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Molecular identification of the invasive cladoceran *Cercopagis pengoi* (Cladocera: Onychopoda) in stomachs of predators

Elena Gorokhova
Department of Systems Ecology, Stockholm University, Stockholm, Sweden

Abstract

Cercopagis pengoi, a recent invader to the Baltic Sea and the Laurentian Great Lakes, is recognized as an important prey for native fish. Its contribution to diets of other zooplanktivores has not been addressed so far, mostly because of the inadequacy of microscopic methods for diet analysis of aquatic invertebrates. To detect remains of *C. pengoi* in the guts of predators, a DNA-based method and new species-specific primers, amplifying a 154-bp fragment of mitochondrial 16S rDNA gene, were developed and tested. This approach allowed identification of *C. pengoi* in stomachs of *Neomysis integer*, a common Baltic mysid, and is likely to be useful for diet analysis of other predators, both invertebrates and vertebrates, in a variety of trophic studies. Apart from obtaining a useful indicator of species identity in animal diets, these findings contribute to a growing body of evidence that *C. pengoi* successfully integrates into food webs in the invaded ecosystems and represents novel prey not only for fish but also for invertebrate zooplanktivores.

Introduction

Since the beginning of the 1990s, *Cercopagis pengoi* (Ostroumov 1884), a cladoceran of Ponto-Caspian origin, occurs in the Baltic Sea and has became one of the most successful colonists in this region (Leppakoski et al., 2002). Shortly after its arrival to the Baltic Sea, *C. pengoi* also appeared in the North American Great Lakes and small inland lakes, where it is spreading rapidly (Therriault et al., 2002). Currently it is recognized as a species with a large potential to affect food webs and fish feeding conditions in both the Baltic Sea and the Great Lakes (Leppakoski et al., 2002; Vanderploeg et al., 2002; Vander Zanden et al., 2004). *C. pengoi* is a zooplanktivore (Rivier, 1998; Laxson et al., 2003; Gorokhova et al., 2005) and may compete for herbivorous zooplankton with pelagic early life stages of fish (Fig. 1). Studies are underway to assess feeding habits of *C. pengoi* and to quantify its impact on native zooplankton communities. Considering the ability of this species to modify the trophic structure of invaded ecosystems (Ojaveer et al., 2004; Gorokhova et al., 2005) and its range expansion during the last decade, it is equally important to examine the whole spectrum of native species in the invaded ecosystems that could potentially prey on this zooplankter or themselves become its prey.

There is solid evidence that zooplanktivorous fish in the Baltic Sea (Antsulevich and Välipakka, 2000; Gorokhova et al., 2004, 2005; Ojaveer et al., 2004) and in the Great Lakes (Bushnoe et al., 2003) prey on *C. pengoi*. No other predators on *Cercopagis* have been reported to date, although a number of invertebrate species are recognized as important zooplanktivores in these ecosystems. A possible reason for this is methodological difficulty in diet analysis of aquatic invertebrates. Conventional methods, such as microscopic stomach content analysis, are not reliable, because invertebrates often macerate their prey, making the gut content amorphous and mainly unidentifiable (Mauchline, 1980). Other direct methods, such as feeding experiments, are difficult to apply when studying interactions between predators and *C. pengoi*, because the species is very sensitive to handling and experimental manipulations, and no feeding experiments using *C. pengoi* as prey have been reported so far.

When dietary information is particularly difficult to obtain by other means, DNA-based methods have been successfully used to identify prey species via species- or group-specific polymerase chain reaction (PCR) assays (Symondson, 2002; Jarman et al., 2004; Harper et al., 2005); the develop-

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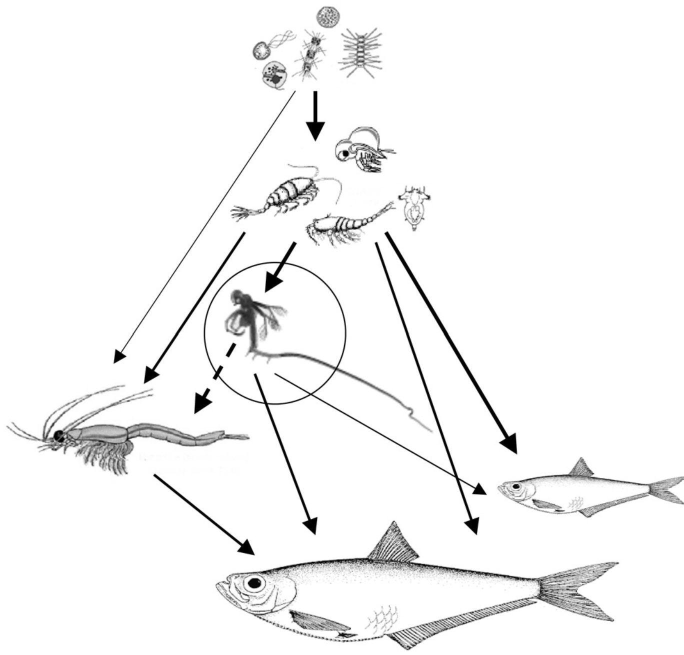

Fig. 1. Trophic structure of the pelagic food web in the northern Baltic Sea after the appearance of *C. pengoi* (circled). Prior to the invasion, the web components were phytoplankton, herbivorous zooplankton, mysids, and juvenile and adult fish. Interactions are depicted by arrows; arrow thickness indicates the relative importance of a particular food source for a consumer. Dashed arrow indicates trophic linkage between mysids and *C. pengoi* addressed in this study.

ment and application of such methods for studying trophic interactions in aquatic ecosystems has been limited, however (but see Nejstgaard et al., 2003). A molecular method for identification of *C. pengoi* in predator stomach content is proposed here. The method is based on identification of a specific region in the mitochondrial 16S rDNA of *C. pengoi*, designing species-specific primers, generating PCR products, and confirming DNA identity by sequencing. The applicability of this method was demonstrated by using it to identify *C. pengoi* in stomachs of *Neomysis integer* (Leach), a common Baltic mysid (Rudstam et al., 1986).

Materials and procedures

Primer design—Species-specific primers for *C. pengoi* were designed on the basis of aligned 16S rDNA regions from *C. pengoi* and 5 other species of Onychopoda (Fig. 2), which have been shown to be its closest living relatives by phylogenetic analysis based on nuclear and mitochondrial DNA (Richter et al., 2001; Cristescu and Hebert, 2002). These were *Podon leuckarti* (Sars 1862), *Podon intermedius* (Lilljeborg 1862), *Pleopis polyphemoides* (Leuckart 1859), *Evadne nordmanni* (Loven 1836), and *Bythotrephes longimanus* (Leydig 1860). These species co-occur with *C. pengoi* in the Baltic Sea (Krylov et al., 1999; Johannson et al., 2004), and their 16S rDNA is highly similar to that of *C. pengoi*—alignments over 494 bp revealed 79% to 90% sequence similarities among the species. Molecular identification of *C. pengoi* from stomach contents is prone to errors due to the possible presence of these genetically similar onychopods in predator stomachs. Hence, it was important to design the assay such that it could distinguish *C. pengoi* from these closely related species. Another important consideration in designing primers was that identification of DNA from partially degraded material requires a small-sized region to be amplified as it would more likely survive digestion (<300 bp; Symondson, 2002). The primers designed, forward: 5′-CCGACTGTCTCTTGAGTGATTG-3′ and reverse: 5′-CCCAACCAAAATTAAACCCTCT-3′, were synthesized (CyberGene, Huddinge, Stockholm) and used to amplify a 154-bp segment of the 16S rDNA.

Collection and preparation of samples—Marine onychopods were sampled, using plankton net, in the summers of 2003 and 2004 at 2 locations (Himmerfjärden Bay: 58° 59′ 07N, 17° 43′ 60E, and Askö Biological Station: 58° 48′ 28 N, 17° 37′ 60 E; 36 zooplankton bulk samples) in a coastal area of the western Baltic proper, preserved in RNA*later* (Ambion, Austin, TX, USA), and stored at 4° C (Gorokhova, 2005). Specimens of *Bythotrephes longimanus* were collected in Lake Abborrtjärn 3, central Sweden, in July 2004, using plankton net, and preserved in 95% ethanol. Mysids were collected in the Askö area on a few occasions in September 2001, using either plankton net or a benthic dredge, and preserved in 95% ethanol within 2 hours of collection. After 2 to 9 months in storage, onychopods were identified using a microscope, transferred to 1.5-mL Eppendorf vials (1 to 10 individuals per vial, depending on body size; 15 samples per species), and used for DNA extraction with *Chelex* (Straughan and Lehman, 2000). In addition, ~0.2 mg (wet weight) of bulk zooplankton containing no *C. pengoi* was used to extract DNA representing "*Cercopagis*-free zooplankton"; this was done for 10 sampling occasions. Assessment of *C. pengoi* presence in the mysid stomachs was based on 36 stomachs from *N. integer* (subadults, 10-13 mm), 14 of which were used for microscopic examination and 22 for PCR analysis. For microscopic examination, stomachs were dissected from the dorsal side under a dissecting microscope. Composite samples of 2 to 5 mysid stomachs were prepared by squashing them on a microscopic slide in a drop of glycerin and examined under an inverted microscope for presence of *Cercopagis* body parts. For DNA extraction, stomachs were placed directly into extraction tubes (1 to 5 stomachs per sample), thoroughly crushed with a plastic pellet pestle (Kontes), and processed according to Asahida and co-workers (1997). As a second negative control (mysid own tissue), a dissected mysid pleopod was used to exclude possibility of contamination with DNA originating from the gut content. *Cercopagis* DNA served as a positive control. The total DNA yields for zooplankton and stomach samples were 0.2 to 1.8 and 1.2 to 10.4 µg $sample^{-1}$, respectively.

Feeding trials—To confirm that DNA of *Cercopagis* in mysid stomach is identifiable after a confirmed predation event, feeding experiments were conducted as follows. Mysids

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                                       10         20         30         40         50         60
                               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C. pengoi AY075067             GAGAAACCGA CTGTCTCTTG AGTGATTGTC GAACTTCACT TCTAGGTGAA AAGGCCTAGA
B. longimanus AY075069         ........TT .........T TA...GAA.T ...T...... .......... ..........
E. nordmanni AY075070          .....GGTA. .........T TAC..C.T.T ...T.C.... ...G...... .....TC...
P. polyphemoides AY075072      .....GGAA. .........T TA..GAAC.T ...T...... ...G...... ......C...
P. intermedius AY075074        .....GGT.. .........T TA...CAC.T ...T...... ...G...... ......C...
P. leuckarti AY075073          .....GGTA. .........T TA...A.C.T ...T...... ...G...... ......C...

                                       70         80         90        100        110        120
                               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C. pengoi AY075067             TAGTTCTGGA GGACGATCAG ACCCTGTAGA GCTTTAATTG ATATCCTTAT TATAGTGTAG
B. longimanus AY075069         .GA....... .......... .......... .........A .C-....AT. ....TCT...
E. nordmanni AY075070          .GAA.T.... .......... .....AA... ....G....T TCC.GACCC. .G.--CT..A
P. polyphemoides AY075072      ..AA.T..A. .......... .....AA... ....G..C.T C.TAGT.... .G.--CT..T
P. intermedius AY075074        ..AA.T.AA. .......... .....A.... ....A....T C.TGAG.... .G.--.T...
P. leuckarti AY075073          ..AG.T.AA. .......... .....AC... ....A.G..T T.T.AAC... .G.--CT..C

                                      130        140        150        160        170        180
                               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C. pengoi AY075067             CAGAATGTTA TA-TAAGTAA GAGGGTTTAA TTTTGGTTGG GGCGACAGAG AGAAATAAAT
B. longimanus AY075069         TG.G-...G. ..-...C..T TTA..G..G. .......... .......... ....TA....
E. nordmanni AY075070          TT..-...G. ..AG..A-GT .TTCTGAAG. ....A..... .......A.. ....TA....
P. polyphemoides AY075072      TT..-A.... ..A...A.G. -.TA.AGAGG .......... .......... ....TA....
P. intermedius AY075074        TT..-A..A. ..AGGG.... ATTCAAGAG. .......... .......A.. ....TA....
P. leuckarti AY075073          TT..-A..G. ..AG..A.GT -TTAAAAAG. .......... .......A.. ....TA....
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Fig. 2. Alignment of mitochondrial 16S rDNA sequences for *Cercopagis pengoi* and other Baltic onychopods based on sequences obtained from GenBank (accession numbers are given along with species names). The position and sequences of primers for amplifying the *C. pengoi* mtDNA fragment in this region (but not the equivalent fragment of the other species) are underlined. Clustal W in the BioEdit 4.5.8. Sequence Alignment Editor (Hall, 1999) was used to align sequences taken from GenBank and to edit sequences obtained in this study. The gap opening and gap extension penalties for multiple alignments were set to 10 and 0.5, respectively.

(7-12 mm) were caught with large plankton net, acclimated for 26 to 28 hours in 0.2 µm filtered seawater to allow for gut clearance, and placed in a 12-well microplate, 1 mysid per well. Each mysid was offered a single *Cercopagis* individual as prey, and the plate was incubated at 17° C; every 6 hours, the wells were checked for presence of prey. When *Cercopagis* disappeared from a well, the mysid was killed; its stomach was dissected and processed as described above. During the 28-hour trial, 5 mysids consumed *Cercopagis*. Their stomachs, analyzed individually, served as a second positive control; those individuals that did not consume any *Cercopagis* were dropped from the experiment.

DNA amplification and sequencing—Amplifications were performed on MJ Research MiniCycler; test PCRs of 25 µL contained 12.5 µL PCR Master Mix (Promega, cat. no. M7502), 2 µL of each primer (final concentration 0.2 µM), 3 µL DNA template (final concentration 15-25 ng), and 5.5 µL nuclease-free water (Gibco, USA). A stepdown cycling regimen (Hecker and Roux, 1996) was performed using the following conditions: initial denaturing period of 7 minutes at 95° C; 11 cycles of 95° C (30 s), 60° C (30 s) with a temperature increment of –1° C, 70° C (45 s); 11 cycles of 95° C (30 s), 55° C (45 s) with a temperature increment of –1° C, 70° C (45 s); 18 cycles of 95° C (30 s), 50° C (45 s), 70° C (45 s); and a 7-minute synthesis period at 72° C. Completed PCRs were separated in 2% (wt/vol) agarose, 1× TBE, 0.5 mg mL^{-1} ethidium bromide gel with a 100-bp ladder at 70V. A sample was considered successful when a band was observed in the expected size range (~150 bp); band intensity was not evaluated. Nucleotide sequences were determined by an automated fluorescence sequencer (ABI, 373A-Stretch; Perkin-Elmer, Norwalk, CT) using a BigDye terminator cycle sequencing kit (PerkinElmer) at CyberGene (Huddinge, Sweden), aligned with the *C. pengoi* 16S rDNA sequence from GenBank (AY075067), and submitted to GenBank (accession no. AY826815). To verify that the overall quality of DNA was sufficient, amplifications were also performed as described above and using 2 universal 16Sar and 16Sbr primers (Palumbi, 1996) for a ~570-bp region of 16S rDNA and following stepdown PCR cycling conditions: 5 cycles of 95° C (30 s), 60° C (45 s), 72° C (45 s); 5 cycles of 94° C (30 s), 55° C (45 s), 72° C (45 s); 27 cycles of 94° C (30 s), 50° C (45 s), 72° C (45 s). For all samples, PCRs with universal primers resulted in positive amplifications (not shown), indicating that the DNA was amenable to amplification.

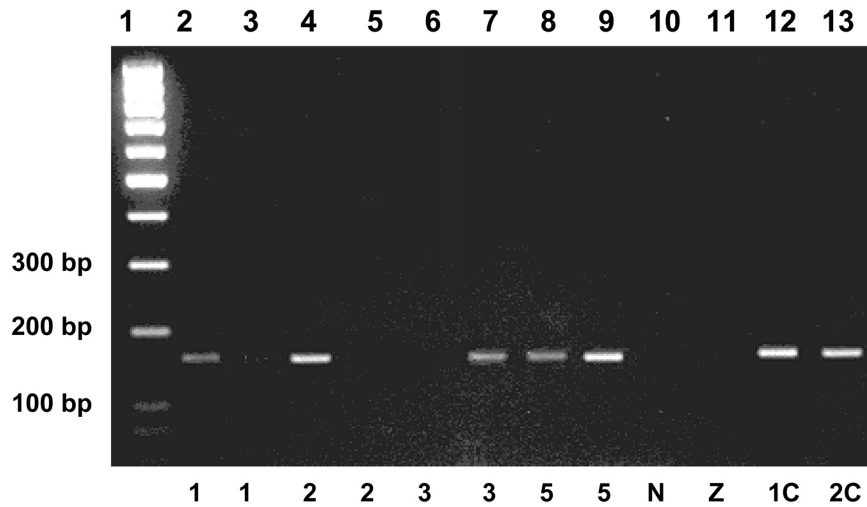

Fig. 3. Agarose gel showing PCR amplification of the 154-bp 16S rDNA fragment of *Cercopagis pengoi* and some of the mysid stomachs, but no amplification from mysid tissue or any of the other zooplankton species tested. Lane 1 contains DNA marker with sizes of bands indicated in bp; lanes 2 to 9 contain PCR from DNA extracted from mysid stomachs (number of stomachs per sample indicated below the bands); lane 10, negative control, a pleopod of *N. integer* (N); lane 11, *Cercopagis*-free zooplankton (this particular sample included all species of Onychopoda except *C. pengoi* tested in this study as well as non-onychopod zooplankton present in the samples: copepods *Acartia bifilosa* and *Eurytemora affinis*, rotifers *Synchaeta* spp., *Keratella cochlearis*, and *K. quadrata*; Z); lane 12, first positive control, *C. pengoi* (1C); lane 13, second positive control, stomach of mysid that consumed *C. pengoi* in feeding experiment (2C).

Assessment

Prey DNA obtained from 1 target (*C. pengoi*) and 5 nontarget zooplankton species was amplified as well as DNA from mysid stomachs. Primers amplified *C. pengoi* DNA at high stringency (100%), whereas no PCR product was obtained when DNA from any other species was used as a template (Fig. 3). The PCR products generated from the mysid stomachs, dissected from both wild-caught mysids and those used in feeding experiments, were the same size as the 16S rDNA fragment from *C. pengoi*, indicating that target DNA was present in the stomachs. Further, these PCR products were sequenced along with PCR product from *C. pengoi* DNA and their identity was confirmed. All mysids that were observed to consume *Cercopagis* in the feeding experiment contained its DNA in their stomachs (Fig. 3). For wild-caught mysids, 5 of 8 samples (22 stomachs altogether) produced positive amplifications, implying that at least 23% of *N. integer* individuals used for the analysis contained *C. pengoi* DNA in their stomachs. In contrast, no recognizable *C. pengoi* remains were observed in stomachs by microscopic examination: samples contained very few identifiable body parts, mainly fragments of copepod mandibles and *Bosmina* rostrums, the rest being highly macerated amorphous material.

Discussion

Microscopic examination of samples composed of as many as 5 stomachs did not reveal any signs of *C. pengoi* in mysid stomachs, but a positive amplification was produced using DNA extracted from as little as a single stomach (Fig. 3). The suitability of this method for in situ studies was confirmed through feeding *C. pengoi* to mysids and analyzing their stomachs for presence of *Cercopagis* DNA. Thus the method provides a sensitive means of detecting *C. pengoi* when morphological identification is hindered either by digestive damage to diagnostic features or by the lack of taxonomic expertise.

The use of the short fragments of mitochondrial 16S rRNA for prey identification in ecology has been advocated (Symondson, 2002; Kasper et al., 2004). The primers designed in this study that amplified the short, species-specific 154-bp fragment of 16S rRNA were able to detect the remains of *Cercopagis* in 100% of cases where consumption of this prey was recorded in the feeding experiment. The fact that only a single prey item needs to be consumed for detection to be successful indicates a high level of assay sensitivity. Probability of detection of DNA after decomposing and diminishing during a digestion process, however, is a function of time elapsed after the ingestion. Therefore, before assay data from field-collected animals can be used, the detectability half-life for a single prey individual must be determined. Considerable differences in half-life of prey have been found between different prey organisms as well as between predator species (Symondson, 2002) and, perhaps, between predators of different size within a species that commonly exhibit ontogenetic variations in assimilation rate and digestion time (Mauchline, 1980). Obviously, further work is needed to verify such speculations.

Apart from obtaining a useful indicator of species identity in animal diets, these findings contribute to a growing body of

evidence that *C. pengoi* successfully integrates into food webs in the invaded ecosystems and represents a novel prey not only for fish but also for invertebrate zooplanktivores. Despite its rarity in plankton (<1% of total zooplankton abundance and biomass; Gorokhova et al., 2004), *C. pengoi* seems to be ingested with a relatively high frequency by mysids (≥23% of analyzed individuals), indicating that this prey is accessible to a significant part of the mysid population. The fact that mysids are able to consume *C. pengoi* has significant implications for pelagic food web structure in the Baltic and possibly also in the Great Lakes, where mysids are also important zooplanktivores (Johannsson et al., 2001), co-occurring with increasingly abundant *C. pengoi* (Vander Zanden et al., 2004). Indeed, if *C. pengoi* is or can become a competitor with zooplanktivorous fish, then an additional predation pressure exerted by mysids might mediate the strength and the net trophic outcome of this competition. From the multiple-enemy prospective, there are well-documented mechanisms whereby predator diversity can be both beneficial and detrimental to the overall rate of predation (Sih et al., 1998). For example, intraguild predation or behavioral interference can result in diminished enemy impact on prey species and dampen cascading effects on basal resources (Finke and Denno, 2004). That is, an effective direct predation by fish on mysids would decrease overall predation on *C. pengoi* and thus increase predation on herbivores. In contrast, an increased overall predation may result from, for example, functional synergy among predators (Cardinale et al., 2003). Indeed, if fish predation on *C. pengoi* forces its population to migrate deeper in the water column, mysids (whose population normally resides below thermocline) would have better access to this prey. This would increase negative impact on *C. pengoi* stock and consequently release herbivores from its predation. It is possible that other zooplanktivores, such as mysids (*Mysis mixta*, *M. relicta*), decapods (*Crangon crangon, Palaemon asterus*), jellyfish (*Aurelia aurita, Cyanea capillata*), and predatory cladocerans (*Leptodora kindtii*, *Bythotrephes longimanus*) are also feeding on *C. pengoi*, but this remains to be tested. A particularly interesting application of the method would be a study of predator-prey interactions between *B. longimanus* and *C. pengoi*. These species co-occur in both the Baltic Sea (Krylov et al., 1999) and the Great Lakes (Vander Zanden et al., 2004), and it has recently been suggested that in Lake Michigan *B. longimanus* can prey on *C. pengoi* (Witt and Caceres, 2004). The occurrence of this predation in situ is difficult to determine, however, because of the difficulty of simulating ambient predator and prey concentrations in the laboratory.

Thus, the method proposed here could be used to qualitatively examine the level of predation on *Cercopagis* by a variety of species, both pelagic and benthic, to assess the broader food-web consequences of the invasion and to understand ecological processes (intra- and interspecific competition, switching behavior, apparent competition, etc.) underpinning food-web changes in the invaded ecosystems. A distinct advantage of a DNA-based approach is not limited to the detection of feeding on *C. pengoi* by analyzing stomach content and/or feces. Another potential application of this method is to use it as a species-specific DNA test for detection of *C. pengoi* presence in environmental and ballast water samples; this may facilitate research on invasion corridors, ballast water screening processes, and ballast discharge management (Hayes et al., 2005).

Comments

There is an alternative explanation for the occurrence of *C. pengoi* DNA in mysid stomachs. The species is a cyclic parthenogen producing diapausing eggs that sink down and overwinter in the sediment (Rivier, 1998). Mysids, on the other hand, are known to perform vertical migrations (Rudstam et al., 1986; Johannsson et al., 2001), and it is possible that they feed on the benthic material, including the diapausing eggs, while staying close to the bottom. If this is the case, the consumption of diapausing eggs by mysids could potentially affect the recruitment of *C. pengoi* the following year. However, the molecular identification proposed here does not allow discrimination between the eggs and free-living individuals. Therefore, there are two possible explanations, not necessarily exclusive, to account for observed presence of *C. pengoi* DNA in the stomachs of *N. integer*, and they both suggest existence of the trophic linkage between the two species, which needs to be further evaluated.

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