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Diagnostic PCR can be used to illuminate meiofaunal diets and trophic relationships

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Abstract

Analysis of the meiofaunal food web is hampered because few prey have features that persist long enough in a predator's digestive tract to allow identification to species. Hence, at least for platyhelminth predators, direct observations of prey preference are almost nonexistent, and where they occur, prey identification is often limited to phylum. Studies using an *in vitro* approach are rare because they are extremely time-consuming and are subject to the criticism that predators removed from their natural environment may exhibit altered behaviors. Although PCR-based approaches have achieved wide application in food-web analysis, their application to meiofaunal flatworms suffers from a number of limitations. Most importantly, the microscopic size of both the predator and prey does not allow for removal of prey material from the digestive tract of the predator, and thus the challenge is to amplify prey sequences in the presence of large quantities of predator sequence. Here, we report on the successful use of prey-taxon-specific primers in diagnostic PCR to identify, to species level, specific prey items of 13 species of meiofaunal flatworms. Extension of this method will allow, for the first time, the development of a species-level understanding of trophic interactions among the meiofauna.

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Authors' Contributions. HM carried out the primer design and conducted most of the laboratory work under the supervision of JSIII. AW designed and conducted the initial blocking-primer experiments. The study as a whole was designed by JSIII in concert with MKL and SRF. All authors read and approved the final manuscript before submission.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Flatworm DNA extracts screened for nematode prey using diagnostic PCR, arranged by taxonomic placement.

Table S2. Primers and protocols for routine 18S sequencing. For each predator species used, a portion of the 18s rDNA gene was recovered by nested PCR and sequenced using the primers and protocol shown below.

Table S3. Nematode and flatworm sequences used for primer design.

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Additional key words

diet analysis; flatworms; food webs; nematodes; predation

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Trophic interactions among organisms determine structuring processes within communities (e.g., competition and predation), as well as the flow of energy and matter through ecosystems. Identifying and quantifying such relationships have been viewed as fundamental and essential goals of ecological research, starting over a century ago (Forbes 1887) and continuing to the present day (Pascual & Dunne 2006). However, elucidating food web relationships has proven to be challenging within all consumer trophic levels and in all habitats (Pascual & Dunne 2006), but especially so for communities dominated by meiofauna (Giere 2009). The lack of both qualitative and quantitative information on meiofaunal trophic structure limits our ability to understand how meiofaunal communities are organized and hampers testing hypotheses about the role of meiofauna in different habitats. Presently, a species-level food web has not been described for any marine meiofaunal assemblage (Giere 2009).

The small body size (generally 50–500 μm) of meiofauna and the complex, three-dimensional, opaque substrates in which most meiofauna live, strongly limit the application of techniques generally used for comparably-sized plankton and for larger organisms. To determine trophic connections, previous investigators have used direct observations (Watzin 1985; Moens et al. 2000), inferences based on spatial distributions (Hochberg 1999), or morphology (Wieser 1953; Doe 1976; Martens & Schockaert 1986; Hochberg 2004; Moens et al. 2006; Uyeno & Kier 2010). Molecular techniques have included antibodies directed at prey (Feller 1979), isotopic tracer studies (e.g., Pascal et al. 2013), stable isotope ratios (Moens et al. 2005; Lebreton et al. 2012), and fatty-acid signatures (Leduc 2009; Leduc et al. 2009; Cnudde et al. 2013). Although each of these techniques has advantages, their disadvantages limit their application and generalization of their results.

Direct observations can provide clear evidence of a species' capability to ingest specific food items (see Watzin 1985 for platyhelminths, and dos Santos et al. 2011 for nematodes), and this method was used to collect what we believe to be the only species-level food web information for a meiofaunal community (Schmid-Araya et al. 2002). However, these studies are so labor-intensive they are rarely carried out, and because all such observations occur in laboratory conditions they are subject to the criticism that they do not allow generalization to processes occurring in undisturbed habitats. Correlations between buccal and pharyngeal morphologies and presumed diet have provided much information about nematode trophodynamics (Wieser 1953; Moens et al. 2006), but morphological information may be misleading (Moens et al. 2005), and morphology only provides a clue to general prey category and subsequent laboratory studies are needed to provide more specific information on species selectivity. Finally, radioactively-labeled tracers, stable isotopes, and fatty-acid signatures can provide insight into dietary connections between, but not within, trophic levels (Olafsson et al. 1999; Maria et al. 2011; Moens et al. 2002).

A variety of PCR-based methods have been used in molecular-ecological diet studies of macrofauna (King et al. 2008; Vestheim & Jarman 2008; Boessenkool et al. 2011; O'Rourke

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et al. 2012; Pompanon et al. 2012). When we employed a predator-sequence blocking primer/universal prey primer approach during preliminary trials with field-collected turbellarian predators, we uniformly recovered predator sequences. As a result, we focused on evaluating the utility of a diagnostic PCR approach (Jarman et al. 2002, 2004) for elucidating trophic relationships among meiofauna. In diagnostic PCR, taxon-specific primers are designed to amplify prey sequences while simultaneously avoiding amplification of predator DNA. Here, we report success with this approach for 13 species of meiofaunal turbellarians.

Methods

Meiofaunal turbellarians were collected from exposed beaches in North Carolina and Tobago (Supporting Information, Table S1). The North Carolina sites were chosen in part because we have an on-going study of long-term changes in meiofaunal community structure at several sites on Bogue Banks, NC (Whitson et al. 2011; Bursey et al. 2012). Specimens were extracted (along with most meiofauna) from sediment samples using $MgCl_2$ anesthesia/decantation (Hulings & Gray 1971), and identified to helping-name (see below) under the stereomicroscope. Specimens used for 18S sequencing and for diet analysis described here were removed from the extraction dish at variable times (from immediately following extraction to up to 36 h later), anesthetized in $MgCl_2$, and preserved in 100% ethanol. Some specimens were isolated from non-conspecifics into watch glasses and starved overnight. A single specimen of *Cicerina* “debrae” was preserved immediately after being observed to capture and engulf a nematode; this specimen served as our positive control.

Prior to and during this study, specimens were processed for microscopy as described in Whitson et al. (2011) and Bursey et al. (2012). As nearly all of the species used are undescribed, the results of the microscopic investigations were used for taxonomic placement to the lowest possible level and to ensure that identifications under the stereomicroscope were correctly made. To avoid creating *nomina nuda*, names of undescribed species listed here are not formally being made available for taxonomic purposes. Accordingly, they are listed by lowest taxonomic rank presently known, or, in some cases, by “helping-name”. The synonymies generated here will be noted in future species descriptions.

Genomic DNA was extracted using the DNeasy Blood and Tissue kit according to the manufacturer’s protocol (Qiagen Inc., Valencia, CA). Nested-PCR amplification for routine 18S sequencing followed a modified version (Table S2) of the protocol used by Noren & Jondelius (1999). Amplicons were purified for sequencing using QIAquick or MinElute columns (Qiagen, Inc., Valencia, CA). DNA concentrations were measured with a Nanodrop 2000 (Thermo Scientific). Purified amplicons were diluted, premixed with sequencing primer, and sent to commercial facilities for sequencing (EnGenCore, Columbia, SC or EurofinsMWG|Operon). Trace files were checked for quality and edited in FinchTV (vers. 1.4.0; Geospiza Inc., Seattle, WA) and assembled into consensus sequences using MacVector (vers. 12.7.4; MacVector Inc., Cary, NC). Putative prey sequences were compared to the 18S sequence of the predator to check for the possibility that our primer

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sets amplified predator, rather than prey DNA. Prey sequences were identified to taxon by blastn (NCBI; http://www.ncbi.nlm.nih.gov/). Putative conspecific sequences recovered from multiple predators were aligned with each other to ascertain that a prey species can indeed be consumed by several different predator species.

Diagnostic primer design

Twenty-three nematode and ten kalyptorhynch 18S rDNA sequences from Genbank were aligned with nine sequences of potential predators collected from our sites. To design nematode-specific primers, we aligned putative prey target sequences representing the marine Chromadorea and Enoplia (Table S3). Within each group, we selected nematode species common in exposed, high-energy beaches, similar to environments from which our flatworms had been collected. As a result, our primers can be used to survey representatives from 9 nematode families. Predator sequences included 8 Kalyptorhynchia and one undescribed prolecithophoran *(Plagiostomum* “corculum”; Table S2). The alignment was used to design primers that were predicted to be nematode-specific in the presence of kalyptorhynch (or plagiostomid) DNA by identifying nematode priming-sequence regions that exhibited 3′–end-mismatches with the flatworm sequences. Primers sets were designed from two non-overlapping regions of the alignment to yield predicted products of ~215 base pairs (bp) and 340 bp, respectively: Nem215Fwd: 5′-GCGAATRGCTCATTACAAC-3′/ Nem215Rev: 5′-GACACTTGAARGAYACRTCRCC-3′ and Nem340Fwd: 5′-CAGCAGCCGCGGTAAT-3′/ Nem340Rev: 5′-CACCTCTMACGYBGSARTACGA-3′. Diagnostic PCR was carried out on DNA samples from 31 turbellarians (Table S1) using the two primer sets and 3–15ng of predator DNA in 25μL reactions containing 2x Taq master-mix (New England Biolabs, Ipswich, MA). An initial denaturation at 95°C for 30 s was followed by 34 cycles of 95°C for 25 s, 52°C for 30 s, 68°C for 1 min, and a final extension at 68°C for 5 min.

Results

Using diagnostic PCR and the “nematode-specific” primer sets, screening of 31 potential predators (Table S1) with both primer sets resulted in 22 positive amplifications (from 15 predator species) that were sequenced (Table 1). Of the 15 predators yielding positive amplifications, slightly more of the shorter 215 bp amplicon and slightly fewer of the 340 bp amplicon were obtained (13 vs. 9). Of these, 16 amplifications could be assembled into consensus sequences of potential prey items using both forward and reverse reads (including nematode prey from the positive control, *Cicerina* “debrae”). With one exception, the 16 samples yielded >86% sequence identities among the top-scoring Blastn results. We identified 12 sequences as nematodes (Table 1).

We also recovered four proseriate sequences (Table 1). Specifically, a member of Monocelidinae was found in the kalyptorhynch *Lehardyia alleithoros* Whitson et al. 2011, the sequence of an undescribed species of Coelogynoporidae (Coelogynoporidae n. sp. 1) was recovered from the kalyptorhynch “ProschizoSpirale,” a sequence of 93% similarity to *Cirrifera dumosa* Sopott 1972 was obtained from an undescribed monocelid (*Paramonotus*? n. sp.), and from the undescribed coelogynoporid (Coelogynoporidae n. sp. 1) sequences that

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were identical to the predator itself were recovered (Table 1). Furthermore, our primer sets also recovered sequences of Acoelomorpha from "ProschizoTertius" and "S&M Schizo" (Table 1). In two instances ("EukalyptoZange" and *L. alleithoros*), PCR products clearly contained multiple sequences that could not be resolved unambiguously.

We were able to recover the same prey species in multiple species of turbellarian predators. Specifically, a 215 bp product amplified from the eukalyptorhynch *Cicerina* "debrae," from one specimen of the schizorynch *Cheliplana* n. sp., and from the macrostomorph *Paromalostomum* "riegeri" was identified as a species of *Daptonema* (Table 1). In contrast, the 215 bp and 340 bp amplicons from "EukalyptoRiese" matched different species of *Enoplolaimus* (Table 1). The 215 bp product from *Prognathorhnychus busheki* Ax 1977 matched an unknown metazoan sequence with 97% identity.

Discussion

We have demonstrated that diagnostic PCR is useful for elucidating species-level trophic connections within a meiofaunal community. Specifically, we have now identified some of the prey species consumed by 13 different meiobenthic turbellarians. In contrast, four years of intensive sampling at our Bogue Banks sites yielded exactly two instances of direct observation of predation among 31 potential predators. Specimens of *Cicerina* "debrae" have been observed to eat nematodes on three occasions, and one *Paramonotus* sp. was observed with an ingested nematode. Clearly, diagnostic PCR provides a more rapid identification of trophic connections. It also facilitates species-level identification of the prey for the non-specialist (our direct observations of the prey in the two instances mentioned above were recorded as "nematodes").

The smaller, 215 bp fragment was recovered more frequently than the larger 340 bp one. This result is expected because DNA had been partially digested. Furthermore, although it was gratifying that the majority of our prey sequences matched known genera, it is clear that the success of diet studies in identifying species-level interactions in any given community will depend initially on the quality of the available genetic databases and ultimately on having known sequences for all of the species in the community.

We are able to use these preliminary data to specify focused hypotheses about ecological interactions for subsequent testing. Although the nematodes from our collecting sites have not been identified to species by microscopic examination, it appears that a species of *Daptonema* (or perhaps *Metadesmolaimus*) is consumed by at least three turbellarian predators (i.e., *Cicernia* "debrae," *Cheliplana* n. sp., and *Paromalostomum* "riegeri"). Because the three species co-occur at low-tide level, and as we have demonstrated, share at least part of their diet, future investigations may test for potential interspecific competition among them. Other results suggest that spatial partitioning of prey species could be playing a role in the distribution of their predators. "EukalyptoRiese" occurs in surface sediment around mid-tide-level, and *Drepanorhynchides hastatus* Ax 1977 is found at depth at the high-tide line. Both predators consume members of *Enoplolaimus* (albeit different species). Although our results only allow for the stipulation of hypotheses, they provide for a first

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attempt at uncovering the interspecific mechanisms structuring the diverse and physically complex meiofaunal community

Because we designed our primer sets specifically to exclude kalyptorhynch and plagiostomid flatworm sequences, results for predators from other taxonomic groups are expected to vary. Sequences from nematode prey were recovered from the macrostomorph *Paromalostomum* “riegeri”. However, our “nematode-specific” primers also amplified sequences from proseriate flatworms, either as prey sequences (e.g., from the kalyptorhynchs *L. alleithoros* and “ProschizoSpirale”, and from the proseriate *Paramonotus*? n.sp.) or as amplified predator sequence (e.g., Coelogynoporidae n. sp. 1). Our primer sequences are a closer match to proseriates, as evidenced by comparison with existing proseriate sequences, than to the other turbellarian sequences used in our design. As a consequence, a different set of primers will be needed to search for nematode prey in proseriate predators.

Similarly, our nematode-specific primer sets also retrieved acoelomorph prey sequences from “S&M Schizo” and “ProschizoTertius.” Both species occur sympatrically with *Pseudaphanostoma smithrii* Hooge & Tyler 2003 and *Kuma flava* Hooge & Smith 2004, as well as other acoelomorphs. Again, the primer sequences are a much closer match to acoelomorphan than to the turbellarian sequences used in our primer design.

We have shown that diagnostic PCR can be used successfully to identify nematode and other prey species in meiofaunal flatworms. The advantages of this approach are that prey can be uniquely identified (except in the cases of cannibalism or secondary predation—see Sheppard et al. 2005), that only short target sequences (as may be present in digested materials) are required, and that the method is both rapid and cost-effective (approximately $10/sample, in our case).

Diagnostic PCR is appropriate for the initial screening stages of whole-community food-web analyses, in which the number of predators to be surveyed is both comparatively large and taxonomically diverse, and when the majority of their prey is unknown. It is a cost-effective method, especially for communities that already are the focus of 18S sequencing for taxonomic identifications. For example, our EI site contains ~70 species of “Turbellaria” and Acoelomorpha that were characterized to helping-names in the late 1960s and early 1970s (Rieger, unpublished). Since then, only two have been described, a situation that is almost universally true for most predatory turbellarians in most meiofaunal communities (e.g., Appletans et al. 2012, for Rhabditophora). Similarly, in most meiofaunal communities, taxonomic surveys of potential prey are equally sparse. Although published studies of gastrotrich (Ruppert 1977, 1979) and copepod (Lindgren 1972) diversity exist for North Carolina beaches, there are no published data for nematodes. Thus, the 18S sequences recovered in our study represent the first data on nematodes from our EI site. Our technique then, provides a way to rapidly and relatively cheaply obtain a molecular barcode (*sensu lato*) for prey items across a wide range of potential predators.

In contrast, next-generation sequencing (NGS), with its high cost/run (~$1000 – see Glenn 2011) is not appropriate for an initial survey of numerous predator species such as the one

described here. However, NGS is appropriate for in-depth studies of trophic connections (Pompanon et al. 2012), and, when coupled with universal primers, is capable of generating a relatively complete picture of the diet of a single predator species (Piñol et al. 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Results of successful PCR amplifications for the two diagnostic primer sets. GenBank accession numbers and percent sequence identities are listed for each prey species identified by Blastn. ACOEL, acoelomorph; NEM, nematode; TURB, turbellarian. The two *Cheliplana* predators represent two different individuals.

Predator	215bp product	340bp product
Cicerina "debrae"	NEM: *Metadesmolaimus* sp. JN968218, *Daptonema hirsutum* AM236231, or *Daptonema setosum* AM234045 (96%)	NEM: *Daptonema hirsutum* AM236231 or *Daptonema setsosum* AM234045 (95%)
Prognathorhynchus busheki	Uncultured metazoan clone AF372734 (97%)	NEM: *Punctodora ratzeburgenis* FJ969138 (78%)
Drepanorhynchides hastatus	NEM: *Enoplolaimus* sp. HM564464 (99%)	NEM: *Enoplolaimus* sp HM564464 (99%)
"EukalyptoRiese"	NEM: *Enoplolaimus* sp. JN968238 (100%)	NEM: *Enoplolaimus* HM564473 or Thoracostomopsidae HM564472 (97%)
"EukalyptoZange"	Multiple sequences	No amplification
Cheliplana n.sp.	TURB: *Cheliplana orthocirra* AJ012507 or NEM: *Neochromadora* JN968215 (80%) (forward read)	No amplification
Cheliplana n.sp.	NEM: *Metadesmolaimus* sp JN968218 or *Daptonema* sp FJ040463 or *D. hirsutum* AM236231 or *D. setosum* AM232405 (76%) (reverse read)	No amplification
Lehardyia alleithoros	Multiple sequences	TURB: Monocelidinae sp HM026567 (95%)
"ProschizoSpirale"	No amplification	TURB: Coelogynoporidae n. sp1
Schizochilus "foxi"	NEM: Draconematidae gen.sp. FJ182219, (96%)	No amplification
"S&M Schizo"	NEM: *Dorylaimopsis punctata* AM234047 (reverse read only) (86%)	ACOEL: Fwd: *Pseudohaplogonaria* FR837750 (86%) Rev: *Kuma albiventer* FR837712 (94%)
"ProschizoTertius"	ACOEL:*Pseudaphanostoma smithrii* AY078375 *Haplogonaria "schillingi*" FR837700 (86%)	No amplification
Paromalostomum "riegeri"	NEM: *Metadesmolaimus* sp. JN968218 *Daptonema hirsutum* AM236321 (94%)	No amplification
Coelogynoporidae n. sp1	TURB: Self	TURB: Self
Paramonotus? n. sp.	No amplification	TURB: *Cirrifera dumosa* AJ270154 (93%)