Universal primers and PCR of gut contents to study marine invertebrate diets

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Abstract

Determining the diets of marine invertebrates by gut content analysis is problematic. Many consumed organisms become unrecognizable once partly digested, while those with hard remains (e.g. diatom skeletons) may bias the analysis. Here, we adapt DNA-based methods similar to those used for microbial diversity surveys as a novel approach to study the diets of macrophagous (the deep-sea amphipods *Scopelocheirus schellenbergi* and *Eurythenes gryllus*) and microphagous (the bivalve *Lucinoma aequizonata*) feeders in the deep sea. Polymerase chain reaction (PCR) in conjunction with 'universal' primers amplified portions of the mitochondrial cytochrome *c* oxidase I (*CO*I) gene for animals ingested by *S. schellenbergi* and *E. gryllus* and the *18S rRNA* gene for lesser eukaryotes ingested by *L. aequizonata*. Amplified sequences were combined with sequences from GenBank to construct phylogenetic trees of ingested organisms. Our analyses indicate that *S. schellenbergi*, *E. gryllus* and *L. aequizonata* diets are considerably more diverse than previously thought, casting new light on the foraging strategies of these species. Finally, we discuss the strengths and weaknesses of this technique and its potential applicability to diet analyses of other invertebrates.

Keywords: *18S rDNA, COI, deep sea, diet, Lucinidae, Lysianassidae*

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Introduction

Understanding the dietary habits of marine invertebrates is central to studies of food webs, ecological processes, energetics, and natural history. Yet, the diets of most marine invertebrates are poorly known. Principle methods of diet analyses include direct observation of feeding and examination of gut contents or faeces for recognizable remains of ingested organisms. However, material in invertebrate guts is often digested beyond visual recognition (Symondson 2002) and observing marine invertebrates in their natural environment is generally difficult, especially in remote settings such as the deep sea.

In his review, Symondson (2002) postulates that polymerase chain reaction (PCR)-based techniques are a promising approach to uncovering the range and diversity of prey taken by highly generalist invertebrates, though these techniques have not yet been applied to wild-caught marine invertebrates. The concept is founded on the assumption that DNA from consumed organisms is not completely degraded during digestion and therefore could be amplified via PCR and analysed (Zaidi *et al.* 1999). DNA sequencing has become prolific, and the concept of a DNA barcode or species identification based on a short sequence of DNA, is now accepted (Hebert *et al.* 2003). Accordingly, DNA sequences recovered from gut contents can be compared against rapidly growing DNA databases to provide taxonomic insight on the consumed organism.

DNA-based analyses of terrestrial arthropod gut contents have been successful when utilizing species-specific primers that target a narrow group of closely related species (Zaidi *et al.* 1999; Agustí *et al.* 2003). With one exception, diet analyses adapting these methods in marine systems also have employed species-specific primers and have been limited to vertebrate hosts (Jarman *et al.* 2002; Rosel & Kocher 2002). Recently, Jarman *et al.* (2004) tested group-specific primers designed to expand the range of dietary items detected in marine vertebrate stomach contents and scats. This tactic is a vast improvement over species-specific primers for revealing the species diversity in dietary samples. Yet, group-specific primers will still miss untargeted dietary taxa and employing these primers requires some a priori knowledge concerning which taxa are

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potentially consumed. The diets of many marine invertebrates are either unknown or consist of a broad array of taxa. Hence, we advocate 'universal' primers designed to amplify DNA from a wider range of eukaryotes.

We incorporate this universal-primer approach to investigate the diets of the deep-sea bivalve *Lucinoma aequizonata* from the Santa Barbara basin and the deep-sea scavenging crustaceans *Scopelocheirus schellenbergi* and *Eurythenes gryllus* from the Tonga trench. The aim of this study is to (i) present our methods and results of the two molecular diet analyses and (ii) discuss and evaluate technique-related issues including contamination, primer selection and DNA sequence analysis.

L. aequizonata is a member of the family Lucinidae. Bivalves from this family feature shorter and less developed guts than other comparably sized bivalves and harbour chemosymbiotic bacteria, which provide the host with the majority of its carbon requirements (Duplessis 2002). Consequently, *L. aequizonata* feeding was downplayed until Duplessis (2002) documented extensive evidence of feeding and incorporation of ingested material in tissues. To ascertain the feeding strategies of *L. aequizonata*, we amplified DNA from gut contents to determine its diet. Analysis of DNA sequences recovered from *L. aequizonata* guts reveals a diverse array of prokaryotic and eukaryotic microbes, suggesting a nonselective feeding mode for this bivalve. A brief account of the *L. aequizonata* diet analysis is published elsewhere (Duplessis *et al.* 2004), but relevant methods and results are expanded upon here and evaluated to further validate the technique.

The crustaceans *S. schellenbergi* and *E. gryllus* belong to a family of amphipods (Lysianassidae) that are well known as deep-sea scavengers. These members of the lysianassid pelagic guild are often considered obligate necrophages, feeding on large carrion (> 1 kg) falling to the deep-sea floor (Sainte-Marie 1992; Britton & Morton 1994; Kaiser & Moore 1999). Rapid and reliable bait attendance in the deep sea, acute chemoreceptor organs for detecting carrion, morphological adaptations to mandibles and guts for carrion feeding, and the ability to withstand long periods of starvation all favour a necrophageous foraging strategy (Smith & Baldwin 1982; Sainte-Marie 1992). As most deep-sea amphipods are captured through baited traps, amphipods with access to bait are usually satiated with it, and amphipods prevented from consuming bait are often retrieved with empty stomachs (Hessler *et al.* 1978; Smith & Baldwin 1982, 1984; Hargrave *et al.* 1995). Consequently, there are few lysianassid diet analyses reported in the literature. However, we note that existing diet studies do provide some evidence for supplemental detritivory or predatory feeding modes in addition to scavenging (Hessler *et al.* 1978; Ingram & Hessler 1983; Smith & Baldwin 1984; Sainte-Marie 1992).

We amplified the contents from the amphipod hindguts with universal primers to determine if the consumed taxa would support our hypothesis that these obligate necrophages may also employ an alternative nutritional strategy. Here, we show that DNA sequences recovered from the gut contents of a juvenile *E. gryllus* and two adult *S. schellenbergi* reflect a diet that includes a variety of invertebrates not likely to be repeatedly encountered as carrion. Thus, this DNA-based analysis of gut contents supports the hypothesis that these lysianassid amphipods may also consume living organisms or detritus.

Materials and methods

Sample collection and DNA extraction

Three fresh *Lucinoma aequizonata* specimens collected by otter trawl from the Santa Barbara Basin (500 m depth, November 2001) were dissected and their guts (lining and contents) excised and cryopreserved *in situ*. Visual observations showed that one gut contained particulate matter (gut A) and two appeared empty (guts B and C). Following storage at –80 °C for nine to 11 months, DNA was extracted from the guts using a DNeasy Kit (QIAGEN, protocol modification for gram-positive bacteria). A blank extraction (substituting gut content tissue with PCR grade water) was performed simultaneously with each gut extraction to control for contamination.

Scopelocheirus schellenbergi and juvenile *Eurythenes gryllus* amphipods were collected with free-vehicle traps baited with bigeye tuna (*Thunnus obesus*) carcasses and deployed to 1 m above the sea floor in the Tonga Trench (SW Pacific Ocean). Amphipods were preserved in cold 70% ethanol *in situ* within 12 h of recovery and stored for up to 14 months at 4 °C. Three specimens were selected from three locations for this study: a juvenile *E. gryllus* from 23°49′90″ S 174°24′85″ W at sea floor depth of 6252 m, two adult *S. schellenbergi*, one each from 17°14′89″ S 172°09′15″ W at sea floor depth of 7349 m and 17°20′11″S 172°16′31″W at sea floor depth of 8732 m. Amphipods were dissected and the contents of their hindguts, which contained the least amount of bait tissue relative to the rest of the gut, were removed and placed into DNA-free tubes. All dissections were performed in a UV-sterilized laminar flow hood with flame-sterilized dissection tools to avoid contamination with extraneous DNA. Gut-content DNA was extracted with a Forensics Kit (MoBio). A blank extraction was performed simultaneously with each set of DNA isolations as a negative control. DNA was also extracted from the bait (bigeye tuna, *T. obesus*) used in the amphipod traps.

Primer selection and PCR amplification

Universal primers amplifying a portion of the eukaryotic *18S rRNA* gene (18SA and 18SB, Medlin *et al.* 1988) successfully

amplified DNA from *L. aequizonata* gut contents following the protocol in Duplessis *et al.* (2004).

For amphipod gut contents, universal primers amplifying portions of the mitochondrial cytochrome *c* oxidase I (*CO*I) gene (LCO1490 and HCO2198, Folmer *et al.* 1994), mitochondrial *16S rDNA* fragments (16Sar and 16Sbr & Simon 1991) and nuclear *18S rDNA* (18SA and 18SB, Medlin *et al.* 1988), respectively, were employed in preliminary PCRs (three *S. schellenbergi* gut contents) to estimate amplification diversity. Results indicate that the *CO*I primers amplified DNA from the widest range of phyla (invertebrates and vertebrates), followed by *16S rDNA* (vertebrates only), followed by *18S rDNA* (*S. schellenbergi* DNA only). Based on this preliminary test, we selected the *CO*I primers for an amphipod diet analysis.

All PCRs were prepared under UV sterilized flow hoods with DNA-free tubes and tips and included two negative reactions to control for contamination during both the DNA extraction and PCR preparation. To amplify amphipod gut content DNA, PCRs combined 25 μL of HotStar*Taq* Master Mix (QIAGEN), 3 μL each of primers LCO1490 and HCO2198 (10 μM), 4 μL of DNA elute and 17 μL of sterile water. The PCR temperature profile for amphipod gut samples: Hotstart (1 cycle of 95 °C for 15 min); 1 cycle of 80 °C for 5 min, and 40 cycles of 92 °C for 1.5 min, 42 °C for 1 min, 72 °C for 2 min (Bio-Rad iCycler).

COI *primer assay for fish*

While the *CO*I primers employed in this study successfully amplify DNA from diverse invertebrates (Folmer *et al.* 1994), they failed to amplify *CO*I genes from bigeye tuna DNA, which was likely present in the amphipod guts. To ascertain the applicability of the primers to fish taxa, we tested these *CO*I primers on nine different fish species. DNA was extracted from fresh fin clips of the honeycomb rockfish (*Sebastes umbrosus*), black surfperch (*Embiotoca jacksoni*), blacksmith (*Chromis punctipinnis*), barred sand bass (*Paralabrax nebulifer*), California halibut (*Paralichthys californicus*), plainfin midshipman (*Porichthys notatus*), swell shark (*Cephaloscyllium ventriosum*), Pacific hagfish (*Eptatretus stoutii*), and sculpin (*Scorpaena guttata*) via the DNeasy Kit (QIAGEN, protocol modified for animal tissues). Whole genomic DNA extract of each fish (including *T. obesus* extract) was visualized on an agarose gel to confirm sufficient DNA quantity and quality for PCRs. The PCRs were completed as described in the previous section (substituting 2 μL of fish DNA extract instead of 4 μL gut DNA), with two replicates for each fish. PCR products were subsequently visualized on an agarose gel and scored as either strong amplification (i.e. bright PCR product band), weak amplification (i.e. very light band) or no amplification (i.e. no band).

Selecting restriction enzymes for enrichment of amphipod diet PCR products

Restriction maps of *CO*I sequences from *S. schellenbergi* and *E. gryllus* revealed endonuclease cut sites that could be applied to PCR products to digest and subsequently remove host amplicons. We only selected cut sites near the centre of the amplified fragment for greater separation of uncut and cut PCR products on an agarose gel. The *S. schellenbergi CO*I map revealed *Hin*fI (five-cutter) and *Bsa*BI (six-cutter) cut sites near the centre of the amplified fragment. We examined restriction maps of *CO*I sequences from 150 diverse metazoan taxa for the presence of the aforementioned restriction sites. Almost 60% of the examined *CO*I sequences carried a *Hin*fI restriction site, while approximately 10% of examined taxa harboured *Bsa*BI restriction sites. Similarly, the *E. gryllus CO*I map revealed *Hin*fI and the six-cutter restriction enzyme *Sac*II; the latter enzyme recognition site was present in 12% of the 150 metazoan *CO*I sequences examined.

Separation and sequencing of PCR products

PCR products [approximately 700 base pairs (bp)] from *S. schellenbergi* gut samples were digested with either the enzyme *Bsa*BI or *Hin*fI to cleave the host amphipod DNA. *E. gryllus* gut PCR products were digested with either the enzyme *Sac*II or *Hin*fI. Digested PCR products were then separated on an agarose gel (0.9%), and undigested PCR products were excised. Clone libraries for all gut samples were prepared from purified PCR products with a TOPO TA Cloning Kit (Invitrogen). Up to 40 clones from each library were selected for sequencing. Sequences with 98% identity or greater were considered representatives of the same species (Meyran *et al.* 1997; Jarman *et al.* 2004). Amplification of DNA from bait was not a concern because the primers failed to amplify *CO*I sequences directly from preserved bait tissue. In contrast, *L. aequizonata* gut PCR products were not digested prior to cloning. Instead, clone plasmids were digested with *Sac*I; clones exhibiting banding patterns identical to plasmids carrying *L. aequizonata 18S rDNA* fragments were discarded (Duplessis *et al.* 2004).

Phylogenetic analysis

Haplotypes recovered from *L. aequizonata*, *S. schellenbergi*, and *E. gryllus* guts were referenced against the GenBank database via BLAST to determine their approximate phylogenetic affiliation. To analyse *18S rDNA* clones from *L. aequizonata* guts, we aligned gut sequences with additional *18S rDNA* sequences obtained from GenBank and included those sequences that appeared in BLAST searches (CLUSTAL_X, default settings). Phylogenetic trees were then constructed

using the neighbour-joining (NJ) methods in PAUP 4.0 (Sinauer) based on 769 bp. *18S rDNA* gut haplotypes were assigned to taxonomic lineages based on their topological position within the phylogenetic trees (Duplessis *et al.* 2004). For *S. schellenbergi* and *E. gryllus* gut sequences, we aligned gut haplotypes with 150 additional sequences retrieved from GenBank (CLUSTAL_X). Selected sequences were comprised of those used in a previous study (Hebert *et al.* 2003) supplemented with representatives from additional marine metazoan and protozoan phyla. Initial NJ distance-based trees allowed us to approximate topological location of gut haplotypes as well as reduce the number of support sequences to 75 without altering tree integrity. Mitochondrial DNA (mtDNA) sequences were translated to their predicted amino acid sequence for deeper phylogenetic approximation (Hall 2001; Hebert *et al.* 2003) and subsequently aligned with CLUSTAL_X (default settings) following manual refinement with MACCLADE (Sinauer). We constructed a NJ distance-based phylogenetic tree based on 232 amino acid positions (PAUP 4.0).

Results

Lucinoma aequizonata *molecular diet analysis*

18S rDNA clone libraries were constructed for all three *Lucinoma aequizonata* guts with 40, 25 and 25 colonies selected from gut A, B and C libraries, respectively. Restriction digests of plasmids showed that five of 40 gut A clones, 22 of 25 gut B clones and 25 of 25 gut C clones contained *L. aequizonata 18S rDNA* inserts and were therefore discarded. Of the remaining clones, a total of 18 unique DNA sequences were detected and included in the phylogenetic analysis (Fig. 1). Based on topological positions within the NJ tree, we identified eight green algae species, one stramenopile, one acantharea, five alveolates, and three euglenozoans (Duplessis *et al.* 2004).

Fish COI *primer assay*

Embiotoca jacksoni, *Paralichthys californicus*, *Paralabrax nebulifer*, *Chromis punctipinnis*, and *Eptatretus stoutii* all showed
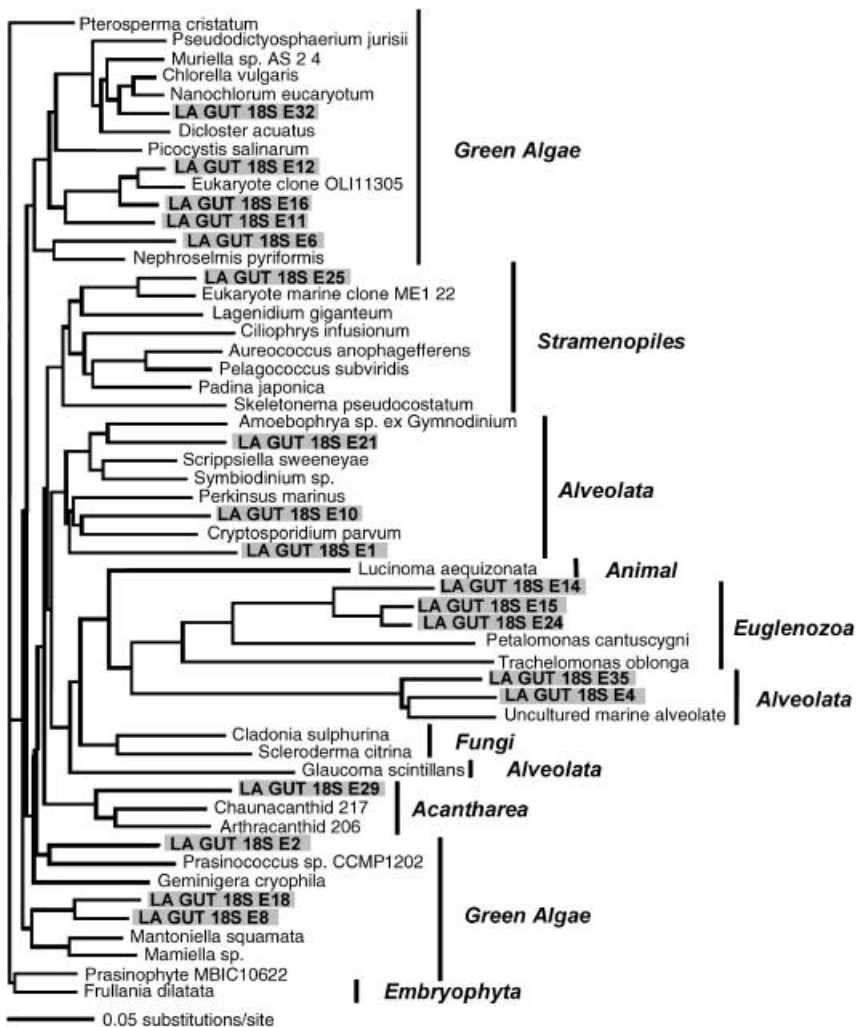

Fig. 1 Distance-based phylogenetic tree of *Lucinoma aequizonata* gut haplotypes from a 769 bp fragment of the *18S rRNA* gene. Highlighted sequences beginning with 'LA GUT' represent those sequences recovered from the *L. aequizonata* gut contents. The remaining eukaryotic sequences, with the exception of *L. aequizonata*, were obtained from GenBank. Support sequences were selected because of their appearance during BLAST searches, or to add support to a particular lineage.

strong PCR amplification in both replicates, while *Porichthys notatus* and *Scorpaena guttata* each had one strong and one weak amplification. *Cephaloscyllium ventriosum* produced weak amplification both times and DNA from the honey-comb rockfish (*Sebastes umbrosus*) and bait (*Thunnus obesus*) did not amplify in either replication.

Amphipod molecular diet analysis

A considerable portion of each PCR product was eliminated during the *Hin*fI digest designed to reduce host DNA prior to cloning. Consequently, cloning efficiency was low and only 10 clones were sequenced from each *Hin*fI library. *Scopelocheirus schellenbergi Hin*fI digest libraries each revealed three unique sequences and four haplotypes were detected from the *Eurythenes gryllus Hin*fI digest library. Of the 25 clones sequenced from each *S. schellenbergi Bsa*BI digest library, we identified three and four unique sequences, respectively. Eight haplotypes were distinguished among the 40 sequenced clones from the *E. gryllus Sac*II digest library. Clones containing the host amplicon constituted less than 10% of each sequenced library.

Sequences recovered from amphipod gut contents assembled into five topological clusters within the phylogenetic tree (Fig. 2). We discerned at least 22 unique sequences in total from the amphipod guts (GenBank Accession nos AY830417–AY830441). Surprisingly, there was no sequence overlap between the two digest libraries constructed from the same amphipod gut sample.

The haplotype represented by SS1-*Hin*fI 1, SS2-*Bsa*BI 10, and EG-*Hin*fI 1 was detected in all three amphipod guts, and likely represent the same species of Teleostei as indicated by their topological position and BLAST searches. The additional teleost haplotype (SS2-*Bsa*BI 24) differed from the former haplotype by only one amino acid substitution, and is therefore either a close relative or the same species.

We detected seven haplotypes affiliated with the Amphipoda lineage; two haplotypes (SS1-*Hin*fI 2, EG-*Hin*fI 3) topologically affiliate with *S. schellenbergi* while the remaining five sequences (SS2-*Bsa*BI 18, EG-*Sac*II 8, EG-*Sac*II 34, SS2-*Bsa*BI 3, EG -*Sac*II 10) associate with the *Eurythenes* genus. DNA-based BLAST searches for all seven amphipod haplotypes found either *S. schellenbergi* or *E. gryllus* to be the closest relative, suggesting that all sequences represent amphipods from the Lysianassidae family.

The sequence SS2-*Hin*fI 1 associates with Nematoda, though the phylogenetic association is not a strong one and may instead be an artefact of long-branch attraction. Likewise, the haplotype SS1-*Bsa*BI 3 is shown under the bivalve class of the mollusks, but DNA and protein BLAST searches actually returned a myriad of protists (stramenopiles, red algae) as the nearest matches, though the identity level (e^{-23}) was unconvincing.

The remaining 12 *CO*I haplotypes do not clearly affiliate with any phylogenetic group, though the sequences Mollusca 12 (*Scapharca broughtonii*) and Brachiopoda 3 (*Lingula anatina*) appear in the interior of this assemblage. We denoted these unidentified haplotypes as 'mysteries of the deep'. For these 12 sequences, *S. broughtonii* was near the top of most BLAST returns, followed by a suite of sequences from an uncultured zooplankton sample.

Discussion

Discerning the dietary habits of marine invertebrates is important to understanding ecological structure and processes in marine systems. In postmortem examination of gut contents, many important food items are missed if remains are unrecognizable (Symondson 2002). We successfully amplified DNA fragments up to 1.8 kilobases from a broad array of organisms in a *Lucinoma aequizonata* gut. Though the same primers failed to amplify DNA from *Scopelocheirus schellenbergi* and *Eurythenes gryllus* hindgut contents, *CO*I primers targeting a smaller 700-bp fragment were successful. This inconsistency may be attributed, in part, to differences in host digestion processes. Lysianassid amphipods retain and digest food for long periods of time, resulting in thorough degradation of macromolecules such as DNA (Sainte-Marie 1992; Hargrave *et al*. 1995). As both *S. schellenbergi* and *E. gryllus* midguts were engorged with bait, we tested only hindgut material, which would exacerbate the problem. In contrast, the gut contents of the suspension feeder *L. aequizonata* likely contained some freshly ingested organisms yielding higher quality DNA. However, differences in preservation techniques (cryo-preservation vs. 70% ethanol), dietary content, and DNA extraction methods may also have contributed to the *18S rDNA* amplification discrepancy.

Kohn & Wayne (1997) recognized that faeces contain DNA that could potentially be detected through PCR. Although they focused on obtaining host genetic information through faeces, many of their method-related considerations are applicable here. Chief among these is the selection of an appropriate molecular marker. Because each cell has multiple copies (often hundreds) of mtDNA, there is a greater chance of amplifying mtDNA sequences from degraded samples (e.g. faeces or gut contents) than single copy nuclear genes. The same idea applies to ribosomal RNA, as there are multiple copies per cell. Fortunately, universal primers are available for mitochondrial genes such as *16S rRNA* (Simon 1991), *12S rRNA* (Simon 1991) and *CO*I (Folmer *et al*. 1994) as well as the nuclear *18S rRNA* encoding gene (Medlin *et al*. 1988). However, the term 'universal' is misleading, as universal primers will still favour DNA from taxa with exact complimentary sequences. Consequently, the primers selected may be contingent upon targeted taxa. For example, we only detected vertebrate
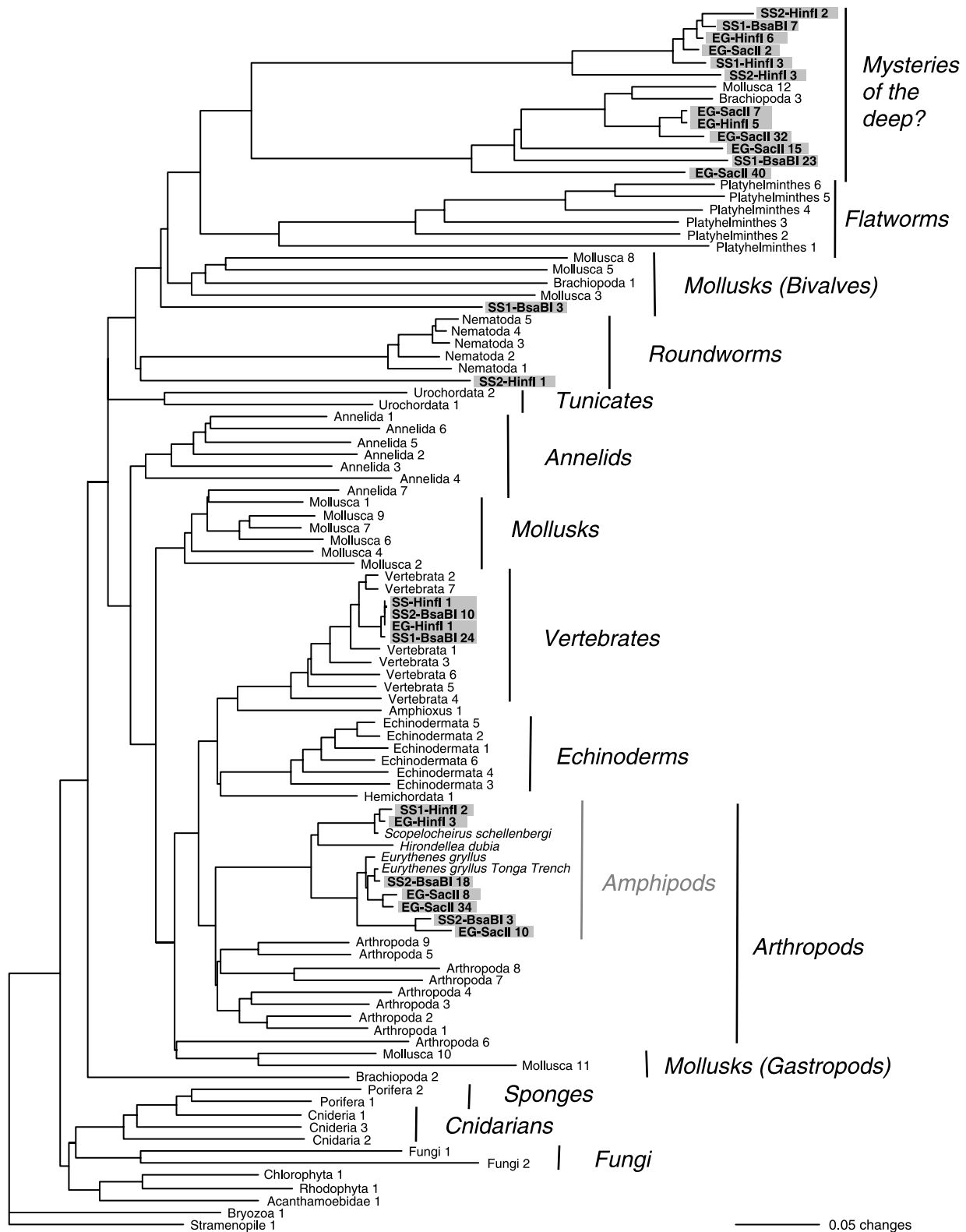
Fig. 2 Distance-based tree of *Scopelocheirus schellenbergi* and *Eurythenes gryllus* gut haplotypes based on 232 *COI* amino acid positions. Gut sequences are highlighted and labelled SS1 (*S. schellenbergi* from depth 7349 m), SS2 (*S. schellenbergi* from depth 8732 m) or EG (*E. gryllus*) followed by their respective digest library (e.g. *Hin*fI) and a counting number. Additional eukaryotic sequences are labelled with corresponding phylum names for ease of comprehension but the species names are available in the supplement. An additional *E. gryllus* sequence (GenBank Accession no U92666) was included because *E. gryllus* from different depth zones show considerable intraspecific variation (France & Kocher 1996). The lysianassid *Hirondellea dubia* was captured in the same vicinity as *S. schellenbergi* and *E. gryllus*, and its sequence is also shown.

sequences when amplifying *S. schellenbergi* gut contents with *mt16S rDNA* primers, yet *CO*I primers amplified mostly invertebrate DNA. For this study, we were most interested in discerning invertebrates, so we selected the latter marker. Additionally, targeting shorter sequences may increase the success of PCR and therefore the diversity of PCR products (Asahida *et al.* 1997).

Assigning an unknown DNA sequence to a species requires: (i) a reference DNA sequence of the same species and preferably, (ii) a genetic study to determine the intraspecific DNA sequence variation of the species. For this method, our sequences were referenced against GenBank, though we did not find exact matches to most gut haplotypes. This problem was partly resolved by combining our sequences with reference sequences in a phylogenetic tree, a common type of analysis for molecular microbial diversity surveys (see Moon-van der Staay *et al.* 2001; Dawson & Pace 2002). This approach worked well for haplotypes recovered from *L. aequizonata* guts and facilitated resolution of most sequences to their respective higher taxonomic lineage (Fig. 1). However, this analysis was problematic for *CO*I sequences recovered from *S. schellenbergi* and *E. gryllus* guts. DNA sequences (retrieved from GenBank) belonging to members of the same phyla were often scattered throughout the initial *CO*I-based trees and did not assemble into one group (data not shown). Translating these DNA sequences into their respective amino acid sequences resulted in better congruency between members of the same phyla (Fig. 2). Yet some taxa, such as the phylum Mollusca, are not effectively resolved by phylogenetic analyses of *CO*I, possibly because the *CO*I region evolves too quickly to recover deep divergences (Hebert *et al.* 2003). Another glaring issue is the presence of unresolved sequences, exemplified by the 'mysteries of the deep' assemblage as well as SS2-*Hin*fI-1 and SS1-*Bsa*BI-3. We offer several possible explanations for an apparent lack of association: (i) few deep-sea invertebrates have been sequenced; (ii) a transposition of the mitochondrial *CO*I gene into the nuclear genome has been reported for some species and may confound results, though we emphasize that no stop codons were evident in the amino acid translation (Zhang & Hewitt 1996); (iii) the *CO*I sequences are actually chimeric artefacts (Hugenholtz & Huber 2003), though their clustering within the tree and the lack of stop codons does not favour this hypothesis; and (iv) the *CO*I GenBank database has few representatives of important marine fauna such as cnidarians, foraminiferans, and poriferans, all of which may be potential food items for deep-sea amphipods. The suite of uncultured zooplankton *CO*I sequences surfacing in our BLAST searches sans any support from identified relatives implies that some eukaryotic clades have yet to be sequenced for *CO*I. However, as the GenBank database continues to expand, the taxonomic level to which gut DNA sequences can be identified will also be refined.

Controlling for contamination is crucial when attempting to amplify degraded DNA with universal primers. Including controls at every step up to the PCR reaction is imperative. For the DNA extraction, we implemented the QIAGEN DNeasy Kit for *L. aequizonata* gut samples and MoBio's Forensic DNA Kit for amphipod gut samples. Reagents from the DNeasy Kit are not packaged individually, and contamination from bacterial DNA was introduced for two of the three bivalve gut samples extracted with the DNeasy Kit (see Duplessis *et al.* 2004). No contamination was evident with the MoBio Forensic DNA Kit; we advocate the MoBio Forensic DNA Kit or any comparable forensic method for DNA extraction. All procedures used DNA-free tubes and pipette tips and were performed in a UV-sterilized laminar flow hood that was swabbed with 10% bleach periodically. For *S. schellenbergi* and *E. gryllus*, dissecting tools were bleached and flame-sterilized and guts were excised in the UV-laminar flow hood. Several studies working with ancient DNA suggest the use of separate rooms or facilities to extract and subsequently amplify highly degraded DNA (Kohn & Wayne 1997; Woodruff 2004). Because such facilities were not available, we used the same flow hood for both DNA extraction and PCR preparation but sterilized it between procedures. The controls customarily yielded a negative result and we therefore conclude that our procedures are sufficient to control for contamination.

We recognize that this technique, like most methods, is subject to bias. As mentioned earlier, universal primers will still favour DNA with exact complimentary sequences. Second, PCRs will preferentially amplify DNA of higher quality. This is particularly problematic because host DNA is less degraded and may be selectively amplified if the primers are compatible. This situation was encountered by Jarman *et al.* (2004) when attempting a molecular diet analysis from the stomach contents of the giant squid. The primers readily amplified squid DNA, and as a result, 78 of 80 clones screened contained the host DNA. Similarly, the majority of clones screened from the two libraries constructed from the empty *L. aequizonata* guts contained host DNA. We eliminated the presence of host amphipod DNA from our amphipod gut clone libraries by first cleaving PCR products with a restriction enzyme designed to cut host *CO*I sequences in half. Following a restriction digest, PCR products were separated via an agarose gel and uncut products extracted and cloned. Unfortunately, this enrichment technique also eliminates any other PCR products with the same restriction enzyme recognition sequence, though this may be a small trade-off if PCR overwhelmingly favours amplification of host DNA. Additionally, selecting more stringent restriction enzymes or rotating between two restriction enzymes can help minimize this problem. For example, the *Hin*fI and *Sac*II digest libraries constructed from the same *E. gryllus* gut sample each

imparted a distinct set of sequences, thereby increasing the overall sequence diversity.

In light of the *COI* primers' affinity for the majority of the fish species tested, its failure to amplify bait (*Thumus obesus*) was both fortuitous and fortunate. However, if these *COI* primers did amplify *T. obesus*, the same restriction digest technique could be applied to remove it from the clone library.

This PCR-based method is a potentially powerful course for expanding the range and diversity of dietary items detected in stomach contents, especially by generalist feeders. When the gut contents of several *L. aequizonata* specimens were analysed via scanning electron microscopy, only few diatom frustules were discernable (Duplessis *et al.* 2004). In contrast, DNA sequences belonging to 33 different species were identified through the molecular diet analysis (Duplessis *et al.* 2004). Likewise, many deep-sea lysianassid amphipods that are caught in baited traps are assumed to feed solely on carrion through scavenging. Thus, one would expect that the majority of DNA sequences recovered from scavenging amphipod guts would represent fish or other animals likely to be carrion. In concurrence, visual inspection confirmed the presence of tissue fillets in each amphipod gut (presumably from the bait) but no other identifiable remain was discerned. Yet, our molecular results indicate that *S. schellenbergi* and *E. gryllus* feed on a variety of invertebrates (including other amphipod species) not likely to be consistently presented as carrion, which suggests that these notorious scavengers may also be predators. However, we acknowledge that other possible contributors to gut content DNA, including resident microflora, parasites, and secondarily ingested organisms such as food in the guts of prey (see Klages *et al.* 2001 for example), could confound results and may not easily be distinguished.

Amplifying stomach contents with universal primers should continue to benefit studies investigating diets in an assortment of both marine and terrestrial organisms. With a fair amount of precaution, careful implementation of controls, and the selection of an optimal molecular marker, this technique has the potential to reveal previously unknown dietary items for many invertebrates. With the continued expansion of DNA databases, we conjecture that PCR-based approaches with universal primers will become increasingly useful in studying invertebrate dietary habits.

Acknowledgements

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