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Short Communication

The use of ISSR markers for species determination and a genetic study of the invasive lionfish in Guanahacabibes, Cuba

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ABSTRACT. The red lionfish (*Pterois volitans*) and devil fire-fish (*Pterois miles*) are invasive species that pose a threat to the biodiversity and stability of coral reefs in the Western Atlantic, Gulf of Mexico and Caribbean Sea. Species identification of lionfish is uncertain in some parts of Cuba, and research has mainly been focused on their biology and ecology. The principal aim of this study was to determine highly polymorphic markers (Inter Simple Sequence Repeat, ISSR) that could be used in research on lionfish population genetics in addition to confirming the presence of *Pterois* species in the Guanahacabibes National Park. The genetic profile or "fingerprint" of individuals collected in Mexico, formally identified as *P. volitans*, was compared with the genetic profile of specimens from Cuba. There were very few "diagnostic bands" and a high number of "common bands", demonstrating that the same species exists in both countries. Furthermore, Nei's genetic distance and the unrooted tree do not show significant differences between both localities. In light of these results, we can confirm the presence of *P. volitans* in the Guanahacabibes National Park, Cuba. This study demonstrates the functionality of ISSR as a molecular tool for species identification and their application for genetic population studies of this invasive fish species.

Keywords: *Pterois volitans*, fish, fingerprint, ISSR, Caribbean Sea.

Uso de marcadores ISSR para la determinación de especies y estudios genéticos del pez león, especie invasora en Guanahacabibes, Cuba

RESUMEN. El pez león rojo (*Pterois volitans*) y el pez fuego diablo (*Pterois miles*) son especies invasoras que amenazan la biodiversidad y estabilidad de los arrecifes coralinos del Atlántico occidental, Golfo de México y Mar Caribe. La identificación del pez león sigue incierta en unas zonas de Cuba y la investigación se ha centrado principalmente en su biología y ecología. El propósito principal de este estudio fue determinar marcadores altamente polimórficos (secuencias repetidas inter simples, ISSR) útiles para estudios de genética poblacional del pez león y aplicarlos para determinar la especie de *Pterois* presente en el Parque Nacional Guanahacabibes. Se comparó el perfil genético de individuos colectados en México, formalmente identificados como *P. volitans*, con el perfil genético de especímenes de Cuba. Los perfiles genéticos mostraron un bajo número de "bandas diagnósticas" y un alto número de bandas comunes lo que demuestra que en ambos países está presente la misma especie. Por otra parte, los resultados de distancia genética de Nei y el árbol no enraizado no muestran ninguna diferencia significativa entre ambas localidades. Estos resultados confirman la presencia de *P. volitans* en el Parque Nacional Guanahacabibes, Cuba, y se demostró la funcionalidad de ISSR como herramienta molecular para la identificación de especies y su aplicación para estudios de genética poblacional de este pez invasor.

Palabras clave: *Pterois volitans*, fingerprint, ISSR, peces, Mar Caribe.

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Invasive species are a major threat to marine ecosystems. They impact the ecosystem structure and function and have a negative effect on biodiversity (Costello *et al*., 2010; Ojaveer *et al*., 2014). During the last decade, considerable efforts were made to understand and eradicate one of the most important invasive predators in the Atlantic, the Indo-Pacific lionfish complex: the red lionfish *Pterois volitans* (Linnaeus, 1758) and the devil fire-fish *Pterois miles* (Bennett, 1828). The invasion has progressed rapidly since the first observation of lionfish in Florida in 1985 (Morris & Akins, 2009). Today, the species has invaded an area over 7 million km^2 (Dahl & Paterson, 2014), including the eastern coast of the USA, Gulf of Mexico, Caribbean Sea (Schofield, 2009) and has extended their range throughout of the eastern coast of South America, mainly in Venezuela and Brazil (Fortunato & Avigliano, 2014; Ferreira *et al.*, 2015).

According to Sakai *et al*. (2001), the study of the genetic diversity and population structure of a new invasive species is essential as the obtained information may contribute to understanding the potential establishment and geographical dispersion of invasive species. This information could also play an important role in the development of appropriate strategies for the management of such species. Another fundamental aspect concerning invasive species is the correct identification of the introduced species and the determination of their origin (Le Roux & Wieczorek, 2009).

An erroneous taxonomic classification may obscure accurate invasion history and preclude management strategies. Because of inter- and intraspecific variations, morphological identification often presents difficulties, whereas molecular markers can accurately, reliably and rapidly identify species as well as variants and cryptic taxa (Holland *et al.*, 2004; Le Roux & Wieczorek, 2009; García-Morales & Elías-Gutiérrez, 2013). These examples show the efficacy and importance of molecular tools when carrying out research on invasive organisms, especially as taxonomic identification is a first important step towards developing effective control and management decisions (Le Roux & Wieczorek, 2009).

The choice of markers is important and depends on the specific research aims. In this context, dominant ISSR markers (Inter Simple Sequence Repeats) use the PCR-method to amplify DNA sequences between two closed but inverted SSR (Simple Sequence Repeats) by means of a single primer, generally composed of 18-20 base pairs from a microsatellite sequence (Zietkiewicz *et al.*, 1994). The great advantage of ISSR is that the primers work universally for most animal and plant species, so prior knowledge of genome sequences is not required. This method finds abundant polymorphisms in many systems and provides genomic information for a wide range of applications (Maltagliati *et al*., 2006; Wink, 2006). Furthermore, ISSR is straightforward, demands fewer experimental steps, and incurred costs are relatively low (Huang & Sun, 2000; Le Roux & Wieczorek, 2009).

Only one recent study (Butterfield *et al.*, 2015), about phylogeographic structure of red lionfish over a wide-range geographical scale, include samples from Cuba (Guantanamo Bay). Otherwise, any other genetic-related information on the lionfish invasion in other parts of Cuba is available (Chevalier *et al*., 2013). The first, albeit non-confirmed, observation of lionfish in Cuba was in 2005 (Schofield *et al*., 2009), and the first official report of its presence was in 2007 (Chevalier *et al*., 2008) at six sites along the Atlantic coast (northern Cuba) and two localities on the Caribbean coast (Santiago de Cuba, southeastern Cuba). These authors identified both collected specimens as *P. volitans* based on morphological characters following Schultz (1986). However, both species of *Pterois* present in Florida, *P. volitans* and *P. miles*, are not 100% distinguishable based solely on morphology (Schofield, 2009; Jud *et al*., 2015).

Consequently, the identification of *Pterois* species in different parts of Cuba requires validation using molecular tools. Recent study (Butterfield *et al.*, 2015) confirms that this species occupy the eastern part of the island, but for other regions, such as the northern coast, no genetic information is available. This region of Cuba is particularly important, because the model of biological connectivity, proposed by Cowen *et al.* (2006), suggests that ocean currents could exchange individuals of *P. miles* between Bahamas and northern Cuba; so the distribution of *P. miles* could be extended to this part of the archipelago.

In this study, we propose to (1) determine the ISSR markers that may be useful for molecular genetic studies of lionfish and (2) use the ISSR technique to confirm the identification of the lionfish species in Guanahacabibes National Park, Cuba.

Cuban lionfishes were collected at Guanahacabibes National Park, the westernmost point of Cuba, within the Biosphere Reserve "Peninsula de Guanahacabibes". Sampling was conducted over a wide area from Cabo Corrientes to La Bajada (Fig. 1), performed by scuba divers, between 9:00-14:00 h at a water depth of 10 to 20 m. Nineteen fishes were captured using a spear, conserved in ice and transported to the laboratory. Muscle tissue was cut from each individual, placed in 96° ethanol, and finally conserved at 4°C for molecular analysis at ECOSUR (El Colegio de la Frontera Sur, Chetumal, Mexico). Reference specimens were obtained

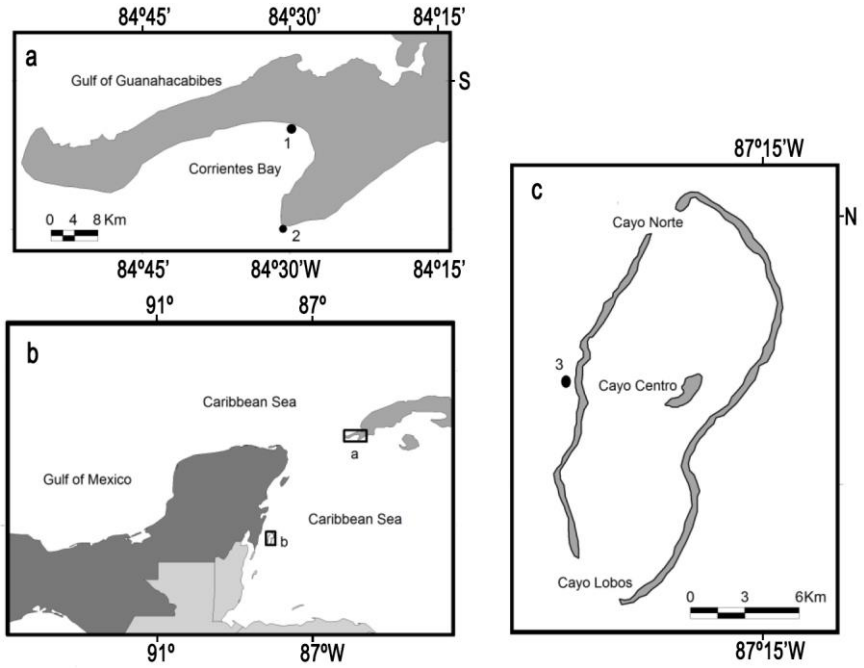

Figure 1. Location of the lionfish samples in the Caribbean Sea. Black dots indicate sampling locations. a) Localities in Cuba: 1: La Bajada and 2: Cabo Corrientes, b-c) locality in Mexico: 3: Banco Chinchorro.

at the "Coral Negro" locality within the Banco Chinchorro Biosphere Reserve in the Mexican Caribbean (Fig. 1). Twenty individuals were captured and preserved in similar conditions to the Cuban specimens. Using the barcoding method, the lionfish from the Mexican Caribbean were formally identified as *P. volitans* (Valdéz-Moreno *et al*., 2012).

Genomic DNA was extracted from a small part of the lionfish muscle tissue using the Wizard Genomic DNA Purification Kit (Promega) following the manufacturer's instructions, stored at -20°C until amplification. DNA-concentration was determined by the Qubit®2.0 fluorometer (Invitrogen). Quality was checked using agarose gel (1% with TAE buffer 1X; Promega) and the post-gel staining method using GelRed™ (Quimica Valaner).

To identify the more reliable ISSR markers for lionfish, we probed 23 sequences (Table 1). All primers were tested using five individuals from each locality and the optimal PCR conditions were determined for each primer. PCR amplifications were performed under the following conditions: 15 µL reaction volume containing: ~20 ng de template DNA, 1.5 µL of 5X Green Buffer (Promega), 200 µM of each dNTP from dNTP mix (Promega), 3 mM $MgCl_2$ (Promega), 1 µM of primer (Integrated DNA Technologies), 1.25 U of GoTaq Flexi DNA Polymerase (Promega) and finally the volume was adjusted by adding ultrapure water. All amplifications were conducted in a T100 Thermal Cycler (Bio-Rad™). The cycling conditions were as follows: initial denaturation step at 94°C for 4 min, 39 cycles of denaturation at 94°C for 45s, annealing temperature (*Ta*) from 57°C to 66°C depending on the ISSR primer (Table 1), extension temperature at 72°C for 2 min, and a final extension at 72°C for 10 min. Amplification products were separated by electrophoresis (110 V for 2 h) using 3 µL of amplified product on agarose gels (2% in a 1X TAE Buffer, Promega) and a 100 bp DNA Ladder (Promega) was used as reference for fragment length. The bands were detected with the GelRed™ under UV light and digitized using an Imaging System (AlphaImager® Mini, ProteinSimple).

A binary matrix was generated, scoring band presence (coded 1) or absence (coded 0) for each individual and primer. Only bands that scored consistently among populations were used, and we assumed that each marker band represented a distinct locus. The following informative parameters were determined at the population level: total number of bands, number of diagnostic bands and number of polymorphic bands. The binary matrix was applied to determine the percentage of polymorphism (*P*) and the Nei's gene diversity (*h*), using corrected allele frequency (Lynch & Milligan, 1994), at the species and localities level. All analyses were carried out using GenAlex 6.501 (Peakall & Smouse, 2006) and Popgen 1.32 (Yeh & Boyle, 1999).

Table 1. ISSR primers screened for ISSR-PCR in lionfish. %GC (proportion of G and C bases in the sequence), T*m* (melting temperature), T*a* (annealing temperature), B = T, C or G; D = A, G or T; R = A or G; Y = C or T and W = A or T. Number of total bands is of 5 individuals from each locality. Primers written in bold were used for the analysis.

Abbreviation	% GC	T*m*	T*a*	Amplification pattern	Total bands	Size range (pb)
$(CA)_7$	50	55.2	54	Poor amplification	-	-
$(CT)_8C$	52.9	55.7	54	Poor amplification	-	-
$(GAA)_6$	33.3	53.9	52	Smeared with bands	4	900-300
$(CA)_8GT$	50	62.5	61	Smeared with bands	5	1100-300
$(AC)_8C$	52.9	62.3	61	Smeared with bands	6	800-600
$(GA)_8G$	52.9	55.7	54	Good	4	800-450
$(AC)_8G$	52.9	62.5	61	Good	5	1100-400
$(GT)_8C$	52.9	60.4	59	Good	5	1500-900
$(GA)_8C$	52.9	56	54	Good	7	900-200
$(CA)_8AC$	**50**	**61.4**	**60**	**Good**	**8**	**1500-200**
$(GACA)_4$	50	57.1	56	Good	8	1500-200
$(AG)_8Y$	50	57.6	56	Good	8	1200-200
$(AG)_8C$	52.9	58.2	57	Good	8	1200-200
$(GTG)_5GC$	70.6	67.8	66	Good	9	1500-400
$(CA)_8AG$	**50**	**61.1**	**60**	**Good**	**9**	**1600-200**
$WB(GACA)_4$	48.1	61.5	60	Good	10	1500-200
$BDB(ACA)_5$	37	58.4	57	Good	11	1700-100
$(AG)_8YC$	**52.8**	**59.5**	**58**	**Good**	**11**	**1600-200**
$(GAG)_5GC$	**70.6**	**64.8**	**63**	**Good**	**12**	**1400-400**
$(AG)_8G$	52.9	57.5	56	Good	13	1600-200
$(ACA)_5BDB$	37	58.6	57	Good	13	1700-200
$(GACA)_4WB$	**48.1**	**59**	**57**	**Good**	**14**	**1500-200**
$(CA)_8RY$	50	62.1	61	Smeared	-	-

In order to describe the genetic structure and variability between localities, a non-parametric analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992, 9999 permutations) and Nei's genetic distance analysis were performed using GenAlex 6.501 (Peakall & Smouse, 2006). In addition, a mean distance analysis using a heuristic search for an optimal tree, performed by TBR (tree–bisection-reconnection) branch swapping, was conducted using Paup 4.0b10 (Swofford, 2001). The tree was displayed using TreeView 1.5 software (Page, 1996). The distance measurement (minimum evolution) was calculated using the mean character difference. Negative branch lengths were allowed, but set to zero for tree-score calculation. Steepest descent options were not in effect. Starting tree(s) were obtained via neighbor-joining and bootstrap values were calculated under the same criteria.

To confirm the reliability of the ISSR technique for identifying species, we sent samples of five individuals from Cuba for "DNA barcoding" analysis. Samples were placed in a lysis 96-well plate with a drop of 96% ethanol. Genomic DNA was extracted from tissue and the extraction process was conducted following Ivanova *et al.* (2006). DNA amplification and sequencing followed the protocols of Ivanova *et al*. (2007). Sequences and all collateral data from specimens are available on the BOLD website (www.boldsystems.org) in the project entitled "PVCU".

We obtained functional DNA in 14 samples from Cuba and 20 from Banco Chinchorro. Out of the 23 primers tested on five individuals from each population, 17 produced clear reproducible fragments (Table 1). For this study, we selected five primers (Fig. 2) which presented a high percentage of G/C bases and a high number of bands. Finally, we obtained 113 bands of ISSR fragments $(GACA)_4WB$: 18 bands; $(CA)_8AC$: 17 bands; $(CA)_8AG$: 23 bands; $(AG)_8YC$: 24 band and $(GAG)_5GC$: 31 bands) using 34 individuals from both localities. Table 2 shows the numbers of bands scored, the polymorphic bands present at each locality and the number of diagnostic bands found in only one population (Luque *et al*., 2002). Polymorphism is high (83.5%), with the Cuban samples presenting the lowest value, Global Nei's gene diversity is 0.26 ± 0.017) with Mexican samples presenting the lowest value. AMOVA revealed that the majority of variability occurred among individuals within populations (98%, $P = 0.08$) and this was supported by Nei's genetic distance value which presented very low values between

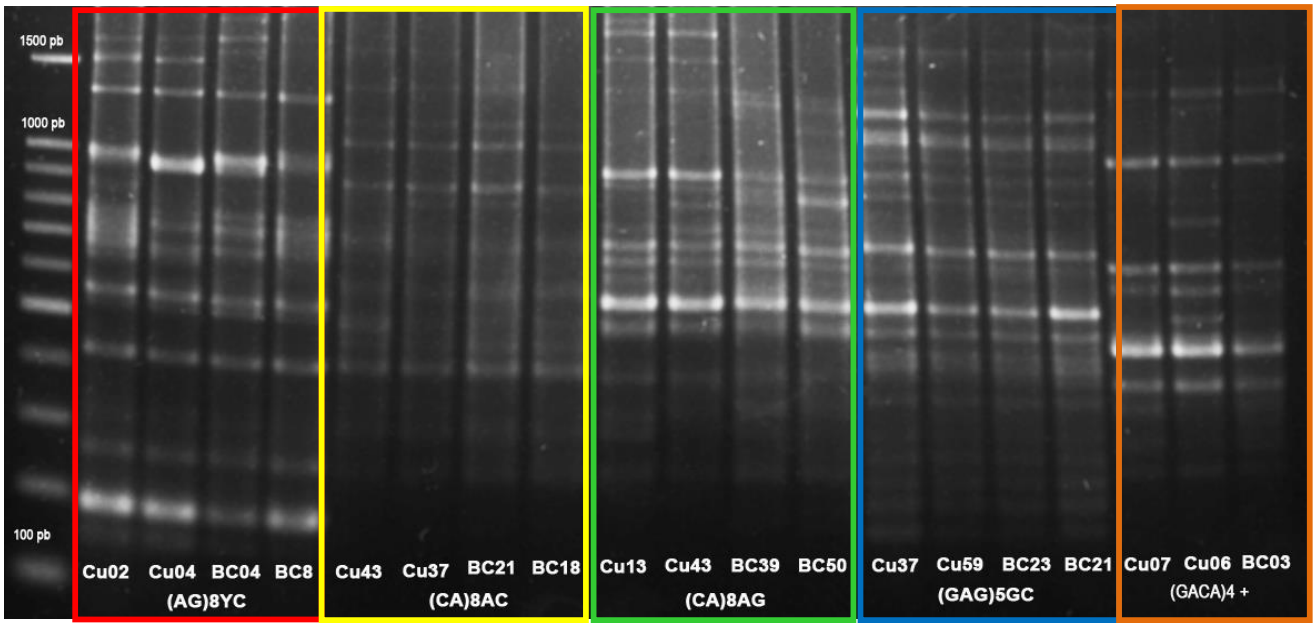

Figure 2. Example of polymorphic banding patterns of the five selected markers [$(AG)_8$YC, $(CA)_8$AC, $(CA)_8$AG, $(GAG)_5$GC, $(GACA)_4$WB] for both localities. The first line corresponds to the DNA ladder (from 100 bp to 1000 bp by steps of 100 bp and one band at 1500 bp), Cu: individuals from Cuba, BC: individuals from Mexico.

both localities (0.035) (Fig. 2). Distance analysis did not demonstrate any significant differences or grouping between our two populations. No clusters were discernible and there were no bootstrap values above 50% (Fig. 3).

Using the tools provided by bold-ids, the obtained DNA barcode confirmed the identification of *P. volitans* in the Guanahacabibes National Park, Cuba, regrouped into the cluster with *P. volitans* individuals from Quintana Roo, previously identified (Valdez-Moreno *et al.*, 2012). The BLAST® tool from GenBank also confirmed the species found in the Guanahacabibes National Park, Cuba, as *P. volitans*.

This first preliminary study identified a high number of ISSR loci for the analysis of genetic variability and population structure of the lionfish in the Caribbean. Due to the high level of ISSR resolution for the study of contemporary events, these markers are excellent candidates for genetic studies of biological invasions.

In this study, the number of bands amplified by five primers were 113 (102 polymorphic) considered sufficient for species determination and studies of population genetics. Indeed, other studies of molecular identification and population genetics in fish species detected a similar number of bands. For example, Casu *et al.* (2009) detected 97 fragments when considering eight primers in *Dentex dentex* L. 1758 (Perciformes, Sparidae). A study on *Cynoglossus semilaevis* Günther, 1873 (Pleuronectiformes, Cynoglossidae) reported 137 fragments using 19 ISSR primers (Liu *et al.*, 2009).

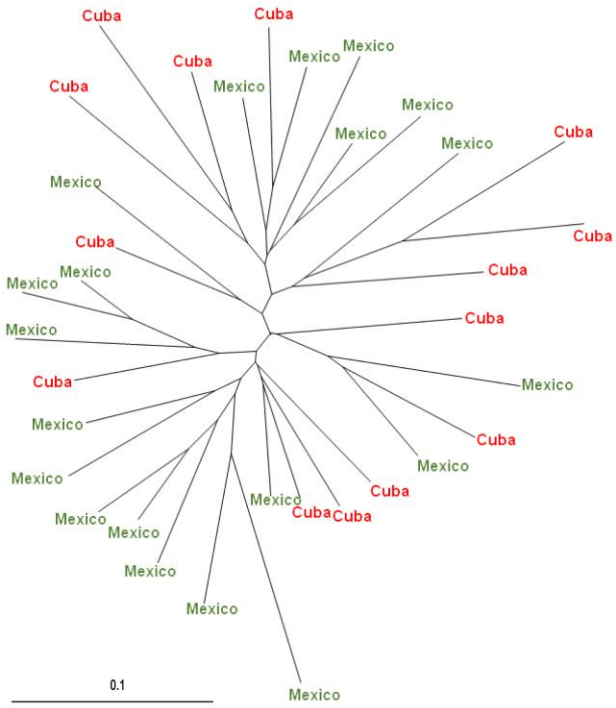

Figure 3. Unrooted tree obtained by distance analysis of lionfish samples from localities in Cuba and Mexico.

Table 2. Lionfish genetic diversity based on ISSR markers in Cuban and Mexican localities. n: number of individuals kept for analysis; N^1: number of bands scored; N^2: number of polymorphic bands; N^3: number of diagnostic bands; *P*: percentage of polymorphism; *h*: Nei's gene diversity; SD: standard deviation.

Locality	n	N^1	N^2	N^3	*P* (%)	*h* (± SD)
Mexico	14	107	82	4	75.2	0.246 (0.018)
Cuba	20	105	79	2	72.5	0.253 (0.019)

Maltagliati *et al*. (2006) recorded 101 bands amplified by nine ISSR primers in cyprinodontiform fish.

This study provides definite confirmation that the species presents in the Guanahacabibes National Park, Cuba is *P. volitans*. Diagnostic bands (Luque *et al*., 2002) are important for the discrimination of species, particularly fish species (Maltagliati *et al*., 2006; Casu *et al*., 2009). The identification of species using the ISSR method always reveals a high number of diagnostic bands (Maltagliati *et al*., 2006; Casu *et al*., 2009). Our study did not identify many diagnostic bands; however, we observed a high number of common bands between both localities, confirming that all the individuals belong to the same species: *P. volitans*. This is further confirmed by the lack of structure in the unrooted tree, the results of AMOVA and the very low genetic distances.

This work is an initial analysis of genetic aspects of the lionfish invasion in Cuba using ISSR. Additionally, we suggest that this technique is an excellent alternative for low-cost genetic monitoring focused on improving control programs of this invasive species in regions with insufficient financial resources.

Given that Guanahacabibes was one of the last places in Cuba to be colonized by lionfish (late 2009) (Chevalier *et al*., 2013), we recommend extending this research to the rest of the Cuban Archipelago. Considering that the first sightings of *P. volitans* were reported on the north coast of Cuba, the genetic analysis of populations both in the north of the archipelago and along the entire Cuban coast is advocated, with the aim of obtaining a complete genetic characterization of this invasive fish in Cuba.

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