Vol. (in press)
doi: 10.3354/meps11738

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published (in press)

Contribution to the Theme Section 'Invasion of Atlantic coral reefs by Pacific lionfish'

Use of DNA metabarcoding for stomach content analysis in the invasive lionfish *Pterois volitans* in Puerto Rico

Chelsea A. Harms-Tuohy*, Nikolaos V. Schizas, Richard S. Appeldoorn

Department of Marine Sciences, University of Puerto Rico at Mayagüez, Call Box 9000, Mayagüez, PR 00681, USA

ABSTRACT: Studies of lionfish feeding ecology seek to document the ecological impact of this invasive predatory species and determine which native prey species are at greatest risk. There are 2 common approaches to feeding ecology through gut content analysis: morphological identification to the lowest possible taxonomic rank and/or DNA barcoding of individual prey components in the stomach. The major disadvantage of both techniques is their inability to use advanced digested material. This study introduces next-generation sequencing to lionfish feeding ecology, employing DNA metabarcoding to analyze all components of the gut contents, including the previously unidentifiable portion. Sixty-three lionfish were caught from the inshore and offshore reefs of La Parguera, Puerto Rico. Stomach contents were separated into 2 sample components — a liquid (i.e. digested) and undigested tissue. A 313 bp region of the cytochrome oxidase subunit I (COI) gene was amplified from extracted DNA using specific primers for Caribbean reef fish. Samples were sequenced with an Illumina MiSeq platform, and the resulting 950+ sequences were compared against GenBank and the Barcode of Life Database to identify specimens at the lowest taxonomic level. Thirty-nine fish species from 16 families were identified (35 each in the digested and tissue fractions), including members of Pomacentridae, Acanthuridae, Gobiidae, Apogonidae, and Scaridae. Using the digested liquiform material proved efficient in detecting prey species, especially those that would have been missed with traditional methods.

KEY WORDS: Reef fish · Feeding ecology · Invasive species · Caribbean · Cytochrome oxidase subunit I · COI · Next-generation sequencing · NGS

INTRODUCTION

Invasive species are capable of altering ecosystems, evolving with their new environment (Mooney & Cleland 2001) and driving native species extinctions (Pimm 1987, Fritts & Rodda 1998). In response, management of invasive species attempts to mitigate their ecological and economic impacts (Buckley 2008). However, marine invasive species present a difficult management scenario where vectors promoting their spread and establishment may be known (i.e. ballast transport, aquarium trade) but cannot be easily regulated or avoided without strict enforcement (Bax et al. 2003). Marine invaders, once established, often become integrated into the ecosystem, whereby complete eradication is unfeasible (Thresher & Kuris 2004). This scenario is exacerbated when their presence extends to areas that remain inaccessible to management, such as mesophotic depths, or in cases where the spread of the invasive species is driven by larval dispersal. Aside from investigating management strategies, invasion ecologists must simultaneously seek to identify which native communities may be at greatest risk, either ecologically or economically.

Invasive species alter ecosystems through competition, niche displacement, hybridization, and predation, among other processes (Mooney & Cleland 2001). In particular, predation in the marine environment is a driving force structuring the fish communi-

*Corresponding author: chelsea.harms@upr.edu

Publisher: Inter-Research · www.int-res.com

ties on coral reefs (Hixon 1991). Aside from observing this predator–prey interaction *in situ*, predation can also be documented using visual inspection or, more recently, DNA barcoding to assess biodiversity in diet from gut contents or feces (Sheppard & Harwood 2005). Over a decade has passed since DNA barcoding first proved useful in biodiversity applications (Hebert et al. 2003), and has recently been promoted as an ecological tool for addressing issues including a species' invasion potential, trophic interactions, and food webs (Joly et al. 2014). With the advancement and lower cost of DNA sequencing and massive growth of reference databases, a metabarcoding approach using next-generation sequencing (NGS) has quickly emerged as a promising method for higher-resolution diet analysis (Pompanon et al. 2012, Taberlet et al. 2012, de Barba et al. 2014, Deagle et al. 2013). Metabarcoding is the combination of DNA-based identification and high-throughput DNA sequencing that reduces sampling effort and maximizes species-level identification of tissue remnants that were previously undetected or underused by traditional methods.

There are known constraints on metabarcoding, including the inability to quantify the species information obtained (Deagle et al. 2010, 2013, Bowles et al. 2011, Murray et al. 2011). Results are limited or biased to the frequency of occurrence, which still provides useful information when seeking to understand localized effects of an invasive predator. However, the underlying variability in DNA quality, differential breakdown of that DNA during digestion, and differences in digestion stages (Deagle & Tollit 2007, Troedsson et al. 2009, Valentini et al. 2009b), as well as the objective of identifying several different organisms within the same sample (i.e. the gut) (Valentini et al. 2009a), still hinder the quantification aspect in metabarcoding of gut contents. Despite these disadvantages, metabarcoding is quickly gaining popularity as a tool for assessing biodiversity in animal diets (Leray et al. 2013, de Barba et al. 2014). NGS allows for the highest degree of confidence in gut content analysis (Pompanon et al. 2012) with significantly reduced sampling effort (Taberlet et al. 2012), but has only recently been applied to fish feeding ecology (Leray et al. 2013, 2015).

Understanding the extent and possible ecological impact of the lionfish *Pterois volitans* invasion of the Western Atlantic, Gulf of Mexico and Caribbean is an issue that employs all facets of lionfish biology and ecology. Of particular interest is how this Indo-Pacific fish will affect native coral reef fauna, especially commercially and ecologically important reef fishes. Researchers have sought to address what lionfish consume, in terms of species and size classes, in an effort to document which species may suffer the greatest level of mortality. Feeding ecology has been a key component in many lionfish studies, resulting in our current understanding of site specificity in dietary preferences (Côté & Maljković 2010, Muñoz et al. 2011, Layman & Allgeier 2012) and overall diversity of diet (Albins & Hixon 2008, Morris & Akins 2009, Green et al. 2011).

There are 2 common approaches to lionfish feeding ecology through gut content analysis: morphological identification to the lowest possible taxon (i.e. using morphological characters to identify whole or only partially digested specimens) or a DNA barcoding approach, which involves sequencing of the mitochondrial 16S rRNA or cytochrome oxidase subunit I (COI) genes 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 digested portion of the stomach contents (the liquids or digested pulp). However, the traditional morphological method is widely applied (Albins & Hixon 2008, Morris & Akins 2009, Alexander & Haynes 2011, Jud et al. 2011, Muñoz et al. 2011, Green et al. 2012, Frazer et al. 2012, Layman & Allgeier 2012, Green & Côté 2014), while the more accurate DNA barcoding approach has been less frequently used (Barbour et al. 2010, Valdez-Moreno et al. 2012, Côté et al. 2013). Despite the higher resolution attained with this approach, traditional DNA barcoding also has disadvantages. This technique does not reduce sampling effort (Coissac et al. 2012) and can be applied only to items in the stomach contents for which barcode information is available either in databases or can be generated during concomitant sequencing of possible prey from the area. However, as opposed to morphological identification, analyzed items can include unrecognizable specimens, liquids, or pulp (Saitoh et al. 2003), but this approach requires molecular cloning and is therefore labor intensive and costly. These digested products may contain underrepresented prey items, or prey items that have yet to be acknowledged within the diet.

In this study, we used metabarcoding analysis of all lionfish stomach contents, regardless of their digestive stage, to provide a more accurate profile of the lionfish prey in Puerto Rico while demonstrating that the methodological approach is applicable to all other regions of the invasion. Metabarcoding resolution of lionfish stomach contents is supported by the

a priori knowledge, albeit site specific, of the lionfish diet (Côté & Maljković 2010, Muñoz et al. 2011, Layman & Allgeier 2012), whereas the use of COI as a marker often allows for identification to the species-level in online reference databases. The specific objectives were (1) to identify the prey of Puerto Rican lionfish in stomach contents through the use of NGS, (2) to compare inshore and offshore diets of lionfish in La Parguera, Puerto Rico, and (3) to assess the general suitability of the NGS metabarcoding approach compared to published studies using other gut content analysis methods.

MATERIALS AND METHODS

Collection and locations

Sixty-three lionfish were used for metabarcoding of entire stomach contents. Approximately half of the lionfish came from inshore reefs of La Parguera (17° 58′ 12.33″ N, 67° 2′ 45.83″ W) while half were collected from offshore shelf-edge reefs in the same region from June 2013 to January 2014 (Fig. 1). La Parguera is a natural reserve on the southwest coast of Puerto Rico that is heavily affected by environmental and anthropogenic stressors resulting in low coral cover, high macroalgal abundance, and diminished populations of large-bodied fish species, resulting in the system being dominated by small-bodied planktivores and piscivores (Pittman et al. 2010). The inshore reefs are subjected to high particle suspension and lower water quality (García-Sais et al. 2005, 2008) and are connected through a series of shallow patch and linear reefs, mangroves, and seagrasses critical for ontogenetic migrations (Aguilar-Perera & Appeldoorn 2007, 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). The inshore and offshore reefs harbor dissimilar fish richness and biomass (Pittman et al. 2010), where inner reefs are comparatively lower in species richness than shelf-edge reefs (Nemeth 2013), thus providing a potential spatial comparison of lionfish diets. Lionfish were collected by pole spear and SCUBA at depths ≤30 m. On the boat, the venomous spines were immediately removed and specimens were placed on ice to slow digestive processes and preserve DNA (Baker et al. 2014). All metrics pertaining to lionfish size, sex, reproductive state, and weight were recorded (see Table S1 in the Supplement at www.int-res.com/articles/suppl/mXXXpXXX_supp.pdf). The stomachs were removed <2 h after lionfish capture, and preserved whole in a −80°C freezer until further processing.

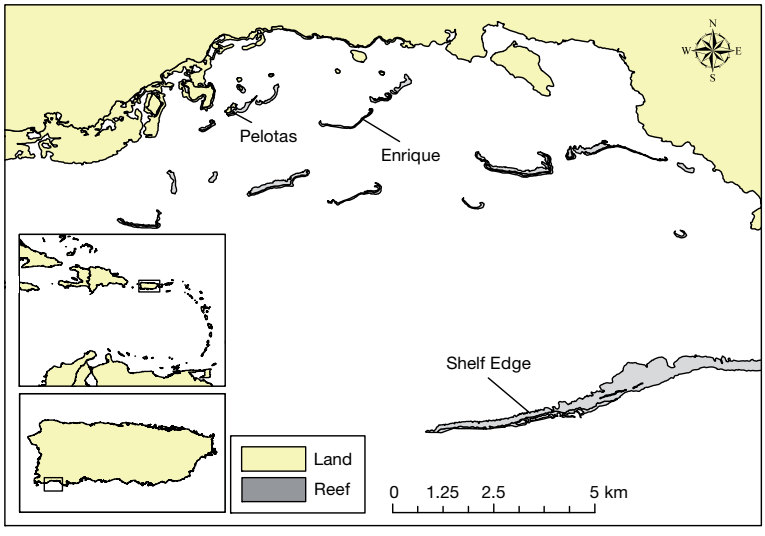

Fig. 1. La Parguera, Puerto Rico, and the insular shelf, with 3 study sites identified. Pelotas and Enrique reefs were the inshore collection sites, while the shelf edge was the offshore location. All sampling was performed up to 30 m depth

DNA extraction and COI amplification

Samples were thawed at room temperature until the liquefied digested materials could be removed. Only a few prey items could be identified with visual inspection, thus morphological identification was not coupled with this study. DNA was extracted (Qiagen DNeasy Blood & Tissue Kit) following the guidelines of the manufacturer from 2 components of the 63 whole stomach contents: (1) the tissues of the remaining partially digested organisms (as with a DNA barcoding approach) and (2) the liquids of completely digested organisms, resulting in 126 samples. Cross contamination was avoided by subjecting dissection utensils to an open flame, followed by an ethanol rinse between each sample, or in some cases new utensils were used for each stomach. The quality and quantity of extracted DNA was measured with the NanoDrop 2000 (Thermo Fisher Scientific). Samples were stored in a −20°C freezer for later analysis.

PCR amplification of a 313 bp COI fragment from prey mtDNA was performed on each of the 126 samples (tissues and liquid). This gene was chosen for its exceptional

coverage of Caribbean fishes (Weigt et al. 2012) and other marine metazoan taxa (Bucklin et al. 2011). It is also the most widely accepted DNA barcode, where its rapid evolution allows for discrimination between closely related species (Hebert et al. 2003). Taxon-specific primers (for fish and invertebrates in coral reef fish guts) were utilized; the mlCOIintF forward primer (5′GGW ACW GGW TGA ACW GTW TAY CCY CC) in conjunction with the jgHCO2198 reverse primer (5′TAI ACY TCI GGR TGI CCR AAR AAY CA) (Leray et al. 2013). The specific region of COI is adequately represented in online databases for Caribbean coral reef fishes and invertebrates (Leray et al. 2013), as well as estimates of relative abundance of species in benthic samples (Leray & Knowlton 2015). The DNA amplification was completed in a total volume of 20 µl on the MyCycler (Bio-Rad Laboratories). The PCR recipe contained 0.6 µl of 10 µM of each forward and reverse primers, 10 µl of MyTaq DNA polymerase mix (Bioline), and 0.5 µl of genomic DNA. This recipe varied slightly depending on the success of the PCR, in which the concentration of DNA was increased up to 1.5 µl and all other ingredients varied accordingly to maintain a 20 µl reaction. We adopted the PCR profile from Leray et al. (2013) and conducted 16 initial cycles: denaturation for 10 s at 95°C, annealing for 30 s at 62°C, and extension for 60 s at 72°C. This initial set of cycles was followed by 25 cycles at 46°C annealing temperature with the same denaturation and extension steps, with a final extension at 72°C for 6 min. Success of PCR amplifications was validated on 1.5% agarose gels. The second step of the PCR process involved addition of the barcode identifiers. COI amplicons were ligated with a unique 3 base identifier (ATG), followed by a specific 6 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 (see Tables S2 & S3 in the Supplement). We produced 126 unique combinations of barcodes from 16 forward primers and 7 reverse primers, including the original PCR primers.

All samples were loaded into a 2% agarose gel with TAE buffer and allowed to run for 45 min. The gel was briefly placed under a low-intensity UV light to identify the presence of the bands. Each sample was then excised from the gel using the 'freeze–squeeze' method (Tautz & Renz 1983), avoiding primer dimers, and was placed into individually labeled 1.5 ml centrifuge tubes. In total, 109 samples were successfully acquired. Successful samples represented 59 offshore samples and 50 inshore samples, divided into 57 tissue samples and 52 liquid samples.

Sequencing and bioinformatics

Samples were multiplexed and sequenced in 1 Illumina MiSeq lane (Scripps Research Institute, CA). Resulting reads were cleaned in the FASTQ filing and extended using FLASH pair software (Mago & Salzberg 2011). Extended fragments were converted to FASTA files. To utilize the insert in both directions, the reverse complement of the extended read ('FASTX') was combined with the original extended fragment. Sequences were then demultiplexed to identify reads back to their original stomach sample.

In total, 966 sequences were obtained. These were manually trimmed of the original PCR primers in Notepad++ v6.8, and each sequence was individually inspected. All sequences shorter than 200 bp length were discarded, along with duplicates and chimeric sequences. The resulting 313 bp COI fragment sequences were blasted (BLASTn) in GenBank (August 2015) to identify matches. A confident match was identified as 98% or higher for vertebrates and 80% or higher for invertebrates. The difference in acceptance of matches is based on the limited availability of invertebrate references in GenBank. Sequences were also referenced in the Barcode of Life Database (BOLD Systems v.3) using known and validated barcode identification numbers corresponding to voucher specimens (Victor et al. 2015) and accepted at a 98% match (September 2015). All cleaned reads were translated into amino acids using ExPASy Translate tool (Artimo et al. 2012) and MEGA 6 (Tamura et al. 2013) to further support accurate matches to references in both databases. Cleaned reads were separated by species and aligned in MEGA 6 to identify insertions, deletions, and frame shifts. If stop codons were present in the sequence, the sequence was rejected. An insertion of an amino acid (3 bases) was accepted, and all reads with 1 or 2 insertions and 1 deletion were accepted. A sequence was discarded if a series of 'N's representing unknown bases were present in the read, indicating sequencing ambiguity. All vertebrate sequences with less than 98% match were removed from subsequent analysis. Species that were represented by only 1 sequence were retained, in an effort to document rare and under-represented items from the gut contents that might previously have been unreported. All sequences obtained from this study are available in GenBank (accession numbers KX140056–KX140702) and a BOLD dataset (DS-PARG2016).

RESULTS

Lionfish diet in La Parguera, Puerto Rico, was diverse, with gut content analysis through metabarcoding revealing 2 phyla, 5 orders, 18 families, 23 genera, and 40 species. We assume that all prey DNA recovered from the gut was prey of lionfish. All fish sequences matched a reference in BOLD and GenBank. Of the 966 sequences recovered, 442 had fish species-level matches to 98% or greater and an additional 205 sequences were the lionfish itself, resulting in a 65% metabarcoding efficiency at the 98% similarity threshold for fish. Of those fish sequences, excluding lionfish, 17 had up to 2 insertions while 8 had 1 deletion and 7 sequences had an additional amino acid. Thirty-seven sequences could not be demultiplexed to the appropriate stomach and were labeled as unclassified. Eleven sequences reported discrepancies in similarities between databases, but were included in the final count if at least 1 match met the acceptance criteria. Forty-six sequences had stop codons present and were discarded, 99 were duplicated sequences from de-multiplexing errors and were discarded. Additionally, 18 chimeric or nonsensical sequences were discarded as well as 2 sequences shorter than 200 bp. Lastly, 99 sequences could not be matched at 98% or higher to either database. At a similarity match of 80 to 100% in GenBank and BOLD, 22 sequences corresponded to invertebrates, with 5 matched at the species level; however, a disagreement of identification occurred when comparing both reference databases. Thus, these species were placed in a higher taxon resulting in 18 Decapoda sequences, 1 Penaeidae, 2 Portunidae, and 1 sequence of the shrimp *Metapenaeopsis gerardoi*.

Fish contributed to the largest portion of the diet (95% of prey DNA recovered). 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 3 species: *Chromis multilineata* (71%), *C. cyanea* (63%), and *Stegastes partitus* (58%) (Table 2).

Four species were observed only in the inshore lionfish stomach contents, while 8 species and 1 family were unique to offshore diets. Furthermore, 3 taxa were detected only in the liquid portion of the diet including the first account of the labrisomid *Starksia williamsi* in Puerto Rico (Table 3).

Table 1. Fish families represented in the diet of lionfish *Pterois volitans* at La Parguera, Puerto Rico. Number of species corresponds to those identified to species level except the Family Lutjanidae. Frequency indicates the number of stomachs in which they were found

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	1[a]	0.55
Pomacentridae	4	34.69
Priacanthidae	1	0.55
Scaridae	5	10.65
Serranidae	3	9.29
Synodontidae	1	2.18

[a]Only identified to Family level

Invertebrates represented a small portion of the diet, accounting for only 5% of the sequences obtained from gut content analysis. All cleaned sequences reported at least an 82% similarity to a reference in GenBank, which was usually complemented by a better match in BOLD. The Order Decapoda was the most abundant taxon (76%) (Table 4). Two families, Penaeidae and Portunidae, were documented only in offshore samples, and only from the tissue. The only species-level identification was the shrimp *Metapenaeopsis gerardoi*.

Lionfish DNA was present in every stomach, indicating the overwhelming abundance of predator DNA in the samples. For this reason, lionfish was not included in the prey profiling.

DISCUSSION

This study presents the first case of DNA metabarcoding for lionfish stomach contents. Overall, fish were identified as the most dominant prey component in the diet of lionfish in La Parguera. Representatives of several fish functional groups were observed within the gut, including herbivores, piscivores, and planktivores. No commercially important species of groupers or snappers were identified, which could be due to their low abundance in the study area resulting from high fishing intensity. Fishermen in the shallow water reef systems of La Parguera typically

Table 2. Number of lionfish *Pterois volitans* stomachs in which fish species were found, by location (inshore and offshore collection sites) and gut fraction. Species could occur in both liquid and tissue samples from the same stomach. Unclassified could not be de-multiplexed back to a particular stomach. Frequency is the frequency of occurrence for all species from all stomachs, including those unclassified

Prey species	Inshore	Offshore	Liquid	Tissue	Unclassified	Frequency (%)
Acanthurus tractus	3	5	4	5	0	12.31
Apogon maculatus	7	11	9	10	2	30.77
Apogon pillionatus	3	10	9	5	0	20.00
Apogon townsendi	0	4	4	1	1	7.69
Bodianus rufus	1	2	1	2	0	4.62
Chaetodon capistratus	2	2	0	4	0	6.15
Chromis cyanea	7	27	16	24	7	63.08
Chromis multilineata	15	28	7	9	3	70.77
Clepticus parrae	0	2	1	1	0	3.08
Coryphopterus glaucofraenum	9	5	8	10	1	23.08
Coryphopterus hyalinus	1	1	1	1	0	3.08
Coryphopterus lipernes	3	8	3	8	2	20.00
Coryphopterus personatus	2	0	1	2	0	3.08
Coryphopterus tortugae	2	0	1	1	0	3.08
Emblemariopsis arawak	0	2	0	2	0	3.08
Emblemariopsis spp.	4	4	5	4	1	13.85
Gnatholepsis thompsoni	0	1	1	0	0	1.54
Gramma loreto	2	0	0	2	0	3.08
Haemulon flavolineatum	2	2	2	3	0	6.15
Halichoeres garnoti	0	6	3	3	0	9.23
Heteropriacanthus cruentatus	1	1	0	2	0	3.08
Hypoplectrus spp.	6	10	8	10	1	26.15
Hypoplectrus aberrans	0	1	1	0	0	1.54
Hypoplectrus nigricans	4	3	3	5	3	15.38
Hypoplectrus puella	2	2	2	2	2	9.23
Lutjanidae sp.	0	1	1	0	0	1.54
Malacoctenus macropus	2	2	1	3	0	6.15
Phaeoptyx conklini	8	11	13	10	1	30.77
Phaeoptyx pigmentaria	2	5	3	5	3	15.38
Sargocentron coruscum	2	1	0	3	1	6.15
Scarus iseri	14	10	14	14	3	41.54
Scarus taeniopterus	0	2	1	1	0	3.08
Scarus vetula	1	1	1	1	0	3.08
Sparisoma radians	3	0	0	3	1	6.15
Sparisoma viride	1	3	2	2	0	6.15
Starksia williamsi	0	2	2	0	1	4.62
Stegastes partitus	8	26	17	15	4	58.46
Stegastes variabilis	1	1	1	1	0	3.08
Synodus intermedius	1	6	4	3	1	12.31

Table 3. Species that were observed in only 1 habitat or type of lionfish *Pterois volitans* stomach content category

Inshore	Offshore	Liquid	Tissue
Coryphopterus personatus	*Apogon townsendi*	*Gnatholepsis thompsoni*	*Emblemariopsis arawak*
Coryphopterus tortugae	*Clepticus parrae*	Lutjanidae sp.	*Chaetodon capistratus*
Gramma loreto	*Emblemariopsis arawak*	*Starksia williamsi*	*Gramma loreto*
Sparisoma radians	*Gnatholepis thompsoni*		*Heteropriacanthus cruentatus*
	Halichoeres garnoti		*Sargocentron coruscum*
	Hypoplectrus aberrans		*Sparisoma radians*
	Lutjanidae sp.		
	Scarus taeniopterus		
	Starksia williamsi		

Table 4. Number of lionfish *Pterois volitans* stomachs in which invertebrate taxa were found, by location (inshore and offshore collection sites) and gut fraction. Stomachs could have taxa represented in both liquid and tissue factions. Frequency is the frequency of occurrence for each taxa from all stomachs. The percent similarity refers to the match to a reference in the Barcode of Life Database

Taxon	Inshore	Offshore	Liquid	Tissue	Frequency	Similarity (%)
Decapoda	11	2	6	10	76.47	97.3
Penaeidae	0	1	0	1	5.88	88.7
Portunidae	0	2	0	2	11.76	100
Metapenaeopsis gerardoi	0	1	0	1	5.88	97.6

target snappers, groupers, grunts, and parrotfishes (Pittman et al. 2010), all of which are potential prey for lionfish. Ecologically important species were identified in the gut, such as *Scarus vetula, S. taeniopterus, S. iseri*, and *Sparisoma viride*, which are known to help prevent macroalgae from displacing corals (Mumby & Steneck 2008). Some of these parrotfishes have been identified to co-occur across all seascapes in La Parguera, including the offshore reefs (Pittman et al. 2010, Nemeth 2013), supporting their presence in the diet of both inshore and offshore lionfish.

Two comprehensive studies of the La Parguera fish assemblages (Pittman et al. 2010, Nemeth 2013) and one island-wide study (NCCOS 2016) provide field-occurrence data for a comparison to observed prey frequencies within the guts (Table 5). In general, lionfish diet is representative of the particular fish assemblages observed in La Parguera and Puerto Rico, which supports the emerging trend observed from other studies in the Caribbean (Côté & Maljković 2010, Muñoz et al. 2011, Layman & Allgeier 2012) that lionfish are trophic generalists and that dietary preferences are site specific and driven by the spatial and temporal dynamics of prey. However, some species are consumed in unequal proportions to what exists in nature (Table 5), represented by the absence of *Thalassoma bisfasciatum* within the guts, and the overrepresentation of both *Chromis cyanea* and *C. multilineata.* These pomacentrids may be preferentially targeted due to their morphology (i.e. small but deep-bodied) and hovering behavior, both of which have been identified as preferred traits for lionfish prey (Green & Côte 2014).

Overall dietary profiles were very similar inshore and offshore, as would be expected given the broad spatial distribution of the dominant prey species observed. Nevertheless, differences were observed between inshore and offshore diets, as revealed by species found only in one of these categories. Eight fish species were identified only in offshore diets. Of these, *Clepticus parrae, Halichoeres garnoti*, and *Scarus taeniopterus* are typically associated with shelf-edge habitats (Pittman et al. 2010, NCCOS 2016) and were not largely represented in the lionfish diet overall. In contrast, *Stegastes partitus* was among the most frequently observed species in the gut, and despite its occurrence across the insular shelf, it showed a strong association with the shelf-edge reef system, with 28 stomachs containing this species in offshore lionfish versus only 8 in the inshore system. In general, more prey species were identified from offshore samples (n = 36 versus inshore n = 31), where their total frequency of occurrence was almost

Table 5. Percent frequency of occurrence of key species within lionfish *Pterois volitans* gut contents (this study) and on the insular shelf of La Parguera (Pittman et al. 2010, Nemeth 2013) and Puerto Rico island-wide (Clark et al. 2015). Nemeth (2013) frequencies refer to abundance in terms of percent mean density of individuals per 100 m^2

Fish species	Frequency				Rank			
	Pittman et al.	NCCOS	Nemeth	Gut	Pittman et al.	NCCOS	Nemeth	Gut
Thalassoma bifasciatum	34	75	11.3	0	5	3	1	8
Chaetodon capistratus	42.2	4	1.8	6	2	7	7	7
Acanthurus tractus	41.9	76	3	12	3	2	6	6
Coryphopterus glaucofraenum	28	21	–	23	6	6	–	5
Scarus iseri	45	49	8.1	42	1	4	3	4
Stegastes partitus	38	80	10.6	58	4	1	2	3
Chromis cyanea	6.1	31	4.7	63	7	5	4	2
Chromis multilineata	3.5	–	3.9	70	8	–	5	1

twice that observed inshore (n = 208 versus inshore n = 119). The shelf edge off La Parguera has the greatest fish species richness and biomass in the region, with up to 41 species identified in a single 100 m^2 transect (Pittman et al. 2010, Nemeth 2013). However, these results may also be due to the significantly (*t*-test, $p < 0.05$) larger size of lionfish found offshore (217.8 g, 200 mm standard length [SL]) compared to inshore (147.6 g, 167.7 mm SL). The gobies *Coryphopterus personatus* and *Coryphopterus tortugae*, the parrotfish *Sparisoma radians,* and the basslet *Gramma loreto* were found only in lionfish sampled from inshore reefs. All were sampled at low frequency, but the distributions of the first 3 species are known to be inshore. Additionally, 2 frequently occurring prey with broad distributions across the shelf, the parrotfish *Scarus iseri* and the goby *Coryphopterus glaucofraenum*, were the only species found more frequently inshore. Juveniles of the former species are common in inshore nursery areas, but are infrequently seen near the shelf edge (Cerveny 2006).

These comparisons between the distribution and frequency of prey species in lionfish stomachs relative to their distribution in the field suggest that both the list of prey species and their frequency of occurrence as determined by metabarcoding can be used to compare diets among different populations or even different habitats and life history stages. In general, smaller or juvenile lionfish have been observed to consume proportionally more invertebrates than larger, adult lionfish (Morris & Akins 2009), and at least 28% of prey by number in stomach contents represent invertebrates (Morris & Akins 2009, Valdez-Moreno et al. 2012). In particular, shrimp are the most common invertebrate observed, representing the families Palaemonidae, Penaeidae (Barbour et al. 2010, Jud et al. 2011, Layman & Allgeier 2012), and Alpheidae (Valdez-Moreno et al. 2012, Layman et al. 2014). In our study, invertebrates were equally consumed by juvenile lionfish (n = 8, 74–181 mm SL) and adult lionfish (n = 7, 190–239 mm SL), and were observed in the guts predominantly from inshore lionfish (n = 11) versus offshore (n = 5). Overall, invertebrates did not contribute to a large portion of the diet, and proved to be the most difficult to identify given the potential number and diversity of available prey species inhabiting Caribbean reefs and the current status of the reference databases. Invertebrates are lacking in species-level identification in both BOLD and GenBank, and occasionally the 2 databases did not agree on the identification based on the submitted DNA sequences. Thus, we had to place our sequence into higher taxa, as our resolution could not be matched by references in both BOLD and GenBank. The crustacean Order Decapoda contributed to the greatest resolution and highest frequency. The diet included both crabs and shrimps, represented by Portunidae and Penaeidae, respectively, which is consistent with the previously known feeding ecology of lionfish (Morris & Akins 2009).

The spatial and temporal distribution of lionfish also affects the prey items detected in the gut. Lionfish are habitat generalists (Cure et al. 2014), and can be found in any natural marine system, or artificial structure, including the seagrass–mangrove–reef continuum within inshore La Parguera. In contrast to other mobile predators (Appeldoorn et al. 2009), lionfish do not typically undertake diurnal feeding migrations between different habitats. However, they have been observed to venture off-structure to feed over sand, perhaps in response to intraspecific competition (Green et al. 2011, Dahl & Patterson 2014). In our study location, lionfish densities are relatively low (C. Harms-Tuohy pers. obs.), and intraspecific competition is likely minimal. A study of lionfish movement on a reef in La Parguera identified that lionfish did not move between nearby forereef habitat of the same depth and characteristics (Harms-Tuohy 2016). Considering that all lionfish were collected from the fore reefs of the sampling sites, we would expect their diets to resemble the prey communities dominant to these areas, and this was evidenced in our results. This further supports that the diet of lionfish observed in this study was driven by the spatial distribution of the prey.

Overall, this study successfully demonstrated the efficiency of the metabarcoding approach to identify the prey profile of lionfish. The most significant contribution of this method is between use of the digested materials in the guts, including what little remains within empty stomachs. We report a comparable resolution of species diversity obtained from the liquefied portion of the guts in comparison with that contributed by the tissues. Given that lionfish collection was performed at times most feasible to divers (08:00–14:00 h), the contents of the lionfish stomachs were almost entirely digested. However, in most cases, partially digested specimens could be identified taxonomically as either fish or invertebrate, but no further. Morphological identification of gut contents relies heavily on the digested state of the prey items (Baker et al. 2014). Regardless, this method has been used widely in lionfish feeding ecology. Visual assessment of gut contents from lion-

fish in the Bahamas reported up to 41 fish species (Albins & Hixon 2008, Morris & Akins 2009), while DNA barcoding of 157 lionfish gut contents in the Mexican Caribbean (Valdez-Moreno et al. 2012), and 130 lionfish gut contents from the Bahamas (Côté et al. 2013), reported 31 and 37 fish species, respectively. Although the yield of new species identified certainly decreases with increased sampling effort (see Morris & Akins 2009), our study reports 39 different fish species from only 63 lionfish stomachs, thus validating the small sampling effort and increased efficiency of DNA metabarcoding (Table 6).

Despite the efficiencies realized using metabarcoding for prey identification, our approach is not without caveats. There is a high initial investment regarding the purchase of primers with enough barcodes to differentiate each sample. However, in subsequent studies, the same barcodes can be reused, thus significantly reducing the cost associated with specimen capturing, DNA processing (e.g. extraction, amplification, gel extraction), and NGS. Additionally, there is no current method to differentiate among prey-of-prey (i.e. items that were consumed by a prey fish that the lionfish subsequently ate) and true prey. However, as our lionfish diet mostly comprised herbivores and planktivores with few piscivores, this scenario likely did not affect our results. There is currently no precise way to quantify prey in the stomach using metabarcoding. Unfortunately, it cannot be assumed that the number of sequences for each particular species represents the amount of DNA (or number of individuals) contributing to the sample because the quality of that DNA depends on many factors including degradation and digestion rates (Deagle & Tollit 2007, Troedsson et al. 2009, Valentini et al. 2009b). Thus, quantitative analyses at this time are limited to the frequency of prey occurrence. Percent composition by number can be calculated by conducting metabarcoding on experimental individuals fed a mixed but controlled number of prey sacrificed over several time periods of digestion, including complete digestion to the liquid phase. Nevertheless, identifying prey and their frequency of occurrence using metabarcoding is a significant step forward, allowing useful information to be obtained from a minimum number of samples (Taberlet et al. 2012) without the need to collect samples immediately after feeding events. To further enhance the resolution of sequences obtained from this method, species-specific primers could be generated to search for the presence of specific prey items that may be of concern (Pompanon et al. 2012). This is particularly useful if the prey are poorly represented in a diet. Predator blocking primers could also assist in a wider range of detected species, in that predator DNA many times overwhelms that of the prey (Pompanon et al. 2012). In addition, it is unlikely that our primers amplified every single prey. Thus, the fish diet presented here is not expected to be exhaustive of all taxa consumed by the lionfish.

Successful mitigation of the impacts of invasive species requires an understanding of how they are affecting native communities. Impacts can be defined as competition or predation with native species, habitat alteration, niche displacement, and hybridization among many other factors. The direct effect of predation can be assessed through gut content analysis and measured in terms of what species may be targeted, or what functional groups are at risk in a broader sense. Feeding ecology will continue to provide temporal and spatial snapshots of lionfish impacts on native communities, which can be compared regionally and annually to assess changes in prey assemblages.

Acknowledgements. Support for this study was provided by the Puerto Rico Sea Grant College Program and the Caribbean Laboratory of Marine Genomics (University of Puerto Rico, Mayagüez). We are grateful to B. Victor and L. Schaffer for assistance with bioinformatics; E. Tuohy, R. Becicka, D. Sanabria, O. Espinosa, and M. Carlo for their assistance with lionfish collection; and A. Galindo and M. Ricaurte for guidance with DNA extraction and PCR amplification.

Table 6. Yield of new fish species identified in different methodological attempts. Comparison of visual identification, DNA barcoding, and DNA metabarcoding methods

No. of stomachs	No. of fish species	Method	Yield	Reference
1069	41	Visual ID	0.038	Morris & Akins (2009)
52	14	Visual ID	0.269	Albins & Hixon (2008)
157	31	Barcoding	0.197	Valdez-Moreno et al. (2012)
130	37	Barcoding	0.285	Côté et al. (2013)
63	39	Metabarcoding	0.619	This study

LITERATURE CITED

Aguilar-Perera A, Appeldoorn RS (2007) Variation in juvenile fish density along the mangrove–seagrass–coral reef continuum in SW Puerto Rico. Mar Ecol Prog Ser 348: 139–148

Aguilar-Perera A, Appeldoorn RS (2008) Spatial distribution of marine fishes along a cross-shelf gradient containing a continuum of mangrove–seagrass–coral reefs off southwestern Puerto Rico. Estuar Coast Shelf Sci 76: 378–394

Albins MA, Hixon MA (2008) Invasive Indo-Pacific lionfish *Pterois volitans* reduce recruitment of Atlantic coral-reef fishes. Mar Ecol Prog Ser 367:233–238

Alexander AK, Haynes JM (2011) Red lionfish (*Pterois volitans*) invade San Salvador, Bahamas: no early effects on coral and fish communities. Int J Bah Stud 17:50–66

Appeldoorn RS, Aguilar-Perera A, Bouwmeester BL, Dennis GD and others (2009) Movement of fishes (Grunts: Haemulidae) across the coral reef seascape: a review of scales, patterns and processes. Caribb J Sci 45:304–316

Artimo P, Jonnalagedda M, Arnold K, Baratin D and others (2012) ExPASy: SIB bioinformatics resource portal. Nucleic Acids Res 40(W1):W597–W603

Baker R, Buckland A, Sheaves M (2014) Fish gut content analysis: robust measures of diet composition. Fish Fish 15:170–177

Barbour AB, Montgomery ML, Adamson AA, Díaz-Ferguson E, Silliman BR (2010) Mangrove use by the invasive lionfish *Pterois volitans.* Mar Ecol Prog Ser 401:291–294

Bax N, Williamson A, Aguero M, Gonzalez E, Geeves W (2003) Marine invasive alien species: a threat to global biodiversity. Mar Pol 27:313–323

Bowles E, Schulte PM, Tollit DJ, Deagle BE, Trites AW (2011) Proportion of prey consumed can be determined from faecal DNA using real-time PCR. Mol Ecol Resour 11:530–540

Buckley YM (2008) The role of research for integrated management of invasive species, invaded landscapes and communities. J Appl Ecol 45:397–402

Bucklin A, Steinke D, Blanco-Bercial L (2011) DNA barcoding of marine metazoa. Annu Rev Mar Sci 3:471–508

Cerveny K (2006) Distribution patterns of reef fishes in southwest Puerto Rico, relative to structural habitat, cross-shelf location, and ontogenetic stage. MSc thesis, University of Puerto Rico, Mayagüez, PR

Coissac E, Riaz T, Puillandre N (2012) Bioinformatic challenges for DNA metabarcoding of plants and animals. Mol Ecol 21:1834–1847

Côté IM, Maljković A (2010) Predation rates of Indo-Pacific lionfish on Bahamian coral reefs. Mar Ecol Prog Ser 404: 219–225

Côté IM, Green SJ, Morris JA Jr, Akins JL, Steinke D (2013) Diet richness of invasive Indo-Pacific lionfish revealed by DNA barcoding. Mar Ecol Prog Ser 472:249–256

Cure K, McIlwain JL, Hixon MA (2014) Habitat plasticity in native Pacific red lionfish *Pterois volitans* facilitates successful invasion of the Atlantic. Mar Ecol Prog Ser 506:243–253

Dahl KA, Patterson WF III (2014) Habitat-specific density and diet of rapidly expanding invasive red lionfish, *Pterois volitans*, populations in the northern Gulf of Mexico. PLoS One 9:e105852

De Barba M, Miquel C, Boyer F, Mercier C, Rioux D, Coissac E, Taberlet P (2014) DNA metabarcoding multiplexing and validation of data accuracy for diet assessment: application to omnivorous diet. Mol Ecol Resour 14: 306–323

Deagle BE, Tollit DJ (2007) Quantitative analysis of prey DNA in pinniped faeces: potential to estimate diet composition? Conserv Genet 8:743–747

Deagle BE, Chiaradia A, McInnes J, Jarman SN (2010) Pyrosequencing faecal DNA to determine diet of little penguins: Is what goes in what comes out? Conserv Genet 11:2039–2048

Deagle BE, Thomas AC, Shaffer AK, Trites AW, Jarman SN (2013) Quantifying sequence proportions in a DNA-based diet study using Ion Torrent amplicon sequencing: Which counts count? Mol Ecol Resour 13:620–633

Frazer TK, Jacoby CA, Edwards MA, Barry SC, Manfrino CM (2012) Coping with the lionfish invasion: Can targeted removals yield beneficial effects?. Rev Fish Sci 20: 185–191

Fritts TH, Rodda GH (1998) The role of introduced species in the degradation of island ecosystems: a case history of Guam. Annu Rev Ecol Syst 29:113–140

García-Sais J, Appeldoorn R, Bruckner A, Caldow C and others (2005) The state of coral reef ecosystems of the commonwealth of Puerto Rico. In: Waddell JE (ed) The state of coral reef ecosystems of the United States and Pacific Freely Associated States: 2005. Tech Memo NOS NCCOS 11. NOAA, Silver Springs, MD, p 91–134

García-Sais J, Appeldoorn R, Battista T, Bauer L and others (2008) The state of coral reef ecosystems of the commonwealth of Puerto Rico. In: Waddell JE, Clarke A (eds) The state of coral reef ecosystems of the United States and Pacific Coral reef ecosystems of Reserva Natural de La Parguera (Puerto Rico): spatial and temporal patterns in fish and benthic communities (2001–2007). Freely Associated States: 2008. Tech Memo NOS NCCOS 73. NOAA, Silver Springs, MD, p 75–116

Green SJ, Côté IM (2014) Trait-based diet selection: prey behaviour and morphology predict vulnerability to predation in reef fish communities. J Anim Ecol 83: 1451–1460

Green SJ, Akins JL, Côté IM (2011) Foraging behaviour and prey consumption in the Indo-Pacific lionfish on Bahamian coral reefs. Mar Ecol Prog Ser 433:159–167

Green SJ, Akins JL, Maljković A, Côté IM (2012) Invasive lionfish drive Atlantic coral reef fish declines. PLoS One 7:e32596

Harms-Tuohy CA (2016) The management and feeding ecology of the invasive lionfish (*Pterois volitans*) in Puerto Rico. PhD dissertation, University of Puerto Rico, Mayagüez, PR

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proc R Soc Lond B Biol Sci 270:313–321

Hixon MA (1991) Predation as a process structuring coral reef fish communities. In: Sale PF (ed) The ecology of fishes on coral reefs. Academic Press, San Diego, CA, p 475–508

Joly S, Davies J, Archambault A, Bruneau A and others (2014) Ecology in the age of DNA barcoding: the resource, the promise and the challenges ahead. Mol Ecol Resour 14:221–232

Jud ZR, Layman CA, Lee JA, Arrington DA (2011) Recent invasion of a Florida (USA) estuarine system by lionfish *Pterois volitans/P. miles.* Aquat Biol 13:21–26

Layman CA, Allgeier JE (2012) Characterizing trophic

ecology of generalist consumers: a case study of the invasive lionfish in The Bahamas. Mar Ecol Prog Ser 448: 131–141

Layman CA, Jud ZR, Nichols P (2014) Lionfish alter benthic invertebrate assemblages in patch habitats of a subtropical estuary. Mar Biol 161:2179–2182

Leray M, Knowlton N (2015) DNA barcoding and metabarcoding of standardized samples reveal patterns of marine benthic diversity. Proc Natl Acad Sci USA 112: 2076–2081

Leray M, Yang JY, Meyer CP, Mills SC and others (2013) A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. Front Zool 10:34

Leray M, Meyer CP, Mills SC (2015) Metabarcoding dietary analysis of coral dwelling predatory fish demonstrates the minor contribution of coral mutualists to their highly partitioned, generalist diet. PeerJ 3:e1047

Mago T, Salzberg SL (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27:2957–2963

Mooney HA, Cleland EE (2001) The evolutionary impact of invasive species. Proc Natl Acad Sci USA 98:5446–5451

Morris JA Jr, Akins JL (2009) Feeding ecology of invasive lionfish (*Pterois volitans*) in the Bahamian archipelago. Environ Biol Fishes 86:389–398

Mumby PJ, Steneck RS (2008) Coral reef management and conservation in light of rapidly evolving ecological paradigms. Trends Ecol Evol 23:555–563

Muñoz RC, Currin CA, Whitfield PE (2011) Diet of invasive lionfish on hard bottom reefs of the Southeast USA: insights from stomach contents and stable isotopes. Mar Ecol Prog Ser 432:181–193

Murray DC, Bunce M, Cannell BL, Oliver R and others (2011) DNA-based faecal dietary analysis: a comparison of qPCR and high throughput sequencing approaches. PLoS One 6:e25776

NCCOS (National Centers for Coastal Ocean Science) (2016) National Coral Reef Monitoring Program: assessment of coral reef fish communities in Puerto Rico from 2014-05-19 to 2014-12-03 (NCEI Accession 0131260). NOAA National Centers for Environmental Information. Dataset. http://data.nodc.noaa.gov/cgi-bin/iso?id=gov.noaa.nodc:0131260

Nemeth M (2013) Distribution of roving herbivorous fishes on coral reefs at multiple spatial scales. PhD dissertation, University of Puerto Rico, Mayagüez, PR

Pimm SL (1987) The snake that ate Guam. Trends Ecol Evol 2:293–295

Pittman SJ, Hile SD, Jeffrey CFG, Clark R and others (2010) Coral reef ecosystems of Reserva Natural La Parguera (Puerto Rico): spatial and temporal patterns in fish and benthic communities (2001–2007). Tech Memo NOS NCCOS 107. NOAA, Silver Springs, MD

Pompanon F, Deagle BE, Symondson WO, Brown DS, Jarman SN, Taberlet P (2012) Who is eating what: diet assessment using next generation sequencing. Mol Ecol 21:1931–1950

Saitoh K, Takagaki M, Yamashita Y (2003) Detection of Japanese flounder-specific DNA from gut contents of potential predators in the field. Fish Sci 69:473–477

Sheppard SK, Harwood JD (2005) Advances in molecular ecology: tracking trophic links through predator–prey food-webs. Funct Ecol 19:751–762

Taberlet P, Coissac E, Pompanon F, Brochmann C, Willerslev E (2012) Towards next-generation biodiversity assessment using DNA metabarcoding. Mol Ecol 21: 2045–2050

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 30:2725–2729

Tautz D, Renz M (1983) An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels. Anal Biochem 132:14–19

Thresher RE, Kuris AM (2004) Options for managing invasive marine species. Biol Invasions 6:295–300

Troedsson C, Simonelli P, Nägele V, Nejstgaard JC, Frischer ME (2009) Quantification of copepod gut content by differential length amplification quantitative PCR (dla-qPCR). Mar Biol 156:253–259

Valdez-Moreno M, Quintal-Lizama C, Gómez-Lozano R, García-Rivas MDC (2012) Monitoring an alien invasion: DNA barcoding and the identification of lionfish and their prey on coral reefs of the Mexican Caribbean. PLoS One 7:e36636

Valentini A, Miquel C, Ali Nawaz M, Bellemain E and others (2009a) New perspectives in diet analysis based on DNA barcoding and parallel pyrosequencing: the trnL approach. Mol Ecol Resour 9:51–60

Valentini A, Pompanon F, Taberlet P (2009b) DNA barcoding for ecologists. Trends Ecol Evol 24:110–117

Victor BC, Valdez-Moreno M, Vásquez-Yeomans L (2015) Status of DNA barcoding coverage for the tropical Western Atlantic shorefishes and reef fishes. DNA Barcodes 3: 85–93

Weigt LA, Baldwin CC, Driskell A, Smith DG, Ormos A, Reyier EA (2012) Using DNA barcoding to assess Caribbean reef fish biodiversity: expanding taxonomic and geographic coverage. PLoS One 7:e41059

Editorial responsibility: Mark Hixon (Guest Editor), Honolulu, Hawaii, USA

Submitted: January 15, 2016; Accepted: April 19, 2016
Proofs received from author(s): May 30, 2016