Molecular Detection of Eukaryotic Diets and Gut Mycobiomes in Two Marine Sediment-Dwelling Worms, **Sipunculus nudus** and **Urechis unicinctus**

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The present study aimed to reveal the eukaryotic diets of two economically important marine sediment-inhabiting worms, **Sipunculus nudus** (peanut worm) and **Urechis unicinctus** (spoon worm), using clone libraries and phylogenetic analyses of 18S rRNA genes. Fungal rDNA was also targeted and analyzed to reveal mycobiomes. Overall, we detected a wide range of eukaryotic phylotypes associated with the larvae of **S. nudus** and in the gut contents of both worms. These phylotypes included ciliates, diatoms, dinoflagellates, euglenophytes, placidids, oomycetes, fungi, nematodes, flatworms, seaweeds, and higher plants. Oomycetes were associated with the planktonic larvae of **S. nudus**. The composition of eukaryotic diets shifted greatly across the larval, juvenile, and adult stages of **S. nudus**, and among different gut sections in **U. unicinctus**, reflecting lifestyle changes during the ontogeny of the peanut worm and progressive digestion in the spoon worm. Malassezia-like fungi were prevalent in mycobiomes. *Epicoccum* and *Trichosporon*-related phylotypes dominated mycobiomes associated with larval individuals and in the gut contents of adults, respectively. The gut mycobiome of **S. nudus** was successively characterized through the midgut, aspiratory intestines, hindgut, and rectum as having a high proportion of *Climacodon-Rhizochaete, Ceriporiopsis, Cladosporium-Pseudomicrostroma*, and Malassezia-related species in the libraries. These results emphasize the dynamics of diets and gut mycobiomes in marine benthic animals.

**Key words:** food source, gut microbiome, life cycle, marine benthos, mycobiome

Animals of the phyla Sipuncula and Annelida are generally diverse and dominant in the marine benthos. Through bioturbation, deposition, and/or suspension feeding, these worms concentrate organic matter and trace elements and regenerate nutrients, thereby playing an important role in sustaining the trophic links of marine benthic food webs (33, 45, 57). A major issue in the functional ecology of these sediment-dwelling worms has been to understand their trophic functions, which include feeding and digestive structures and modes, behavior, and the actual diets ingested and assimilated (33, 45). During the last several decades, biochemical, isotope tracing, and microscopic observation methods revealed that besides bacteria (13, 18, 29), eukaryotic microalgae (mostly diatoms) are important carbon sources for many sediment-dwelling species (*e.g.*, 5, 12, 13) and whole macrofaunal communities in coastal and estuarine sedimentary environments (*e.g.*, 22, 32, 34).

Microscopic observations of the gut contents typically identify dietary eukaryotes at high taxonomic ranks, and may overlook some small, morphologically indistinct, and fragile eukaryotes, potentially resulting in generally low diet breadths (*e.g.*, 12, 61). This is the case for worms living in coastal and estuarine sedimentary environments, at which the diversity of microbial eukaryotes (including microalgae, protozoa, and fungi) is large (24, 66). Eukaryotic diets other than microalgae have yet to be revealed for benthic animals. Ribosomal RNA (rRNA) gene-based molecular methods have recently been increasingly employed to reveal the diets and microbiota of many coastal invertebrate predators with higher classification resolutions. For example, by using the PCR amplification and sequencing of 18S rRNA genes, algal prey or eukaryotic diets in whole guts were identified and quantified for planktonic copepods (49), the larvae of the red rock lobster (50), the adult individuals of marine suspension-feeding bivalves *Mytilus* spp. (46), and mariculture shrimps (16). However, the molecular detection of eukaryotic diets in coastal sediment-dwelling worms has not yet been extensively conducted.

In the present study, we investigated the molecular diversity of eukaryotic diets in two marine species of sediment-dwelling animals, the peanut worm *Sipunculus nudus* Linnaeus, 1766 (Sipuncula, Sipunculidea, Sipunculidae), and the spoon worm *Urechis unicinctus* von Drasche, 1881 (Annelida, Echiura, Urechidae), both of which are economically important in China (43). *S. nudus* is mainly distributed in tropical and subtropical coasts (30) and has been successfully bred artificially in China for decades. The development of *S. nudus* includes three stages: larval, juvenile, and adult. During these successive stages, the food source is markedly altered by changes in behavior and feeding structures and modes, as previously observed using microscopy (61). However, the molecular characterization of gut contents across developmental phases has yet to be performed in detail for this species and other marine worms (29, 33).

*U. unicinctus* is distributed in the coasts of China, Korea, Russia, and Japan (44, 65). Since the artificial breeding of *U. unicinctus* is not that successful, most of these worms are harvested from their natural habitat and available from local seafood markets. Unlike all other deposit-feeding echinurans, adult *Urechis* species are active filtering feeders in that they...
form a mucous net within a U-shaped burrow through which they draw water by peristaltic contractions to collect food particles (33). The alimentary tract of this species is five-fold longer than its body length, including the midgut, respiratory intestines, hindgut, and rectum (56), which allows for the sufficient digestion of ingested food and absorption during the gut passage (42, 56). Variations in eukaryotic diets across gut sections need to be investigated in this species and many other marine worms.

The present study investigated compositional variations in major eukaryotic groups across the larval, juvenile, and adult stages of *S. nudus*, and in different gut sections of adult *U. unicinctus*. We also examined the diversity of and changes in the community composition and structure of gut fungi (gut mycobiomes) for the following reasons: 1) among eukaryotes, fungi represent a highly specialized ecological group that may mediate the transformation of non-living organic matter, thereby enhancing nutrient uptake in the anaerobic gut environment (1, 6, 29); 2) sipunculans and echinurans both have markedly longer gut residence times (>1 d) than other worms (mostly between 0.5 and 2 h), which provide longer periods for food caching and re-ingestion. Furthermore, their guts have mildly alkaline pH values, resulting in high digestive efficiencies in extracting organic matter in their diets (33); 3) gut fungi have been isolated from a few marine invertebrates, such as irregular sea urchins (60) and crabs (47, 52), but have rarely been studied in coastal marine benthic invertebrates using culture-independent approaches. The hypotheses that developmental stages and locations in the long alimentary tract affect gut fungal diversity have yet to be tested.

**Materials and Methods**

**Specimens and gut content collection**

The test organisms of *S. nudus* were originally collected from the coast of Beihai (Beibu Gulf, South China Sea), Guangxi, southern China. The artificial breeding of peanut worms has been successfully conducted in China for a decade; therefore, sampling of the different life stages of this species was easily performed at a breeding base affiliated with the Guangxi Institute of Oceanology in 2014. Three stages of the peanut worm (referred to as SN-I, SN-j, and SN-a) were collected: planktotrophic pelagosphera larvae (body length 0.3–0.4 cm), sediment-dwelling juveniles (2.5–4.0 cm), and adults (10–15 cm). Larvae were obtained from an indoor nursery. Despite a mixed solution of microalgal species being routinely supplied as the main food source to larvae, other unidentified protists/microeukaryotes in the system may mediate the transformation of non-living organic matter, thereby enhancing nutrient uptake in the anaerobic gut environment (1, 6, 29) and may mediate the transformation of non-living organic matter, thereby enhancing nutrient uptake in the anaerobic gut environment (1, 6, 29).

Larvae were obtained from an indoor nursery. Despite a mixed solution of microalgal species being routinely supplied as the main food source to larvae, other unidentified protists/microeukaryotes in the system may mediate the transformation of non-living organic matter, thereby enhancing nutrient uptake in the anaerobic gut environment (1, 6, 29).

The body surfaces of the organisms were repeatedly washed with sterilized seawater and 75% (v/v) ethanol solution before dissection. All dissecting tools were autoclaved before use. Gut sections were sliced into pieces, flushed with sterile seawater, and centrifuged and re-suspended repeatedly in order to discard large pieces of tissue and sediment gravel. The suspension solution containing fine intestinal contents was filtered onto polycarbonate membranes with a pore size of 0.22 μm (47 mm in diameter; Millipore, Burlington, MA, USA), and then placed into cryotubes and stored at –80°C for further processing.

**DNA extraction, PCR amplification, cloning, and sequencing**

The extraction and purification of DNA were performed using the FastDNA spin kit (MP Biomedical, Santa Ana, CA, USA) according to the manufacturer’s instructions. The quality of extracted DNA was assessed using gel electrophoresis (1% agarose gels) and quantified using a Nanodrop 2000c spectrophotometer (ThermoFisher, Waltham, MA, USA).

In order to inspect eukaryotic diversity in the gut contents of deposit-feeding marine worms, 18S rRNA genes were PCR amplified using extracted DNA as the template and the eukaryote-specific primers EuA and EuB (48). The PCR program was as follows: 94°C for 3 min, and 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2.5 min, with a final extension at 72°C for 10 min. Since no PCR product bands were detected via gel electrophoresis, a nested PCR strategy was applied: 1 μL of first-round PCR product solution was used as a template in second-round PCR amplification using the eukaryotic-specific primer sets EuA1 and EuB516r (17). The thermocycling procedure for the second amplification was 94°C for 3 min, 35 cycles of 94°C for 30 s, 52°C for 1 min, 72°C for 30 s, and a final extension at 72°C for 10 min.

Regarding mycobiomes, nested PCRs targeting fungal 18S rRNA were similarly conducted using first-round PCR solution as a template and the fungi-specific primers nu-SSU-0817 and nu-SSU-1536 (8). Twenty-five microliters (final volume) of the PCR reaction solution contained 2.5 μL of 10× Ex Taq PCR buffer, 2.5 μL MgCl₂ (25 mM), 0.5 μL dNTP (10 μM each), 1 μL of each primer (10 μM), 1 μL template, and 0.2 unit of Taq DNA polymerase (Fermentas, ThermoFisher). PCR was run under the following conditions: 35 cycles of denaturation at 94°C for 30 s, annealing at 52.5°C for 45 s, extension at 72°C for 1 min, preceded by 5 min of denaturation at 94°C and followed by a 10-min extension at 72°C. In each sample, triplicate PCR products were pooled and purified with a Tian Quick Midi extraction kit (Tiangen, Beijing, China) in order to minimize PCR biases in cloning.

Clone libraries were constructed using the InstAclone PCR cloning kit (ThermoFisher) according to the manufacturer’s instructions. The resulting plasmids were transformed into *Escherichia coli* DH5α-competent cells (Tiangen). Approximately 100 and 150 clones of each library were randomly selected for eukaryote and fungi, respectively. The presence of the 18S rDNA insert in colonies was checked by PCR reamplification with the vector-specific primers M13F and M13R using the eukaryote-specific primer sets EuA1 and EuB516r (17). PCR amplification products of fungal clones containing the right insert size were digested with 1 U of the restriction enzyme *Msp* I (Fermentas, ThermoFisher) at 37°C for 1 h. Clones that produced the same RFLP pattern (DNA fragments of the same size) were considered to be members of the same phylotype and 4 clones of each phylotype were randomly selected for subsequent sequencing (Sangon Biotech, Shanghai, China). Putatively chimeric sequences were identified using Bellerophon (31) and removed from the libraries before subsequent analyses.

The eukaryotic and fungal 18S rRNA gene sequences obtained in the present study have been deposited in the NCBI database under the accession numbers MG753800–MG753972 and MG711921–MG712280, respectively.

**Phylogenetic analyses**

Operational taxonomic units (OTUs) for eukaryotes and fungi (specifically designated as FOTUs to distinguish them from those of
Eukaryotes and Fungi in Guts of Marine Worms

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Higher classification of these representative sequences was initially each OTU and FOTU were selected using Mothur v. 1.39.5 (55). The more practical in ecological surveys. Representative sequences of intragenomic variations of microeukaryotes (11, 23), and, hence, is important to note that species-level rDNA differences vary among eukaryotic species. We selected the 97% OTU definition because it is moderately stringent, able to overcome the major

Results

Detection of eukaryotic phylotypes in guts of the peanut and spoon worms

In order to detect gut eukaryotes in two marine worm species, 301 clones of 6 clone libraries were screened and sequenced. After discarding the 18S rRNA genes of the host species and 25 eukaryotic OTUs were obtained for the two worms (Table 1). The OTU-level composition of gut eukaryotes markedly varied among the development stages of S. nudus or across gut sections in U. unicinctus. BLASTing against GenBank showed that gut fungi had 6 OTUs, representing the most diverse eukaryotic group in the guts of these two animals. In terms of the proportion of reads, Fungi was the most abundant (28.9%), followed by Stramenopiles (26%), Archaeplastida (20.2%), and Metazoa (16.8%) and a few Euglenozoa (5.9%) and Amoebozoa (1.7%, Fig. 1 and Table 1). The results of the phylogenetic analysis indicated that half of these OTUs were closely related to members of the genus Malassezia and the remaining fungal OTUs belonged to Saccharomyces (Ascomycota) (Fig. 2A).

Metazoan phylotypes were detected in both worms. There were 3 metazoan OTUs (Table 1), of which OTU2 was a member of the order Enoplida (Nematoda) and only detected in the aspiratory intestines of the spoon worm. Another nematode (OTU20) belonging to the order Desmodorida, together with a rhabditophoran flatworm (OTU9), were detected in the adults of S. nudus (Fig. 2B).

Unicellular eukaryotes (protists) including autotrophs and heterotrophs were detected in the animal guts. A single phylotype closely related to Nitososolenus (Euglenida) was also present in adult S. nudus (Fig. 2C and Table 1). An Actinocyclus-like diatom (OTU10) and placidid nanoflagellate (OTU4) were present in the aspiratory intestine samples of S. nudus (Fig. 1 and Table 1). The results of the phylogenetic analysis indicated that half of these OTUs were Metazoa (1.7%, Fig. 1 and Table 1). The results of the phylogenetic analysis indicated that half of these OTUs were

| Table 1. Classification and distribution of gut eukaryotic OTUs in libraries and results of BLASTing against GenBank. |
|---------------------------------------------------------------|
| Classification                  | OTU ID | SN-1 | SN-j | SN-a | UU-m | UU-a | UU-h | Closest relative (accession number) | Identity (%) |
|---------------------------------|--------|------|------|------|------|------|------|-----------------------------------|--------------|
| Fungi, Basidiomycota            | OTU1   | 0    | 0    | 0    | 0    | 0    | 21   | Sclerotium sp. (AF10303)           | 100          |
|                                 | OTU5   | 0    | 0    | 0    | 0    | 0    | 13   | Sclerotium sp. (JX132785)          | 99           |
|                                 | OTU12  | 0    | 0    | 0    | 0    | 0    | 6    | Sclerotium sp. (AF10303)           | 99           |
| Fungi, Ascomycota               | OTU13  | 0    | 5    | 0    | 0    | 0    | 0    | Archaeospora sp. (KT923272)        | 99           |
|                                 | OTU15  | 0    | 4    | 0    | 0    | 0    | 0    | Archaeospora sp. (KT923272)        | 99           |
|                                 | OTU23  | 0    | 0    | 0    | 1    | 0    | 0    | Closidium cladosporioides (MF522178) | 99           |
| Metazoa                         | OTU2   | 0    | 0    | 0    | 0    | 19   | 0    | Tripyloides sp. (AY854202)         | 96           |
|                                 | OTU9   | 0    | 0    | 9    | 0    | 0    | 0    | Duplominona sp. (KJ682379)         | 89           |
|                                 | OTU20  | 0    | 0    | 1    | 0    | 0    | 0    | Desmodora ovigera (Y16913)         | 94           |
| Archaeplastida, Streptophyta    | OTU11  | 0    | 0    | 0    | 0    | 7    | 0    | Lygodemia juncea (KT179674)        | 99           |
|                                 | OTU14  | 0    | 0    | 0    | 4    | 0    | 0    | Grevillea sp. (JX132698)           | 100          |
|                                 | OTU19  | 0    | 0    | 1    | 0    | 0    | 0    | Grevillea sp. (JX132698)           | 99           |
|                                 | OTU24  | 0    | 0    | 1    | 0    | 0    | 0    | Grevillea sp. (JX132698)           | 99           |
| Archaeplastida, Chlorophyta     | OTU6   | 0    | 0    | 0    | 0    | 11   | 0    | Ulva pertusa (AB425961)            | 97           |
|                                 | OTU8   | 0    | 0    | 0    | 0    | 0    | 10   | Ulva lactuca (AB425960)            | 99           |
|                                 | OTU25  | 0    | 0    | 0    | 0    | 1    | 0    | Ulva pertusa (AB425961)            | 97           |
| Alveolata, Dinophyceae          | OTU18  | 0    | 0    | 2    | 0    | 0    | 0    | Amphidinium steinii (LC054921)     | 96           |
|                                 | OTU22  | 0    | 0    | 1    | 0    | 0    | 0    | Peridiniose parudnii (AB353771)    | 98           |
|                                | OTU1   | 0    | 0    | 0    | 0    | 0    | 1    | Paramecium bursaria (KC495068)     | 99           |
|                                | OTU10  | 0    | 0    | 0    | 0    | 0    | 8    | Actinocyclus sp. (KC395022)        | 99           |
|                                | OTU17  | 0    | 0    | 3    | 0    | 0    | 0    | Pseudoetraedraella kamillae (EF044311) | 95         |
|                                | OTU17  | 0    | 0    | 3    | 0    | 0    | 0    | Pseudoetraedraella kamillae (EF044311) | 95         |
|                                | OTU18  | 0    | 0    | 2    | 0    | 0    | 0    | Amphidinium steinii (LC054921)     | 96           |
|                                | OTU22  | 0    | 0    | 1    | 0    | 0    | 0    | Peridiniose parudnii (AB353771)    | 98           |
|                                | OTU21  | 0    | 0    | 0    | 0    | 0    | 1    | Paramecium bursaria (KC495068)     | 99           |
|                                | OTU10  | 0    | 0    | 0    | 0    | 0    | 8    | Actinocyclus sp. (KC395022)        | 99           |
|                                | OTU17  | 0    | 0    | 3    | 0    | 0    | 0    | Pseudoetraedraella kamillae (EF044311) | 95         |
|                                | OTU3   | 0    | 0    | 3    | 0    | 0    | 0    | Pseudoetraedraella kamillae (EF044311) | 95         |
|                                | OTU4   | 0    | 0    | 0    | 0    | 0    | 15   | Saugettaurus clinomigrationis (AB976561) | 87         |
|                                | OTU16  | 0    | 0    | 0    | 0    | 0    | 0    | Nitososolenus urceolatus (KJ778682) | 89           |
|                                | OTU16  | 0    | 0    | 0    | 0    | 0    | 0    | Arcyria cinerea (AY145523)         | 89           |

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Amoebozoa (Fig. 3E) were observed in the sediment-dwelling stages of S. nudus; one OTU of a Paramecium ciliate was detected in the hindgut of U. unicinctus only (Fig. 3D).

The detritus of higher plants appeared to be ingested by these two deposit-feeding animals. Four streptophyte OTUs were found to be affiliated with the phylum Tracheophyta (vascular plants) (Fig. 3B). Among these, OTU11 was closely related to Lygodesmia juncea and observed in the aspiratory intestines of U. unicinctus (Table 1), whereas the remaining 3 OTUs (OTU14, OTU19, and OTU24) were Grevillea-like and only found in juvenile S. nudus. Three OTUs of Chlorophyta were detected in the aspiratory intestines and hindgut and showed higher sequence similarities (97–99%) with Ulva spp. (Table 1 and Fig. 3B), indicating that spoon worms feed on these macroalgae.

Structural variations in gut eukaryotic phylotypes were evident across the growth stages of peanut worms. The most abundant group changed from Oomycota (100%) at the larval stage, to saccharomycetous fungi (50%) at the juvenile stage, then to metazoans (38%) and euglenids (38%) at the adult stage. Fig. 1. Variations in proportions of clones of eukaryotic groups based on a clone library analysis. Fig. 2. Maximum likelihood (ML) trees with a similar topology of the Bayesian tree based on 18S rRNA genes, showing the systematic positions of dietary eukaryotic OTUs detected in guts of S. nudus and U. unicinctus. Major clades of Fungi (A), Metazoa (B), and Euglenozoa (C) are presented. Asterisks indicate different topologies from the Bayesian trees. Only nodal supports with bootstrap values >50%, Bayesian posterior probability >0.8 are shown.

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stage (Fig. 1). Fungal groups that dominated the gut eukaryotic phylotypes were abundant in the midgut and hindgut of spoon worms (Fig. 1).

Composition of fungal communities associated with two marine worms

Seven clone libraries of 18S rRNA genes were constructed for gut fungal communities based on fungus-specific primers and PCR amplifications. A total of 1,005 clones were screened, 95 types were identified using an RFLP analysis, and 360 sequences were obtained after removing possible chimeras. At a cut-off of 97% sequence similarity, 48 FOTUs were identified (Table 2). Calculations of library coverage and rarefaction curves demonstrated that the sampling effort was sufficient to recover the most fungal phylotypes in these gut samples (Table S1 and Fig. S1). All FOTUs were affiliated with the subkingdom Dikarya, with 31 FOTUs of Basidiomycota and 17 FOTUs of Ascomycota. Basidiomycetous phylotypes were present in all 7 clone libraries, whereas ascomycetous phylotypes were absent in SN-j and UU-r.

Gut fungi shared no common OTUs among the different growth stages of peanut worms. Nevertheless, two FOTUs (FOTU1 and FOTU2) were consistently detected in 3 out of the 4 gut sections of spoon worms (Table 2). In the gut of S. nudus, FOTU richness varied slightly; it was the highest in the adult (13) and the lowest in the juvenile (7) (Fig. 4A). FOTU richness among different gut sections of U. unicinctus varied widely, with markedly fewer FOTUs (1) in the rectum than in the midgut (10), respiratory intestines (8), and hindgut (8) (Fig. 4B).

By BLASTing the representative sequences against GenBank, 18 out of the 48 FOTUs were found to have <97% sequence similarities with the described fungal species (Table 2), indicating that the guts of these marine worms host many fungal species yet to be described. This was the case for the class Malasseziomycetes, 7 FOTUs of which were potentially new (Table 2). A phylogenetic analysis of these FOTUs resolved their systematic placements at lower ranks with reasonable confidence (Fig. 5). Overall, the gut fungi of these two marine animals were mainly represented by the classes Malasseziomycetes (15 FOTUs), Tremellomycetes (10 FOTUs), and Dothideomycetes (9 FOTUs). The classes Agaricomycetes, Agaricomycetes, and Saccharomycetes each had 5 FOTUs, whereas only 1–2 FOTUs were affiliated with Exobasidiomycetes, Sordariomycetes, or Leotiomycetes (Fig. 5 and Table 2). A large proportion of fungal phylotypes (52.5% clones) were yeasts closely related with Malassezia spp., Trichosporon cutaneum, T. sporotrichoides, Debaryomyces hanseni, Cryptococcus zeae, Pichia sorbitophila, Cladosporium sp., Bullera alba, and Bandooniomyza tunnelae (Table 2). These yeast-like FOTUs were mainly from Malasseziomycetes (62.5%), Tremellomycetes (30.6%), and Saccharomycetes (6.4%).

Fungal community structures in the guts of the two animals were characterized based on the clone numbers of the FOTUs...
Table 2. Number of clones belonging to each FOTU in genetic libraries and phylogenetic affiliations of representative clone sequences obtained using BLASTing against GenBank

| Classification          | OTU ID | SN-1 | SN-j | SN-a | UU-m | UU-a | UU-h | UU-r | Closest relative (accession number) | Identity (%) |
|-------------------------|--------|------|------|------|------|------|------|------|------------------------------------|--------------|
| Basidiomycota           |        |      |      |      |      |      |      |      | Malassezia restricta (EU192367)    | 100          |
| Malasseziomycetes       |        |      |      |      |      |      |      |      | Malassezia globosa (EU192364)      | 99           |
|                         |        |      |      |      |      |      |      |      | Malassezia pachydermatis (EU192367)| 100          |
|                         |        |      |      |      |      |      |      |      | Malassezia pachydermatis (EU192366)| 95           |
|                         |        |      |      |      |      |      |      |      | Trichosporon sp. (KF036721)        | 96           |
|                         |        |      |      |      |      |      |      |      | Malassezia restricta (EU192367)    | 95           |
|                         |        |      |      |      |      |      |      |      | Malassezia globosa (EU192364)      | 97           |
|                         |        |      |      |      |      |      |      |      | Malassezia globosa (EU192364)      | 96           |
|                         |        |      |      |      |      |      |      |      | Malassezia globosa (EU192364)      | 97           |
|                         |        |      |      |      |      |      |      |      | Malassezia globosa (EU192364)      | 97           |
|                         |        |      |      |      |      |      |      |      | Malassezia globosa (EU192364)      | 97           |
| Exobasidiomycetes       |        |      |      |      |      |      |      |      | Pseudomicrostroma glucosipilum (KR912075)| 100        |
| Tremellomycetes         |        |      |      |      |      |      |      |      | Trichosporon cutaneum (KF036712)   | 98           |
|                         |        |      |      |      |      |      |      |      | Trichosporon cutaneum (KF036712)   | 98           |
|                         |        |      |      |      |      |      |      |      | Trichosporon sp. (JN939434)        | 98           |
|                         |        |      |      |      |      |      |      |      | Cryptococcus zeae (FH153134)       | 100          |
|                         |        |      |      |      |      |      |      |      | Bullera alba (AY344034)            | 94           |
|                         |        |      |      |      |      |      |      |      | Trichosporon cutaneum (KF036712)   | 96           |
|                         |        |      |      |      |      |      |      |      | Sirobasidium sp. (LC03430)         | 93           |
|                         |        |      |      |      |      |      |      |      | Malassezia globosa (EU192364)      | 97           |
|                         |        |      |      |      |      |      |      |      | Malassezia globosa (EU192364)      | 97           |
|                         |        |      |      |      |      |      |      |      | Cladosporium sp. (KU1512834)       | 96           |
| Agaricomycetes          |        |      |      |      |      |      |      |      | Climacodon septentrionalis (AY705964)| 99          |
| Dothideomycetes         |        |      |      |      |      |      |      |      | Ceriporiopsis sp. (EU670846)       | 99           |
|                         |        |      |      |      |      |      |      |      | Ceriporiopsis sp. (EU670846)       | 98           |
|                         |        |      |      |      |      |      |      |      | Ceriporiopsis sp. (EU670846)       | 96           |
|                         |        |      |      |      |      |      |      |      | Ceriporiopsis sp. (EU670846)       | 96           |
|                         |        |      |      |      |      |      |      |      | Ceriporiopsis sp. (EU670846)       | 96           |
| Ascomycota              |        |      |      |      |      |      |      |      | Cladosporium sp. (KU1512834)       | 100          |
| Dothideomycetes         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 98           |
|                         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 95           |
|                         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 97           |
|                         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 97           |
|                         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 97           |
|                         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 97           |
|                         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 97           |
|                         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 97           |
|                         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 97           |
|                         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 97           |
|                         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 97           |
|                         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 97           |
|                         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 97           |
|                         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 97           |
|                         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 97           |
|                         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 97           |
|                         |        |      |      |      |      |      |      |      | Epicoccum nigrum (KM096239)        | 97           |
| Sordariomycetes         |        |      |      |      |      |      |      |      | Simplicillium lanoosineum (KY075854)| 99          |
| Leotiomycetes           |        |      |      |      |      |      |      |      | Thelebolus microsporus (KJ939313)   | 99           |
|                         |        |      |      |      |      |      |      |      | Thelebolus microsporus (KJ939313)   | 99           |
|                         |        |      |      |      |      |      |      |      | Thelebolus microsporus (KJ939313)   | 99           |
| Saccharomycetes         |        |      |      |      |      |      |      |      | Debaryomyces Hansenii (LC041126)   | 98           |
|                         |        |      |      |      |      |      |      |      | Bandoniozyma tunnelae (KF036608)    | 94           |
|                         |        |      |      |      |      |      |      |      | Trichosporon cutaneum (KF036712)   | 92           |
|                         |        |      |      |      |      |      |      |      | Pichia sorbitiphila (FO082054)      | 94           |
|                         |        |      |      |      |      |      |      |      | Peltula farinosa (MF766273)         | 93           |

Fig. 4. Venn diagrams showing numbers of fungal operational taxonomic units (OTUs) shared between clone libraries among life stages of *S. nudus* (A) and among gut regions in *U. unicinctus* (B).

(Fig. 6). Overall, the phylogenotypes of Basidiomycota (67.2%) appeared to be more abundant than those of Ascomycota (32.8%, Fig. 6). At the class level, Malasseziomycetes (40.1%) were also the most abundant, followed by Dothideomycetes (23.7%), Tremellomycetes (16.1%), and Agaricomycetes (8.5%). The other groups in the gut fungal communities were minor (<4%) (Fig. 6). Malasseziomycetes were presented in 6 libraries, except for adult peanut worms. Saccharomycetes were detected in *S. nudus*, but not in *U. unicinctus*. In contrast, Exobasidiomycetes, Sordariomycetes, and Leotiomycetes were only present in *U. unicinctus*. Across the larval, juvenile, and adult stages of *S. nudus*, the most abundant group in the gut fungal community shifted from Dothideomycetes (71.5%) to Malasseziomycetes (81.1%) and Tremellomycetes (93.2%). Nevertheless, in the 4 gut sections...
Fig. 5. The maximum likelihood (ML) tree with a similar topology of the Bayesian tree, showing positions of FOTUs obtained from fungal DNA-targeted clone libraries. Asterisks indicated different topologies observed from the ML and Bayesian trees. Only nodal supports with bootstrap values >50%, Bayesian posterior probability >0.8 are shown. The size of the circle scales with the proportion (in percentage) of the FOTU in the fungal clone library.
of *U. unicinctus*, Malasseziomyces was consistently the most abundant (midgut, 47.5%; hindgut, 60.0%; rectum 100%), except for in the aspiratory intestines (21.5%).

**Discussion**

**Eukaryotic diets in larval, juvenile, and adult stages of *S. nudus* and different gut sections of *U. unicinctus***

To the best of our knowledge, the present study is the first to reveal eukaryotic diet compositions in coastal sediment-dwelling sipunculan and annelid animals using molecular approaches. We detected a wide range of eukaryotic groups associated with the larvae of *S. nudus* and in the guts of both worms. The diets detected in juvenile and adult *S. nudus* included benthic microalgaee (microphytobenthos) and macrophytes, which is consistent with previous findings obtained using a microscopic gut analysis (61). Nevertheless, our study using an 18S rRNA gene-based clone library analysis provided higher taxonomic resolutions to these prey in the peanut worm, which contributed to the interpretation of the trophic function of the predator. For example, dinoflagellates closely related to *Amphidinium steinii* and *Peridiniopsis*, eustigmatophytes, and *Notosolenus*-like euglenids were detected (Table 1). *Amphidinium* and *Notosolenus* species have both been commonly observed in marine sediments (2), and *Peridiniopsis* spp. are members of freshwater plankton (63). These findings provide evidence that peanut worms feed not only on benthic, but also planktonic microalgae, even including some from freshwater run-off, at sediment-dwelling stages. Eustigmatophytes are yellow-green algae, typically with small (<30 μm) cell sizes and abundant amounts of polysaturated fatty acids; some cultured species of this class are commonly used as food for larvae in mariculture (19), which was the case in the present study. The presence of eustigmatophytes in the gut of adult *S. nudus* implies that these small algae are still a valuable food source for the worm in its natural habitat. Sequences of *Grevillea* sp., a flowering tree mainly distributed in tropical and subtropical areas of China, were detected in the gut of juvenile *S. nudus*, indicating the ingredients of the artificial diet supplied during the pond culture period.

The observation of marked changes in eukaryotic diets at separate life stages of *S. nudus* is intuitive and straightforward because of the contrasting lifestyles (planktonic vs. benthic) and feeding modes (filtering vs. suspension+surface deposit-feeding) across the larval, juvenile, and adult stages of this species, as well as aquaculture management during the first two stages. Nevertheless, our molecular analysis revealed some novel aspects on what and how diets change. An important point is that *Anisolpidium* and *Olpidiopsis*-related oomycetes (pseudofungi) were detected exclusively at the planktonic pelagosphera stage. Oomycete phylotypes were also found in the larvae of the red rock lobster (50). *Anisolpidium* and *Olpidiopsis* species were brown and red algae-infecting pathogens that have the potential to cause great economic losses in the seaweed-farming industry (21, 37). Therefore, our results suggest that the flagellated zoospores of these oomycete pathogens may be filtered and ingested by the larvae of *S. nudus*, a potential trophic link between the macroalgal pathogens and planktonic larvae of cultivated animals, which may provide an insight into reducing the risk of an outbreak of oomycete diseases in seaweed farming. None of the microalgae (i.e., 3 chlorophytes, 3 chrysophytes, and 3 diatoms) or baker’s yeast, which are good prey and generally applied for the larvae of *S. nudus* (40), were detected in the present study. These artificially prepared diets may have been easily digested or egested due to the short gut passage time in small-sized larvae. Apart from protists, higher sequence proportions of fungi and animals (i.e., nematodes and flatworms) were found in the clone libraries for adult *S. nudus* and *U. unicinctus*, indicating a markedly broader food spectrum and the omnivorous nature of these two suspension-feeding worms.

The burrow-dwelling species *U. unicinctus* uses a mucus net to filter food particles in the water, and, thus, is a typical suspension or filtering feeder. It is commonly considered to be a non-selective feeder, whereas microscopic observation-based information on the gut content of the spoon worm has not been available (56). The present study is the first to characterize its gut contents. Our molecular analysis of midgut, aspiratory intestine, and hindgut samples collectively showed that the diet included the enoploid nematodes, *Actinocyclos* diatoms, of *Ulva* seaweeds, detritus of *L. juncea* (a typical sand dune-inhabiting plant near the coastal sites of sampling), and flagellates of the stramenopile class Placididea, indicating the omnivory of this worm. Our detection of *Ulva* species in the gut content samples of *U. unicinctus* collected at Yantai suggests that spoon worms feed on seaweed detritus. This trophic relationship is easily explained by the green-tide species *Ulva* frequently blooming in the summer at the Yellow Sea in recent years (64), resulting in some seaweed detritus drifting to and being buried in the intertidal zones of the coastal line of Yantai and then being consumed. This finding supports the food value of macroalgae to the sediment-dwelling macrofaunal community (e.g., 41).

The described placidids are all heterotrophic nanoflagellate (HNFs), of which the bacterivorous species *Suigetsumonas clinomigrationis* inhabits anoxic environments (39, 51), suggesting that the placidid phylotypes identified in the present
study are sediment-dwelling, distributed in the pore water of sediments, pumped into the burrow by peristaltic contractions, intercepted by the mucus net, and eventually ingested by *U. uncinutus*. Approximately 30% of the clones in the library of the aspiratory gut were from placid HNFs, which contrasts with the rarity of this group in coastal and marine benthic microeukaryotes (24, 66), suggesting that placid HNFs were selectively filtered and predated. Alternatively, placid flagellates may be commensal, adapted to the anoxic gut environment. Further investigations are needed in order to clarify this issue.

During gut passage from the aspiratory gut to the hindgut, most protists and metazoan diets decrease, whereas *Ulva* and some basidiomycetous fungi remain, highlighting the high digestive efficiency of this worm and the residential nature of many fungal phylotypes in the animal’s gut. Fungal phylotypes appear to be important because they dominated the two libraries of eukaryotic diets in peanut and spoon worms in the present study. The dominance of fungi in eukaryotic diets has also been reported in other marine animals, e.g., the anterior caecum of the irregular sea urchin (60) and the larval midgut of the red rock lobster (50). Nevertheless, it is still premature to examine the function of these gut fungi in order to establish whether they are ingested, inactive, commensal, or parasites (50).

*Varying gut mycobiomes at larval, juvenile, and adult stages of the peanut worm*

The variation patterns of fungal diversity and composition across time and space need to be clarified in order to obtain a better understanding of their functions in the gut. The high coverage (≥97%) of all fungal clone libraries and rarefaction curves indicate that most fungal phylotypes have been recovered, which allows for alpha diversity comparisons of these fungal communities. Among the three stages of *S. nudus*, adults took a longer time to develop and had the longest alimentary tract, which enabled more fungal species to colonize, resulting in the highest gut FOTU richness. At the larval, juvenile, and adult stages, the fungal community was dominated by *Epiconcium nigrum*, *Malassezia* spp., and *Trichosporon* related phylotypes, respectively. *Epiconcium* fungi are saprophytic and capable of producing antifungal and antibacterial compounds (9). It currently remains unclear whether the exclusive presence of these phylotypes plays a role in protecting larvae from other microbial pathogens.

*Malassezia*-like fungi are ecologically diverse and widespread yeasts (4), but not a numerically abundant group in the fungal communities of coastal water and sediment environments (27, 62, 66). In the present study, *Malassezia* spp. were prevalent in the juveniles and adults of these worms, indicating that this group of fungi are specifically enriched in the gut and, hence, appear to be residents rather than ingested transients. This group has also been detected in the guts of other marine animals, such as in humans (25) and earthworms (10). Members of this genus are capable of degrading cellulose and lignin (38). Although less abundant than *Malassezia* and *Cladosporium*-related phylotypes, the exobasidiomycetous *Pseudomicrostroma glucosiphilum* also appeared to be numerically important in the hindgut of *S. nudus*. This species was originally isolated using a glucose air-exposed agar plate, and relevant ecological information is currently not available, except for physiological knowledge that it assimilates a range of carbon sources including cellobiose, trehalose, arabinose, and ribose and grows on vitamin-free medium (36). The present results add the gut environment as an additional ecological niche of this fungus.

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