Pioneering the Use of DNA Metabarcoding for Stomach Content Analysis in the Invasive Lionfish (*Pterois volitans*) in Puerto Rico

Nuevos Caminos para el Uso de DNA Metabarcoding para el Análisis de Contenido Estomacal en el Pez León Invasiva *(Pterois volitans)* en Puerto Rico

Première à Utiliser des DNA Metabarcoding pour l'Analyse du Contenu de l'Estomacdans la Rascasse Volante Invasive (*Pterois volitans*) à Porto Rico

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KEY WORDS: Lionfish, *Pterois volitans,* DNA, Metabarcoding, stomach content, Puerto Rico

EXTENDED ABSTRACT

Introduction

Two common approaches to lionfish feeding ecology through gut content analysis are: (1) identification to the lowest possible taxon (i.e., using morphological characters to identify whole or partially digested specimens) or (2) a DNA barcoding approach, which involves sequencing of mitochondrial cytochrome oxidase subunit I (CO1) gene from all distinct prey components of the stomach. Morphological identification relies heavily on the ability to identify digested organisms to the species level, which is not possible in many cases (Baker et al. 2014). This technique discards useful information that could be obtained in the semi-digested portion of the stomach contents (small unidentifiable pieces from prey and the liquids or digested "mush"). DNA barcoding allows taxonomic access to small unidentifiable tissue pieces, but despite the higher resolution, it also has some disadvantages. It can still only be applied to identifiable items in the stomach contents and does not reduce sampling effort (Coissac et al. 2012). The digested products may contain under-represented prey items, or prey items that have yet to be acknowledged within the diet.

Metabarcoding is the amalgamation of DNA based identification and high-throughput DNA sequencing that reduces sampling effort and maximizes species-level identification of organism remnants, previously undetected or underutilized in traditional methods. In this study, metabarcoding analysis of all lionfish stomach contents, regardless of their digestive stage, is used to provide a more accurate profile of the lionfish prey in Puerto Rico while demonstrating that the approach is applicable to all other regions of the invasion. The specific objectives are:

i) To identify the prey of Puerto Rico lionfish in stomach contents through metabarcoding, and
ii) To assess the effectiveness of this approach compared to published studies utilizing other gut content analysis methods.

Questions to be addressed were:

i) Can this metabarcoding method identify prey items within the digested contents of the gut? and
ii) Do the diets of the lionfish differ spatially?

Materials and Methods

Sixty-three lionfish were utilized for metabarcoding of entire stomach contents. Approximately half of the lionfish came from near-shore reefs of La Parguera, southwest Puerto Rico, (17°58′12.33″N, 67°2′45.83″W) while half were collected from off-shore reefs in the same region from June 2013 to January 2014. The two reef locations are characterized by distinctly different habitats, where inshore reefs are connected through a series of shallow patch and linear reefs, mangroves, and seagrasses critical for ontogenetic migrations (Aguilar-Perera and Appeldoorn 2008). The offshore shelf edge reefs are characterized by spur and groove formations and better water quality, with exposure to stronger currents (Pittman et al. 2010). Lionfish were collected by pole spear on scuba at depths equal to or less than 30 m, with venomous spines being immediately removed. On the boat, specimens were immediately placed on ice to slow digestion and preserve DNA (Baker et al. 2014). All metrics pertaining to lionfish size, sex, reproductive state and weight were recorded. The stomachs were removed in less than two hours after lionfish capture, and preserved whole in a -80°C freezer until further processing.

DNA was extracted following the guidelines of the manufacturer (Qiagen DNeasy Blood & Tissue Kit) from two components of the 63 whole stomach contents:

i) The tissues of remaining partially digested organisms (as with a DNA barcoding approach), and
ii) The liquids of completely digested organisms, resulting in 126 samples.

Polymerase chain reaction (PCR) amplification of a 313 bp CO1 fragment from prey mtDNA was performed on each of the 126 samples (tissues and liquid). Taxon-specific primers (for fish and invertebrates in coral reef fish guts) and the PCR profile were utilized from Leray et al. (2013).

CO1 amplicons were ligated with a unique three base identifier (ATG), followed by a specific six base barcode added to the forward and/or reverse primer that would allow for identification of each sequence back to a particular lionfish stomach, as well as whether it was sampled from the liquid or tissue portion of the diet. In total, 109 samples were successfully amplified. Successful samples represented 59 offshore stomachs, 50 inshore stomachs, 57 tissue samples and 52 liquid samples. CO1 amplicons were multiplexed and sequenced in one Illumina MiSeq lane (Scripps Research Institute, CA), resulting in a total of 966 sequences, with 669 sequences retained after quality control. These sequences were blasted (BLASTn) in GenBank (August 2015) and referenced in the Barcode of Life Data Systems (BOLD) (September 2015) to identify matches. A confident match for fish was identified as 98% or higher on both databases.

Results

Lionfish diet in La Parguera, Puerto Rico was dominated by fish. Fish families with the greatest number of species represented in the diet included Gobiidae (6), Apogonidae and Scaridae (5) and Pomacentridae (4). By frequency of occurrence, Apogonidae made up 18%, while Gobiidae (9%) and Scaridae (10%) were less frequently found (Table 1). Pomacentridae had the greatest frequency of occurrence (35%), which was dominated by three species: *Chromis multilineata* (71%), *Chromis cyanea* (63%), and *Stegastes partitus* (58%).

Four species were only observed in the inshore lionfish stomach contents, while eight species and one family were unique to offshore diets. Furthermore, three taxa were only detected in the liquid portion of the diet including the first account of *Starksia williamsi* in Puerto Rico.

Table 1. Fish families represented in the lionfish (*Pterois volitans*) diet. Number of species corresponds to those identified to species level. Frequency indicates the number of stomachs in which they were found. Scorpaenidae refers only to *P. miles* haplotype.

Family	No. of Species	Frequency
Acanthuridae	1	2.18
Apogonidae	5	18.58
Chaenopsidae	1	3.00
Chaetodontidae	1	1.09
Gobiidae	6	9.56
Grammatidae	1	0.55
Haemulidae	1	1.09
Holocentridae	1	1.09
Labridae	3	3.00
Labrisomidae	2	1.91
Lutjanidae	0	0.23
Pomacentridae	4	34.69
Priacanthidae	1	0.55
Scaridae	5	10.65
Serranidae	3	9.29
Synodontidae	1	2.18

Discussion

The spatial comparison of lionfish diet revealed similarities consistent with native fish distribution in the region. Two dominant members of the diet, *Scarus iseri* and *Stegastes partitus,* are found across the insular shelf in La Parguera (Pittman et al. 2010). However, there were species that were unique to inshore or offshore diets, such as *Clepticus parre* and *Halichoeres garnoti,* which are typically observed on the shelf edge in this region. There were no commercially important species, but many ecologically important members of the family Scaridae. This family is a key player in the native fish assemblage of La Parguera, likely due to the lack of large-bodied piscivores and overabundance of macroalgal reefs (Pittman et al. 2010). These results are consistent with previous studies indicating lionfish have a site specific diet (Côté and Maljkovic 2010), with locally abundant species often dominating. However, lionfish could also be targeting specific prey, given that the most abundant fish on the reefs of La Parguera is *Thalassoma bisfasciatum* (Nemeth 2013), which was absent from the gut contents. This hypothesis can be further supported by the frequency of occurrence of Pomacentridae and Apogonidae within the guts. These families demonstrate specific prey characteristics that have been found to contribute to their susceptibility to predation: small bodied, hovering behavior, solitary and nocturnal (for Apogonidae) (Green and Côté 2014).

The most significant contribution of this metabarcoding approach includes the utilization of the digested materials in the guts, including what little remains within empty stomachs. Some taxa were only detected within the digested components of the gut, indicating the contribution of this material which was previously neglected in the other two methods of gut content analysis. For example, the first account of the blenny *Starksia williamsi* was reported for Puerto Rico.

Overall, this study successfully demonstrated the efficiency of the metabarcoding approach to identify the prey profile of lionfish. When compared to studies utilizing morphological identification, reduction in sampling effort was significantly improved while resolution was retained. In comparison with traditional DNA barcoding, two studies utilizing over 100 lionfish stomachs resulted in 31-37 species identified within the guts (Valdez-Moreno et al. 2012, Côté et al. 2013), while this study used 63 lionfish and identified 39 species of fish.

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