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Forensic Application of *Cytb* and *COI* Genes for Freshwater stingray Identification: A Seizure Case in Bogotá, Colombia

Aplicación forense de los genes *Cytb* y *COI* para la identificación de rayas de agua dulce: Un caso de incautación en Bogotá, Colombia

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ABSTRACT

Freshwater stingrays of the Potamotrygonidae family are highly valued in the international market as ornamental fish, leading to growing demand and economic significance. In Colombia, despite legal regulations aimed at protecting these animals and their habitats, the effectiveness of these norms is not always assured. This is partly due to export regulations focusing on morphological criteria for species identification, which may result in inaccuracies, prompting the search for a more precise method based on genetic aspects such as DNA barcoding. This study aimed to assess the potential of the *Cytb* and *COI* genes as molecular markers for identifying a species within the Potamotrygonidae family in Colombia using the barcoding technique. DNA was extracted from blood samples of various stingray species from the Puerto Inírida basin, using PCR to amplify specific regions of the *COI* and *Cytb* genes, which were then sequenced using the Sanger method. The sequences were read in BioEdit, manually cleaned, analyzed in the BLAST program, and aligned in MEGA, where a neighbor–joining tree was constructed using the Kimura 2–parameter model (K2P). Results indicated no genetic identity between the obtained sequences and the unidentified stingray sequence compared to those in GenBank, nor with other analyzed species, both for *COI* and *Cytb*. Phylogenetic analysis showed that the confiscated individual exhibited genetic proximity to *Potamotrygon motoro*. Additionally, a close genetic relationship between *Potamotrygon orbignyi* and *Potamotrygon schroederi* was identified. In conclusion, the phylogenetic method suggests that the seized stingray likely belongs to *Potamotrygon motoro*. Furthermore, the barcoding method alone is not ideal for identifying species of the Potamotrygonidae family

Key words: DNA–Barcoding; Potamotrygonidae; forensic sciences

RESUMEN

Las rayas de agua dulce de la familia Potamotrygonidae son muy valoradas en el mercado internacional como peces ornamentales, lo que lleva a una creciente demanda y significancia económica. En Colombia, a pesar de las regulaciones legales para proteger a estos animales y sus hábitats, la efectividad de estas normas no siempre está asegurada. Esto se debe en parte a que las regulaciones de exportación se centran en criterios morfológicos para la identificación de especies, lo que puede resultar en inexactitudes. Este estudio evaluó el potencial de los genes *Cytb* y *COI* como marcadores moleculares para identificar una especie dentro de la familia Potamotrygonidae en Colombia usando la técnica de código de barras. Se extrajo ADN de muestras de sangre de varias especies de rayas del río Puerto Inírida, utilizando PCR para amplificar regiones específicas de los genes *COI* y *Cytb*, que luego se secuenciaron con el método Sanger. Las secuencias se leyeron en BioEdit, se limpiaron manualmente, se analizaron en BLAST y se alinearon en MEGA, donde se construyó un árbol neighbor–joining usando el modelo de 2 parámetros de Kimura. Los resultados indicaron que no hubo identidad genética entre las secuencias obtenidas y la secuencia de la raya no identificada en comparación con las depositadas en GenBank, ni con otras especies analizadas. El análisis filogenético mostró que el individuo confiscado exhibía proximidad genética a *Potamotrygon motoro*. Además, se identificó una estrecha relación genética entre *Potamotrygon orbignyi* y *Potamotrygon schroederi*. En conclusión, el método filogenético sugiere que la raya incautada probablemente pertenece a *Potamotrygon motoro*. Además, se concluye que el método de código de barras por sí solo no es ideal para identificar especies de la familia Potamotrygonidae.

Palabras clave: Código de barras de ADN; Potamotrygonidae; veterinaria forense

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INTRODUCTION

The Potamotrygonidae family, commonly known as river stingrays or freshwater stingrays, comprises a group of cartilaginous fish found exclusively in South America [1]. These species are distributed in rivers throughout the region, with a notable concentration in the Amazon River basin and its extensive network of tributaries. Among these water bodies are not only the Amazon River itself but also the Orinoco River, Paraná River, Paraguay River, and other smaller river systems that traverse the vast Amazon region and the Southern Cone of South America [1]. Species of *Potamotrygon* reported for Colombia include *Potamotrygon constellata*, *Potamotrygon magdalenae*, *Potamotrygon motoro*, *Potamotrygon orbignyi*, *Potamotrygon schroederi*, *Potamotrygon scobina*, and *Potamotrygon yepezi*, with the genus *Potamotrygon* being the most diverse with 36 species [2, 3, 4, 5, 6]. These species play an important ecological role in regulating fish, crustacean, and insect populations in their aquatic habitats, thus maintaining the balance of the ecosystems where they thrive [7]. In Latin America, the export of these species is a frequent activity, driven by the growing demand in sectors such as aquaristics, gastronomic, artisanal manufacturing (e.g., drums and sandpapers), and alternative medicine. On the other hand, in the commercial sphere, the funds generated through the capture of these stingrays play a significant role in local communities living near rivers, providing a supplementary income of value during several months of the year [8].

To preserve and protect these animal populations, some regulations and laws have been implemented aiming to conserve their habitats, considering that several of them are categorized as near threatened (NT) and vulnerable (VU) [9]. Despite the efforts made by the Colombian government, effective monitoring of stingray populations is not always ensured due to limited information on their handling, taxonomy, human impact (overfishing and bycatch), and the sustainable management of ornamental fishery resources [10, 11]. As an example, fishermen are still allowed to operate under a regime of open access, neglecting the importance of educating about the conservation status of the species and the benefits of sustainable fishing [12]. The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), has played a crucial role in classifying species of the *Potamotrygon* genus subject to stricter regulation regarding their international trade [8]. These species include *P. constellata*, *P. magdalenae*, *P. schroederi*, *P. motoro*, *P. orbignyi*, *P. scobina*, and *P. yepezi*, all included in Appendix III. In turn, the National Action Plan (NAP) established by the Ministry of Environment and Sustainable Development for the Colombian Amazon assigns a very high priority level to obtaining information about the species *P. motoro* and *P. orbignyi* [12]. Restricting the export of species is crucial to mitigate the negative impact on aquaculture populations and their ecosystems. However, accurate species identification during individual capture is crucial. This underscores the importance of employing effective and understandable methods for fishermen, who often distinguish species primarily through morphological aspects, such as the number and arrangement of fins, individual size, and color pattern [13]. However, relying solely on these characteristics for identification can lead to significant errors, prompting the current consideration of molecular factors.

To prevent of illegal exportation, it is necessary for authorities to implement a more precise identification method that allows species determination genetically, such as mitochondrial DNA barcode analysis, a method that has been increasingly adopted by the scientific community for this purpose, as it enables species and subspecies identification [14]. In the field of forensic genetics, barcoding is employed to analyze DNA polymorphisms. This method has been successfully applied in biodiversity conservation, enabling the identification of species at risk or present in protected areas. This information significantly contributes to decision–making for species preservation. Additionally, barcoding has applications in the trade of biological products, such as species recognition in the food market, control of invasive species, or detection of species in traditional medicine products [14]. Furthermore, it has been used in species characterization in ecological and evolutionary studies, enabling the investigation of genetic diversity patterns, phylogenetic relationships, and species migration. Additionally, it has been employed in the identification of a wide range of animal species, including insects such as members of the genus *Spodoptera* (Lepidoptera), as well as Diptera, Psychodidae, Phlebotominae, and Hemiptera. It has also been applied to birds and mammals in forensic investigations. [15, 16, 17, 18, 19]. The most commonly used molecular markers in this type of study are mitochondrial DNA (mtDNA) markers, favored for their high polymorphism, lack of recombination, efficient isolation from minimal biological tissue, and resistance to degradation. As mtDNA is maternally inherited, it is particularly useful for tracing evolutionary relationships and providing unique population–level data [4, 20]. The mitochondrial gene Cytochrome c Oxidase 1 (*COI*) is widely employed in barcoding due to its conserved regions, which allow for universal primer design across diverse organisms, and its effectiveness in species discrimination [20]. Similarly, the cytochrome b gene (*Cytb*) is valuable for differentiating closely related species and providing robust phylogenetic insights [17].

The aim of this study was to assess the potential of the *Cytb* and *COI* genes as molecular markers for species identification within the Potamotrygonidae family in Colombia through the barcoding technique.

MATERIALS AND METHODS

Case history

Fishermen from Guainía, Colombia, involved in artisanal ornamental fishing, sold a batch of freshwater stingrays (*Potamotrygon* spp.) to a Bogotá–based export company. After quarantine protocols by ICA, stingrays were selected for export to Vietnam, complying with CITES permits issued by AUNAP, ICA, and SDA. During a routine inspection at El Dorado International Airport, a stingray specimen exhibited atypical morphological traits, lacking the characteristic ocelli of *P. motoro* (brown–olive to dark gray with yellow–orange spots) [9] (FIG. 1). Due to concerns of illegal trafficking, its export was halted, and genetic analysis was initiated to confirm its species.

Blood samples were extracted from six specimens belonging to the species *P. motoro* (n=2), *P. orbignyi* (n=2), and *P. schroederi* (n=1), as well as from one taxonomically unidentified individual (n=1), which was confiscated at El Dorado International Airport, Bogotá, Colombia. These individuals were acquired from the same ornamental fish establishment in Bogotá, Colombia, where the stingray was previously seized. This center reported that the stingrays originated from the Puerto Inírida basin. This information

FIGURE 1. Confiscated individual at El Dorado Airport, Bogotá, Colombia

is relevant for achieving a more precise approach if conclusive results are not obtained through the BLAST (Basic Local Alignment Search Tool) search in GenBank. Blood samples were obtained by immersing the stingrays in a solution of 1 liter of water and Eugenol at a concentration of 40 mg·L^{-1} for 5 min to sedate them. Subsequently, a 3 to 5 mL blood sample was extracted from the caudal vein located under the tail. These samples were promptly transferred to a Vacutainer tube containing Ethylenediaminetetraacetic acid (EDTA) for preservation and storage.

Genomic DNA extraction was performed using the Monarch® Genomic DNA Purification Kit (New England Biolabs, Ipswich, MA, USA) following the manufacturer's instructions. The collected genetic extract was quantified using a DeNovix® DS–11 FX+ nanodrop (Denovix Inc. Wilmington, USA). In preparation for Polymerase Chain Reaction (PCR), the DNA extracts from each sample were diluted to a concentration of 40 ng·μL^{-1}. Subsequently, a mixture of the reagents to be used was prepared in 1.5 mL conical tubes, with each tube containing 4.5 µL magnesium chloride ($MgCl_2$) (2.25 mM), 4 µL dNTPs (0.8 mM), 0.62 µL of each primer (1.25 mM), 5 µL buffer, 1 µL Taq (1U), 33.2 µL water, and 1 µL of DNA, resulting in a total volume of 50 µL per tube. The processing of the samples, extraction, and amplification were carried out in the Laboratory of Molecular Population Genetics and Evolutionary Biology at the Pontificia Universidad Javeriana.

The primers FPmagCOI531 (5'–GCAATCTCTCAATACCAAACACCAC–3') and RPmagCOI1432 (5'–CGTTTTGATGCAAATGCTTCTCAGAG–3') [21], were used for the *COI* gene, while the primers FPmagCYTB182 (5'–CATCAGCCTTCTCCTCMATCGCAC–3') and RPmag932 (5'–CGGAAGGTGAGGCTTCGTTGTTTGG–3') [21] were used for the *Cytb* gene. The amplification protocol was performed using a T100® Thermal Cycler from BioRad (Bio–Rad Laboratories, California, USA) under the following conditions: an initial denaturation at 94°C for 5 min, followed by 25 cycles comprising denaturation at 94°C for 30 seconds (s), an annealing phase at 60.1°C (*COI* gene) or 58°C (*Cytb* gene) for 30 s, an extension at 72°C for 30 s, and a final extension at 72°C for 7 min. The resulting samples were stored at 4°C in a refrigerator (Supernordico, Colombia). Subsequently, the amplicons were verified through agarose gel electrophoresis (Thermo EC 330, USA) at 0.8% using the Quick–Load® Purple 1 kb Plus DNA Ladder molecular weight marker. Once amplification was confirmed, the resulting DNA was sent to the GenCore laboratory at University of the Andes in Bogotá, Colombia.

The purified products after PCR amplification were automatically sequenced in both directions (5' to 3' and 3' to 5') using the tagDye Deoxy Terminator Cycle–sequencing method on a 3730XL sequencer (Applied Biosystems, USA). The generated electropherograms were analyzed using BioEdit® v.7.7.1 and PeakTrace® v.6.6 software to obtain the DNA sequences. These sequences were aligned using MEGA® v.11 software. Intra–and interspecific genetic distances were calculated using the neighbor–joining (NJ) evolutionary method and the Kimura 2-parameter (K2P) substitution model [22]. The resulting dendrogram was obtained in MEGA® v.6.0 with 1000 bootstrap replicates [23]. All analyzed sequences were submitted to the National Center for Biotechnology Information (NCBI) and assigned unique accession numbers: PP873634 to PP873638 for *COI* and PQ015594 to PQ015598 for *Cytb*. The GenBank BLAST tool was used to identify species with the highest similarity and alignment length percentage. For phylogenetic analyses involving other Colombian stingray species, including those obtained in this study, the following GenBank sequences were considered: MW482070.1 (*P. schroederi – Cytb*), MW482065.1 (*P. orbignyi – Cytb*), MW481862 (*P. magdalenae – Cytb*), MW481840.1 (*P. hystrix – Cytb*), MW482068.1 (*P. motoro – Cytb*), MW482173.1 (*P. magdalenae – COI*) [24], NC_023116 (*P. motoro* – complete genome) [25], EF532673.1 (*P. schroederi – COI*), EF532666.1 (*P. orbignyi – COI*) [26], and MK520996.1 (*P. hystrix – COI*) [2]. Finally, the two analyzed genes were concatenated to obtain more reliable information, considering the sequences obtained from the Puerto Inírida individuals.

RESULTS AND DISCUSSION

Amplification for the *COI* and *Cytb* genes for the *Potamotrygon* genus was successful using primers originally designed for the species *P. magdalenae* [21]. Manual filtering of the sequences yielding the following results regarding sequence lengths for the *COI* gene, ranging from 612 bp for *P. schroederi* to 689 bp for one of the individuals of the unknown species. For the *Cytb* gene, sequence sizes ranged from 620 bp for *P. schroederi* to 728 bp for one of the two individuals of the species *P. orbignyi*. From these, 34 variable positions were found in the *COI* gene sequence (FIG. 2) and 50 in the *Cytb* gene sequence (FIG. 3). In the former, a similarity was observed between *P. orbignyi* and *P. schroederi*, as well as between *P. motoro* and the unknown stingray species. In both cases, the sequences exhibited significant similarity due to a higher number of shared nitrogenous bases among the species. However, for the *Cytb* gene, 3 positions (2 transitions and 1 transversion) were found to have changes between the sequences of the species *P. orbignyi* and *P. schroederi*. Additionally, the sequence of the unknown species stingray showed variation in 5 transversions compared to the sequences of *P. motoro*. Finally, it was identified that the sequences belonging to *P. orbignyi* showed 2 transitions and 1 transversion compared to the other species, found from position 594 to 596, suggesting that this change may be significant in differentiating this species from the others studied in this family.

COI																																				
Posición (pb)	191	194	215	239	242	251	260	305	311	323	353	371	377	386	410	413	419	422	425	431	434	452	470	482	488	506	509	563	566	579	581	584	599	605
P. orbignyi 2	C	C	C	A	C	A	T	T	C	C	C	C	T	T	G	T	G	T	C	C	C	G	C	C	T	T	T	C	T	T	A	A	C	C
P. orbignyi 1	C	C	C	A	C	A	T	T	C	C	C	C	T	T	G	T	G	T	C	C	C	G	C	C	T	T	T	C	T	T	A	A	C	C
P. motoro 2	T	T	T	C	T	G	C	C	T	T	T	T	C	C	A	C	A	C	A	T	T	A	T	T	C	C	A	A	A	C	G	C	T	T
P. motoro 1	T	T	T	C	T	G	C	C	T	T	T	T	C	C	A	C	A	C	A	T	T	A	T	T	C	C	A	A	A	C	G	C	T	T
P. schroederi	C	C	C	A	C	A	T	T	C	C	C	C	T	T	G	T	G	T	C	C	C	G	C	C	T	T	T	C	T	T	A	A	C	C
Sp. desconocida	T	T	T	C	T	G	C	C	T	T	T	T	C	C	A	C	A	C	A	T	T	A	T	T	C	C	A	A	A	C	G	C	T	T

FIGURE 2. Variable positions (bp) in the *COI* gene sequences obtained from individuals of the genus *Potamotrygon* and the unknown stingray species (generated using MEGA software)

CYTB																																						
Posición (pb)	94	110	115	128	136	142	171	181	193	199	202	223	243	257	263	265	271	276	322	325	334	352	394	445	451	454	484	502	511	514	517	533	542	545	551	556	561	565	575	592	594	595	596	601	609	610	625	652	661	673
P. orbignyi 2	C	C	C	T	T	C	T	T	A	T	T	C	T	A	T	T	A	C	C	G	T	C	A	C	T	T	T	T	G	T	A	A	A	G	G	C	C	T	G	T	C	A	T	C	C	C	T	C	T	C
P. orbignyi 1	C	C	C	T	T	C	T	T	A	T	T	C	T	A	T	T	A	C	C	G	T	C	A	C	T	T	T	T	G	T	A	A	A	G	G	C	C	T	G	T	C	A	T	C	C	C	T	C	T	C
P. motoro 2	C	T	T	C	C	T	C	C	T	C	T	T	C	A	C	C	G	A	T	A	C	T	C	T	A	C	C	C	A	C	C	C	G	A	A	A	T	C	A	C	T	C	C	T	T	T	C	T	C	T
P. motoro 1	C	T	T	C	C	T	C	C	T	C	T	T	C	A	C	C	G	A	T	A	C	T	C	T	A	C	C	C	A	C	C	C	G	A	A	A	T	C	A	C	T	C	C	T	T	T	C	T	C	T
P. schroederi	C	C	C	T	T	C	T	T	A	T	T	C	T	A	T	T	A	C	C	G	T	C	A	C	T	T	T	T	G	T	A	A	A	G	G	C	C	T	G	T	T	C	C	C	C	C	T	C	T	C
Sp. desconocida	T	T	T	C	C	T	C	C	T	C	C	T	C	G	C	C	A	A	C	A	C	T	C	T	A	T	C	C	A	C	C	C	G	A	A	A	T	C	A	C	T	C	C	T	T	T	C	T	C	T

FIGURE 3. Variable positions (bp) in the *Cytb* gene sequences obtained from individuals of the genus *Potamotrygon* and the unknown stingray species (generated using MEGA software)

BLAST analysis for the *COI* Gene

According to the results obtained using the BLAST tool to analyze the *COI* gene sequences in the GenBank database, there is significant genetic similarity between the unidentified stingray and the species *P. falkneri* (98.55%) and *P. motoro* (98.40%), based on the percentage of identity. Although *P. falkneri* shows a higher identity percentage, there are no records of its presence in the Puerto Inírida basin, the location where the stingrays were obtained [8]. Therefore, it is inferred that the unidentified stingray is more closely related to *P. motoro*. For the other individuals analyzed with the BLAST tool in this study, where the species were identified, it was found that the sample from the *P. schroederi* stingray shows the highest genetic similarity, with a 99.76% identity percentage with *P. schroederi* and 94.9% with *P. magdalenae*, with coverages of 68% and 100%, respectively.

Regarding one of the samples of the *P. orbignyi* species, identity percentages of 94.29% with *P. magdalenae*, 91.90% with *P. motoro*, and 91.75% with *P. orbignyi* were identified, each with a coverage of 100%. For the second sample of *P. orbignyi* analyzed, a similarity was observed with the same species identified in the previous sample, with identity percentages of 94.45%, 92.21%, and 92.06%, and coverages of 99%, 99%, and 98%, respectively.

Concerning the two samples analyzed for the species *P. motoro*, both align with the species' identification according to the BLAST results from GenBank. However, it is noteworthy that BLAST reveals a significant genetic similarity to *P. falkneri*. In one of these samples, an identity percentage of 98.20% and a coverage of 100% were recorded for both *P. motoro* and *P. falkneri*. In the second sample, an identity percentage of 98.52% with a coverage of 99% was observed for *P. falkneri*, while for *P. motoro*, it showed an identity percentage of 98.05% with a coverage of 100%.

The high genetic similarity between *P. falkneri* and *P. motoro*, reported by Cruz *et al.* [2], includes documented cases of hybridization, attributed to ecological proximity, genetic relationship, and the same number of chromosomes. Additionally, Sanches *et al.* [24] indicate hybridization events among other species of *Potamotrygon* (*P. motoro*, *P. orbignyi*, *P. scobina*, and *P. leopoldi*) in the Xingu River basin, Brazil. Therefore, further research is suggested to determine whether *P. falkneri* constitutes an independent species or, conversely, a subspecies of *P. motoro*.

BLAST analysis for the *Cytb* Gene

Contradictory results were observed in the genetic analysis using BLAST to determine the species of the stingray under investigation. For the *Cytb* gene, the first result indicated *P. orbignyi* with an identity percentage of 98.78%, while the second result was *P. motoro* with 97.56%. In contrast, for the *COI* gene, the primary result was *P. falkneri*, followed by *P. motoro*. Although both analyses converge on identifying *P. motoro*, the discrepancy between the top species indicated for each gene complicates the accurate determination of the stingray species. Upon analyzing the other individuals, a significant match was observed in the genetic sequence of the *P. schroederi* individual with a GenBank sequence corresponding to *P. schroederi* and another to *P. orbignyi*, sharing a coverage of 79% and an identity percentage of 100% with both. However, identity percentages of 93.23, 93.06, and 92.58% were obtained with the species *P. magdalenae*, *P. hystrix*, and *P. yepezi*, respectively, with a coverage of 100% for these three. Although a high identity percentage was found, the low coverage makes the data inconclusive for this sample. Similarly, the high identity percentage with various species within the genus complicates the correct classification of the animal into one of these.

Regarding the two analyzed individuals of *P. orbignyi*, a match is evident with the GenBank sequences corresponding to *P. orbignyi*, revealing an identity percentage of 99.66% with a coverage of 81% for the first specimen and an identity percentage of 100% with a coverage of 80% for the second. These results represent conclusive findings, indicating a high match of the samples with the GenBank sequences for this species.

Exploring barcoding in Colombian freshwater stingrays

The findings of this study align with the observations of Toffoli *et al.* [25], where the application of barcoding did not yield conclusive results. During the BLAST analysis, the identity percentages of the samples matched various species of *Potamotrygon*, suggesting that this genus may maintain a high degree of genetic homogeneity, which complicates the precise identification of its species through barcoding. Additionally, it is noted that mtDNA sequences are not effective for accurately establishing species boundaries, especially in recently evolved species. Contrary to these conclusions, Cerutti *et al.* [26] argue that this method is highly useful for stingrays and sharks. Similarly, Pereira *et al.* [27] support this stance by demonstrating the efficacy of barcoding in identifying recently evolved megadiverse fauna, successfully discriminating 99.2% of the species analyzed in their study. In line with the issues identified by Toffoli *et al.* [25], Li *et al.* [28] propose barcoding as a strategy to detect potential errors in GenBank data, specifically using the *Cytb* gene.

On the other hand, an internationally established species–specific barcode for fish, based on the mitochondrial *COI* gene, has been developed. This barcode consists of DNA fragments ranging from 600 to 650 base pairs. However, it has been suggested that only 100 base pairs in a DNA sequence are sufficient to adequately identify and distinguish species [29, 30]. In the present study, the obtained sequences ranged from 500 bp to 900 bp, theoretically providing an adequate size for species identification. However, the desired results were not achieved, as not all samples matched their respective species during the BLAST analysis. In some cases, they matched with more than one species depending on the identity percentage. This suggests that sequences with a greater number of base pairs or the study of additional genes may be necessary to effectively identify the known or unknown species of the genus *Potamotrygon* found in the Inírida area.

Phylogenetic analyses for the COI gene

Phylogenetic analysis of *COI* gene sequences strongly supports a monophyletic clade containing *P. orbignyi* and *P. schroederi* (bootstrap=100), confirming their close evolutionary relationship. The topology further resolves *P. motoro* and an unidentified stingray as sister taxa (Fig. 4). Comparative analysis revealed greater phylogenetic utility of *Cytb* over *COI*, with *Cytb* exhibiting higher nucleotide variability despite conservative substitution patterns in both markers.

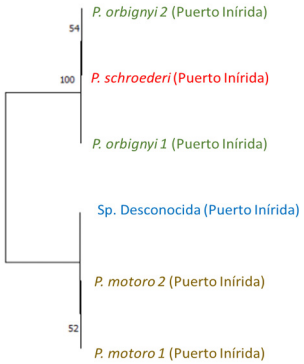

FIGURE 4. Resulting neighbor–joining tree for the analysis of *COI* gene sequences from individuals originating from Puerto Inírida, using the Kimura 2-parameter (K2P) model

Phylogenetic analyses using sequences downloaded from GenBank for the *COI* gene

Phylogenetic analysis of *COI* gene sequences demonstrated clustering of our *P. motoro* specimens with GenBank references (bootstrap=51), while *P. schroederi* formed an intermediate clade with *P. orbignyi* (bootstrap=47). Notably, *P. orbignyi* references exhibited stronger affinity to *P. motoro* (bootstrap=59), suggesting potential biogeographic or mutational influences. In contrast, *P. magdalenae* and *P. hystrix* occupied phylogenetically distinct positions (Fig. 5), consistent with their allopatric distribution.

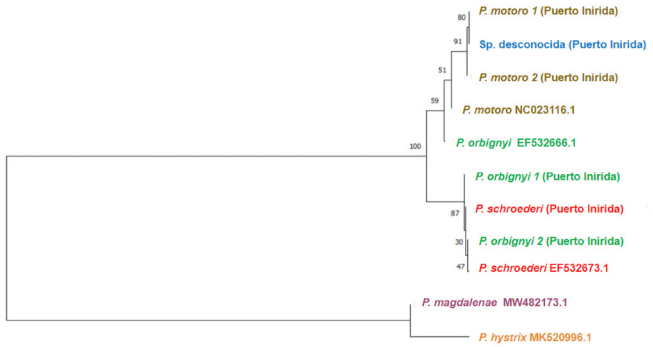

FIGURE 5. Phylogenetic relationship between GenBank sequences and individuals from the present study originating from Puerto Inírida for the *COI* gene, based on a neighbor–joining tree using the K2P model

Phylogenetic analysis of the *Cytb* gene

Phylogenetic analyses of the *Cytb* gene resolve *P. orbignyi* and *P. schroederi* as sister taxa (Fig. 6), a relationship corroborated by *COI* gene topology. Notably, an unidentified stingray specimen clusters with *P. motoro* in both gene trees, reinforcing the consistency of these phylogenetic associations.

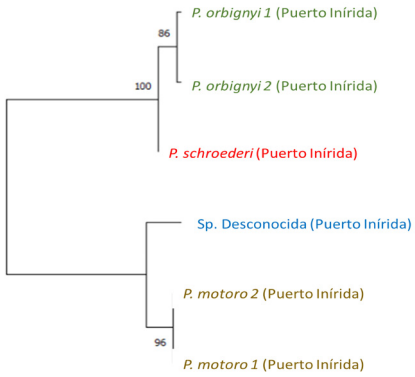

FIGURE 6. Resulting neighbor–joining tree for the analysis of the *Cytb* gene with individuals from Puerto Inírida, using the K2P model

Phylogenetic analyses using sequences obtained from GenBank for the *Cytb* gene

Based on the analysis of the results obtained through phylogenetic trees for the *Cytb* gene, the association of *P. schroederi* and *P. orbignyi* in the same clade (BP=100) was observed. Additionally, the association among individuals of *P. motoro* in a separate clade (BP=71) was highlighted (FIG. 7). However, unlike the *COI* gene tree, a closer relationship of *P. hystrix* (BP=54) to the sequences of *P. motoro* and the unknown stingray from Puerto Inírida (BP=100) was evidenced, while *P. magdalenae* was placed in a separate clade between the two main clades. This finding is of particular importance, as it reveals an association between the unknown species and *P. motoro* in the phylogenetic tree of the *Cytb* gene, consistent with previous analyses.

In this study, it was observed that the sequences in the *COI* gene are more conserved, which may hinder species classification. In contrast, the *Cytb* gene showed greater affinity for this task by presenting a higher number of variable positions among species, highlighting its utility for more accurate species classification and analysis methods (BLAST and phylogeny). Additionally, a marked genetic closeness was found among the sequences of the genus *Potamotrygon* for the two genes studied. Therefore, the use of a greater number of genes and sequences from other species is suggested to improve classification at this level, supporting the results presented by Toffoli *et al.* [25].

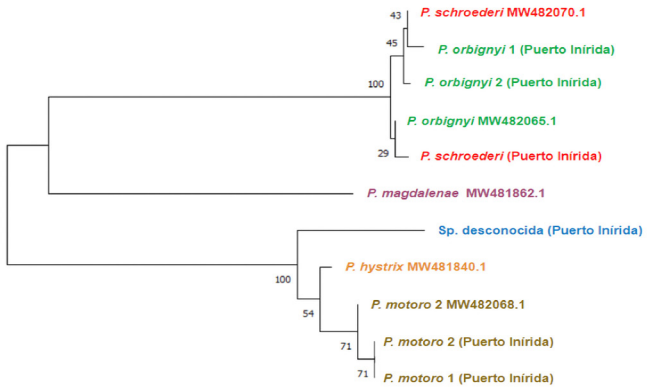

FIGURE 7. Phylogenetic relationship between GenBank sequences and the individuals analyzed in the present study for the *Cytb* gene, based on a neighbor–joining tree using the K2P model

Phylogenetic analysis for concatenated *COI* and *Cytb* genes

The concatenation of the *COI* and *Cytb* genes confirms the observations from the individual gene trees, supporting the relationship between the unknown stingray species and *P. motoro*, suggesting the possibility that it belongs to this species. Furthermore, the tree shows that *P. orbignyi* and *P. schroederi* cluster in the same clade, indicating a close evolutionary relationship between these species (FIG. 8). Regarding the phylogeny of *Potamotrygon*, Lovejoy [31] suggests that neotropical freshwater stingrays share a common ancestor that invaded these ecosystems during a Miocene invasion (which began 23.03 million years ago and ended 5.332 million years ago). The diversification of the Potamotrygonidae family is attributed to an endemic evolutionary radiation in South America, linked to geological events that formed the Amazon and Orinoco basins. This research justifies the low diversification of the family due to the endemic evolutionary radiation, which established physical barriers and intraspecific variation (phenotypic differences within the same species).

It is important to consider that phylogenetic relationships are influenced not only by time but also by biogeography and the distribution patterns of freshwater stingray groups [32]. As a result, the phylogenetic tree not only sheds light on the evolutionary history of species with a common ancestor but also reveals the temporal progression of their evolutionary trajectories [33]. This phenomenon is clearly manifested in the associations observed in the trees, where it is possible to identify the grouping of more than one species in the same clade.

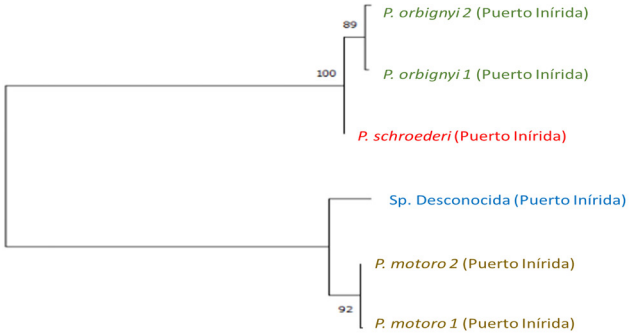

FIGURE 8. Resulting neighbor–joining tree from the concatenation of the *COI* and *Cytb* genes for individuals from Puerto Inírida, using the K2P model

Regarding the coloration of the confiscated species

Regarding the unidentified stingray, the possibility of pathological processes affecting the phenotype of the studied individual is considered, as it presents depigmented areas of skin (FIG. 1). One potential pathology is infection by *Flexibacter columnaris*, caused by a group of bacteria belonging to the Myxobacteria, which manifests as an opportunistic disease, particularly under stress or poor water quality conditions. These bacteria are common in aquatic environments, colonizing the gills and skin of fish. Lesions often appear as small white spots on the caudal fin, progressing towards the head. The caudal and anal fins can be severely affected, showing erosions. In more advanced stages of the disease, the fish's skin may present numerous white or grayish ulcers, potentially compromising the gills [34].

Additionally, this finding is consistent with a possible infection by *Mycobacterium*, which manifests on the fish's surface with clinical signs including emaciation, inflamed skin with loss of color, vertebral deformation, open lesions, and ulcers [34]. Similarly, infection by *Aeromonas* causes hemorrhagic ulcerative formations on the skin, typically around the periocular area, edema

in different parts of the body, desquamation, and in some cases, depigmentation [35]. Nutrient deficiencies could also be related to the appearance of skin spots. For instance, linoleic acid and linolenic acid cannot be synthesized by the fish, and their deficiency can lead to depigmentation, fin erosion, and fatty infiltration of the liver [34].

Another important consideration is that the coloration of fish skin results from the interaction of various types of chromatophores. A possible genetic pathology affecting the individual could be leucism, attributed to the mutation of recessive genes during embryonic development, inhibiting the migration of melanoblasts (pigment–producing cells), resulting in partial or total absence of pigmentation in the skin while maintaining normal retinal pigmentation. However, the etiology of these chromatic variations can be multifactorial, involving non–pathological genetic mutations, population isolation, environmental stress, and heavy metal exposure [36].

CONCLUSION

The results obtained in this study suggest limitations in the effectiveness of the *Cytb* and *COI* genes as molecular markers for identifying species within the Potamotrygonidae family. Based on the analysis conducted using the BLAST tool from GenBank and the phylogenetic relationships established through the neighbor–joining methodology, it is suggested that the stingray in question likely belongs to the species *Potamotrygon motoro*, indicating that its trade is legal and does not involve illegal trafficking of wild species. Furthermore, the analysis of the *Cytb* gene, due to its greater genetic variability, demonstrates a stronger association and suitability for phylogenetic identification compared to the *COI* gene. It is also emphasized that combining both genes yields more reliable results. However, it is important to note that barcoding alone is not the ideal technique for identifying species within the Potamotrygon genus; integration with other disciplines, such as ecology and biogeography, along with knowledge of morphological and behavioral characteristics, is necessary.

Conflict of interest

The authors declare that they have no conflict of interest to disclose.

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