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Morphology and genetics reveal the occurrence of *Girardinus falcatus* (Eigenmann, 1903) (Cyprinodontiformes, Poeciliidae) in eastern Cuba

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Abstract
Girardinus is a Cuban genus of poeciliid fishes. It comprises 7 freshwater-restricted species most of them inhabiting specific ecosystems or regions within the island. *Girardinus falcatus*, the Gold-bellied Topminnow, is common in lowlands of western and central Cuba but thought not to be present in the East. Herein we provide the first well-supported evidence of the occurrence of this species in eastern Cuba. Sixteen specimens were collected and identified primarily by body shape and coloration, and corroborated by gonopodial morphology and by sequences of the mitochondrial gene cytochrome *b*.

Key words
Cytochrome *b*; freshwater ecosystems; Gold-belly Topminnow; gonopodium; range extension.

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Introduction

Poeciliid fishes exhibit high endemism in the Caribbean islands (Rosen and Bailey 1963). There are 3 genera exclusively distributed in these islands, and 2 of them—*Girardinus* Poey, 1854 with 7 species and the monotypic *Quintana* Hubbs, 1934—are restricted to Cuba (Lara et al. 2010). The distribution of species of *Girardinus* is associated to specific habitats. For example, *G. uninotatus* Poey, 1860, *G. creolus* Garman, 1895, *G. cubensis* (Eigenmann, 1903), and some populations of *G. microdactylus* Rivas, 1944 inhabit streams and rivers in the mountain ranges of western Cuba, that is, in Pinar del Río province (Doadrio et al. 2009, Lara et al. 2010, Ponce de León and Rodríguez 2010). *Girardinus denticulatus* Garman, 1895 occurs in mountain rivers of central and eastern Cuba, whereas, *G. metallicus* Poey, 1854 and *G. falcatus* (Eigenmann, 1903) are mostly widespread in lowland freshwater ecosystems in the western and central regions.

The distribution of species of *Girardinus* is known from Poey (1854), Eigenmann (1903), and Rivas (1944a, 1944b, 1958). However, recent samplings for molecular (Doadrio et al. 2009, Lara et al. 2010) and ecology studies (Ponce de León et al. 2011, 2012) provided new information about the distribution of *Girardinus* species. In this work, we present new distribution records of *G. falcatus* in eastern Cuba and analyze the relationships among populations based on cytochrome *b* sequences. In addition, an updated list of localities for this species is provided.

Methods

A total of 29 rivers and streams were sampled during a survey to Holguín province (northeastern Cuba) in January 2016. Adult specimens of *Girardinus falcatus* were found in some of these water bodies. The specimens were collected using seines and hand nets (Perrow et al. 1996), anaesthetized with MS 222 (Tricaine Methanesulphonate) and preserved in 90% ethanol. Voucher specimens were deposited in independent lots according to the new localities in the collection of the Acuario Nacional de Cuba in La Habana, Cuba (ANC).

We followed the morphological and gonopodial features described by Rivas (1958) to distinguish the *Girardinus* species. We also used the photographic evidence of body and gonopodium morphology depicted in Ponce de León and Rodríguez (2010) for the identification of the male specimens collected in the field. To that purpose, and after the primary identification by body morphology, we captured images of the gonopodia using a stereoscopic microscope (40×) and a Nikon Coolpix digital camera.

The genetic identification of *G. falcatus* specimens from Holguín province was based on the mitochondrial gene cytochrome *b* (cyt*b*). DNA was extracted, PCR amplified and sequenced following Lara et al. (2010). The primers used for amplification were Glufish (5′-CCAATGACTTGAAGAACCACCGTTG; Meyer et al. 1990) and CB3 (5′-GCCAAATAGGAARTATCATTC; Palumbi 1996). We compared the sequences of 1 individual from each new locality with those of *G. falcatus* included in Doadrio et al. (2009), and a sequence of *G. uninotatus* was used as outgroup (accession number: FJ178722.1). The new sequences were sent to GenBank database under the accession numbers KY606767–KY606770. Sequences were aligned using Clustal W (Thompson et al. 1994). The model of nucleotide substitution was selected using the Bayesian information criterion (BIC; Schwarz 1978) with jModelTest 2.1.7 (Darriba et al. 2012). The Maximum Likelihood method based on the HKY model was used for the inference of *G. falcatus* phylogenetic relationships using MEGA 7.0.18 (Kumar et al. 2016). The haplotype and nucleotide diversity (*h* and π, respectively) were estimated using DnaSP 5.10 (Librado and Rojas 2009). The genetic divergence (*p*-distance) between each pair of clades was also estimated using MEGA 7.0.18 (Kumar et al. 2016).

A list of localities from which *G. falcatus* has been reported was made by reviewing previous records from 3 major ichthyology collections in Cuba: Museo Nacional de Historia Natural (MNHNCu), Instituto de Ecología y Sistemática (CZACC), and Museo de Historia Natural "Felipe Poey" (MFP). Additionally, the distribution information from published studies available online and from the Global Biodiversity Information Facility (GBIF) database (http://data.gbif.org, consulted on 4 July 2016) were used to update the list of known localities for *G. falcatus*.

Results

Sixteen adult specimens of *Girardinus falcatus* were sampled at 4 different basins in Holguin province: Mano river (9♀) (21.0815 N, 076.3568 W; 35 m a.s.l.), Cacoyugüín river (1♀, 2♂) (21.0537 N, 076.2304W; 25 m a.s.l.), Gibara river (2♀, 1♂) (20.9425 N, 076.1047 W; 50 m a.s.l), and Camazán river (1♂) (20.7889 N, 076.1022 W; 150 m a.s.l). These records as well as the previous ones gathered from museum collections, published studies and GBIF database are represented in Figure 1 and summarized in Table 1.

The segments of the four rivers in which specimens

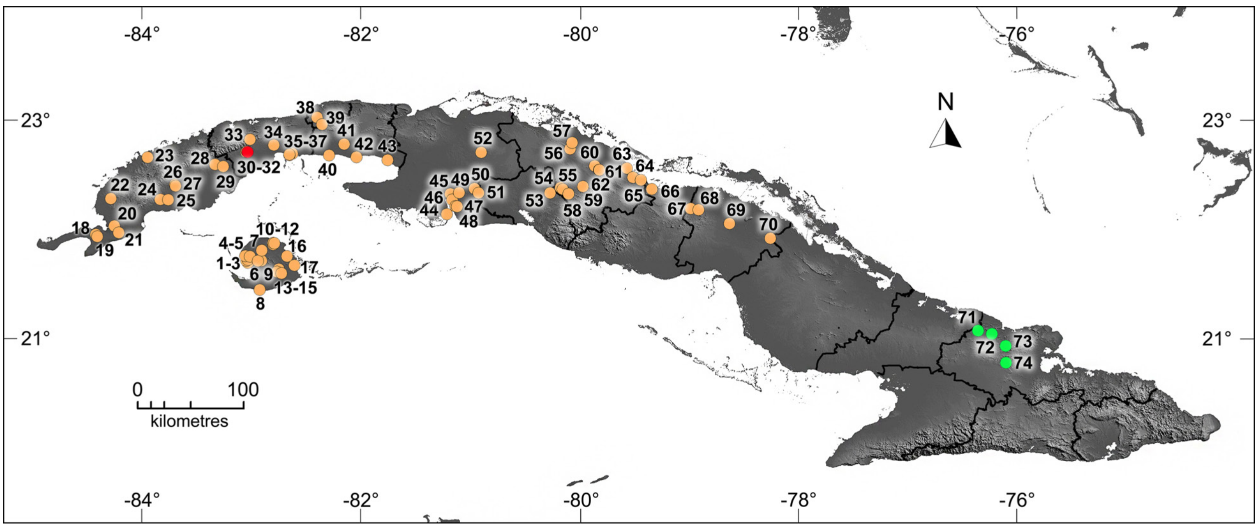


Figure 1. Map depicting the geographic distribution of *Girardinus falcatus* in the Cuban archipelago. Red dot: type locality; orange dots: known localities; green dots: new localities. Locality numbers as in Table 1.

Table 1. Known localities of *Girardinus falcatus* in Cuba (geographic coordinates in decimal degrees; datum WGS84). "Voucher" refers to specimens deposited in museum collections and "Source" refers to publications where the locality was obtained from. AMNH: American Museum of Natural History, New York; ANC: Acuario Nacional de Cuba, La Habana; CAS: California Academy of Sciences, San Francisco; CZAAC: Instituto de Ecología y Sistemática, La Habana; MCZ: Museum of Comparative Zoology, Harvard University, Cambridge; MFP: Museo de Historia Natural "Felipe Poey", Universidad de La Habana, La Habana; MNHNCu: Museo Nacional de Historia Natural de Cuba, La Habana; UMMZ: University of Michigan Museum of Zoology, Ann Arbor; and USNM: National Museum of Natural History, Smithsonian Institution, Washington, DC. The asterisk represents specimens deposited in collection without a catalogue number assigned.

	Province	Locality	Latitude	Longitude	Voucher/Source
1	Isla de la Juventud	Estero La Majagua	21.7047	−083.0328	CAS-SU-(ICH)-54152
2	Isla de la Juventud	Stream 200 m from Punta El Soldado	21.7220	−083.0450	AMNH-96367
3	Isla de la Juventud	Stream crossing the road to Punta El Soldado	21.7253	−083.0392	AMNH-96407; MNHNCu-22-000630
4	Isla de la Juventud	Lagoon close to the road after the mine of gold	21.7301	−083.0310	AMNH-96362
5	Isla de la Juventud	Stream close to the mine of gold, road to the coast	21.7488	−083.0263	AMNH-96355
6	Isla de la Juventud	Stream in Los Indios	21.7192	−082.9409	MCZ-Ich-33624
7	Isla de la Juventud	Las Nuevas river	21.8175	−082.9053	AMNH-96377; MNHNCu-22-000628, MNHNCu-22-000702
8	Isla de la Juventud	Carapachibey	21.4565	−082.9255	GenBank accession numbers: FJ178757 and FJ178758 (Doadrio et al. 2009)
9	Isla de la Juventud	Sierra La Cañada	21.7220	−082.9075	MFP18.000103
10	Isla de la Juventud	River S Nueva Gerona	21.8693	−082.8002	MCZ-Ich-33619
11	Isla de la Juventud	Dos Alcantarillas stream	21.8833	−082.8000	USNM-246560
12	Isla de la Juventud	Lagoon W Nueva Gerona	21.8807	−082.7891	MCZ-Ich-33620
13	Isla de la Juventud	Mella	21.6438	−082.7481	GenBank accession number: FJ178759 (Doadrio et al. 2009)
14	Isla de la Juventud	4 km NW Cayo Piedra	21.6211	−082.7656	AMNH-96387; MNHNCu-22-000716
15	Isla de la Juventud	Ciénaga de Lanier	21.6078	−082.7261	CZAAC*/ GenBank accession number: FJ178760 (Doadrio et al. 2009)
16	Isla de la Juventud	Guayabo dam	21.7623	−082.6744	GenBank accession number: FJ178756 (Doadrio et al. 2009)
17	Isla de la Juventud	16 km SE La Fe	21.6783	−082.6061	AMNH-96394; MNHNCu-22-000668
18	Pinar del Río	Las Coloradas lagoon	21.9606	−084.4214	GenBank accession number: FJ178761 (Doadrio et al. 2009)
19	Pinar del Río	Laguna Lugones	21.9443	−084.4077	ANC-12.2322
20	Pinar del Río	La Fe	22.0395	−084.2517	GenBank accession number: FJ178762 (Doadrio et al. 2009)
21	Pinar del Río	El Cayuco	21.9783	−084.2113	GenBank accession number: FJ178763 (Doadrio et al. 2009)
22	Pinar del Río	Mantua	22.2889	−084.2844	MCZ-Ich-33622, MCZ-Ich-33626
23	Pinar del Río	Santa Lucía and Cayo Hutía	22.6667	−083.9500	USNM-246558
24	Pinar del Río	Bautista river	22.2811	−083.8343	GenBank accession number: FJ178750 (Doadrio et al. 2009)
25	Pinar del Río	San Luis	22.2786	−083.7611	MCZ-Ich-33625
26	Pinar del Río	Guarna river	22.4175	−083.6981	USNM-246559, USNM-120229
27	Pinar del Río	Pinar del Río river	22.4055	−083.6926	CAS-ICH-22762
28	Pinar del Río	San Diego river	22.6019	−083.3336	AMNH-96331
29	Pinar del Río	Los Palacios	22.5835	−083.2571	CAS-Ich-22763; MCZ-Fish-34186/ GenBank accession number: FJ178751 (Doadrio et al. 2009)
30	Artemisa	San Cristóbal river	22.7142	−083.0481	AMNH-96317; MNHNCu-22-000685
31	Artemisa	San Cristóbal	22.7203	−083.0410	GenBank accession number: FJ178753 (Doadrio et al. 2009)
32	Artemisa	San Cristóbal-holotype	22.7156	−083.0353	CAS-ICH-22548; CAS-SU-(ICH)-8500
33	Artemisa	Las Terrazas	22.8307	−083.0126	GenBank accession number: FJ178752 (Doadrio et al. 2009)
34	Artemisa	La Canoa lagoon	22.7798	−082.7957	CAS-SU-(ICH)-32023; UMMZ-103342
35	Artemisa	S La Habana	22.6883	−082.6536	AMNH-96403
36	Artemisa	Guanímar channel	22.6945	−082.6516	MFP18.000271
37	Artemisa	2 km E road to Guanímar	22.7019	−082.6261	MNHNCu-22-000676
38	La Habana	Cristal river	23.0333	−082.4010	ANC-12.2323
39	La Habana	La Habana	22.9672	−082.3531	ANSP-167809; MCZ-33624
40	Mayabeque	Batabanó	22.6853	−082.2908	CZAAC*
41	Mayabeque	Melena del Sur	22.7881	−082.1514	MCZ-Ich-33632
42	Mayabeque	Cajío	22.6692	−082.0383	AMNH-96416
43	Mayabeque	4 km N Tasajeras	22.6411	−081.7553	AMNH-96437, AMNH-96431; MNHNCu-22-000650
44	Matanzas	Las Salinas	22.1461	−081.2133	Barus and Libosvarsky (1988)
45	Matanzas	Pálpite	22.3323	−081.1812	MFP18.000173 (Lara et al. 2010)
46	Matanzas	Playa Larga	22.2789	−081.1642	Barus and Libosvarsky (1988)
47	Matanzas	Laguna de Facundo	22.2194	−081.1322	CZAAC 9.30; MFP18.000164 (Lara et al. 2010)
48	Matanzas	Los Hondones village	22.2169	−081.1203	CZAAC*
49	Matanzas	Laguna del Tesoro	22.3433	−081.1008	Barus and Libosvarsky (1988)

Table 1. *Continued.*

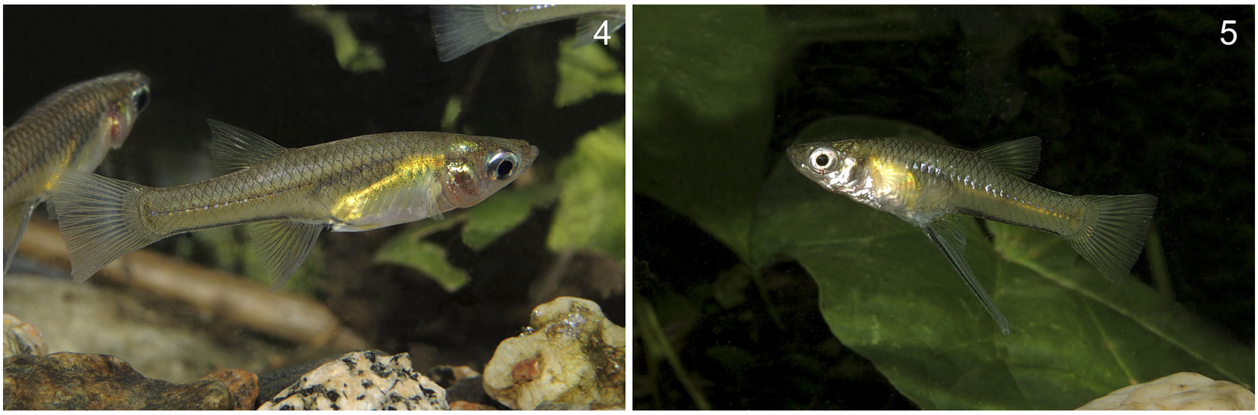
	Province	Locality	Latitude	Longitude	Voucher/Source
50	Matanzas	Hanabana river	22.3817	–080.9608	MCZ-Ich-30618
51	Matanzas	Unión de Reyes	22.3408	–080.9228	AMNH-96572; MNHNCu-22-000703
52	Matanzas	Colón	22.7130	–080.8989	MFP18.000139 (Lara et al. 2010)
53	Cienfuegos	1km S Cruces	22.3414	–080.2703	AMNH-96511
54	Villa Clara	Ranchuelo river	22.3900	–080.1739	AMNH-96489
55	Villa Clara	Níspero river	22.3747	–080.1528	AMNH-96483
56	Villa Clara	Sagua La Grande	22.7453	–080.0856	USNM-246561
57	Villa Clara	2 km S Sagua La Grande	22.8000	–080.0667	AMNH-96343; MNHNCu-22-000686
58	Villa Clara	San Juan de los Yeras	22.3333	–080.1000	UMMZ-103343
59	Villa Clara	Santa Clara	22.4003	–079.9675	AMNH-3115; UMMZ-158698
60	Villa Clara	S Encrucijada	22.5886	–079.8611	Barus et al. (1987)
61	Villa Clara	Near Vega Alta	22.5500	–079.8192	USNM-120228
62	Villa Clara	Vega Alta	22.5500	–079.8169	UMMZ-103341
63	Villa Clara	8 km W Remedios	22.5667	–079.5667	AMNH-96451; MNHNCu-22-000680
64	Villa Clara	Bartolomé river	22.4826	–079.5120	MCZ-Ich-33600
65	Villa Clara	S Caibarién	22.4603	–079.4394	Barus et al. (1987)
66	Sancti Spiritus	W Yaguajay	22.3753	–079.3381	Barus et al. (1987)
67	Ciego de Ávila	Arroyo Blanco	22.1988	–078.9844	USNM-246571 (Rivas 1944b)
68	Ciego de Ávila	Aguas Azules	22.1871	–078.9113	GenBank accession number: FJ178755 (Doadrio et al. 2009)
69	Ciego de Ávila	Laguna La Jagua	22.0620	–078.6289	MCZ-Ich-33990
70	Ciego de Ávila/ Camagüey	Caonao river	21.9273	–078.2565	GenBank accession number: FJ178749 (Doadrio et al. 2009)
71	Holguín	Mano river	21.0815	–076.3568	ANC-12.2324/ GenBank accession number: KY606767 (This study)
72	Holguín	Cacoyugüín river	21.0537	–076.2304	ANC-12.2325/ GenBank accession number: KY606768 (This study)
73	Holguín	Gibara river	20.9425	–076.1047	ANC-12.2326/ GenBank accession number: KY606769 (This study)
74	Holguín	Camazán river	20.7889	–076.1022	ANC-12.2327/ GenBank accession number: KY606770 (This study)

Figures 2, 3. Examples of habitats where specimens of *Girardinus falcatus* were found in Holguín province. **2.** Cacoyugüín river. **3.** Mano river. Photos by S. Rodríguez-Machado.

of *G. falcatus* were found were shallow and with perceptible water flow. The depth varied between 10 and 40 cm and the surrounding areas were composed by secondary forest with herbaceous riparian vegetation (Cyperacea) or secondary grassland and shrubby vegetation (Figs 2, 3). Aquatic vegetation was abundant and dominated by vascular plants (Hydrocharitaceae and Nelumbonaceae). Together with *G. falcatus*, we found specimens of *Gambusia punctata* Poey, 1854, *Gambusia puncticulata* Poey, 1854, and *Limia vittata* (Guichenot, 1853). In Mano river, we also found *Girardinus denticulatus*.

All specimens found in Holguín province showed the unique morphological features that distinguish *G. falcatus* from its congeners: yellowish translucent body, golden belly, lateral scales with black edges, lower body edge with a black line, from the anal fin to the caudal peduncle, the origin of the dorsal fin above end of anal base (Rivas 1958), and males with a dark line along the gonopodium (Eigenmann 1903, Ponce de León and Rodríguez 2010) (Figs 4, 5).

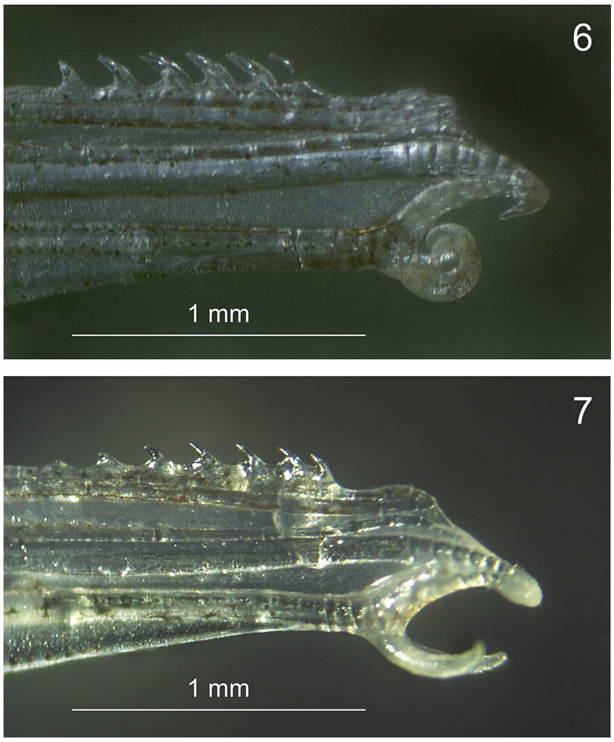
Intra-specific variation in gonopodial structure from the reference individual from Ponce de León and Rodríguez (2010) and a male from Cacoyugüín river is shown in Figures 6 and 7, respectively. The following are some of the most relevant features of the gonopodium of the specimens collected in Holguín province. The gonopodia of the fish identified as *G. falcatus* showed similar coloration, and the proportion gonopodium length/body total



Figures 4, 5. Specimens of *Girardinus falcatus* from Holguín province. **4.** Female from Gibara river. **5.** Male from Mano river. Photos by T.M. Rodríguez-Cabrera.

length was also similar (0.4–0.5) among them. They also had the posterior branch of ray 5 with 6 or 7 serrae and its 2–4 terminal segments unarmed. These features are consistent with the gonopodial size and structure described by Rivas (1958), and used by Rodríguez et al. (1992) for *G. falcatus* identification. The reference specimen in Ponce de Leon and Rodríguez (2010) also presents the same gonopodium size, color, and the number of serrae in 5p. However, despite we found some unarmed segments distal to the serrae in 5p, the actual number of them is not clearly quantifiable in the photograph from Ponce de León and Rodríguez (2010).

The analysis of 804 base pairs of the cyt*b* gene sequences from the 4 samples of Holguín and the 15 samples from Doadrio et al. (2009) showed 14 haplotypes and high haplotype ($h = 0.965 \pm 0.028$) and nucleotide ($\pi = 0.017 \pm 0.003$) diversities. The phylogenetic relationships are presented in Figure 8. We found 4 main clades which correspond to discrete geographical regions within the Cuban archipelago: 1- Western Pinar del Río, 2- Isla de la Juventud, 3- Pinar del Río/Artemisa/Matanzas/Western Central Cuba, and 4- Eastern Central Cuba/Eastern Cuba. The genetic distance between each main clade ranged from $d = 0.012 \pm 0.003$ to $d = 0.038 \pm 0.006$. The lowest values were obtained between populations from Pinar del Río/Artemisa/Matanzas/Western Central Cuba and Eastern Central Cuba/Eastern Cuba, whereas the higher values were found between populations from Western Pinar del Río and Isla de la Juventud.



Figures 6, 7. Gonopodium of *Girardinus falcatus* (40×). **6.** Reference specimen from Ponce de León and Rodríguez (2010). Photograph by J.L. Ponce de León. **7.** Cacoyugüín river, Holguín province (ANC-12.2325). Photograph by T.M. Rodríguez-Cabrera.

Discussion

Our results show that the distribution of *G. falcatus* reaches the northeastern region of Cuba. The westernmost locality among the 4 new ones, Mano river (number 71 in Fig. 1), is about 220 km southeast of Caonao river, Esmeralda (number 70 in Fig. 1), in the boundaries between Ciego de Ávila and Camagüey provinces, the most easterly location known for the species (Doadrio et al. 2009). We found a record referred to as *G. falcatus* (MCZ-Ich-33607) from Alto Songo, Santiago de Cuba province deposited in the fish collection of the Museum of Comparative Zoology, Harvard University. The voucher was collected by Carlos de La Torre in 1910. Unfortunately, no description is available and the specimens are damaged and not identifiable (K. Hartel, in litt., September 2016). Also, the geographic coordinate and the site name are unclear, which makes this record dubious. Moreover, despite the exact location is unknown, we conducted an extensive survey across eastern Cuba, including Granma, Santiago de Cuba and Guantánamo provinces, and we did not found *G. falcatus* in any of the 39 sampling localities; some of which are in the same area reported by Carlos de La Torre. All this increases the value of the present report as verifiable evidence for the occurrence of this species in eastern Cuba.

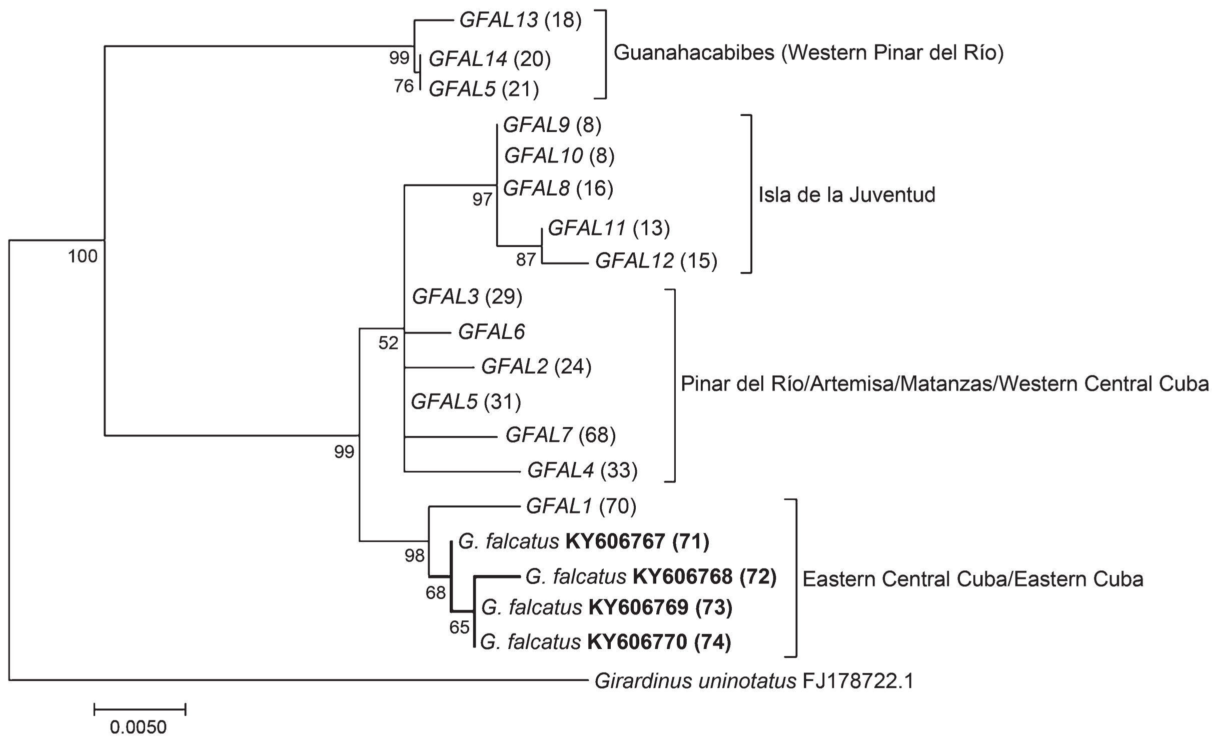


Figure 8. Maximum likelihood phylogenetic tree based on cytochrome *b* (cyt*b*) sequences showing the identification of the Holguín samples (characters in bold) as *Girardinus falcatus*. *GFAL1-GFAL15* are cyt*b* gene sequences reported in Doadrio et al. (2009). Numbers in parentheses are locality numbers as in Table 1.

All the sequences from Holguín cluster together in a node shared basally with the sample from Caonao river in Ciego de Ávila-Camagüey provinces boundary. This relationship is coherent with the geographic location of these sites. Regarding this, the tree topology provides new information about the geographic differentiation of *G. falcatus*. Although human-mediated translocations cannot be discarded (A. Fong and R. Teruel, pers. comm.), the divergence of Holguín sequences from Caonao river sequence ($d = 0.075 \pm 0.003$) and the outgroup condition of the later, suggest that its distribution have followed a natural historical process.

The populations from Guanahacabibes region (western Pinar del Río) are highly differentiated compared to main western ($d = 0.037 \pm 0.007$) and eastern ($d = 0.038 \pm 0.006$) populations. These values are close to the cut-off criterion for species level divergence of Hebert et al. (2003). On the contrary, the clades composed by the rest of the western to central distributed populations (Pinar del Río/Artemisa/Matanzas/western Central Cuba, including Isla de la Juventud) and the eastern populations (eastern Central Cuba/Eastern Cuba) diverge only by $d = 0.014 \pm 0.003$. According to our analysis of cyt*b* sequences, *G. falcatus* seems to be composed by allopatric populations, as found in other Cuban freshwater fishes (Ponce de León et al. 2014, Hernández et al. 2016). Further multi-loci analyses should be addressed to fully understand the genetic diversity and the relationships among *G. falcatus* populations.

The new localities reported in this work provide valuable information about the geographic distribution of the species and bring new insights on the biogeography of *Girardinus*. The geographic discontinuity of the new localities with respect to the previous eastern limit of the species distribution raises interesting questions concerning dispersion mechanisms and ancient distribution of the species. Future studies of population genetics on this species will help in answering these questions and will provide information about the evolutionary history of the genus in Cuba, in particular about possible patterns of dispersion of the populations and the events involved.

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Authors' Contributions

SRM collected the specimens and reviewed the data from Cuban ichthyology collections; SRM, JLPL, DC and EGM contributed to writing the manuscript.

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