ORIGINAL RESEARCH

Fishery and conservation implications of molecular characterization and traceability of ceviche samples from Pacific Panama

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ABSTRACT. Genetic analysis of 111 samples from ceviche cocktails and fish fillets used for ceviche, obtained from fish markets and processing plants in the Pacific zone of Panama were conducted to determine species composition, trace origin (native, nonnative or imported frozen species) and CITES species status. A total of 21 species were detected (20 fishes and one invertebrate): *Coryphaena hippurus* (dolphin fish), *Pangasianodon hypophthalmus* (basa), *Trachinotus falcatus* (pompano), *Cyclopsetta querna* (toothed flounder), *Atheresthes stomias* (arrow-tooth flounder), *Lobotes pacificus* (Pacific tripletail), *Bagre panamensis* (Chihuil sea-catfish), *B. bagre* (Coco sea-catfish), *Ariopsis seemanni* (Tete sea-catfish), *Aspistor luniscutis* (yellow sea-catfish), *Centropomus viridis* (white snook), *C. undecimalis* (Union snook), *Sphyrae naensis* (Mexican barracuda), *Oreochromis niloticus* (Nile tilapia), *O. mossambicus* (Mozambique tilapia), *Cynoscion praedatorius* (Boccone weakfish), *Protonibea diacanthus* (blackspotted croaker), *Gadus chalcogrammus* (Alaska pollock), *Sphyrna lewini* (scalloped hammerhead shark), *Makaira nigricans* (blue marlin) and *Dosidicus gigas* (giant Humbolt squid). Native species found in ceviche samples were reduced in numbers compared with imported and cultivated ones. Thus, the most common detected fish species was basa, followed by the Nile tilapia and the dolphin fish. This is a positive result in terms of sustainability of local fisheries, since basa is imported as frozen fish meat from Asia. The same applies for Nile tilapia, a cultivated freshwater species not captured from local fisheries. For the dolphin fish, despite being common and exploited in Pacific waters, previous studies suggest its fishery is sustainable in Panama waters. In terms of conservation status, one species cataloged by IUCN as vulnerable (VU), the blue marlin (*M. nigricans*) and one as critically endangered (CR), the scalloped hammerhead shark (*S. lewini*) were detected. *Sphyrae lewini* is also catalog as CITES appendix II. The giant Humbolt squid (*D. gigas*), classified by IUCN as data deficient (DD), was the only invertebrate detected in samples obtained from a ceviche processing plant. Two sets of primers and dual labeled probes were designed for qPCR eDNA detection of the only CITES species, *S. lewini*. These represent the first qPCR markers for eDNA detection of *S. lewini*. Results from this project promote the sustainable use of fishery resources and might provide ceviche producers with a certificate from MarViva Foundation certifying that their ceviche is free of sharks or species threatened/protected by law, giving added value to their product. Molecular detection and molecular traceability are sensitive and species specific, what makes of this tool a reliable method to combat IUU (illegal, unreported and undocumented) fishing.

Key words: CITES species, molecular traceability, overexploitation, supply chain, elasmobranchs, native species, non-native species, environmental DNA.

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Implicancias para la pesca y la conservación de la caracterización molecular y la trazabilidad de muestras de ceviche del Pacífico de Panamá

RESUMEN. Se realizaron análisis genéticos de 111 muestras de cócteles de ceviche y filetes de pescado utilizados para ceviche, obtenidas en lonjas y plantas de procesado de la zona Pacífica de

Panamá para determinar la composición por especies, el origen y la trazabilidad de éstas (especies autóctonas, no autóctonas o importadas congeladas) y el estatus de especies CITES. Se detectó un total de 21 especies (20 peces y un invertebrado): *Coryphaena hippurus* (dorado), *Pangasianodon hypophthalmus* (basa), *Trachinotus falcatus* (pompano), *Cyclopsetta querna* (platija dentada), *Atheresthes stomias* (platija diente de flecha), *Lobotes pacificus* (berrugate del Pacífico), *Bagre panamensis* (bagre, chihuil), *B. bagre* (bagre doncella), *Ariopsis seemanni* (bagre tete), *Aspistor luniscutis* (bagre amarillo), *Centropomus viridis* (róbalo plateado), *C. undecimalis* (róbalo blanco), *Sphyrae naensis* (barracuda mexicana), *Oreochromis niloticus* (tilapia del Nilo), *O. mossambicus* (tilapia de Mozambique), *Cynoscion praedatorius* (corvina bocona), *Protonibea diacanthus* (corvina negra), *Gadus chalcogrammus* (abadejo de Alaska), *Sphyrna lewini* (tiburón martillo), *Makaira nigricans* (merlín azul) y *Dosidicus gigas* (calamar gigante de Humbolt). Las especies autóctonas encontradas en las muestras de ceviche eran menos numerosas que las importadas y cultivadas. Así, la especie de pescado detectada con más frecuencia fue el Basa, seguida de la tilapia del Nilo y el dorado. Se trata de un resultado positivo en términos de sostenibilidad de la pesca local, ya que el Basa se importa de Asia como carne de pescado congelada. Lo mismo ocurre con la tilapia del Nilo, una especie cultivada de agua dulce que no se captura en las pesquerías locales. En cuanto al dorado, a pesar de ser común y explotado en aguas del Pacífico, estudios previos sugieren que su pesca es sostenible en aguas panameñas. En cuanto al estado de conservación, se detectó una especie catalogada por la UICN como vulnerable (VU), el Merlín azul (*M. nigricans*) y otra en peligro crítico (CR), el tiburón martillo festoneado (*S. lewini*). *Sphyrna lewini* también está catalogado como CITES: apéndice II. El calamar gigante de Humbolt (*D. gigas*), clasificado por la UICN como de datos insuficientes (DD), fue el único invertebrado detectado en las muestras obtenidas en una planta procesadora de ceviche. Se diseñaron dos conjuntos de cebadores y sondas de doble etiquetado para la detección qPCReDNA de la única especie CITES, *S. lewini*. Estos representan los primeros marcadores qPCR para la detección eDNA de *S. lewini*. Los resultados de este proyecto promueven el uso sostenible de los recursos pesqueros y podrían proporcionar a los productores de ceviche un certificado de la Fundación MarViva de ceviche libre de tiburones o especies amenazadas/protegidas por la ley, que dan un valor añadido a su producto. La detección y trazabilidad molecular son sensibles y específicas para cada especie, lo que hace de esta herramienta un método fiable para combatir la pesca Ilegal, No declarada e Indocumentada (INDNR).

Palabras clave: Especies CITES, trazabilidad molecular, sobreexplotación, cadena de suministro, elasmobranquios, especies autóctonas, especies no autóctonas, ADN ambiental.

INTRODUCTION

Traceability is defined as the group of procedures that allow stakeholders, business owners, and scientists to determine origin of a species or a product since its capture or processing along the supply chain until this species or product is imported or consumed by local population (Díaz-Ferguson et al. 2023). Traceability gives a track of the production process, origin or capture zone and exportation of a product or species that must be available in a traceability certificate and a certificate of origin of the product that warrants the quality, identity, and safety (Ogden 2008). This information is also useful for conservation, commercialization, understanding market preferences and new trends and protection of marine species and provides to consumers the best quality products minimizing misleading to consumers by labeling fraud (Wong and Hanner 2008).

There are three types of traceability: 1) physical (use of electronic devices or physical tags), 2) chemical (using chemical isotopes), and 3) molecular (using molecular markers). Molecular traceability is defined as the use of molecular markers for determine species composition and tracking a species or product (Díaz-Ferguson 2012). Molecular characterization through barcode have demonstrated to be an effective way to identify and track natural, imperil, and cultivated populations (Galimberti et al. 2013). The use of barcode of life in fish populations started in 2003 (Hebert et al. 2003). Since then, more than 5,000 species of fishes have been characterized by this method (Ward et al. 2009). Genetic diversity data obtained by barcode method allowed to understand genetic structure and connectivity of marine populations (Díaz-Ferguson 2012; Díaz-Ferguson et al. 2012. This genetic information is key for sustainable fisheries (Hauser et al. 2002; Díaz-Ferguson et al. 2010). In addition, barcode have demonstrated being effective in species detection of processed

food and fishery products providing important information about the fishery and determining new trends in use and consumption of natural marine resources (Khaksar et al. 2015). The traceability certificate also allows us to track and register a record of species composition and origin from an elaborated product like ceviche (Borit and Olsen 2012). Ceviche is a raw fish cocktail that consist of small pieces of fish assorted with lemon and spices. This dish represents one of the most common and traditional dishes sell in Central America, Panama, Colombia, Ecuador, and Peru. However, in some cases, CITES (The Convention on International Trade in Endangered Species of Wild Fauna and Flora), banned and endangered species such as sharks and marlins are used to create this dish. This practice can be regulated and penalized using the right detection method.

Through this research, we sought to provide information on the main teleost species used for ceviche in the Pacific Panama using barcode as molecular traceability method. This analysis will be effective in checking the origin and conservation status of detected species. This information promotes good conservation and sustainability practices among *cevicheros* providing a seal of sustainability and good practices to those markets, restaurants and ceviche processing plants that sell ceviche free of endangered, VU or CITES species. This seal of sustainability and good practices will be an added value to their products and a support for conservation of marine species.

Sequence data of analyzed samples create a genetic database of teleost fish that could be useful to establish a pilot program of molecular traceability of main commercial and consumed fish species from the Pacific Panama. It will also be useful to understand the stock genetic structure, connectivity patterns and fishery status of commercialized species, as well as helping us trace the history and location of the fishery, detect mislabeled products, finding new trends in fish commercialization and potential uses. Additionally, fishery biologists will be able to determine the origin (native, nonnative, imported frozen species and fishery area) and conservation status of detected species by identifying species composition. This information is essential for fisheries transparency, helping to enforce regulations and combat IUU (illegal, unregulated, and unreported fisheries) (Costa Leal et al. 2015). Currently, molecular traceability and genetic studies of teleost fish populations and cartilaginous fishes (sharks and rays) are reduced in the Pacific Panama and Latin America. Thus, for cartilaginous fish in Panama, studies are mainly focused on pelagic species along the eastern tropical Pacific such as the whale shark (*Rhincodon typus*) and the hammerhead shark (*Sphyrna lewini*) by examining connectivity and migratory patterns (Nance et al. 2011; Guzmán et al. 2021). Recently, two demersal species have been studied genetically the white tip reef shark (*Triaenodon obesus*) (Díaz-Ferguson and Guzman in preparation), the brown smoothhound (*Mustelus henlei*) and the sicklefins mooth hound (*Mustelus lunulatus*) (Justo 2022). Results from this research will allow understanding the commercialization process of fishing products in Panama, promote new trends in commercialization and help visualize new consumer preferences as well as quality control and traceability practices. This information is key for sustainable fisheries and conservation of fish populations and fishery stocks.

MATERIALS AND METHODS

Sample collection

Sample sites consisted of ceviche processing plants (2), restaurants (3), and the largest fishery market of the country, (San Felipe) in Panama City. Furthermore, 10 additional samples were collected randomly in small restaurants inside San Felipe Fish Market. Tissue samples of 10 mg of prepared ceviche chunks (2 subsamples per ceviche porcion/cup) and also fillets were collected from all

these sites between 2021 and 2022. A total of 111 samples were collected and tissue samples were preserved in 70% ethanol solution. Analyses were carried out in the Laboratory of Genetics and Molecular Biology of the Department of Genetics of University of Panama.

DNA extraction

DNA was extracted using the DNeasy® Blood and Tissue (QIAGEN) kit. After extraction, DNA was preserved in 1.5 ml Eppendorf tubes at -20 °C. DNA quality and quantity were checked using a NanodropTM 2000 spectrophotometer (Thermo Scientific).

DNA amplification and sequencing

A fragment of 650 bp of the mitochondrial gene Cytochrome Oxidase I (COI) was amplified by PCR reaction using fish universal primers, FishF1 (Forward) and FishR1 (Reverse), with the following sequences: Forward FishF1 5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3' and Reverse FishR1 5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3' (Ward et al. 2005). PCR reaction was performed under the following conditions: an initial denaturation for 1 min at 95 °C, follow by 5 cycles of denaturation at 95 °C for 30 s, hybridization at 50 °C for 40 s and extension at 72 °C for 1 min. Followed by 35 cycles of denaturation at 95 °C for 30 s, hybridization at 55 °C for 40 s with and extension of 72 °C for 1 min.

Positive PCR product amplification was verified through electrophoresis in 1% agarose gel (QA-Agarose/MP Biomedicals), TBE 1X buffer and dye using Gel RedTM Dropper Bottle (Orelup SSP®).

Data analysis

Positive PCR products were sent to the Psomagen Service Center in Maryland, USA. Sequences and chromatograms thus obtained were visualized in the Geneious Prime software (Geneious Prime2022). Sequences were edited, ends trimmed and aligned in Geneious Prime as well. Sequence ID was determined using the software BLAST and confirmed by the Barcode of Life Data System (BOLD) (Ratnasingham and Hebert 2007). Afterwards, sequences were group by species and accession numbers, where sequence haplotype were assigned by species using the Sequin software.

Primer and probe design for *Sphyrna lewini* eDNA qPCR detection

Primers and probes for taqman qPCR environmental DNA detection and quantification of the hammerhead shark were developed *in silico* using two programs: Genescript Real-Time PCR (TaqMan) primer and probe design tool and Primer Express 3.0. Two sets of primer and probes for eDNA qPCR detection of *S. lewini* were designed. Amplicon segments were selected from a 625 bp COI region and tested through BLAST to corroborate species specificity as well as primer and probe set. Primer and probes were designed within an amplicon size range between 70 and 250 bp located in the initial sequence region (Table 1). As part of the protocol for a species-specific primer and probe design for *S. lewini*, five sequences of the target species were aligned and compared to other shark species to find a conserved and variable region among species. Using the Geneious prime software, an alignment of 13 sequences was performed, including the nine not commercial species of sharks in Panama and 5 sequences of *S. lewini* found in our analysis.

RESULTS

Species composition, barcode identification, origin and traceability

A high-quality DNA (OD = 2.0) with a range

Table 1. Species-specific primers and probes developed for *Sphyrna lewini.*

Name	Sequence	Strand	Position	Tm °C	Modification
Primer Set 1: Amplicon Size = 177					
L1 Low	TTGTAACTGCCCACGCTTTC	Forward	73	56.15	
R1 Reverse	CGGAAGCTAGGAGGAGAAGG	Reverse	230	56.02	
Pr1 Probe	AGGCCATGTCTGGCGCACCA	Reverse	164	64.05	5' 6-FAM-3' 6-TAMRA
Primer Set 2: Amplicon Size = 66					
L1 Low	TCTGGCTTCTTCCACCATCA	Forward	208	55.62	
R1 Reverse	TACCTGCTCCAGCTTCTACC	Reverse	254	55.54	
P1 Probe	CCTTCTCCTCCTAGCTTCCGCTGG	Forward	230	62.56	5' 6-FAM-3' 6-TAMRA

concentration between 100 a 200 ng μl^{-1} was extracted. A total of 88 high-quality sequences out of 111 DNA samples were obtained. Accession numbers to 21 identified species were successfully assigned (Table 2). Results showed that 62% of species found were natives of the Pacific waters of Panama, 33% were exotic (23% non-native species imported frozen and 10% non-native cultured species), and 5% were considered CITES species or in a particular conservation status (vulnerable, treated, or critically endangered) and the most frequent is *Pangasianodon hypophthalmus*, a non-native species imported frozen (Figure 1). Results showed the reception of imported and cultured goods from industrial fisheries (imported frozen fish) and the most common non-native species cultured in the country (tilapias) (Figure 2).

Primers and probe development for *Sphyrna lewinie* DNA qPCR detection

For each set of markers, probes were design in a conserved amplicon region of the COI gene of *S. lewini* (Table 1) using as template the 3 haplotypes of *S. lewini* found among the analyzed sequences with the following accession numbers: h1 OQ360658, h2 OQ623177 and h3 OQ623178. Specificity of the *S. lewini* probe was compared among the 8 most captured elasmobranch species in the country with COI sequences available in BLAST: *M. henlei*, *S. corona*, *S. tiburo*, *Carcharhinus limbatus*, *C. leucas*, *M. lunulatus*, *Galeocerdo cuvier* and *Alopias pelagicus* (Figure 3 A and 3 B) (Rodriguez Arriatti et al. 2021) using the Geneious Prime 2022 software.

DISCUSSION

Seafood and fish has become one of the most traded food commodities in the world. Thus, fishery products are among the main sources of protein consumed globally (Håstein et al. 2001; FAO 2020). Because of the increased global demand for seafood, supply chains are becoming more complex in quantity and diversity is increasingly difficult to monitor and trace (Velez-Zuazo et al. 2021). Understanding the species composition of a fish product contribute to sustainable fishery management by unraveling key information about local fisheries by determining the origin and conservation status of the catch and categorizing the product as imported, farmed. This information also provides new trends in the consumer market and product commercialization (Helyar et al. 2014), con-

Table 2. Species composition (origins, cultivate and CITES), and common name found in ceviche samples.

Scientific name	Common name	Total number of sequences	Common haplotype accession number
Coryphaena hippurus+	Mahi Mahi	11	OQ248021
Lobotes pacificus+	Triple tailfish	1	OQ703989
Cyclopsetta querna+	Flatfish	4	OQ286171
*Pangasianodon hypophthalmus**	Iridescent cat fish/basa	23	OQ286520
Bagre bagre+	Catfish	1	OQ286515
Bagre panamensis+	Chilhuil sea catfish	4	OQ286045
Ariopsis seemanni+	Tete sea catfish	2	OQ286391
Centropomus viridis+	White snook	1	OQ286057
Notarius luniscutis+	Sea catfish	1	OQ287044
*Gadus chalcogrammus**	Alaska pollock	1	OQ442221
*Oreochromis niloticus***	Nile tilapia	18	OQ248008
Makaira nigricans+	Blue marlin	3	OQ296411
Cynoscion praedatorius+	Boccone weakfish	4	OQ248252
Trachinotus falcatus+	Pompano	1	OQ286530
Centropomus undecimalis+	Union Snook	1	OQ286043
*Oreochromis mossambicus***	Mossambique tilapia	1	OQ286133
Sphyrae naensis+	Barracuda	2	OQ286528
*Protonibeadi acanthus**	Black spotter croaker	2	OQ248032
*Sphyrna lewini****	Hammer head shark	5	OQ360658
*Dosidicus gigas**	Humbolt giant squid	1	OQ651129
*Atheresthes stomias**	Arrowtooth flounder	1	OQ286384

+Native.

*Non-native imported frozen species.

**Cultured non-native species.

***CITES (The Convention on International Trade in Endangered Species of Wild Fauna and Flora).

tributing to fish transparency by helping to enforce and combat IUU fishing (Jacquet and Pauly 2008). In Panamá, 223 species of commercially important fish have been cataloged for the Pacific (Garcés 2021). Nonetheless, species composition of the main processed fishery products remains uncertain. Studies using molecular markers and barcode for detection of species in processed fish products and for ceviche are limited (Velez-Zuazo et al. 2021). Our results showed up to 21 teleost fishes, 1 elasmobranch and 1 invertebrate would be part of the traditional ceviche dish in Panama City. The species identified were principally native. Species richness was greater in native species; while the overall number of non-natives or exotic species were higher. For instance, the most abundant species in ceviche samples was the basa fish (*P. hypophthalmus*) imported from Asia followed by the Nile tilapia (*O. niloticus*) a cultivated invasive species originally from Africa.

By providing species information, this research contributes to build a binomial species nomencla

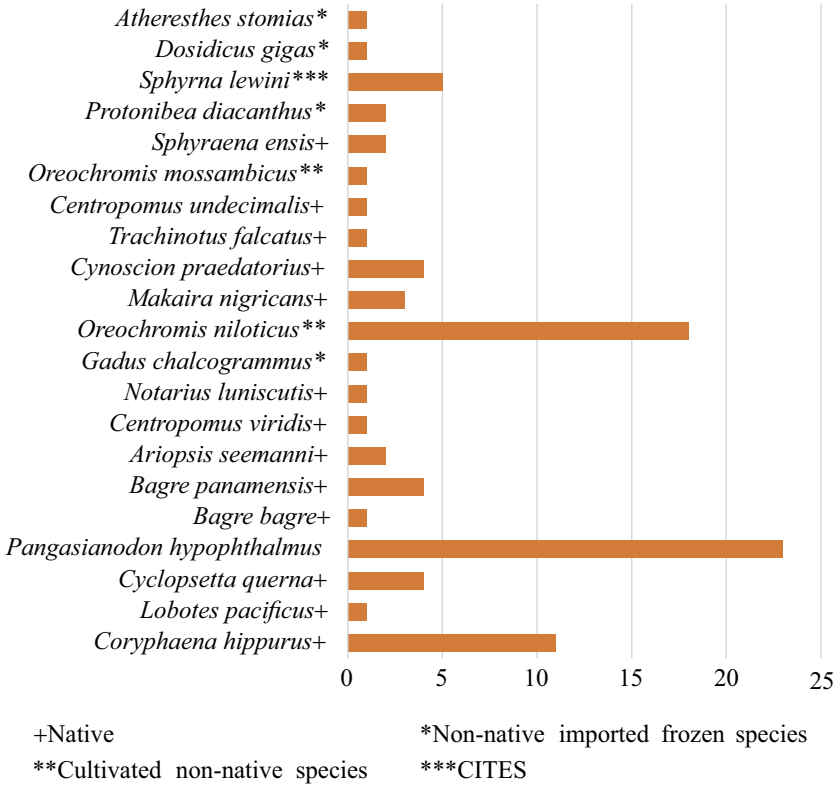

Figure 1. Frequency of species by sample. CITES: The Convention on International Trade in Endangered Species of Wild Fauna and Flora.

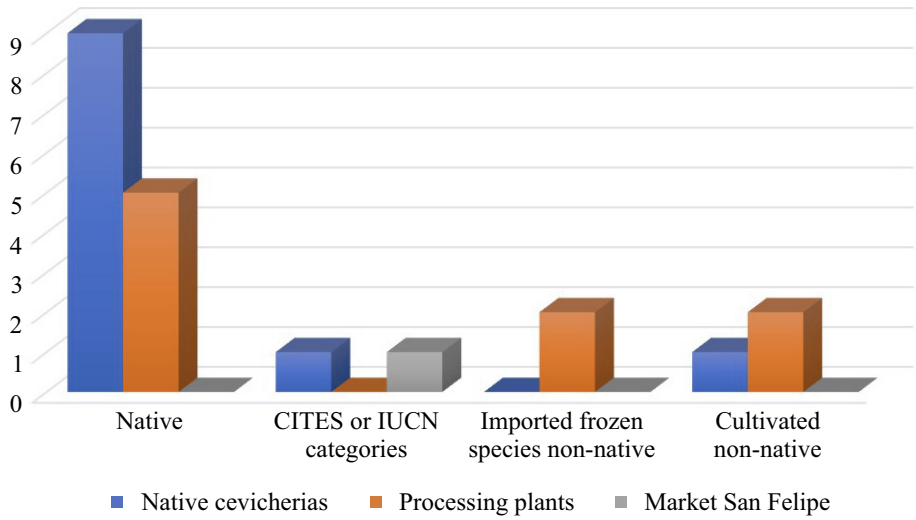

Figure 2. Total number of species per sampling site and categories of origin. IUCN: The International Union for Conservation of Nature, Red List of Threatened Species. CITES: The Convention on International Trade in Endangered Species of Wild Fauna and Flora.

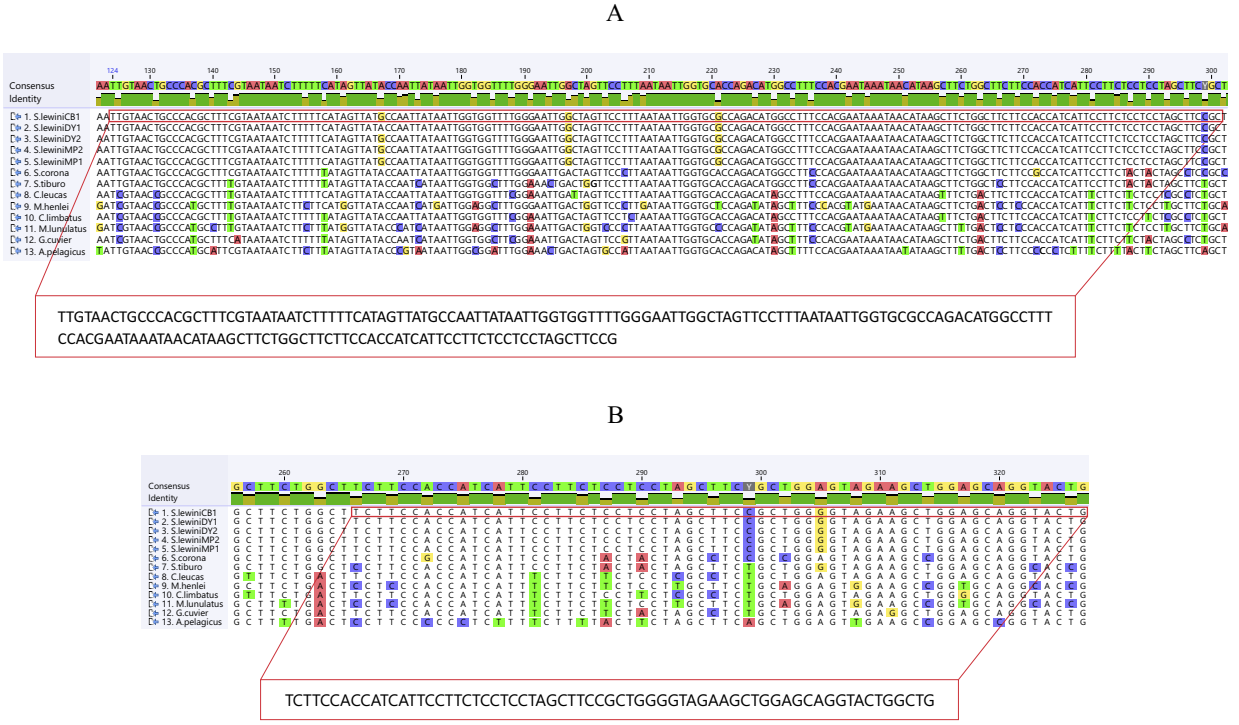

Figure 3. A) Primer and probe set 1. Conserved region of *Sphyrna lewini* where the amplicon of 177 base pair length containing primers and dual labeled probe is showed within the red square. The shaded base pairs show variation among species. B) Primers and probe set 2. Conserved region of *S. lewini* where a 66 base pair amplicon containing primers and probe was designed. Shaded base pairs show variation among species.

ture system that is essential to track the history, location, and composition of our exported food commodities (Naaum and Hanner 2016; Hosch and Blaha 2017). Thus, an important finding from this research was the reduced number or absence of species that have traditionally been used in ceviche such as snappers and corvinas. Only two corvina species were reported and not a single snapper species was found in the analysis. Results indicate that markets are open to offer other teleost species at a lower cost such as flatfish, catfish, tilapia or a mixture of species for ceviche. This information suggests that consumers do not distinguish the difference in flavor between species nor are they selective or concerned about the species composition of the product they purchase. Thus, results show a reduction in fishery pressure for snappers and corvinas and demonstrate that the market is open to other species that are not even listed as commercially important among native fishes species such as *Notarius luniscutis*, *Ariopsis seemanni* and *C. querna*.

Most of the native species were not considered as CITES or in any of the nine IUCN categories. Nonetheless, three positive samples of the blue marlin, a vulnerable billfish according to IUCN categories, and five positive samples of the hammerhead shark, a CITES elasmobranch, were detected among the analyzed samples. *Sphyrna lewini* is the most captured elasmobranch species in the Pacific Panama (Rodriguez Arriatti et al. 2021). Like other shark species, *S. lewini* has low reproductive success and reduced number of pups making them more susceptible to fishery pressure than bony fishes (Cailliet et al. 2005; Hosch and Blaha 2017). One of the most well-known game fishes is

the blue marlin, a highly migratory species found mostly in tropical waters around the world. The last IUCN assessment in 2010 classified the species globally as VU (Collette et al. 2022). Molecular analysis (Graves and McDowell 2003) and fishery catch rates data suggest the existence of a unique stock for blue marlin in the Pacific Ocean, currently corroborated by genetic data (Kleiber et al. 2003; Williams et al. 2020).

Additionally, important information that allows establishing a relationship between sampling site, origin categories, and trends in commercialization of ceviche was obtained. Based on our results, processing plants are the main receptor of species from industrial and local fisheries and showed a higher number of species than any other sampling site. Processing plants seems to be the main distributor of fish fillets for ceviche commercialization. The absence of CITES species in processing plants and the reduced number in local *cevicherias* evidence the existence of quality control and inspection of local authorities in these sampling sites. In contrast, the absence of control and traceability in local markets was evident, indicating that artisanal fisheries and informal sellers are also providers of fishery products for these markets.

Results from this research showed that molecular analysis through barcode can provide important information for traceability of wild and fishery important fish populations. This information is key for effective management and conservation policies of fishery and ecologically important species. This research is also pioneer in the generation of a new qPCR eDNA detection primers and dual labeled probes that will contribute to establishing the status of fishery products (as free of shark meat), and as sustainable traceable resource unit (TRU) at the earliest stage of commercialization and before they are incorporated into the supply chain. One of the newly developed markers is able to discriminate between *S. lewini* and the eight most commercial shark species of the Pacific Panama. Whilst the other set, an amplicon of 177 bp region can discriminate between *S. lewini* and seven other shark species from the Pacific Panama since its sequence is similar in base pair composition with *S. corona*.

CONCLUSIONS

This research demonstrated that molecular traceability using barcode is an effective method for vertebrate and invertebrate species detection in ceviche samples. This is the first molecular characterization of ceviche samples conducted in Panama. This research not only provides a list of commercial fish species (natives, non-natives and imported frozen species) used for this traditional dish; by understanding species composition we can combat IUU, enforce regulations of fish transparency, avoid species mislabeling, determine conservation status of the species, provide new trends in commercialization, and identify new consumer preferences in a particular processed product.

Results indicated a higher abundance of two invasive species (basa and tilapia) in the samples followed by a native species with a demonstrated sustainable fishery such as the dolphin fish (Guzman et al. 2015). This finding shows that ceviche consumption does not represent a treat to local fisheries. Nonetheless, the existence of VU species such as the blue marlin, critically endanger (CR), and CITES species such as *S. lewini*, is an indication of the need for a national and international molecular traceability program to control and regulate fishery products.

Finally, the development of a new set of markers for the detection of *S. lewini* provides a new tool for shark conservation and the development of a species-specific tariff code system (binomial species nomenclature system) supported by molecular data that enforce the fight against IUU fishing. Results from this project also contribute to the sustainable use of fishery resources and provide *cevicheros* with a certificate of good practices that add value to their product.

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Author contributions

Edgardo Díaz-Ferguson: conceptualization, sequences editing aligning, primer and probe design, lab work and accession numbers to Genebank, writing and editing. Sara C. Justo: lab work and accession numbers to Genebank. Vicente del Cid: sampling coordination. Juan Posada: writing and editing.

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