between groups of *Paragonimus* species.

Species/group	1	2	3	4	5	6	7
1. *P. mexicanus* (12)	0.7						
2. *P. kellicotti* (1)	16.0	N/A					
3. *P. caliensis* (6)	19.0	17.0	0.0				
4. *P. ohirai* Gp (2)	27.9	25.0	29.5	18.0			
5. *P. westermani* Gp (2)	40.9	41.0	44.5	45.8	27.0		
6. *P. miyazakii* (1)	19.3	19.0	24.0	28.0	44.0	N/A	
7. *P. heterotremus* (1)	13.2	11.0	18.0	22.0	38.0	10.0	N/A

The number of sequences within each group is shown in parentheses following the name of the group. On the diagonal are average numbers of differences over all sequence pairs within each group. N/A indicates that the calculation was not applicable (only one sequence in the group). The *P. ohirai* group (Gp) contains *P. ohirai* and *P. harinasutai*. The *P. westermani* group contains *P. westermani* and *P. siamensis*.

Our new molecular data prove that *P. mexicanus* and *P. caliensis* are distinct species, and not synonymous as proposed by Tongu [10] and Veléz et al. [11]. Tongu [10], in particular, proposed that *P. mexicanus* was the only valid *Paragonimus* species in Latin America, citing apparently trivial differences in body shape and dimensions, and in branching patterns of gonads in adults, as well as the impossibility of counting numbers of flame cells in metacercariae (which might otherwise provide species-specific diagnostic features). Veléz et al. [11] added to this the possibility that presence (as in *P. caliensis*) or absence (as in *P. mexicanus*) of a metacercarial cyst wall might be an intra-specific polymorphism. These and other difficulties in finding consistent taxonomic characters for the genus *Paragonimus* have been noted by Blair [1], who emphasized the value of a molecular approach. Given the relatively small number of metacercariae that were studied further investigations may determine more morphotypes and new molecular data.

In this study in Costa Rica, we have demonstrated the importance of supplementing morphological analyses, including light microscopy and SEM, with molecular analyses.

5. Conclusion

This study determined the presence of two species of lung flukes in Costa Rica through molecular analyses of the 28S rDNA, ITS2 and *cox*1 regions. One of these species was certainly *P. mexicanus*, for which previous molecular data were available. The other we regard as *P. caliensis* on the basis of metacercarial/cyst morphology. No previous molecular data were available for this species. Both species have morphological variation in ventral sucker papillae, as seen using SEM. *Paragonimus caliensis* is the third species of lung fluke from the Americas for which molecular data are available. The molecular confirmation of a second *Paragonimus* species, *P. caliensis*, in Costa Rica raises several questions about its epidemiology, ecology and biology. Is *P. caliensis* a pathogen of humans? Does this species use the same first, second and final hosts as *P. mexicanus*? If it is a pathogen for humans, would disease caused be similar to that caused by *P. mexicanus*?

Limitations of the study

Sample sites were different in their environmental characteristics. We observed contrasts and variation in the number of crabs collected and rates of infection with metacercariae of the genus *Paragonimus*. After all it was very difficult to find enough specimens of *P. caliensis*, and in some cases of *P. mexicanus*, nevertheless, for each locality we were able to characterize at least one metacercaria for each purpose (light microscopy, SEM and molecular analysis).

Acknowledgements

This work was carried out as part of the research project "*Parasitismo letal a través del consumo de cangrejos y camarones de agua dulce: identificación de zonas de riesgo y desarrollo de un plan de prevención de la paragonimiasis en Costa Rica*" [*Lethal parasitism through consumption of freshwater crabs and shrimps: identification of risk zones and development of a paragonimiasis prevention plan in Costa Rica*]. We are really grateful to Consejo Nacional de Rectores (CONARE) and Vicerrectoría de Investigación, Universidad Nacional (0390-14), for providing funds and financing for this research (FEES Funds), and to the Deutscher

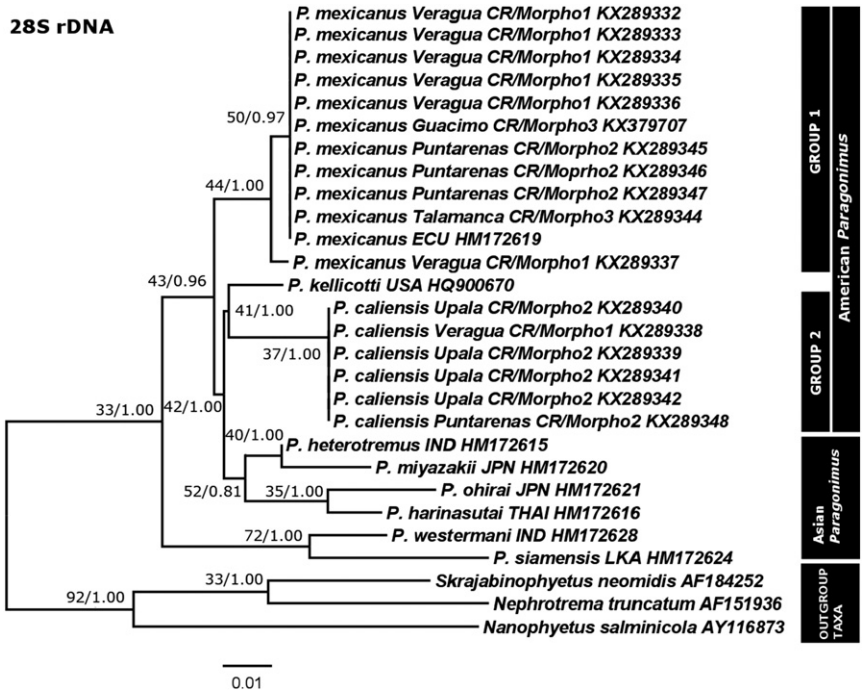

Fig. 4. Bayesian phylogenetic tree reconstructed from 28S rDNA sequence alignment. Sequences were analyzed utilizing maximum likelihood (ML) and Bayesian inference (BI), using *Skrajabinophyetus noemidis, Nephrotrema truncatum* and *Nanophyetus salminicola* as outgroup taxa. The topology of this BI tree is the same as that obtained from ML analysis. Numbers assigned to each node are bootstrap support values in the ML analysis, followed by the BI posterior support values. (CR = Costa Rica, MEX = Mexico, ECU = Ecuador, USA = United States of America, THAI = Thailand, JPN = Japan, LKA = Sri Lanka and IND = India.

Akademischer Austauschdienst (DAAD) for granting a scholarship to Roderico Hernández Chea. We thank Dr. Ingo S. Wehrtmann, Fresia Villalobos and Raquel Romero of the CIMAR, Universidad de Costa Rica, for their technical support, and collection of samples and data. Thanks also to the technicians and office personnel from Veragua Rainforest Research and Adventure Park, Limón, Costa Rica. We also want to thank Jorge Picado, Instituto Costarricense de Electricidad, Puntarenas for his technical support and helping collect samples. Our special thanks to Dr. Paron Dekumyoy, Mahidol University, Bangkok, Thailand for donating positive controls for the PCR.

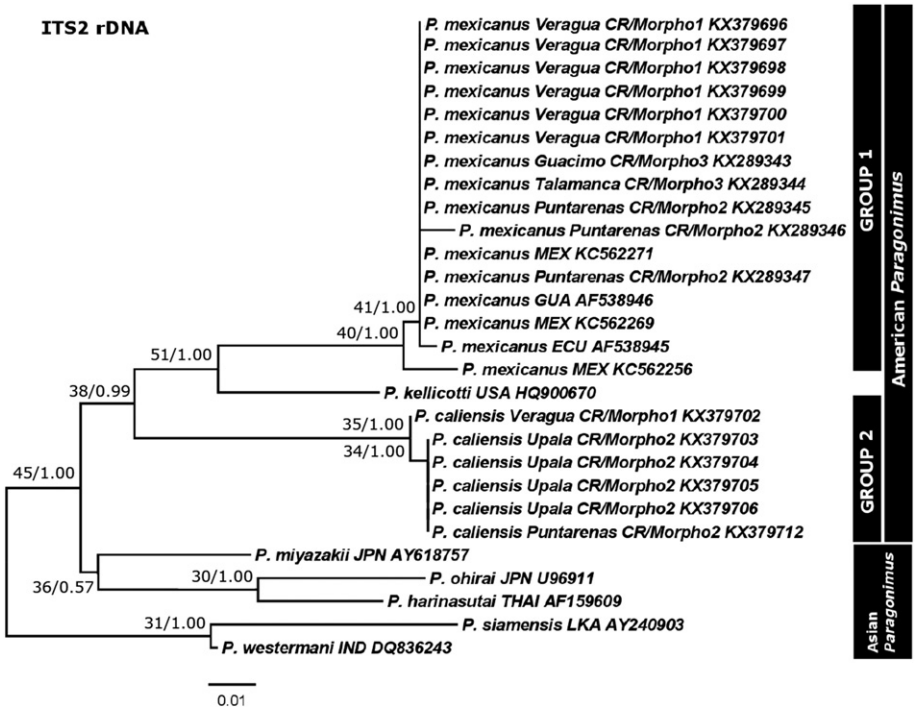

Fig. 5. Bayesian phylogenetic tree reconstructed from ITS2 sequence alignment. Sequences were analyzed utilizing maximum likelihood (ML) and Bayesian inference (BI). The topology of this BI tree is the same as that obtained from ML analysis. Numbers assigned to each node are bootstrap support values in the ML analysis, followed by the BI posterior support values. (CR = Costa Rica, MEX = Mexico, ECU = Ecuador, GUA = Guatemala, USA = United States of America, THAI = Thailand, JPN = Japan, LKA = Sri Lanka and IND = India). The tree is midpoint-rooted.

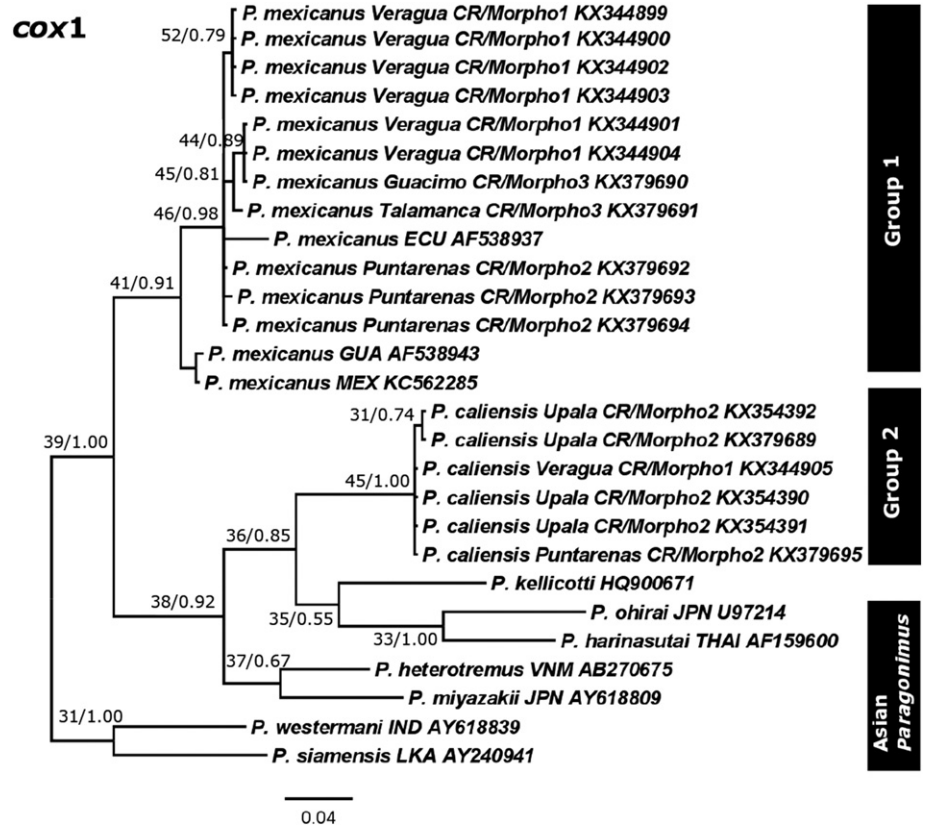

Fig. 6. Bayesian phylogenetic tree reconstructed from the *cox*1 sequence alignment. Sequences were analyzed utilizing Maximum Likelihood (ML) and Bayesian inference (BI). The topology of this BI tree is the same as that obtained from ML analysis. Numbers assigned to each node are bootstrap support values in the ML analysis, following the BI posterior support values. (CR = Costa Rica, MEX = Mexico, ECU = Ecuador, GUA = Guatemala, USA = United States of America, THAI = Thailand, VNM = Vietnam JPN = Japan, LKA = Sri Lanka and IND = India). Members of the *P. westermani* group were used to root the tree.

References

[1] B.D. Paragonimiasis, R. Toledo, B. Fried, Digenetic Trematodes, Advances in Experimental Medicine and Biology, Springer, New York, 2014 115–152.
[2] G.W. Procop, North American paragonimiasis (caused by *Paragonimus kellicotti*) in the context of global paragonimiasis, Clin. Microbiol. Rev. 22 (2009) 415–446.
[3] I. Miyazaki, An Illustrated Book of Helminthic Zoonoses, International Medical Foundation, Japan, Tokyo, 1991 369–409.
[4] J. Voelker, G. Müller, A. Prata, What is *Paragonimus rudis* (Diesing, 1850)? Report on a field study in Mato Grosso, Brazil. Mem Inst Oswaldo Cruz. 76 (1981) 409–414.
[5] E. Caballero, Presencia de *Paragonimus rude* (Diseing, 1850), Braun, 1899 en mamíferos silvestres en Centroamérica, Ann. Inst. Biol. Méx. 27 (1956) 397.
[6] R. Brenes, R. Zeledón, G. Rojas, The finding of *Paragonimus* sp. in mammals, crabs and snails in Costa Rica, Bol. Chil. Parasitol. 23 (1968) 164.
[7] I. Miyazaki, Y. Ishii, Studies on the Mexican lung flukes, with special reference to a description of *Paragonimus mexicanus* sp. nov. (Trematoda: Troglotrematidae), Jpn. J. Parasitol. 17 (1968) 445–453.
[8] G. Rojas, E. Monge, R. Brenes, *Paragonimus caliensis* en Costa Rica, 58, Fourth Parasitology Latin American Congress, Fourth Microbiology and Parasitology Central American Congress, Costa Rica, 1976.
[9] M.D. Little, *Paragonimus caliensis* sp. n. and paragonimiasis in Colombia, J. Parasitol. 738-746 (1968).
[10] Y. Tongu, The species of *Paragonimus* in Latin America, Bull Fac Health Sci Okayama Univ Med Sch. 12 (2001) 1–5.
[11] I. Vélez, C. Lenis, L.E. Velásquez, *Paragonimus mexicanus* (Digenea: Troglotrematidae) de Valle de Pérdidas, Urrao, Antioquia. Revista de Ciencias. 13 (2009) 49–56.
[12] C. Komalamisra, S. Boonchuen, J. Waikagul, E. Pongponratn, Chaetotaxy of newly excysted metacercariae among five species of Thai *Paragonimus*, J. Trop. Med. Parasitol. 28 (2005) 1–7.
[13] F. Hernandez, E. Monge, Cuticular ultrastructure of *Paragonimus* from Costa Rica, J. Electron Microsc. 38 (1989) 41–46.
[14] Y. Kong, P.N. Doanh, Y. Nawa, Paragonimus, in: L. Xiao, U. Ryan, Y.Y. Feng (Eds.), Biology of Foodborne Parasites, CRC Press 2015, pp. 445–462.
[15] P. Morera, Trematodiasis pulmonar. Estudio de dos casos encontrados en Costa Rica, Acta Med. Costarric. (1968) 11–225.
[16] D.T.J. Littlewood, D.A. Johnston, Molecular phylogenetics of the four *Schistosoma* species groups determined with partial 28S ribosomal RNA gene sequences, Parasitology 111 (1995) 167–176.
[17] J. Bowles, D. Blair, D.P. McManus, A molecular phylogeny of the human schistosomes, Mol. Phylogenet. Evol. 4 (1995) 103–109.
[18] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.
[19] T.A. Hall, BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT, Nucleic Acids Symp. Ser. 41 (1999) 95–98.
[20] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.
[21] D. Darriba, G.L. Taboada, R. Doallo, D. Posada, jModelTest 2: more models, new heuristics and parallel computing, Nat. Methods 9 (2012) 772.
[22] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol. 30 (2013) 2725–2729.
[23] J.P. Huelsenbeck, F. Ronquist, MRBAYES: Bayesian inference of phylogeny, Bioinformatics 17 (2001) 754–755.
[24] F. Ronquist, J.P. Huelsenbeck, MRBAYES 3: Bayesian phylogenetic inference under mixed models, Bioinformatics 19 (2003) 1572–1574.
[25] Rambaut A. FigTree v1.4.2, A Graphical Viewer of Phylogenetic Trees. Available from http://tree.bio.ed.ac.uk/software/figtree/, 2014.
[26] Y. Tongu, Y. Iwanaga, H. Hata, M. Tsuji, M. Yokogawa, P. Morera, M. Conejo, Morphological features of *Paragonimus* metacercariae from Costa Rica, Kiseichugaku Zasshi. 36 (1987) 236–241.
[27] T. Aji, H. Oh, Y. Tongu, S. Inatomi, H. Hata, M. Kobayashi, M. Yokogawa, H. Miranda, N. Ibanez, Ultrastructure of tegumental surface of the metacercaria of *Paragonimus peruvianus*, Jpn. J. Parasitol. 331 (1984) 15–21.
[28] H. Higo, Y. Ishii, Scanning electron microscopy of the newly excysted juveniles *Paragonimus westermani* (Kerbert, 1878) Braun, 1899 (parthenogenetic type) and *P. miyazakii* Kamo, Nishida, Hatsushika and Tomimura, 1961, Jpn. J. Parasitol. 33 (1984) 421-127.
[29] D. Blair, G.M. Davis, B. Wu, Evolutionary relationships between trematodes and snails emphasizing schistosomes and paragonimids, Parasitology 123 (2001) 229–243.
[30] D. Blair, Y. Nawa, M. Mitreva, P.N. Doanh, Gene diversity and genetic variation in lung flukes (genus *Paragonimus*), Trans. R. Soc. Trop. Med. Hyg. 110 (2016) 6–12.
[31] T.S. Singh, H. Sugiyama, A. Rangsiruji, *Paragonimus* & paragonimiasis in India, Indian J. Med. Res. 136 (2012) 2–192.