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Culturable gut microbiota of marine wood boring invertebrates *Dicyathifer manni* (wright, 1866), *Sphaeroma terebrans* (Bate, 1866) and *Cirolana* sp.

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Abstract

The cultured aerobic c*opiotrophic* bacteria and fungi from the digestive tracts of *Dicyathifer manni* (Wright, 1866), *Sphaeroma terebrans* (Bate, 1866) and a *Cirolana sp.* were investigated. The objective of the present study was to determine the bacterial and fungal diversity within the digestive tracts of the woodborers. Bacteria isolated on nutrient agar and fungi isolated on *sabouraud* dextrose agar (SDA) were identified by 16S rRNA and ITS gene barcoding respectively, with subsequent phylogenetic analysis. Four strains of bacteria, namely *Lysinibacillus boronitolerans* (from *D. manni* and *S. terebrans*), *Lysinibacillus fusiformis* (from *S. terebrans* and *Cirolana sp.), Lysinibacillus sphaericus* and *Lysinibacillus xylanilyticus* (both from *Cirolana sp.)* had similarity to known 16S rRNA sequences of 98–99%. A neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showed that the bacteria are closely related members of the genus *Lysinibacillus*. Different strains of *Ascomycetes* fungi were also isolated. *Aspergillus niger* was isolated from the digestive tracts of *D.manni* and *S. terebrans*. In addition, *Neosartorya fischeri, A. fumigatus* and *Penicillium sp.* were isolated from *D. manni* whereas *Botryotinia fuckeliana* was isolated form *S. terebrans* digestive tract. *A. costaricaensis* and *A. fumigatus* were isolated from *Cirolanna sp.* digestive tract. The fungi had similarity to known ITS sequences of 95–100%. Existence of bacterial and fungal groupings symbiotically associated with woodborers gut is proposed. Investigation of the functional characteristics and role in the host organism is required to confirm the symbiotic status of the microorganisms associated with the woodborers digestive tract.

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Introduction

The marine woodborer *Dicyathifer manni* (Wright, 1866) belong to class bivalvia, family Teredinidae. *Sphaeroma terebrans* (Bate, 1866) belong to phylum Arthropoda, class crustacea, family Sphaeromatidae whereas *Cirolana sp*. belongs to phylum Arthropoda, class crustacea, family Cirolanidae.

Teredinidae ingests a certain amount of wood, but they also possess the filter feeding mechanism found in other bivalves (George and George, 1979). Digestion in some members of Teredinidae is extracellular in the stomach, and absorption occurs in the digestive glands. Others use finer particles as food in filter feeding on gills, extracellular digestion in the stomach, as well as within the digestive gland. Digestion in carnivorous species occurs in the muscular stomach lined with chitin that acts as a crushing gizzard. Proteases from the digestive gland initiate extracellular digestion in the stomach. The digested material is conveyed into the ducts of the digestive diverticula for further digestion (intracellular). Wood-boring bivalves attack mangrove wood and timber swept into the sea by rivers, and they play an important ecological role in the reduction of sea-borne wood. They excavate sawdust for food and the stomach is provided with a caecum for sawdust storage, and a section of digestive gland is specialized for handling wood particles. Symbiotic bacteria housed within a special organ that opens into the oesophagus provide cellulose digestion and also, by fixing nitrogen, compensate for the low-protein diet (Barnes, 1987).

Marine wood-boring Sphaeromatidae feed on wood as they tunnel and bore into stems and roots of mangroves. Their hepatopancreatic secretions include cellulase. They are thought to be attracted to the fungi in the wood and the fungi is thought to add nitrogen to their largely cellulose diet (Geyer and Becker, 1980). The role of Cirolanidae in the wood-boring community is uncertain. Keable (2001) describes scavenging species of *Cirolana* from the Australian coast. Bowman *et al.* (1981) also reported that an exotic *Cirolana sp*ecies, *C. arcuata*, was found in the company of a *Sphaeroma sp*ecies, *S.quoyana* in the San Francisco bay, North America. They could be either woodborers or scavengers. The present investigation determined the bacterial and fungal diversity within the digestive tracts of the woodborers.

Materials and Methods

Woodborers

Adult woodborers were collected from submerged parts of roots (proproots, pneumatophores), stems and branches of mangroves in the intertidal region of Mida creek (North coast), Tudor creek (island) and Gazi bay (south coast) along the Kenyan coast. They were identified as *Dicyathifer manni*, *Sphaeroma terebrans* and *Cirolana sp.* by morphological and molecular identification in our earlier work (unpublished). The host plants were *Rhizophora mucronata* (for *D. manni*) and *Avicennia marina* (for *S. terebrans and Cirolana sp*.).

Culturing of microorganisms from the gut of the woodborers

One *D. manni*, three *S. terebrans,* and three *Cirolana sp*. were surface sterilized with 70% ethanol, rinsed in distilled water and allowed to air dry for 1 minute. Under aseptic conditions, the entire gut of *D. manni* and the pooled guts of the 2 isopoda species were separately removed with a blade and a pair of tweezers and mixed with 1 ml 0.85% sodium chloride to obtain inoculums.

Bacteria were cultivated in nutrient agar and subsequent culturing and plating carried out to obtain pure colonies. The cultures were incubated at 37^{o}C for 3 days. Fungi were cultivated on sabouraud dextrose agar (SDA) medium solidified with 1.6% w/v agar. They were incubated at 30^{o}C for 2-5 days. A single agar disc was cut from the actively growing colony margin of a culture to inoculate each assay medium in subsequent culturing and plating to isolate pure colonies.

Morphological & Molecular identification of the bacteria.

A light microscope with Leica ICC 50 camera (Leica Microsystems) connected to a windows computer was used to view the cells of each culture, and Gram stain was used in identifying the cultures. A colony was picked from a nutrient agar plate, placed in a drop of water on a slide and fixed by passing the slide through a flame. The Gram stain involved staining with crystal violet and then iodine for 30 seconds each, and counterstaining with safranin for 1 minute. This resulted in Gram-positive cells appearing purple and Gram-negative cells appearing pink.

DNA preparation and 16S rRNA fragment analysis

Bacteria were lysed and proteins removed by digestion with proteinase K. Cell wall debris, polysaccharides and residual proteins were removed by selective precipitation with cetyltrimethylammonium bromide (CTAB), and purification completed by phenol: chloroform extraction. Finally the DNA was recovered from the resulting supernatant by isopropanol precipitation, resuspended in nuclease free water and stored at -20°C.

The 16S rRNA gene was amplified using DNA polymerase (Invitrogen) using the following two bacterial specific primers; 27F GAGTTTGATCCTGGCTCAG and 1492R GGTTACCTTGTTACGACT. The polymerase chain reaction (PCR) was performed in a total volume of 25 µl using 13.7 µl distilled water, 1.0 µl dNTPs (10 mM), 2.5 µl PCR buffer, 2 µl MgCl (25 mM), 1.5 µl forward primer (10 mM), 1.5 µl reverse primer (10 mM), 0.8 µl Taq polymerase(5U/ µl) and 2.0 µl DNA template. The PCR profile included initial denaturation step at 95°C for 5 minutes followed by 45 cycles of 95°C (30 sec) denaturation, 50°C (30 sec) annealing, 72°C (2 min) extension and a final 72°C (10 min) extension.

The PCR product was analysed on 1% agarose gel electrophoresis containing 0.5 mg/ml ethidium bromide. The amplification patterns were viewed with DNA gel viewer (Herolab) and cleaned using QIAquick PCR purification kit according to manufacturer's instruction. The clean PCR product was sequenced with an ABI sequencing kit (Big Dye Terminator Cycle Sequencing, Applied Biosystems). Preparation reaction for big dye PCR consisted of big dye (0.5 µl), 5X seq buffer (1.75 µl), 10mM primer (1.0 µl), PCR product (4.0 µl) and water (2.75 µl). The PCR profile was 96°C for 1 min followed by 25 cycles of 96°C for 30 seconds, 50°C for 30 seconds and 60°C for 4 minutes. Clean up (ethanol/ sodium acetate precipitation of extended sequencing products) consisted of nuclease free water (24.5 µl), 3M NaOAc pH 5.2 (3.0 µl) and absolute ethanol (62.5 µl). To analyse the gene, 10 µl of HI-DI formamide (Applied Biosystems) was added and samples denatured at 96°C for 3 min. The samples were electrophoresed on 3130xl Genetic Analyzer (Applied Biosystems®).

Bacterial DNA Sequence Analysis

The electropherograms were viewed, aligned and edited using BioEdit software. The 16S rRNA sequences were compared with the closest sequences deposited in the GenBank (NCBI) public database (http://www.ncbi.nlm.nih.gov/BLAST) using the BLASTn software.

Morphological & Molecular identification of the fungi

Morphological identification of fungal isolates on SDA plates was done using macromorphological features (observed with naked eye or stereo microscope) and micromorphological features (seen through compound microscope).

Macromorphological features included conidial colour, mycelia colour, reverse colour (colour under colonies), type of hyphae, and fruit bodies. Fungal isolates from SDA plates were stained with lactophenol blue stain for studying

micromorphological characteristics. The slides were observed under a microscope (Olympus BX51). The photographs of the slides were used in identification of the fungi. The shape and size of vesicles, shape, size and surface texture of conidia, stipe and ascospores differentiated various isolates from each other.

DNA preparation and internal transcribed spacer (ITS) fragment analysis

Extraction of DNA was performed using Quick-gDNA™Miniprep extraction kit (Zymo Research) according the method described by the manufacturer. The eluted DNA was stored at -20°C.

The rDNA internal transcribed spacer (ITS) regions were amplified using Dream Taq DNA polymerase (Thermo Scientific) following the manufacturer's instructions with slight modifications. ITS primers used were; ITS1 5'-tccgtaggtgaacctgcgg-3' and ITS4 5'-tcctccgcttattgatatgc-3'. The PCR was performed in a total volume of 25 µl using 13.7 µl distilled water, 1.0 µl dNTPs (10 mM), 2.5 µl PCR buffer, 2 µl MgCl (25 mM), 1.5 µl forward primer (10 mM), 1.5 µl reverse primer (10 mM), 0.8 µl Taq polymerase(5U/ µl) and 2.0 µl DNA template. The PCR profile included initial denaturation step at 95°C for 5 minutes followed by 45 cycles of 95°C (30 sec) denaturation, 50°C (30 sec) annealing, 72°C (1min) extension and a final 72°C (10 min) extension.

The PCR product was analysed on 1% agarose gel electrophoresis containing 0.5 mg/ml ethidium bromide. The amplification patterns were viewed with DNA gel viewer (Herolab) and cleaned using QIAquick PCR purification kit according to manufacturer's instruction. The clean PCR product was sequenced with an ABI sequencing kit (Big Dye Terminator Cycle Sequencing, Applied Biosystems).

Preparation reaction for big dye PCR consisted of big dye (0.5 µl), 5X seq buffer (1.75 µl), 10mM primer (1.0 µl), PCR product (4.0 µl) and water (2.75 µl). The PCR profile was 96°C for 1 min followed by 25 cycles of 96°C for 30 seconds, 50°C for 30 seconds and 60°C for 4 minutes. Clean up (ethanol/ sodium acetate precipitation of extended sequencing products) consisted of nuclease free water (24.5 µl), 3M sodium acetate pH 5.2 (3.0 µl) and absolute ethanol (62.5 µl). To analyse the gene, 10 µl of HI-DI formamide was added and samples denatured at 96°C for 3 min. The samples were electrophoresed on 3130xl Genetic Analyzer (Applied Biosystems®).

Fungal DNA Sequence Analysis

The electropherograms were viewed, aligned and edited using BioEdit software. To search for available matches with published sequences of other fungi, we aligned the sequences on BLASTn at the NCBI web site.

Phylogenetic analysis

Fungal and bacterial nucleotide sequences were aligned with ClustalW and phylogenetic trees were constructed using the neighbour-joining method provided in the MEGA 5.2.2 software using maximum likelihood algorithm (Tamura *et al.*, 2011). The significance of the junctions was established using the bootstrap method (1000 replicates) and junctions with greater than 50% significance were labelled.

Results

Morphological & Molecular identification of the bacteria

Four different species of bacteria were identified; *Lysinibacillus fusiformis* from *S. terebrans* and *Cirolana sp.*, *L. sphaericus* and *L. xylanilyticus* from *Cirolana sp* whereas *L. boronitolerans* was obtained from *D. manni* and *S. terebrans* guts. They were identified by the fact that they were aerobic, rod-shaped and gram-positive cells. *Lysinibacillus* are Gram-positive, rod-shaped, and round-spore-forming bacterial genus of the family Bacillaceae. 16S rRNA fragment analysis confirmed the morphological identification of the bacteria (Table 1.), and their gene sequence phylogenetic analysis is shown in Fig. 1.

Table 1. Identification of bacteria from woodborers' gut

Best blastn 16S rRNA Match			
Isolate	**NCBI ACCESSION**	**SPECIMEN**	**% IDENTITY**
St 1	NR 041276.1	*Lysinibacillus boronitolerans*	98
St 2	NR 042072.1	*Lysinibacillus fusiformis*	98
St 3	NR 042072.1	*Lysinibacillus fusiformis*	98
Csp 1	NR 042072.1	*Lysinibacillus fusiformis*	98
Csp 2	KF 208475.1	*Lysinibacillus xylanilyticus*	99
Csp 3	JX 144693.1	*Lysinibacillus sphaericus*	99
Dm 1	NR 041276.1	*Lysinibacillus boronitolerans*	98

Best blastn matches of the 16S region to database sequences are shown; bacteria isolated from St, *Sphaeroma terebrans*, Csp, *Cirolana sp.*, and Dm, *Dicyathifer manii*.

Morphological & Molecular identification of the fungi

Aspergillus niger strains and *Botryotinia fuckeliana* were isolated from *S. terebrans* gut while *A. costaricaensis* and *A. fumigatus* were isolated from *Cirolana sp. A. niger, Neosartorya fischeri, A. fumigatus* and *Penicillium sp.* were isolated from *D. manni*. The rDNA internal transcribed spacer (ITS) region analysis confirmed the morphological identification of the fungi (Table2.). Their subsequent phylogenetic analysis is shown in Fig. 2.

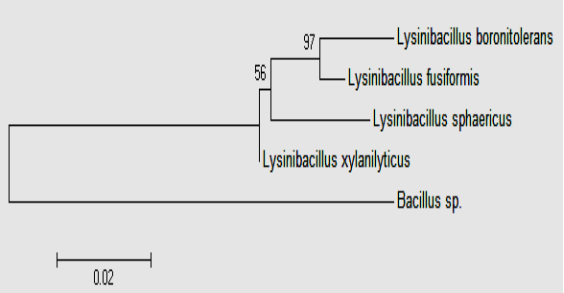

Fig. 1. Neighbour-Joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of the bacterial isolates and some other related taxa. Bootstrap values (>50 %) based on 1000 replications are shown at branch nodes. *Bacillus sp.* (Accession No. AJ000648.1) was used as an out-group. Bar, 0.02 substitutions per nucleotide position.

Table 2. Identification of fungi from woodborers' gut

Best blastn ITS Match				
Isolate	**Taxon**	**NCBI ACCESSION**	**SPECIMEN**	**% IDENTITY**
St 1	*Aspergillus sp.*	NT 166520.1	*Aspergillus niger*	96
St 2	*Aspergillus sp.*	NT 166520.1	*Aspergillus niger*	99
St 3	*Aspergillus sp.*	NT 166520.1	*Aspergillus niger*	95
St 4	*Botryotinia sp.*	NW 001814456.1	*Botryotinia fuckeliana*	100
St 5	*Aspergillus sp.*	NT 166520.1	*Aspergillus niger*	95
Csp 1	*Aspergillus sp.*	NR103604.1	*Aspergillus costaricaensis*	99
Csp 2	*Aspergillus sp.*	NC 007197.1	*Aspergillus fumigatus*	99
Dm 1	*Aspergillus sp.*	NT 166520.1	*Aspergillus niger*	98
Dm 2	*Aspergillus sp.*	NT 166520.1	*Aspergillus niger*	98
Dm 3	*Aspergillus sp.*	NW 001509767.1	*Neosartorya fischeri*	99
Dm 4	*Aspergillus sp.*	NT 166520.1	*Aspergillus niger*	98
Dm 5	*Aspergillus sp.*	NC 007197.1	*Aspergillus fumigatus*	99
Dm 6	*Penicillium sp*	nd	nd	nd

Best blastn matches of the internal transcribed spacer (ITS) region to database sequences are shown; fungi isolated from St, *Sphaeroma terebrans*, Csp, *Cirolana sp*, and Dm, *Dicyathifer manni*; nd, not determined due to consistent failure to obtain good sequences despite repeated attempts with various reaction conditions.

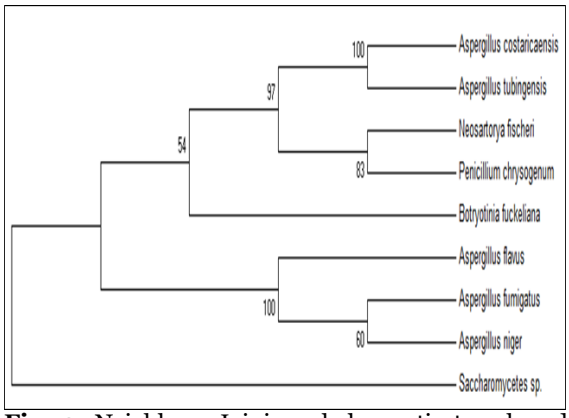

Fig. 2. Neighbour-Joining phylogenetic tree based on ITS gene sequences showing the positions of the fungal isolates and some other related taxa. Bootstrap values (>50 %) based on 1000 replications are shown at branch nodes. *Saccharomycetes sp*. (Accession No. JQ993372.1) was used as an out-group.

Discussion

Bacteria isolated from the digestive tracts of the woodborers belong to the genus *Lysinibacillus*. Four strains, namely *L. boronitolerans* (from *D. manni* and *S. terebrans*), *L. fusiformis* (from *S. terebrans* and *Cirolana sp.*), *L. sphaericus* and *L. xylanilyticus* (both from *Cirolana sp.*) had similarity to known 16S rRNA sequences of 98–99% (Table 1.).

It is possible that bacteria establish a mutual symbiosis within the digestive tract of the woodborers. Bacteria inhabit the soil or wood and develop considerably when there are easily degradable organic nutrients. The bacterial community inside the digestive tract of woodborers may pertain to at least four physiological groups: plant growth promoters, free-living nitrogen fixers, biocides and phosphate solubilizers. Lysinibacillus is commonly found in soil (Ahmed *et al.*, 2007) and has been isolated from plant tissues (Melnick *et al.*, 2011), from fermented plant seed products (Parkouda *et al.*, 2010, Nam *et al.*, 2012), from insect gut (Maji *et al.*, 2012), and even from puffer fish liver specimens (Wang *et al.*, 2010). *L. xylanilyticus* is associated with forest humus (Lee *at al.*, 2010). The diversity of bacterial communities within the digestive tracts of woodborers may depend on host plant, climate, soil type and organic matter. Therefore, existence of bacterial groupings symbiotically associated with woodborers gut is proposed.

Fungi isolated from the digestive tracts of the woodborers were different strains of Ascomycetes. *A. niger* was isolated from the digestive tracts of *S. terebrans* and *D.manni*. In addition, *N. fischeri*, *Penicillium sp*. and *A. fumigatus* were isolated from *D. manni* digestive tract whereas *A. costaricaensis* and *A. fumigatus* were isolated from *Cirolanna sp*. digestive tract. They had similarity to known ITS sequences of 95–100% (Table 2.).

The fungi in the woodborers gut may have originated from the wood they ingest or the soil in which the host plants grow. Many *Penicillia* are soil fungi, and grow in a variety of organic substances, particularly dead plant materials (Hamlyn *et al.*, 1987). Lee *et al.* (2011) has reported wood-inhabiting *Penicillium* strains. Lindblad, 2000; Gilbert *et al.*, 2002; Gilbert & Sousa, 2002 and Ferrer & Gilbert, 2003 have reported a large number of fungi that inhabit wood where they play the role of wood decay. El Shanshoury *et al.* (1994) reported a number of cellulolytic microorganisms associated with wood boring marine isopods; amongst these were *A. niger* and *A. candidus*. Ectomycorrhizal fungi improve host performance by enhancing nutrient and water uptake from the soil and protect host roots from pathogens and toxic compounds (Smith and Read, 1997).

Not many species of symbiotic microorganisms were found in the gut of woodborers in this study. The mechanism of wood digestion in marine bivalves differs from that found in terrestrial wood consumers. Terrestrial organisms that consume wood as food contain within their digestive tracts communities of symbiotic microorganisms that are thought to aid in the digestion and metabolism of wood (Haigler and Weimer, 1991). Wood-boring bivalves appear to lack such highly developed

microbial communities within their guts (Liu and Walden, 1970). In the case of teredinids, the ability to feed on wood is thought to depend on intracellular bacterial endosymbionts contained within specialized cells (bacteriocytes) of their gills. These bacterial endosymbionts are thought to produce cellulolytic enzymes that aid the host in digestion of wood (Distel, 2003). The bacteria are also known to fix nitrogen (Lechene *et al*., 2007; Waterbury *et al*., 1983) that may supplement the host's nitrogen deficient diet. These intracellular bacteria constitute a consortium of closely related species (Distel *et al*., 2002; Luyten *et al*., 2006), only one (*Teredinibacter turnerae*) has been grown in pure culture. This species was however not isolated in our study. Members of Xylophagainae have also been shown to harbor bacterial endosymbionts within their gills (Distel and Roberts, 1997) although none has yet been cultivated (Distel *et al*., 2011).

Cragg *et al.* (1999) reported wood degrading tunnelling bacteria, and soft-rotting ascomycete and deuteromycete fungi in the wood ingested by boring crustaceans. The gut contents of *Limnoria* (a wood-boring isopod) consisted of wood particles and associated microorganisms (bacteria and fungi) that were associated with tunnelled wood. However, the authors did not specify whether these microorganisms were wood-degraders.

The growth of bacteria and fungi inside the digestive tracts of woodborers is of industrial importance in biodegradation of lignocellulose. Investigation of the functional characteristics and role in the host organism is required to confirm the symbiotic status of the microorganisms associated with the woodborers digestive tract.

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