Introduction

Since the first appearance of the exotic lionfish in the western Atlantic [1], there has been great concern about the potential impact on coral reefs in the Caribbean region. A number of studies have recently been published on the lionfish invasion, in particular, the geographical distribution [2], the feeding behavior in the Bahamas [3], an analysis of cytochrome B mtDNA sequences to examine founder effects and for species identifications [4], establishing a molecular phylogeny [5], use of nursery habitats [6], and evaluating native predator species [7].

Two species of lionfishes have been recorded as invaders in the western Atlantic: *Pterois volitans* (Linneo, 1758) and *Pterois miles* (Bennet, 1828). Although once considered to be synonyms, sequence differences in cytochrome b have confirmed the separation of the two species [8]. Nevertheless, despite cytochrome b is an important marker for species determination and it was successfully used to discriminate both, the barcodes, based in sequences of the cytochrome oxidase I, are becoming a wider standard in species identification (see www.fishbol.org).

At present, the lionfish invasion has spread to all along the coastal Yucatan Peninsula, including the entire Mesoamerican coral reef and has been recorded throughout the Caribbean as far as Venezuela [2,9]. Recently, and for the first time, a larval lionfish was collected and reported in the Atlantic Ocean [10]. In the beginning, seem to this species was introduced as an ornamental fish, and later it escaped from an aquarium located in Florida [1,2,11].

DNA barcodes have proven to be more than 90% successful in the identification of marine fish species in studies from Australia [12] and Mexico, where they also were used to connect
developmental stages unidentified with adults [13]. One of the first and more important applications of this technique has been to detect exotic species in a fast, reliable and cost-effective way [14]. For example, exotic moths have been detected among field-caught populations [15] and an invasive microcrustacean, as the cladoceran Daphnia lumholtzi, has been discovered in Mexican freshwaters [16]. Another useful application of this method is the analysis of dietary habits. This approach has recently been used for an analysis of bat feces, since DNA barcoding permits the identification of prey in the absence of morphological evidence after digestion [17]. In case of fishes, two previous studies have used this technique, one to analyze herbivorous fish diets [18], and the other confirming the utility of the technique for piscivorous fishes, but in the laboratory [19].

In this study, we apply the DNA barcoding method to analyze the prey composition for the carnivorous lionfish. The material studied comes from several collections of lionfish in Cozumel, along the Mexican portion of the Mesoamerican Coral Reef. Our primary goals were to establish, based on DNA barcodes, which species of Pterois is present on the Mexican Caribbean reef and which species comprise the diet of lionfish, based on the analysis of stomach contents.

Results

This study is the first report of the application of DNA barcoding to determine the prey composition for the invasive lionfish in the Atlantic Ocean. Partially-degraded biological material, such as stomach contents, can yield small PCR DNA fragments, sometimes less than 200 bp in length. Nevertheless, DNA barcoding can identify species with fragments as short as 100 bp with at least 90% efficiency [20]. The development of these mini-barcodes permits the species identification. This opens a great possibility to obtain sequences from short DNA fragments, quickly and cheaply [21].

DNA Barcode Identification of Lionfish Adults

Pterois volitans and P. miles overlap in most morphological and meristic characters but do have different DNA sequences [8]. All sequences we obtained from 30 adult lionfish in the Mexican Caribbean matched with Indo-Pacific Pterois volitans with over 99% similarity. The average K2P distance among individuals was 0.054%. The mean sequence composition was guanine 19.75%, cytosine 26.98%, adenine 23.22%, tyrosine 30.06%, GC 46.73%. GC% Codon position 1, 56.07, GC% Codon position 2, 42.81 and GC% Codon position 3, 40.83 (Table 1).

Identification of Prey Based in DNA Barcoding

Of the 157 stomachs examined, 144 had measurable contents (Table 2). In total 330 prey items were obtained but about 90% were mostly digested specimens. These included fish, typically only body parts or fragments of skeleton and tissue. As a result, most prey items were impossible to visually identify, even to order level. Some crustaceans were almost complete and could be identified before barcoding. All of the prey tissue fragments were barcoded, but only 168 yielded readable sequences. The read lengths in the majority (85%) were more than 600 bp long, while the remaining sequences had segments between 500 and 300 bp (mainly crustaceans) and only two sequences were less than 200 bp. There were no insertions, deletions or stop codons in any sequence. The sequences had segments between 500 and 300 bp (mainly crustaceans) and only two sequences were less than 200 bp. There were no insertions, deletions or stop codons in any sequence. The sequences were compared to the reference library of sequences in the Barcode of Life Database (BOLD). Of the 168 sequences, 125 matched with fishes and 43 with crustaceans. In case of the fish sequences, 94% matched with greater than 99.38% similarity to reference sequences in BOLD, allowing identification to the species level. The remaining 6% could be identified only to genus (Table 3).

Five orders of fishes comprising 14 families, 22 genera and 34 species were identified. The families with the greatest number of species were Gobiidae (7) and Apogonidae (6) followed by Scaridae (4), Labrisomidae (3), Labridae, Pomacentridae, Tripterygiidae, Serranidae (2), Holocentridae, Grammatidae, Haemulidae, Scorpaenidae, and Monacanthidae (1) (Figure 1, Table 3).

The total fishes species identified in the stomach contents (Table 3, Figure 1) include 27 species previously reported in the

| Table 1. Pterois volitans COI sequences composition (from 30 samples). |
|----------------------------------------------------------|
| **Sequence composition (%)** | Min | Mean | Max | SE  |
|-----------------------------|-----|------|-----|-----|
| Guanine                     | 18.96 | 19.75 | 20.19 | 0.028 |
| Citocynne                   | 26.66 | 26.98 | 27.01 | 0.009 |
| Adenine                     | 23.16 | 23.22 | 23.61 | 0.017 |
| Tyrosine                    | 29.7  | 30.06 | 30.41 | 0.013 |
| Guanine-Citocynne           | 45.97 | 46.73 | 46.97 | 0.024 |
| Guanine-Citocynne codon position 1 | 54.2  | 56.07 | 56.13 | 0.047 |
| Guanine-Citocynne codon position 2 | 41.86 | 42.81 | 44.01 | 0.041 |
| Guanine-Citocynne codon position 3 | 40.03 | 40.83 | 41.52 | 0.047 |

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| Table 2. Lionfish (Pterois volitans) specimens collected in the different localities from Mexican Caribbean. |
|----------------------------------------------------------|
| **Locality** | **Specimens collected** | **Specimens with stomach content** | **Collecting date (year)** | **Min-Max length of the specimens (mm)** |
|--------------|-------------------------|-----------------------------------|-----------------------------|------------------------------------------|
| Cozumel      | 58                      | 47                                | 2009                        | 28–216                                   |
| Xcalak       | 59                      | 54                                | 2009, 2010                  | 40–262                                   |
| Mahahual     | 35                      | 21                                | 2010                        | 70–320                                   |
| Isla Contoy  | 10                      | 6                                 | No data                     | 10–90                                    |
| Banco Chinchorro | 13              | 11                                | 2009                        | 60–282                                   |
| Puerto Morelos| 1                       | 1                                 | 2009                        | 76–308                                   |
| Playa del Carmen | 2                   | 1                                 | 2009                        | 330                                       |
| Isla Mujeres | 9                       | 3                                 | No data                     | 20–70                                     |

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Mexican Caribbean: Sargocentron coruscum, Apogon lachneri, A. maculatus, A. townsendi, Astrapogon puniculatus, Coryphopterus venezuelae, C. hyalinus, Priolepis hipoliti, Gramma loreto, Haemulon flavolineatum, Halichoeres garnoti, Thalassoma bifasciatum, Malacoctenus triangulates, Abudefduf saxatilis, Stegastes partitus, Scarus iseri, S. taeniopterus, Sparisoma aurofrenatum, S. viride, Cephalopholis cruentata, Liopropoma rubre, Enneanectes altivelis, Enneanectes boehlkei, Bothus lunatus, Pterois volitans, Monacanthus tuckeri, and seven species unreported before: Apogon mosavi, Coryphopterus venezuelae, C. thrix, C. tortugae, Lythrypnus minimus, Starkia langi and S. ocellata.

In terms of percent composition by number (%N) fishes dominated the lionfish diet (74.4%). The fish families with highest %N were Labridae (26.4%), comprising Halichoeres garnoti (17.6%) and Thalassoma bifasciatum (8.8%); Gobiidae (20%), comprising Coryphopterus venezuelae (4.8%), C. tortugae (4.8%) Lythrypnus (4.8%), C. eilodin (1.6%), C. hyalinus (1.6%), C. thrix (0.8%), and Priolepis hipoliti (0.8%); Scorpaenidae (12.8%) comprised the one species Pterois volitans; and Scaridae (10.4%) comprising Sparisoma aurofrenatum (6.4%), Scarus iseri (1.6%), S. viride (1.6%), and S. taeniopterus (0.8%).

The overall percent composition by number of crustaceans in lionfish stomach contents was 25.6%, with Decapoda the most frequent prey (93%) followed by Stomatopoda (4.6%) and Euphausiacea (2.4%). Of the 43 crustacean prey sequences, we identified 20 different taxa of which twelve were decapods. Only four showed more than a 90% similarity to reference sequences on BOLD, while the remainder showed similarities between 79 and 89% (Table 4). Three crustacean orders were identified: Euphausiacea with only one species, Euphausia americana; Stoma-

**Table 3.** List of fishes prey identified in the stomach contest of lionfish (Pterois volitans) by DNA barcoding analysis.

| Order            | Family          | Genus       | Species                        | No. of specimens | Similarity (%) |
|------------------|-----------------|-------------|--------------------------------|------------------|----------------|
| Beryciformes     | Holocentridae   | Sargocentron| Sargocentron coruscum          | 1                | 100            |
| Perciformes      | Apogonidae      | Apogon      | Apogon lachneri                | 2                | 100            |
| Perciformes      | Apogonidae      | Apogon      | Apogon maculatus               | 2                | 100            |
| Perciformes      | Apogonidae      | Apogon      | Apogon mosavi*                 | 1                | 99.68          |
| Perciformes      | Apogonidae      | Apogon      | Apogon townsendi               | 2                | 100            |
| Perciformes      | Astrapogonidae  | Astrapogon  | Astrapogon puniculatus         | 1                | 99.84          |
| Perciformes      | Gobiidae        | Coryphopterus| Coryphopterus venezuelae*      | 6                | 99.69          |
| Perciformes      | Gobiidae        | Coryphopterus| Coryphopterus eilodin          | 2                | 100            |
| Perciformes      | Gobiidae        | Coryphopterus| Coryphopterus hyalinus         | 2                | 100            |
| Perciformes      | Gobiidae        | Coryphopterus| Coryphopterus thrax*           | 2                | 99.85          |
| Perciformes      | Gobiidae        | Priolepis   | Priolepis hipoliti             | 1                | 99.69          |
| Perciformes      | Gobiidae        | Lythrypnus  | Lythrypnus minimus*            | 6                | 99             |
| Perciformes      | Grammatidae     | Gramma      | Gramma loreto                  | 3                | 99.84          |
| Perciformes      | Haemulidae      | Haemulon    | Haemulon flavolineatum         | 3                | 100            |
| Perciformes      | Labridae        | Halichoeres | Halichoeres garnoti            | 22               | 100            |
| Perciformes      | Labridae        | Thalassoma  | Thalassoma bifasciatum         | 11               | 100            |
| Perciformes      | Labrisomidae    | Malacoctenus| Malacoctenus triangulates       | 2                | 99.69          |
| Perciformes      | Labrisomidae    | Starkia     | Starkia ocellata*              | 1                | 99.38          |
| Perciformes      | Labrisomidae    | Starkia     | Starkia langi*                 | 1                | 99             |
| Perciformes      | Pomacentridae   | Abudelfduf  | Abudelfduf saxatilis           | 1                | 100            |
| Perciformes      | Pomacentridae   | Stegastes   | Stegastes partitus             | 6                | 99.85          |
| Perciformes      | Scaridae        | Scarus      | Scarus iseri                   | 2                | 100            |
| Perciformes      | Scaridae        | Scarus      | Scarus taeniopterus            | 1                | 100            |
| Perciformes      | Scaridae        | Sparisoma   | Sparisoma aurofrenatum         | 8                | 100            |
| Perciformes      | Scaridae        | Sparisoma   | Sparisoma viride               | 2                | 100            |
| Perciformes      | Serranidae      | Cephalopholis| Cephalopholis cruentata       | 3                | 100            |
| Perciformes      | Serranidae      | Liopropoma  | Liopropoma rubre               | 2                | 100            |
| Perciformes      | Tripterygiidae  | Enneanectes | Enneanectes altivelis          | 3                | 100            |
| Perciformes      | Tripterygiidae  | Enneanectes | Enneanectes boehlkei           | 1                | 100            |
| Pleuronectiformes| Bothidae        | Bothus      | Bothus lunatus                 | 1                | 100            |
| Scorpaeniformes  | Scorpaenidae    | Pterois     | Pterois volitans               | 16               | 100            |
| Tetraodontiformes| Monacanthidae   | Monacanthus | Monacanthus tuckeri            | 1                | 100            |

Also is showing percent of closest matches to reference sequences on BOLD.

*New range extension for Mexican Caribbean.

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Mexican Caribbean: Sargocentron coruscum, Apogon lachneri, A. maculatus, A. townsendi, Astrapogon puniculatus, Coryphopterus edolon, C. hyalinus, Priolepis hipoliti, Gramma loreto, Haemulon flavolineatum, Halichoeres garnoti, Thalassoma bifasciatum, Malacoctenus triangulates, Abudefduf saxatilis, Stegastes partitus, Scarus iseri, S. taeniopterus, Sparisoma aurofrenatum, S. viride, Cephalopholis cruentata, Liopropoma rubre, Enneanectes altivelis, Enneanectes boehlkei, Bothus lunatus, Pterois volitans, Monacanthus tuckeri, and seven species unreported before: Apogon mosavi, Coryphopterus venezuelae, C. thrix, C. tortugae, Lythrypnus minimus, Starkia langi and S. ocellata.

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topoda with two samples that apparently belong to Gonodactylidae and Pseudosquillidae, and the remaining matches were all to Decapoda. Among the latter, one specimen matched the genus Synalpheus, another matched to Hippolytidae and two groups, of three and seven specimens respectively, matched to two clades within Palemonidae. All remaining taxa could not be resolved to a finer level beyond Decapoda (Figure 2).

Discussion

Adults

It is possible to distinguish nine species of Pterois, including P. miles and P. volitans from the reference sequences of COI mtDNA in the BOLD database. All of our specimens matched with Pterois volitans and the low divergence values among them are consistent with a recent invasion from a small number of specimens. Our results support the idea that P. volitans is the only species which has spread into the Caribbean, including the Mexican region [4,9,10,22–24].

Prey Composition

In the Mexican Caribbean the lionfish (Pterois volitans) feeds on a wide diversity of prey, primarily reef-fish species and secondarily crustaceans. These results are concordant with the findings for prey composition of lionfish in the Bahamas [3,22,25].

Our values of %N in fishes and crustaceans are similar to those reported by Morris and Adkins in the Bahamas, who found that fishes comprised 71% of the prey items and crustaceans comprised 28.5% [3].

Seven of the identified species constitute range extensions into this area: Apogon mosavi, Coryphopterus venezuelae, C. thrix, C. tortugae, Lythrypnus minimus, Starksia langi and S. ocellata. The first five species are listed for the western and eastern Caribbean even in Belize [26–28], so their presence in this region is expected. The lionfish...
whose stomach contents included C. tortugae were collected from Banco Chinchorro and Xcalak. Recently we collected two adults of this species in the same locality confirming the presence of this species here. Coryphopterus cyanclus were detected in three lionfish stomachs from Xcalak. Vasquez Yeomans (Pers. comm.) collected a larva of this species in 2006 in East Cayo Centro, Chicharrón, confirming the presence of this fish in this area. Our six specimens of Lythrypnus matched in BOLD with L. minimus, one adult from Dominica (LIDMA 726-11) identified by Benjamin Victor (Pers. comm.) and 62 more Lythrypnus unidentified sequences. S. langi was described recently and is the Belizean species for the species complex of S. sluiteri [29] therefore their presence in the Mexican Caribbean is also expected. Finally, Starkia ocellata is part of a species complex, named S. occidentalis in the Caribbean and the Western Caribbean [30]. This species is a representative with a known range from North Carolina to Florida and the northern Gulf of Mexico. In Mexico there is only a single report in the literature, from Isla Conoy, but there is no voucher specimen. [31].

In the list of prey species, there are five fishes economically important in local markets: Haemulon flavolineatum (Chak-chi or French grunt), Scarus iseri (loro listado or striped parrotfish), Sparisoma aurofrenatum (loro manchado or red band parrotfish), S. viride (loro brilloso or stoplight parrotfish) and Cephalopholis cyanata (cabra, cherna enjambre or graysby). Although these species have not high value in the markets, they are an important source of food for local people.

The yellowhead wrasse, Halichoeres garnoti was the most frequent species in the analyzed stomachs, no doubt reflecting its common occurrence in the region [31]. In contrast, Coryphopterus cyanclus and Gramma loreto have been reported as the most frequent prey of lionfish in the Bahamas, likely reflecting habitat differences in the two locations. [3].

The barcoding of prey species revealed 16 specimens of Pterois volitans, although the majority of the samples showed a high degree of digestion (incomplete skeletons with little tissue). Nevertheless, we found one specimen (MXIV3660) almost completely intact and morphologically identifiable as Pterois. This is the first confirmation of cannibalism among invasive lionfish, a phenomenon that had been previously suggested as likely but with an absence of evidence [22]. The lionfish specimens found in the stomachs were small the intact specimen measured 25 mm SL indicating a preference for juveniles (Figure 3).

Only one specimen in the prey list did not match to any species in the BOLD or GenBank databases. It could be identified to the genus Astrapogon. This genus is represented in the Caribbean by three species, all are sequenced in BOLD: A. punticulatus was found in the prey samples in this study and the other species, A. stellatus and A. alatus did not match our sequence, raising the possibility of a cryptic species of Astrapogon in the region.

Prior studies in the Bahamas recorded 50 species of fishes in the diet of lionfish based on morphological examination of stomach contents as well as field observations [3,22,23]. We found 31 of those species and 17 not previously recorded. Considering the sampling effort for morphological and behavioral analyses, it is evident that DNA barcoding is a more efficient technique, limited for the present only by the incompleteness of the reference databases.

Among crustaceans, the only euphausiid identified was Euphausia americanus (CRU124.1) from a fish collected in Mahahual (Figure 2). This species has been reported from Xcalak to north of Cozumel Island [32,33]. Two specimens were identified as stomatopods. The sample CRU190 from Playa del Carmen,
matched (95% similarity) near the species Pseudosquilla ciliate, but with more than 3% divergence, the specimen was considered Pseudosquilla sp. The species reported in the Mexican Caribbean are P. ciliate [34,35] and P. oculata (IBUNAM:CNCR: CR10740) in the database of the National Collection of Crustaceans from the National Autonomous University of Mexico (UNAM, http://test.unibio.unam.mx), therefore it is possible that this specimen represents P. oculata. The other stomatopod (CRU238 from Contoy Island), matched 87.2% nearest to the Gonodactylidae.

There are three species reported in the literature of this family in Quintana Roo [34,35] and the National Collection of Crustaceans in UNAM, Neogonodactylus bredini and N. oerstedii are in the BOLD database but do not match this sequence, therefore the specimen may represent the third species N. spinulosus.

The most frequent crustacean order preyed on by lionfish was decapods, comprising 95% of the crustaceans. Most of the decapods sequences did not match closely to sequences in the reference databases (Table 3), in which case we applied "strict criteria" [36]. Only one sample (CRU 118) was 93% similar to Thor ambioensis, and thus considered Thor sp. Four species of this genus have been reported in the Mexican Caribbean: T. ambioensis, T. dobbini, T. floridanus and T. manningi [34,37,38]. The sample CRU120 was assigned to the snapping shrimp genus Synalpheus (with 99% of similarity), in the Mexican Caribbean there are six species: Synalpheus fritzmuelleri, S. hemphilli, S. longicarpus, S. minus, S. townsendi and S. apioceros [35,39]. There are 19 genera of snapping shrimps in BOLD, none of which matched with our specimen. Two sets of sequences matched to Palemonidae with 88% similarity (CRU136, 138 and 140 and CRU 107, 153, 155, 202,101,141,213). Little is known of the palaemonid fauna of this region and about 32 species of these shrimps have been reported from the shallow waters from Quintana Roo [38].

Crustaceans are an important component of stomach contents studied in most marine fishes, but their identification using morphology is difficult. For example, from 264 crustaceans found in lionfish stomachs from the Bahamas, 246 could not be identified [3]. In contrast, we could identify our 45 crustacean samples to at least the order level. Species level identifications are usually not feasible because of the present incomplete state of the reference databases.

In this study, the methodology yielded 51% efficiency for sequencing. However, most specimens of lionfish were placed into ethanol, with no injection into the viscera or thick muscle. Then, tissues of stomach contents were subsampled one year later, for pcr amplification. In contrast, from 35 tissues taken directly from fresh stomach contents, 29 of them gave good quality sequences, i.e. 83% efficiency, indicating the importance of the fixation process for the samples.

Our results suggest that lionfish are mostly opportunistic predators, eating any prey of appropriate size, consistent with findings from the native range in the Indo-Pacific [40,25] and including cannibalistic predation on smaller conspecifics as well.

Figure 2. Neighbour joining tree for 20 clades representing crustaceans in the stomach contents of the lionfish. Each clade represents a different species, only one could be identified with no doubts; the base of the triangle gives a rough idea of the most consumed crustaceans.

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Figure 3. Specimen morphologically identifiable as a lionfish, from the stomach content.

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Materials and Methods

For determination of the *Pterois* species, a small piece (about 1–3 mm³) of muscle was removed from 30 specimens collected from Cozumel (25), Xcalak (2), Puerto Morelos (2) and Playa del Carmen (1) and placed in 100% ethanol. To avoid DNA contamination, all tools were flame sterilized before sampling each specimen. The remainder of each fish was retained as a reference voucher in the Fish Collection of El Colegio de la Frontera Sur, Chetumal Unit (ECOCHP).

For the stomach contents analysis, we extracted the stomach from 122 lionfish from whole specimens previously fixed in alcohol, from Banco Chinchorro, Cozumel, Isla Contoy, Isla Mujeres, Puerto Morelos, Playa del Carmen, Xcalak. In case of Mahahual, the digestive tract from 35 fresh lionfish were dissected and placed in 96% ethanol and kept on ice. In total 157 stomachs were analyzed (Table 2). The specimens were collected by personnel from Secretaría del Medio Ambiente y Recursos Naturales (SEMARNAT, Mexico) working in protected areas or volunteers. Collecting methods varied from hand nets, harpoons to plastic bags.

From all stomach contents 1 mm³ tissue plugs were extracted from all recognizable material as a prey item under a binocular microscope, after that, the tissue was cleaned with alcohol to avoid contamination from other material.

To extract DNA, the plugs were placed in vertebrate lysis buffer with Proteinase K and digested overnight at 56°C. Genomic DNA was subsequently extracted using a membrane-based approach on the Biomek FX® liquid handling station and AcroPrep 96,1 mL filter plates with 1.0 μM PALL, glass fiber media [41]. A 652–658 bp segment of COI was amplified using different fish primers: FishF1, FishR1, FishF2, FishR2 (Ward et al. 2005) or a M13-tailed fish primer cocktail [42].

The 12.5 μL PCR reaction mix included 6.25 μL of 10% trehalose, 2 μL of ultrapure water, 1.25 μL of 10× PCR buffer, 0.625 μL of MgCl₂ (50 mM), 0.125 μL of each primer (0.01 μM), 0.0625 μL of dNTP mix (10 μM), 0.625 μL of Taq polymerase (New England Biolabs or Invitrogen), and 2.0 μL of DNA template. Amplification protocols followed those described in earlier publications [43]. PCR products were visualized on precast agarose gels (E-Gels®, Invitrogen) and the positives, represented by a band were selected for sequencing.

Products were labelled by using the BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.) as described [43] and sequenced bidirectionally using an ABI 3730 capillary sequencer following manufacturer’s instructions. Sequence data, electropherograms, trace files, primer details, photographs and collection localities for specimens are available within the project MXLionfish in BOLD (http://www.barcodinglife.org). Sequencing protocols were carried out at the Canadian Centre for DNA Barcoding using standard protocols [43]. Sequences were aligned using SEQUENCE v.2.1.1 software (Applied Biosystems, Inc.). All COI sequences have also been deposited in GenBank (http://www.ncbi.nlm.nih.gov/, See Table S1 for accession numbers).

The sequences obtained were submitted and identified with the ID engine provided in the Barcode of Life Database (BOLD: www.boldsystems.org) to establish whenever possible the identification of the ingested material. Sequence divergences were calculated using the tools provided by BOLD, the Kimura two parameter (K2P) distance model [44]. Neighbour-joining (NJ) trees based on K2P distances were created to provide a graphic representation of the patterning of divergence between species [45] and a simplified tree was constructed using the MEGA 3 software [46]. The criteria to assign identification to a specie level, was based on less than 3% divergence between the unknown and the reference sequence.

When a sequence match was not found in the DNA barcode reference library, we applied the method for visualization of two trees and based our taxonomical assignment following the strict criteria proposed, and consist in nest the “unknown” within a clade comprising of members of a single taxon. This criterion was used previously only with moths and 75% of the queries were correctly assigned to genus [30].

Supporting Information

Table S1 accession codes from specimens of the public database in BOLD and GenBank (XLS).

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Author Contributions

Conceived and designed the experiments: MVM. Performed the experiments: MVM CQL. Analyzed the data: MVM CQL. Contributed reagents/materials/analysis tools: CQL RGL MCGR. Wrote the paper: MVM. The former also included several comments that improved our original version, and especially to Paul Hebert for his support.

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