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Fish Hybridization Leads to Uncertainty Regarding Ciguatera Fish Poisoning Risk; Confirmation of Hybridization and Ciguatoxin Accumulation with Implications for Stakeholders

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Abstract: Globally, ciguatera fish poisoning (CFP) avoidance efforts rely primarily on local knowledge of the fish being consumed, its collection location, and association with illnesses. In 2016, several fish that appeared to be hybrids between a local commercially prized species, *Ocyurus chrysurus*, and a regionally prohibited species *Lutjanus apodus* (due to CFP concerns), were caught nearshore in United States Virgin Islands waters, leading to confusion regarding the safety of consuming the fish. The hybrid status of the fish was verified as *O. chrysurus* (male) × *L. apodus* (female) by comparing two sets of gene sequences (mitochondrial CO1 and nuclear S7). Using an in vitro mouse neuroblastoma (N2a) assay, one of the hybrid fish exhibited a composite cytotoxicity of 0.038 ppb Caribbean ciguatoxin-1 (C-CTX-1) equivalents (Eq.); a concentration below the US Food and Drug Administration (FDA) guidance level for safety in fish products for CFP (0.1 ppb C-CTX-1 Eq.) but approximately 2× above the maximum described in the commercially prized parent species (0.019 ppb C-CTX-1 Eq./g). C-CTX-1 was confirmed in the hybrid sample by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The second hybrid fish tested negative for CTXs. This research confirms hybridization between two species with contrasting commercial statuses, discusses CTX accumulation implications for hybridization, and provides a methodology for future studies into novel CFP vectors, with the goal of providing critical information for fishermen and consumers regarding CFP risk management.

Keywords: ciguatoxin; ciguatera poisoning; hybrid; DNA-based species identification; LC-MS; N2a assay; seafood poisoning

1. Introduction

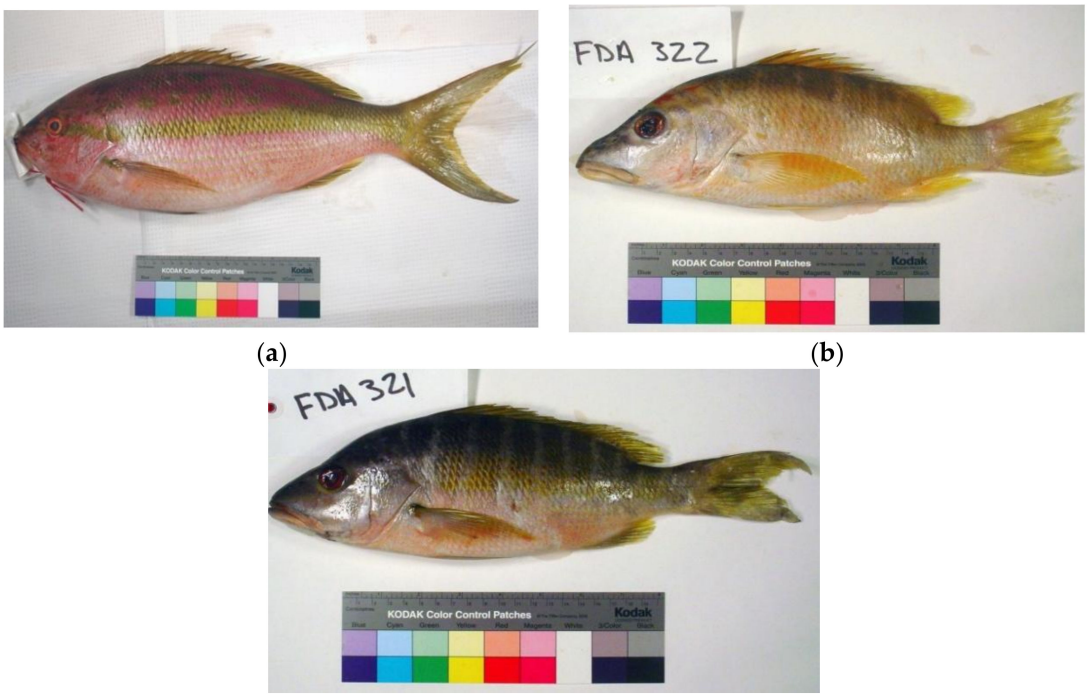
CFP is a circumtropical seafood-toxin illness, resulting from consumption of seafood contaminated with cyclic polyether toxins, classified as ciguatoxins (CTXs) [1]. These toxins are metabolites of less polar and less potent CTX precursors (formally known as gambiertoxins), produced by benthic dinoflagellates in the genus *Gambierdiscus* and *Fukuyoa* [2]. When herbivores, omnivores, or detritivores

feed on the benthos they can consume these dinoflagellates [3] as part of their diet, inadvertently ingesting the toxin precursors. These ingested toxins are then biotransformed into more polar CTXs by oxidative metabolism and spiroisomerisation in the liver of marine animals, leading to bioaccumulation of CTXs in the fish [1,4]. It is not possible to identify CTXs in a specific fish visually or organoleptically. Additionally, reliable rapid chemical tests are not yet available. Therefore, to avoid CFP, consumers rely on local knowledge regarding the identification of fish species frequently associated with CFP, in conjunction with the development of consumption risk profiles based on species and location of harvest [5,6]. The clinical syndrome of 'ciguatera' is diagnosed based on a history of eating reef fish and on a clinical presentation of complaints defined by gastrointestinal, neurological, cardiovascular, or a mixed pattern of these symptoms. The pharmacological targets of CTXs are voltage-dependent sodium channels and the pharmacology of CTXs is characterized by persistent activation of voltage-gated sodium channels, increasing neuronal excitability and neurotransmitter release, impairing synaptic vesicle recycling, and causing cellular swelling [7].

St. Thomas of the United States Virgin Islands (USVI), located within the Caribbean among the Leeward Islands of the Lesser Antilles, is considered a hyperendemic region for CFP, where annual illness rates are high (2–44 incidences per 1000 people per year) [8–10]. In 2016, fishermen in the south-west waters of St. Thomas caught two fish that appeared to be the result of hybridization between species of historically low *O. chrysurus* (regionally known as yellowtail snapper) and medium risk *L. apodus* (regionally known as schoolmaster snapper) for CFP (Figure 1). The south-western region of the USVI was found to be the second lowest site (by percentage) for CTXs and the most commonly fished area in the territory [11,12]. *O. chrysurus* is one of the most common reef fishes in the tropical Western Atlantic, a highly prized fish for consumers and commercial fishermen alike. *O. chrysurus* is considered a 'safe' species for consumption regarding CFP [10,13,14]. However, it has been listed as a potential CFP species in a 1969 assessment of USVI fishing potential, but not confirmed [15]. *L. apodus* is associated with CFP outbreaks and is considered 'a frequent poisoner' [6,15,16]. The island of Guadeloupe (approx. 180 nautical miles to the southeast of the study location) has implemented measures to protect the population against the threat of CFP posed by *L. apodus*, and 15 other species, through a Prefectural order (N° 2002–1249) restricting their harvest and sale. *O. chrysurus* have no sales restrictions regarding CFP [17,18]. According to hospital emergency department cases and regional monitoring networks in Guadeloupe, there were 16 cases of CFP between 2013–2016 where either *L. jocu* or *L. apodus* were implicated in the poisoning (species confirmation was unavailable due to a lack of genetic identification) [18]. *O. chrysurus* was implicated in only one case of CFP out of 234, however, genetic confirmation was not performed and this species was labeled as 'not considered to be responsible for CFP at the time of reporting' [18]. To prevent misidentification for CFP outbreaks, it is important to identify what species are responsible for episodes of CFP in order to build the library of CFP vector information required to inform decision makers regarding the risks a given species may present. The FDA has established a guidance level for Caribbean ciguatoxins at 0.1 ppb C-CTX-1 equivalents (Eq.) [14]. This guidance level, which is based on adverse effects, has a 10× safety factor applied in order to address individual human risk factors and inherent uncertainties [5]. *O. chrysurus* was investigated in a study of commercially important fish species in the USVI for the prevalence of CTXs, the results of which found no *O. chrysurus* individuals exceeding the USFDA guidance level [11].

O. chrysurus and *L. apodus* are found throughout the greater Atlantic and individual adults have relatively small spatial home ranges, normally maintaining a home reef with a small hunting range (typically <10 km) [19–21]. Since their foraging range is relatively localized, they may serve as indicators of the local CTX burden for the invertivore and invertivore/piscivore finfish trophic levels that they represent. *L. apodus* and *O. chrysurus* begin their life cycle similarly, in protected mangroves or seagrass beds, feeding on small prey, such as crustaceans and plankton, then move towards coral reefs as they get larger. As these snappers undergo an ontogenetic diet shift from the nursery habitat to the coral reef their diets diverge, generally, *L. apodus* hunts fish near the reef while *O. chrysurus* can be found higher in the water column feeding on zooplankton [13,22–24]. *L. apodus* is a generalized carnivore,

feeding primarily on nekton (finfish) and, to a lesser extent, the zoobenthos (crustaceans, worms). Its diet composition trophic level is 4.25 [13,25]. Whereas *O. chrysurus* are considered planktivores, or zooplankton feeders, maintain a diet composition trophic level of 4.0, and their meal remnants indicate a diverse diet consisting of zooplankton, bottom detritus, coral fragments, sand grains, mud, algae, sponges, polychaete worms, crustaceans, and small fishes, notably anchovies [13,25]. Since CTXs have bioaccumulative properties, higher order predators generally have higher toxin burdens and, within a species, prey selection and location can be determining factors for CTX content. It is not currently known what potential hybrids between two species with different feeding ecologies, such as these, would mean for their risk for CTX accumulation.



(**a**) (**b**)

(**c**)

Figure 1. (**a**) *Ocyurus chrysurus* (Yellowtail snapper) (FDA51/USNM394166), (**b**) *Lutjanus apodus* (Schoolmaster snapper) (FDA322/USNM444948), (**c**) Hybrid (FDA321/USNM444947) between: *O. chrysurus* (male) + *L. apodus* (female).

The FDA currently uses DNA barcoding to identify seafood, to confirm accurate labeling, and to determine the species identity of meal remnants involved in outbreaks of illness associated with seafood consumption. The application of this technology provides data to better understand species-specific seafood consumption risks for consumers [26,27]. This includes the identification of meal remnants involved in cases of CFP. This information is also used to refine FDA guidance on the identification of species-specific seafood hazards, which is critical to complying with the Hazard Analysis Critical Control Point (HACCP) regulation [14], one of the FDA's primary tools to assure seafood safety in the US. DNA barcoding exploits species-specific diversity within the mitochondrial cytochrome-*c* oxidase I (COI) gene to identify seafood products, but a hybrid between two fish species will only be identified as to the maternal lineage using any mitochondrial gene marker. To confirm the paternal lineage of hybrid fish species, an additional nuclear gene marker is required. Therefore, the presumed hybrid will be evaluated using a novel sequencing approach combining analysis of a nuclear gene (S7) and the DNA barcoding mitochondrial target (CO1). The primers for the nuclear gene analysis were originally described by Chow and Hazama [28] and used by Yaakub, et al. [29] as a tool to diagnose hybrids via sequencing. This, combined with the fish DNA barcoding method refined by Handy, et al. [26] will allow for the determination of both the maternal and paternal fish species.

Fishermen and consumers apply historical risk assessments to base their decisions regarding food safety for CFP. Therefore, a hybrid fish displaying the morphological characteristics of multiple species with variable historical CFP risk profiles poses a unique problem for preventing a human health hazard and is in need of elucidation. In the present study we sought two goals, as follows: (1) Present a methodology applicable for identifying suspected hybrid fish species, and (2) provide methodology that can be used to determine the risks these hybrids pose for causing CFP. In tandem, these goals can aid with the development of improved CFP risk profiles for various fish species in CFP endemic areas. To accomplish this, we tested two fish suspected to be hybrid offspring between a historically low risk (*O. chrysurus*) and medium risk (*L. apodus*) species for CTXs. A low-risk species is defined as 'seldom responsible' for CFP events, while a medium risk species is recognized as a 'frequent poisoner' [6]. We further assessed the potential for a hybrid fish, between *O. chrysurus* and *L. apodus* to contain CTXs, and compared those levels with historical samples and reports of CFP illnesses for the parental species. The CTX testing procedure chosen for the present study includes a 2-tiered protocol involving the following: (1) An in vitro mouse neuroblastoma (N2a) assay used as a semi-quantitative screen for neurotoxicity consistent with the CTX mode of action at voltage-gated sodium channels and (2) LC-MS/MS for molecular confirmation of C-CTX-1 [30].

2. Materials and Methods

2.1. Sample Preparation and Toxin Extraction

Sample collections, preparations, extractions, and analyses for CTXs were conducted as previously described, with slight modifications [11]. Frozen, suspected hybrid fish (n = 2) were thawed and skinless boneless muscle tissue (5 g) was extracted as previously described [30,31]. Importantly, these extraction methods remove potential interferences (e.g., nonpolar lipids, proteins, and hydrophilic compounds) and concentrate potential CTXs in a final chloroform extract. Solid-phase extraction (SPE) using a Bond Elut SI cartridge (500 mg/3 mL, Agilent, Santa Clara, CA, USA) was then performed to clean extracts prior to toxicity assessments. An additional SPE step was also performed prior to LC-MS/MS analysis using an aminopropyl (NH2) cartridge (500 mg/3 mL, Agilent), as previously described [31]. The fish extracts were prepared at a concentration of 50 g wet tissue equivalent (TE)/mL for LC-MS/MS analysis and 5 g TE/mL for N2a analysis. Caribbean ciguatoxin-1 standards used in the cytotoxicity assay were prepared at the US FDA Gulf Coast Seafood Laboratory, Dauphin Island, AL, and purity was verified by LC-MS/MS before use.

2.2. In Vitro N2a Cytotoxicity Assay

An in vitro N2a cytotoxicity assay was used to screen for sodium channel–specific toxins in fish extracts as previously described [30,32]. This functional assay exploits the binding of CTXs to voltage-gated sodium channels, which, in the presence of ouabain and veratridine, results in a dose-dependent loss in cellular viability. Neuroblastoma cells were propagated from cryostorage and maintained in Roswell Park Memorial Institute (RPMI-1640) medium, supplemented with antibiotics (50 mg/mL streptomycin, 50 units/mL penicillin), glutamine (2 mM), sodium pyruvate (1 mM), and heat-inactivated fetal bovine serum (10% *v*/*v*). Cells were harvested for assay when cultures were approximately 80% to 90% confluent and seeded at 4×10^4 cells/well (200 µL volume) into sterile 96-well plates. Concentration–response curves (8-dilutions) of Caribbean ciguatoxin-1 standards and sample extract were prepared with sensitized (at 20 µL each of 2.5 mM ouabain and 0.25 mM veratridine (+OV)) and non-sensitized (–OV) cells as described, with a final well volume was 230 µL [30]. Sensitized cells were used to determine the concentration of extract required to reduce cell viability by 50% (EC_{50}) and compared with a Caribbean ciguatoxin-1 standard. The limit of detection (estimated at EC_{30}) for the assay using *O. chrysurus* was determined to be 0.002 ng Caribbean ciguatoxin-1 Eq./g. The limit of quantification (where a full concentration–response curve was evident without matrix interference) for spiked negative tissue controls was 0.005 ng C-CTX-1 Eq./g. The maximum sample tissue equivalent

concentration used for the hybrid was 870 mg TE/mL and, without a response at this level, it was deemed negative. All samples (including those deemed negative during screening) were further analyzed by LC-MS/MS.

2.3. Analysis of C-CTX-1 by LC-MS/MS

Confirmation of C-CTX-1 in the hybrid that tested positive by the N2a assay was performed by LC-MS/MS, using an Agilent 1260 liquid chromatography system coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems, Inc., Foster City, CA, USA) as previously described [31]. The samples found to be negative by N2a, were also deemed negative by LC-MS/MS. Briefly, analytes were separated by gradient elution using a Kinetex C8 column (75 × 2.1 mm; 2.6 µm particle size; Phenomenex), held at 40 °C, and 10 µL injections were made using the LC's autosampler. The mobile phase consisted of (A) water and (B) 95:5 acetonitrile:water, both with 0.1% formic acid. The chromatographic conditions were as follows: Isocratic gradient of 10% B (1 min), a gradient from 10 to 95% B at 1.5 min, followed by an isocratic hold at 95% B for 3.5 min. At 5.2 min the system conditions were returned to 10% B. Between each sample, the column was re-equilibrated to starting conditions for 4.3 min, yielding a total run time of 9.5 min. The mass spectrometer was operated in positive electrospray ionization mode. Principal instrument settings were as follows: Ionspray voltage 5500 V, declustering potential 75 V, entrance potential 10 V, cell exit potential 15 V, collision energy 35 eV, temperature 400 °C, curtain gas 20 psi, GS1 and GS2 both at 60 psi, and collision gas medium. C-CTX-1 was confirmed with three selected reaction monitoring transitions (*m/z* 1123.6 > 1105.6, *m/z* 1123.6 > 1087.6, and *m/z* 1123.6 > 1069.6) using the dehydrated C-CTX-1 ion (M + H-H_2O^+) as the precursor, as previously described [30,33]. Analyst 1.6.1 software (Applied Biosystems) was utilized for instrument control and data acquisition.

2.4. Species Identification

Samples of fish tissue (10 mg) were taken from 12 fish, including *L. apodus* (n = 2), *O. chrysurus* (n = 8), and the two presumed hybrids, then extracted with a DNeasy® Blood & Tissue Kit (Qiagen Ltd., West Sussex, United Kingdom) for genetic analysis. A portion of the cytochrome c oxidase I (COI) mitochondrial gene was amplified and sequenced following Handy et al., 2011 [26], then compared with COI sequences from FDA-authenticated reference standards [34], as well as publicly available sequences found in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and the Barcode of Life Data System (BOLD) (http://www.boldsystems.org/). In addition, the nuclear gene S7 was amplified using universal primers [28] S7RPEX1F, 5′-TGGCCTCTTCCTTGGCCGTC-3′ and S7RPEX2R, 5′-AACTCGTCTGGCTTTTCGCC-3′, sequenced following Handy et al., 2011 [26], and compared to FDA standards, whose sequences were generated during this project. Alignments of these sequences were constructed separately using the program GeneiousPro version 11 (http://www.geneious.com [35]) and were either analyzed using a UPGMA tree with a Jukes–Cantor genetic distance model for CO1 or by a direct pairwise base comparison for S7.

3. Results

3.1. Identification of Maternal and Paternal Lineages in Suspected Hybrids

To confirm that these individual fish were hybrids, the mitochondrial and maternally derived CO1 gene was sequenced, along with the nuclear S7 gene, which is inherited from both parents. All samples were sequenced until 2× or greater coverage was achieved. All generated sequences have been deposited in Genbank, along with CO1 sequences used from the FDA reference sequence database (NCBI Accessions MK300530-MK300541 (S7) and MK297415-MK297439 (CO1), Supplementary Table S1, metadata for CO1 reference sequences can be found here: https://www.accessdata.fda.gov/scripts/fdcc/?set=seafood_barcode_data&sort=Scientific_Name_display&order=ASC&startrow=1&type=basic&search (accessed 12 June 2018)). The CO1 sequences generated an alignment that was 655 bases in length, which was then compared to the FDA Reference Standard Sequence Library for Seafood Identification (Figure 2).

The two suspected hybrids (FDA321 and H2) matched the *L. apodus* vouchers with a 99.8% pairwise identity score. The yellowtail snapper individuals matched an *O. chrysurus* voucher with a 100% pairwise identity score. This indicates that the maternal species of both suspected hybrids was *L. apodus*.

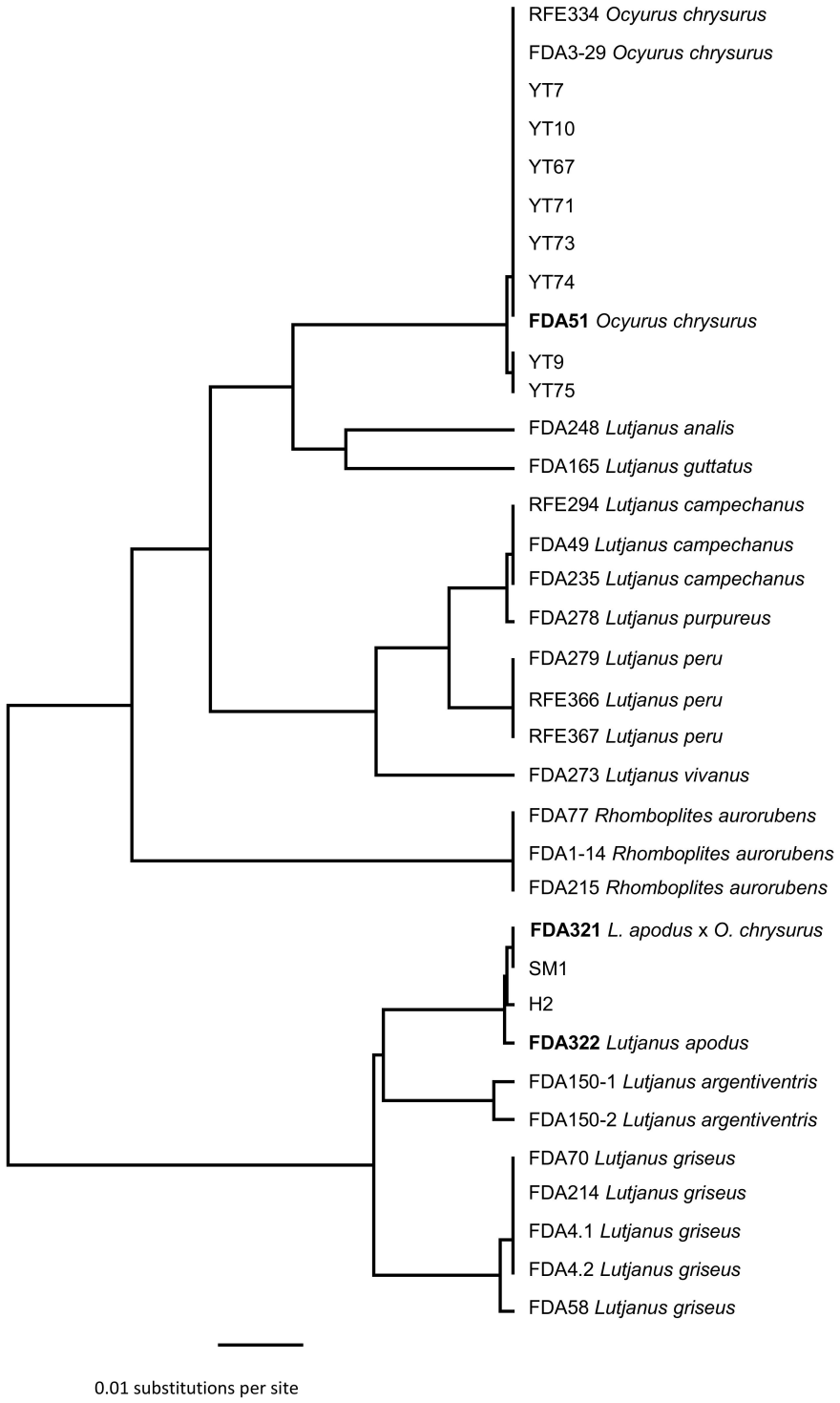


Figure 2. UPGMA (Unweighted Pair Group Method with Arithmetic Mean) tree based on DNA barcoding results (CO1). The results of CO1 sequences for hybrids will only indicate the mother and, for the individuals tested in this study, the sequences match the schoolmaster snapper genetically by 99.8%. FDA51, FDA321, and FDA322 pictured in Figure 1. YT = Yellow Tail Snapper, H = hybrid, SM = School Master Snapper, additional fish collected but not vouchered for this study.

For the suspected hybrids, 322 bases of the S7 gene were successfully sequenced after trimming for primers and quality, while the voucher species varied from 376–779 bases for *O. chrysurus* and 771–776 bases for *L. apodus*. Within the 322 bases of matching sequence, there were 16 positions that were ambiguous for two different bases. In each case, one base matched *O. chrysurus* and the other matched *L. apodus* (Figure 3). The results of the S7 dataset confirm that the two individuals are indeed hybrids and that the paternal species was *O. chrysurus*.

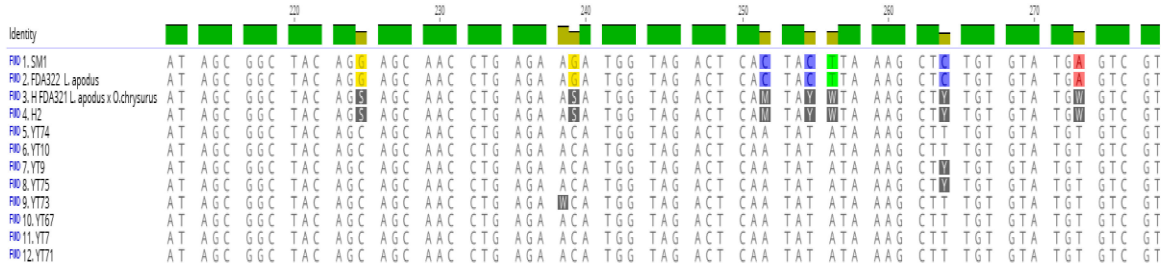


Figure 3. A portion of sequences aligned from the S7 nuclear genes of yellowtail snapper (YT), schoolmaster snapper (SM), and hybrid (H). This gene has been known to show ambiguous bases in hybrids (boxed), indicating a mixture of the parent species. In the ~322 base section from the two hybrids (Sequences 3 and 4), there were 16 instances where the hybrids showed ambiguous bases (S = C or G, M = C or A, Y = C or T, W = T or A). Each one had a base from each of the suspected parental species.

3.2. Ciguatoxin Analysis

Previous investigations into *O. chrysurus* toxicity in St. Thomas (n = 17) found maximum levels in the flesh, at 0.019 ng C-CTX-1 Eq./g. toxins consistent with sodium channel activity [11], confirming its classification as a low-risk CFP species in the region. Data regarding CTX levels for *L. apodus* are currently lacking, however, local fishermen provided interviews and information implicating this species as a CFP risk for consumption [6,15]. Among the two hybrids tested in this study, one contained a 0.038 ppb C-CTX-1 Eq. concentration, with the presence of C-CTX-1 confirmed by LC-MS/MS (Figures 4 and 5), while the second hybrid fish (FDA321/USNM444947) contained no detectable levels of CTXs at the maximal concentration of wet weight fish tissue tested (870 mg TE/mL).

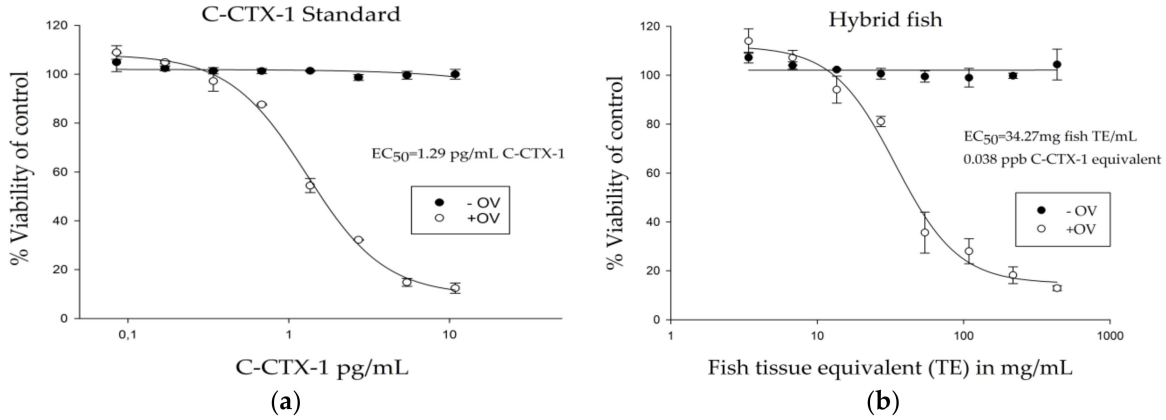


Figure 4. In vitro cell assay of (**a**) C-CTX-1 reference standard, the 8-pt concentration-response curve was applied starting at 10.9 pg/mL (EC_{50} = 1.29pg/mL), and (**b**) hybrid fish tissue extract, the 8-pt concentration-response curve was applied starting at 434.8 mg/mL (EC_{50} = 34.27 mg/mL). In addition to the samples, +OV wells received 20 µL ouabain and veratridine solution, −OV wells received 20 µL buffer solution (PBS).

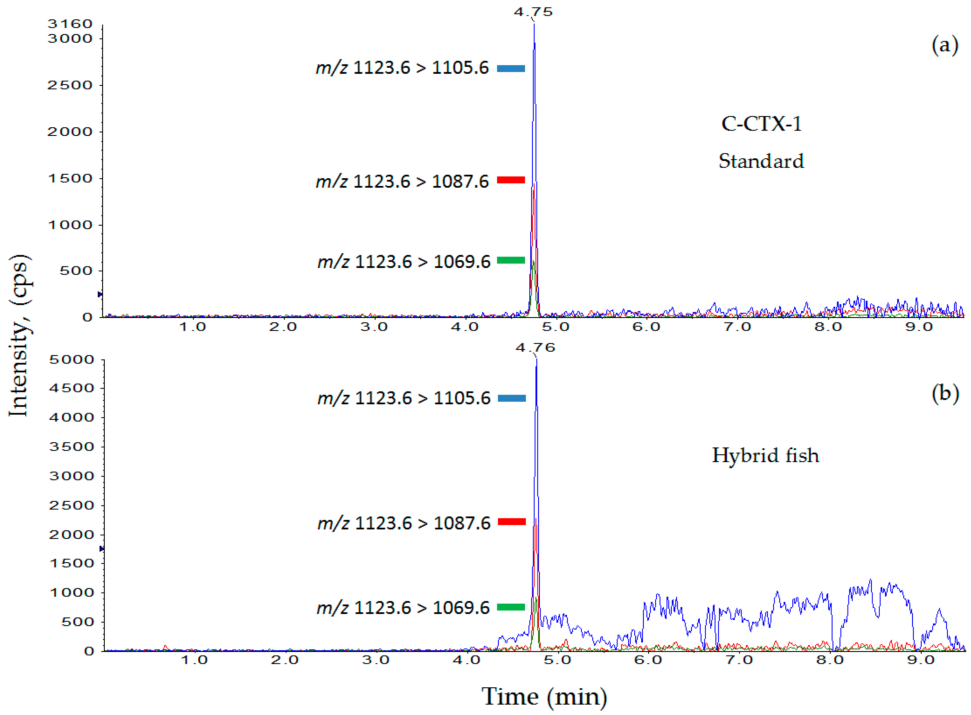


Figure 5. LC-MS/MS (MRM) chromatograms of C-CTX-1: (**a**) C-CTX-1 reference standard of 15 pg on column; (**b**) hybrid fish extract.

4. Discussion

This is the first confirmation, to our knowledge, of hybridization between the highly commercial species, *O. chrysurus*, and a regionally prohibited species, due to the risk of CFP, *L. apodus.* These hybrids were opportunistically caught and sampled. Therefore, efforts at obtaining additional individuals in order to fully assess the CFP risk for this hybrid species were unsuccessful. However, now that a description of this hybridization is available, these findings will be communicated to fishermen with the goal of obtaining additional samples in the future. There is a history of hybridization within the snapper family and these species have a strong overlap in life history and habitat utilization. Therefore, additional hybrids should become available and obtaining samples with a reversal of parental lineage would be of interest [36].

A CTX risk profile for hybrid species has not yet been reported. Therefore, methods applicable for the determination of CFP risk profiles of a hybrid between two species with conflicting historical risk is of ecological and toxicological importance. The biotransformation pathways of CTXs are complex and the results of this study, coupled with additional studies into differences in CTX distributions within the reef ecosystem and feeding ecology, would provide insight into the effects that feeding behaviors/biomechanics inherited from different parental species (e.g., prey selection, movement patterns, etc.) have on the CTX trophic transfer; and ultimately how this parental combination impacts CFP risk. The list of prohibited species from fishing or sale in Guadeloupe is regularly updated with new knowledge regarding CTX contaminated species. Currently, *O. chrysurus* has no fishing restrictions and *L. apodus* is prohibited, regardless of fishing location, when the weight exceeds 1 kg [37]. The results of this study are in agreement with the sales prohibition protections established in Guadeloupe, as the hybrid fish analyzed were below the 1 kg restriction on *L. apodus* and were below the FDA guidance level for C-CTX-1. Therefore, even subjected to the strongest possible restrictions, these hybrids could be sold. However, low CTXs were present in the hybrid (and *O. chrysurus*), raising the issue of chronic low dose exposure or effects of CTX accumulation in humans. CTXs have bioaccumulative properties and consecutive meals containing low-level CTXs can result in CFP symptomology [6].

Various fish markets take an approach based on size, location, and species to serve as predictors for CTXs [14,38,39]. These predictors have shown merit in assessing CTXs in mid-level trophic fish of the USVI [11]. This study presents evidence highlighting the relevance of behavior or physical feeding advantages/biomechanisms as an additional factor to consider when investigating CTX accumulation predictors. In their juvenile life stage *O. chrysurus* and *L. apodus* share the same habitat with a similar prey selection and neither Guadeloupe nor the USVI place any restrictions on their sale for human consumption during this life stage (i.e., size, location, and species as predictors). However, as these fish grow and undergo their ontogenetic diet migration, only one of these species is considered a risk for consumption and banned from sale, while the other, of similar size and age, is not. Therefore, size by itself is not the issue for market sale, as both fish reach >1 kg and only *O. chrysurus* are permitted. The restriction target is what the size represents, i.e., CFP risk. This risk, associated only with *L. apodus*, occurs after it shifts its diet towards finfish. Finfish tend to represent a biomagnification of CTXs, whereas the prey items of *O. chrysurus* are commonly associated with low CTX concentrations. This change in predation is either behavioral or biomechanical, and for *L. apodus*, this shift has resulted in enough CFP events over time to warrant government intervention prohibiting their sale in Guadeloupe.

Additional testing of both confirmed hybrids, between *L. chrysurus* and *L. apodus*, as well as additional *L. apodus* (to confirm its status as a higher risk species), will be required before the CTX associated consumer risk for these fish can be determined. However, this study was an initial opportunity to investigate how the crossing of two species is reflected in CTX bioaccumulation, compared with the historical CTX risk profiles of the parent species. Here we make an assumption that CTX bioaccumulation is an indicator for prey selection or feeding behavior. Assuming CTXs are an indicator of prey selection, then *O. chrysurus*, when hybridized with a more predatory species, gained a trophic feeding advantage over the normal *O. chrysurus*, i.e., by hybridizing with a more apex predator, their offspring were more effective at accumulating CTXs. This can be described by comparing the maximum CTX toxin content found in *O. chrysurus*, throughout the St. Thomas region (including individuals in excess of 1 kg), with the maximum found in the hybrid (a 2× increase). The hybrid was from the west, a relatively lower toxin location for the region [11]. Whether this gain in CTX accumulation 'advantage' is due to behavioral or a physical advantage is unresolved, but this hybrid demonstrated the ability to seek and consume, or accumulate, higher levels of CTXs than its lower risk parental species. It is this implication that we present for consideration when determining the risk of an unknown or novel potential CTX vector. In short, for locations with a history of CFP, a hybrid fish can accumulate CTXs and classifying a hybrid with the higher risk parental species is a conservative approach for preventing CFP. Even though this study serves only as a demonstration of potential, when human health is at risk, especially with a highly incapacitating disease, caution is warranted and should be considered [40].

5. Conclusions

This work provided methodology that was used to confirm the suspected hybridization and parental cross between two species of snapper with differing historical risk in a CFP endemic region and further revealed the presence of C-CTX-1 in a hybrid between *O. chrysurus* (male) and *L. apodus* (female), collected from nearshore USVI waters. In the Caribbean, CTXs poison thousands of people annually and influence consumer demand, current information regarding species, and regions of concern. In Guadeloupe, between 2011–2016, as many as 6.8% of outbreaks of CFP were possibly attributed to *L. apodus*, whereas *O. chrysurus* is 'not considered responsible for CFP'. The results of this and future studies using this methodology will provide additional information for fishermen regarding the CTX content of these hybrids. While additional testing will be necessary, we demonstrated that a hybrid can accumulate CTXs and at levels above those currently observed for the 'lower risk' parental species. While the single CTX-contaminated hybrid found in this study exceeded the maximal CTX values observed for the lower risk species from previous studies, the concentration was still below the level considered to potentially cause illness. Additional work will be required to determine the risk

this hybrid actually holds for consumers and what this may mean for hybrids between other differing historical risk species in other regions of the world.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-1312/7/4/105/s1, Table S1: Supplementary Table S1.

Author Contributions: C.R.L., S.M.H., and J.R.D. conceived the project; S.M.H. conducted the species identification, H.A.F.Q. and C.R.L. performed the HPLC analysis, C.R.L. performed the cell assay. All authors were involved in the data analysis, data interpretation, and preparation of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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