RESEARCH ARTICLE

A tale of two chitons: is habitat specialisation linked to distinct associated bacterial communities?

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Abstract

Although most chitons (*Mollusca: Polyplacophora*) are shallow-water molluscs, diverse species also occur in deep-sea habitats. We investigated the feeding strategies of two species, *Leptochiton boucheti* and *Nierstraszella lineata*, recovered on sunken wood sampled in the western Pacific, close to the Vanuatu Islands. The two species display distinctly different associations with bacterial partners. *Leptochiton boucheti* harbours *Mollicutes* in regions of its gut epithelium and has no abundant bacterium associated with its gill. *Nierstraszella lineata* displays no dense gut-associated bacteria, but harbours bacterial filaments attached to its gill epithelium, related to the *Deltaproteobacteria* symbionts found in gills of the wood-eating limpet *Pectinodonta* sp. Stable carbon and nitrogen isotope signatures and an absence of cellulolytic activity give evidence against a direct wood-feeding diet; both species are secondary consumers within the wood food web. We suggest that the distinct associations with bacterial partners are linked to niche specialisations of the two species. *Nierstraszella lineata* is in a taxonomic family restricted to sunken wood and is possibly adapted to more anoxic conditions thanks to its gill-associated bacteria. *Leptochiton boucheti* is phylogenetically more proximate to an ancestral form not specialised on wood and may itself be more of a generalist; this observation is congruent with its association with *Mollicutes*, a bacterial clade comprising gut-associated bacteria occurring in several metazoan phyla.

Introduction

Despite its terrestrial origin, huge amounts of wood end up on the seafloor after unknown periods of drifting and water-logging. These 'wood falls' are home to opportunistic as well as endemic faunas that have been documented since the 19th century (Agassiz, 1888; Murray, 1895; Wolff, 1979). Recently, the study of sunken-wood ecology has enjoyed a renewed interest because organic falls were shown to shelter close relatives of the large, well-studied, chemosymbiotic metazoans occurring at hydrothermal vents and cold seeps. Among this shared fauna are siboglinid annelids and mussels in *Bathymodiolinae* (Dando *et al.*, 1992; Deming *et al.*, 1997; Distel *et al.*, 2000; Gros & Gaill, 2007; Duperron *et al.*, 2008). Indeed, the process of wood degradation produces reduced compounds, including hydrogen sulphide, which attract organisms associated with sulphur- or methane-oxidising symbionts (Leschine, 1995; Laurent *et al.*, 2009; Treude *et al.*, 2009; Gaudron *et al.*, 2010). Yet, sunken wood remains an underexplored habitat in the deep sea, and their significance in the marine carbon cycle has not been evaluated.

Although wood degradation is often mediated by prokaryotes or fungi in the deep sea (Kohlmeyer, 1968; Holt & Jones, 1983; Blanchette, 2000; Jurgens *et al.*, 2003), dense metazoan communities populate many wood samples, and at least some clearly benefit from the wood not only as a substrate, but also as a food source (Wolff, 1979). Although evidence of direct wood digestion by metazoan enzymes is still lacking in the deep sea, recent work has emphasised the importance of metazoan-associated bacterial communities in some species endemic to

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wood habitats. Among wood-eating deep-sea metazoans, pholadid bivalves of the subfamily *Xylophagainae* are the best documented example (Turner, 1973, 1977, 2002). They are associated with cellulolytic, nitrogen-fixing *Gammaproteobacteria*, localised in their gills, which help to digest the wood and sustain host nitrogen metabolism, although details of the interactions remain obscure (Distel *et al.*, 2002; Lechene *et al.*, 2007). Besides their specific ability to degrade wood, these bivalves can quickly colonise isolated wood substrates, even very far from continents, this last point being suggestive of highly efficient dispersal strategies (Turner, 1973; Gaudron *et al.*, 2010). More recently, a potential wood-feeding lifestyle was suggested for limnorid isopods (Cragg *et al.*, 1999) and for the decapod *Munidopsis andamanica*, possibly with the help of fungi occurring in its gut (Hoyoux *et al.*, 2009). Among molluscs, the patellogastropod *Pectinodonta* sp. may digest wood using prokaryotic symbionts (Zbinden *et al.*, 2010). The *Pectinodonta* digestive gland examined by Zbinden *et al.* (2010) contained at least three bacterial 16S rRNA phylotypes representing strains of *Bacteroidetes*, *Alpha-* and *Gammaproteobacteria*, and it displayed a high cellulolytic activity, and carbon stable isotope signatures of the gland were very close to signatures measured in the wood substrate. A second bacterial community in that gastropod consisting of *Delta-* and *Epsilonproteobacteria* occurred outside the gill epithelium, possibly linked with sulphur metabolism. The gastropods *M. andamanica* and *Pectinodonta* sp. were once (mis)classified as predator and grazer, respectively, which demonstrates that attributing a feeding strategy to a wood-associated animal based only on sister groups and from other habitats can be misleading. Investigating feeding strategies in a wide range of wood-associated animals is thus essential if we are to understand sunken-wood trophic ecology and its connections with the rest of the deep sea.

Deep-sea polyplacophorans represent an interesting target group. Chitons in the sub-order *Lepidopleurida* are recurrent on sunken wood and represent a significant fraction of the metazoan communities (Sirenko, 2004; Sigwart & Sirenko, 2012). In a recent exploration of the waters around Vanuatu islands, within the framework of the SANTO 2006 program, several species were collected from wood samples. Two of the most abundant species in those samples were *Leptochiton boucheti* and *Nierstraszella lineata*. Both species are in a large clade of predominantly wood-dwelling South Pacific deep-sea chitons, which apparently is derived from a nonwood dwelling ancestor (Sigwart *et al.*, 2011). The polyphyletic *Leptochiton* in particular has affinities to many species not inhabiting sunken wood. So although *L. boucheti* and its closest known relatives all inhabit wood substrates, its morphology is allied to many species that do not. *Nierstraszella* is the single genus in the taxonomic family *Nierstraszellidae*, which is thought to be restricted to sunken wood (Sirenko, 1992; Sigwart, 2009; Sigwart & Sirenko, 2012); this suggests that *N. lineata* in particular could be a wood-eating organism.

In the present study, we address the feeding strategies in two polyplacophoran species occurring on sunken wood, that is, *L. boucheti* and *N. lineata*, and the possible involvement of bacterial communities. For this, we analyse morphological characters such as radula, gut and gills using light and electron microscopy (EM) to visualise details of the structures involved. Stable isotope analyses and cellulose enzyme assays are used to test whether wood could be a significant carbon source to the chitons. Because bacterial communities are often involved in nutrition or adaptation to high sulphide habitats (Dubilier *et al.*, 2008), the bacterial diversity associated with the gut and gills of both species is investigated using comparative 16S ribosomal RNA gene sequence analysis and fluorescence *in situ* hybridisation (FISH). Altogether, this study provides the first detailed investigation of feeding strategies in sunken-wood *Polyplacophora* and further documents the complex trophic ecology of these unusual habitats.

Materials and methods

Sampling and conditioning

Chiton specimens were collected in 2006 during the SantoBOA cruise around the Vanuatu Islands (Chief scientist: B. Richer de Forges) aboard the RV *Alis*, in the framework of the Santo 2006 scientific expedition (IRD, MNHN, Pro-Natura International) dedicated to a survey of total biodiversity, from sea bottom to ridge crests, of the Santo Island, Vanuatu. Specimens identified as *L. boucheti* and *N. lineata* used in this study were recovered from wood fragments trawled around Vanuatu Islands on two locations: Big Bay, north of Santo Island (15.00°S; 166.87°E, depth 722–780 m, *L. boucheti* and *N. lineata* specimens) and south east of Malekula Island (*N. lineata* specimens). Specimens were fixed in lots (see Supporting Information, Table S1, first column), according to the different experiments planned, that is, frozen in liquid nitrogen (enzyme assays and isotopes), in ethanol (DNA and some stable isotope analyses) and in 2.5% glutaraldehyde (EM). Specimens for FISH were treated with 2–4% formaldehyde in filtered seawater, rinsed and dehydrated up to 70% ethanol.

Molecular characterisation of chitons and associated bacteria

As the sunken-wood fauna remains largely unknown and taxonomic identification can be contentious, a barcoding

approach was used to confirm the species affinities of the collected chitons. Several specimens in each lot were subsequently sequenced (Table S1), for a total of 43 specimens. In most cases, a piece of tissue was clipped from the girdle and used for DNA extraction. DNA was extracted using the AB PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems) or the QIAmp DNA Microkit (QUIAGEN) for smaller specimens. Two gene fragments were analysed: (1) a fragment of 658 bp of cytochrome oxidase I (COI) mitochondrial gene using universal primers LCO1490 and HCO2198 (Folmer *et al.*, 1994) and (2) a fragment of 700 bp of the 28S gene, using the primers C1′ (5′-ACCCGCTGAATTTAAGCAT-3′) and D2 (3′-TCCGTGTTTCAAGACGGG-5′) (Dayrat *et al.*, 2001; Jovelin & Justine, 2001). All PCRs were performed in 25 μL, containing 3 ng of DNA, 1× reaction buffer, 2.5 mM MgCl2, 0.26 mM dNTP, 0.3 μM of each primer, 5% DMSO and 1.5 units of Q-Bio Taq, QBiogene for both genes. Thermocycles for COI gene consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 40 s and extension at 72 °C for 1 min. The final extension was at 72 °C for 10 min. Thermocycles for 28S gene were identical, except for the annealing temperature (56 °C) and the number of cycles (32). PCR products were purified and sequenced by Genoscope. In all cases, both directions were sequenced and assembled to contigs to confirm the accuracy of each sequence. All specimens and sequences are registered in BOLD (www.boldsystems.org) and GenBank (Table S1, accession numbers JQ950235–JQ950320).

Gills (G) and digestive tract (DT) were dissected from three specimens of *L. boucheti* and two specimens of *N. lineata*. DNA was extracted from each tissue sample and from a fragment of the wood on which *L. boucheti* specimens were recovered, using a standard phenol–chloroform–isoamylacohol protocol (Duperron *et al.*, 2005). A fragment of the gene-encoding bacterial 16S rRNA was PCR-amplified using primers 27F and 1492R following the protocol described in Duperron *et al.* (2005), with 32–35 cycles. Three PCRs were pooled together prior to purification using a QIAquick Kit (Qiagen, CA). PCR products were cloned using a TOPO TA Kit (Invitrogen, CA), and positive inserts were sequenced one-way using the M13R primer. Abundant sequences, potentially representing significant bacterial partners, were sequenced both directions to obtain full-length sequences. Sequences were deposited in GenBank (accession numbers HE663241–HE663393).

Sequence analysis

COI and 28S sequences obtained were combined with all the sequences already available in GenBank for the genera *Leptochiton* and *Nierstraszella* in four different data sets (Sigwart *et al.*, 2011), one for each combination of gene and species. In total, the *Leptochiton* COI, *Leptochiton* 28S, *Nierstraszella* 28S and *Nierstraszella* COI data sets included 66, 71, 5 and 6 sequences respectively (only three COI representative of the diversity among the 47 available from the same publication in GenBank were retained). A sequence of *Leptochiton* was used as outgroup to artificially root the *Nierstraszella* data sets, and vice-versa. DNA sequences were manually (for the COI gene) or automatically (for the 28S gene) aligned using Clustal W implemented in BIOEDIT version 7.0.5.3 (www.mbio.ncsu.edu/BioEdit). Bayesian analyses were performed running two parallel analyses in MRBAYES (Huelsenbeck & Ronquist, 2001), consisting each of four Markov chains of 10 000 000 generations each with a sampling frequency of one tree per thousand generations. A nucleotide substitution model with six substitution categories, a gamma-distributed rate variation across sites approximated in four discrete categories and a proportion of invariable sites were applied for each data set. Convergence of each analysis was evaluated using TRACER 1.4.1 (http://beast.bio.ed.ac.uk/Tracer), and analyses were terminated when effective sample size (ESS) values were all superior to 200. A consensus tree was then calculated after *a priori* omitting the first 25% trees as burn-in.

Bacterial sequences were compared to the GenBank database content using the SeqMatch function of Ribosomal Database Project II and BLAST (Altschul *et al.*, 1990; Cole *et al.*, 2009). The most abundant sequences in clone libraries from both chiton species were included into a data set along with best BLAST hits and relevant sequences. Sequences were aligned using SINA Web Aligner (Pruesse *et al.*, 2007), alignments were manually checked and truncated, and a 1275-bp alignment was used for phylogenetic reconstruction using the same approach described above for COI and 28S genes. Additional bootstrap support values were obtained using PHYLIP package (Felsenstein, 2002) running a maximum likelihood (ML) method on 1000 ML bootstrap replicates.

In situ hybridisation

After removing the dorsal plates, two whole specimens of *L. boucheti* and two of *N. lineata* were embedded in polyethylene glycol (PEG) distearate: 1-hexadecanol (9 : 1) after dehydration in increasing ethanol series. Semi-thin (10 μm) sections were cut using a Jung microtome and deposited on Superfrost Plus slides. Wax was removed and tissue rehydrated in decreasing ethanol series. Tissue sections were hybridised as described in Zbinden *et al.* (2010) using probes Eub338 (5′-GCTGCCTCCCGTAGG AGT-3′, specific for *Eubacteria*), Gam42 (5′-GCCTTCCCA

CATCGTTT-3′, specific for Gammaproteobacteria), Del495a (5′-AGTTAGCCGGTGCTTSTT-3′, specific for *Deltaproteobacteria*), Alf968 and Alf166 (5′-GGTAAGGTT CTKCGCGTT-3′ and 5′-ATCTTTCCCCAAAAGGGC-3′, specific for *Alphaproteobacteria*) in a 30% formamide buffer (Amann *et al.*, 1990; Manz *et al.*, 1992; Neef, 1997; Loy *et al.*, 2002). Probes were labelled with either Cy-3 or Cy-5 depending on the experiments. Two new probes were designed to target *Mollicutes* sequences in the gut of *L. boucheti*, modified from probe PsSym352 (Wang *et al.*, 2004, 2007). Probe MolliA-350 (5′-AAAATTCCT**T**TACT-GCTG-3′) targets phylotype LBDT0517-A and displays a 1-base mismatch (in bold) with phylotype LBDT3651-B. The second probe, MolliB-350 (5′-AAAATTCCCTA CTGCTG-3′), is modified from MolliA-350 so as to display no mismatch with phylotype LBDT3651-B and thus a 1-base mismatch with LBDT0517-A. Both probes were hybridised for 3 h at 46 °C with 30–40% formamide and rinsed for 15 min at 48 °C.

Sections were mounted using SlowFade medium with or without DAPI (Invitrogen) and a coverslip, and observed under an Olympus BX6A fluorescence microscope.

Enzyme assay

Cellulase activity was tested on three whole specimens of *L. boucheti* and two for which foot and visceral mass were separated. Two whole specimens of *N. lineata* and one dissected specimen (foot and visceral mass) were tested. Analyses were performed using the protocol described previously (Zbinden *et al.*, 2010).

Stable isotope analysis

Two wood fragments from Big Bay, Vanuatu (in triplicates), 18 specimens of *L. boucheti* and seven specimens of *N. lineata* without their dorsal plates were rinsed in distilled water prior to stable isotope analyses. Half of the individuals' tissues were dissected separating the visceral mass from the foot. The two half specimens (one each of the dissected and whole individuals) from the two species were dried (4 days, 60 °C) and ground to powder using a mortar and pestle. Due to the small volume of material after drying, dissected tissues were pooled from several specimens of the same species. To avoid significant changes in $\delta^{15}N$ isotopic composition, no HCl was used to remove carbonates (Kaehler & Pakhomov, 2001). Preservation in ethanol can lead to bias in the stable isotopic analyses, as ethanol may increase $\delta^{13}C$ (Kaehler & Pakhomov, 2001). Therefore, we used a *t*-test to compare mean $\delta^{13}C$ in samples of *L. boucheti* tissue stored in ethanol and frozen (MINITAB version 15). We did not perform any lipid treatment because the C : N ratios of all chitons studied were around 3 and 4 (Post *et al.*, 2007).

Analyses were performed at Iso-Analytical (Crewe, UK) for determination of $\delta^{13}C$ and $\delta^{15}N$. Stable isotope data are expressed as the relative per mil (‰) differences between the samples and the conventional standard Pee Dee Belemnite for carbon and air N_2 for nitrogen, according to the following equation:

$$\delta(X) = \left[\left(\frac{R_{\text{sample}}}{R_{\text{standard}}}\right) - 1\right] \times 1000$$

where X (‰) is ^{13}C and ^{15}N abundance and R is the $^{13}C : {}^{12}C$ and $^{15}N : {}^{14}N$ ratio. Standard deviations were ±0.11‰ and ±0.12‰ for carbon and nitrogen, respectively.

EM

Gut and gill tissues of three specimens of *L. boucheti* and two of *N. lineata* were dissected, postfixed in osmium, dehydrated in increasing ethanol series (50, 70, 95 and 100 °C) and embedded in Epon resin (48 h, 60 °C). Sections were cut using a Reichert–Jung ultramicrotome. Semi-thin (600 nm) sections were stained with toluidine blue, and thin (60 nm) sections were mounted on copper grids, contrasted using uranyl acetate and observed using a HITACHI H-7100 transmission electron microscope, operated at 80 kV. Gut, gill and radula, from the same specimens (*L. boucheti*, $n = 3$; *N. lineata*, $n = 2$), were postfixed in osmium for scanning electron microscopy. Fixed samples were dehydrated through an ethanol series and dried in a critical-point dryer (CPD7501), gold-coated (scancoat six sputter coater) and observed in a scanning electron microscope (SEM, Cambridge S260 at 10 kV).

Results

Specimen identification

Representatives of both species measured between 0.8 and 1 cm length and displayed typical characteristics of *Polyplacophora*, including the eight dorsal plates surrounded by a thick girdle (Fig. 1a and b). Polyplacophoran specimens in general, and particularly members of the order *Lepidopleurida* (including our study species), are differentiated by very detailed morphoanatomical features, and the species look superficially very similar to nonspecialists. The present two species are easily differentiated from each other in that *N. lineata* has a protenacious covering of the dorsal valves that is brown in colour and

forms raised pustules in zig-zag patterns; *L. boucheti* has pale valves that are usually visible unless coated in dark mineral deposits. *Nierstraszella lineata* has 12–18 gills per side; *L. boucheti* has four per side in the posterior region of the pallial cavity. Radulae of both species (as most chitons) are dominated by the large, mineralised second lateral cuspids, but differ in the construction of the central and marginal teeth (Fig. 1c and d). Dissection and transverse sections displayed the typical digestive structures found in chitons, including a nonreduced gut (Fig. 1e and f).

Phylogenetic analyses performed on the COI and 28S genes, including many specimens from each of the two collected species as well as several congeneric sequences, confirmed that all our specimens identified as *L. boucheti* were conspecific, as they shared almost identical COI and 28S, all clustered in a highly supported clade (Fig. S1A and B) different from the *Leptochiton* sequences. Similar results were found for the specimens of *Nierstraszella* (Fig. S1C and D). For both *N. lineata* and *L. boucheti*, specimens from GenBank identified with the same names do not cluster with the specimens in the present analysis. This would suggest that several cryptic species may be hidden behind the names *N. lineata* and *L. boucheti* and require further study. Consequently, we consider that the two sets of specimens analysed here, including the specimens also analysed for bacterial diversity, certainly correspond to two, and only two, species.

Distinct bacterial communities are associated with DT and gill of *L. boucheti* and *N. lineata* and with wood

Libraries from *L. boucheti* were dominated by *Mollicutes* which represented 49.6% and 70.1% of 135 and 117 clones in the gut and gill, respectively (Fig. 2). Two sequences were particularly abundant, namely LBDT0517-A, which represented 39.3% and 33.3% of all clones, and LBDT3651-B, which represented 3.7% and 36.8%, respectively. LBDT0517-A and LBDT3651-B displayed 77% identical positions. Best BLAST hits of both sequences included various *Mollicutes* associated with abalone, sea anemones, ascidians and *Spiroplasma* spp. from guts of various insects, but with only 80% and 84% sequence identity. LBDT0517-A occurred in both gill and gut samples of all three *L. boucheti* specimens, meanwhile LBDT3651-B occurred in the gut of a single specimen and in the gill of two. In the phylogenetic tree (Fig. 3),

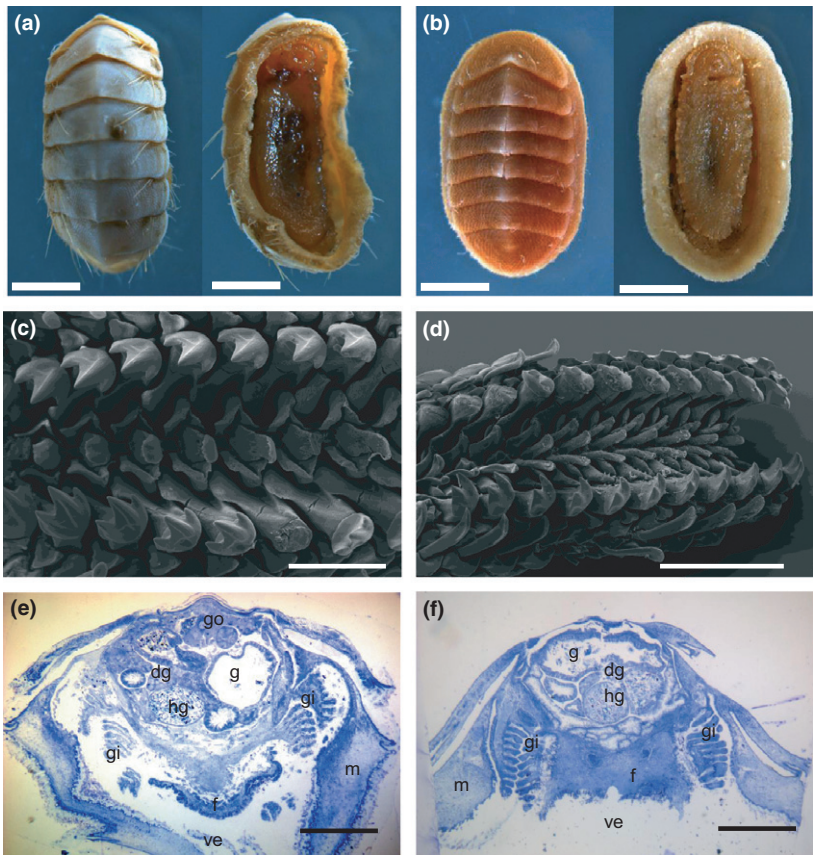

Fig. 1. Dorsal and ventral view of *Leptochiton boucheti* (a) and *Nierstraszella lineata* (b), scale bar (SB) = 2 mm. Radulae of *L. boucheti* (c, SB = 100 μm) and *N. lineata* (d, SB = 200 μm). Transverse section through the posterior body region of *L. boucheti* (c) and *N. lineata* (d), SB = 500 μm; Labels: gut (g), hindgut (hg), digestive gland (dg), gonad (go), gill (gi), girdle (m), foot (f), ventral side (ve).

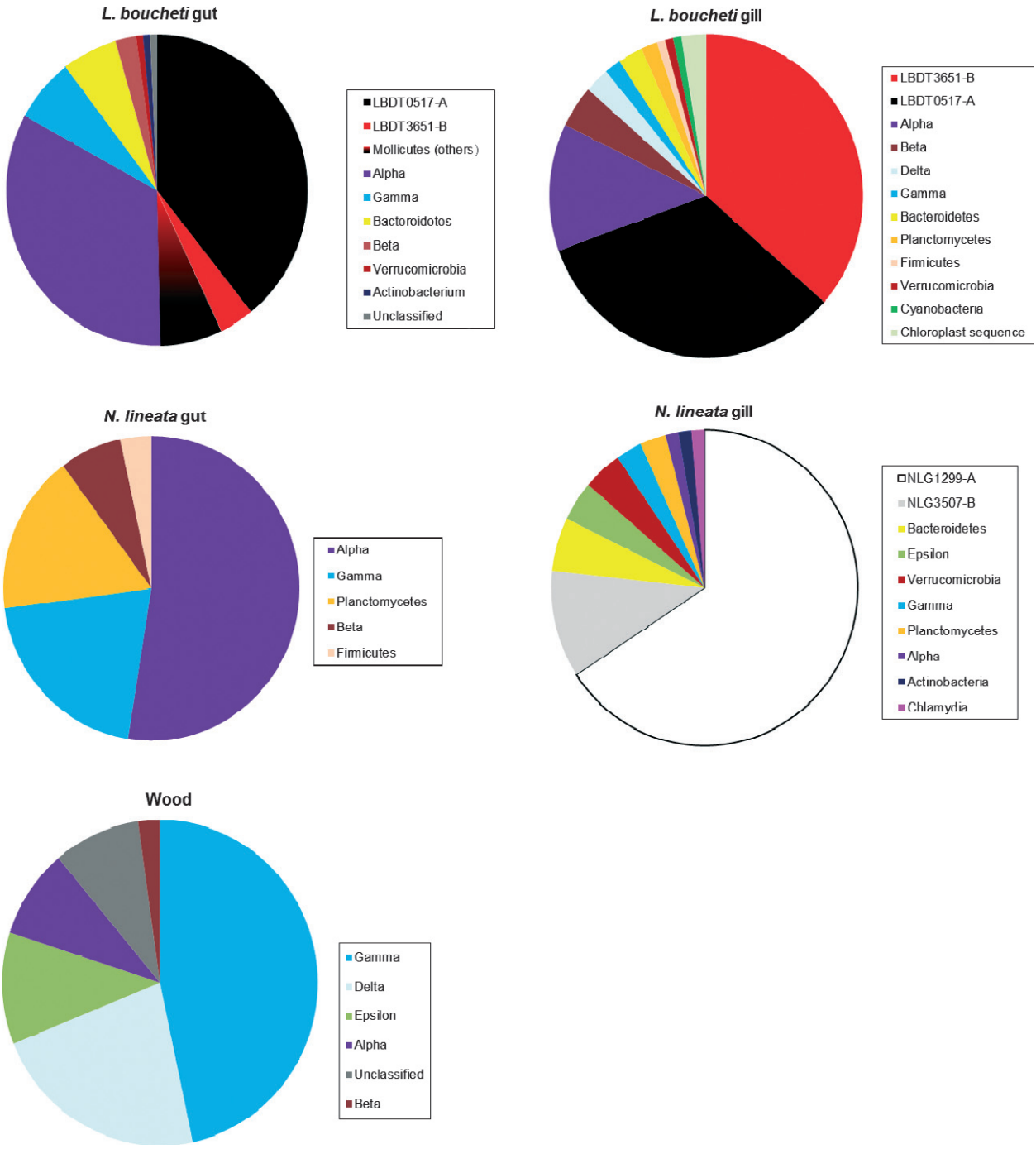

Fig. 2. Proportions of the different bacterial groups identified in the 16S rRNA gene clone libraries from *Leptochiton boucheti* gut and gill, *Nierstraszella lineata* gut and gill and wood. See Table S2 for details of sequences recovered and their closest relatives identified in GenBank.

the closest relative of LBDT0517-A was sequence Mm2 recovered from the gonad of the ascidian *Molgula manhattensis* (Tait *et al.*, 2007). LBDT3651-B clustered with a sequence from the intestinal flora of the abalone *Haliotis diversicolor* (Z. Huang, W. You, F. Guo, J. Zhao and C. Ke, unpublished data), with moderate statistical support. Other sequences included diverse *Alphaproteobacteria* sequences which represented 33.3 and 12.8% of clones in the gut and gill, respectively. Each of the other groups represented less than 7% of the clones (Fig. 2).

The gut-based library from *N. lineata* was dominated by diverse *Alphaproteobacteria*, *Gammaproteobacteria* and

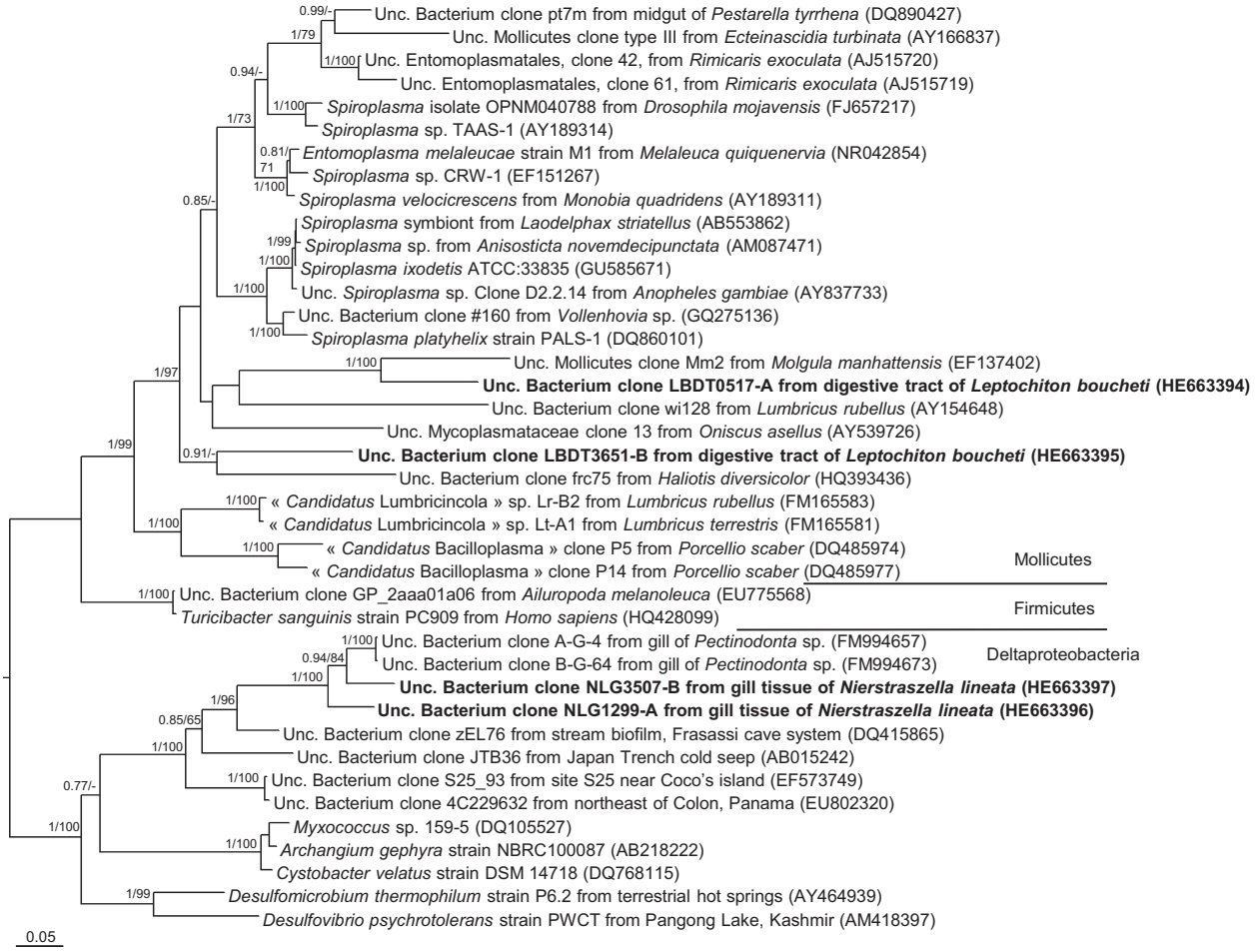

Fig. 3. Bayesian phylogenetic tree displaying the dominant 16S rRNA gene sequences identified in *Leptochiton boucheti* and *Nierstraszella lineata*. Scale bar represented estimated 5% differences, posterior probabilities (pp) > 0.5 and bootstrap values (bs) > 60, shown as 'pp/bs' on nodes. In boldface: LBDT, *L. boucheti* DT; LBG, *L. boucheti* gill; NLDT, *N. lineata* DT; NLG, *N. lineata* gill.

Planctomycetes (52.5%, 20.3% and 16.9% of 59 clones). A single alphaproteobacterial sequence accounted for 10 clones, related to a sediment bacterium from the south Atlantic Bight shelf. Interestingly, two sequences were closely related (97–98% similarity) with alphaproteobacterial sequences from the digestive gland of the wood-eating limpet *Pectinodonta* sp. (Zbinden *et al.*, 2010). In the gill, the clone library was dominated by two deltaproteobacterial sequences (Fig. 2). NLG1299-A and NLG3507-B represented 65.8% and 11.0% of 73 gill clones, respectively. They shared a 90% sequence identity and were mostly similar to *Deltaproteobacteria* recently identified as epibionts in the gills of *Pectinodonta* sp. collected from the same area (91% and 93% identity). Both sequences occurred, dominated by of NLG1299-A, in clone libraries from the two specimens investigated. In phylogenetic trees (Fig. 3), they formed a well-supported clade with *Pectinodonta* symbionts, related to sequences from sulphide-rich environments, a biofilm from a sulphidic stream in the Frasassi cave and a cold seep in the Japan Trench (Macalady *et al.*, 2006). Each other bacterial group represented < 5.5% of the sequences.

The 16S rRNA clone library built from the wood sample itself was dominated by *Gammaproteobacteria*, *Deltaproteobacteria* and *Epsilonproteobacteria*, with 46.7%, 22.2% and 11.1% of 45 sequences. Neither *Mollicute* sequences nor NLG1299-A and NLG3507-B were retrieved in the wood library. Overall, only six sequences were shared (> 99% identical positions) between clone libraries from two distinct sample types (Table S2).

Morphology and ultrastructure of gut and gill

The intestine of *L. boucheti* contained degraded wood fragments as well as debris of planktonic origin (Fig. 4a and c). Long filaments, corresponding to cilia and

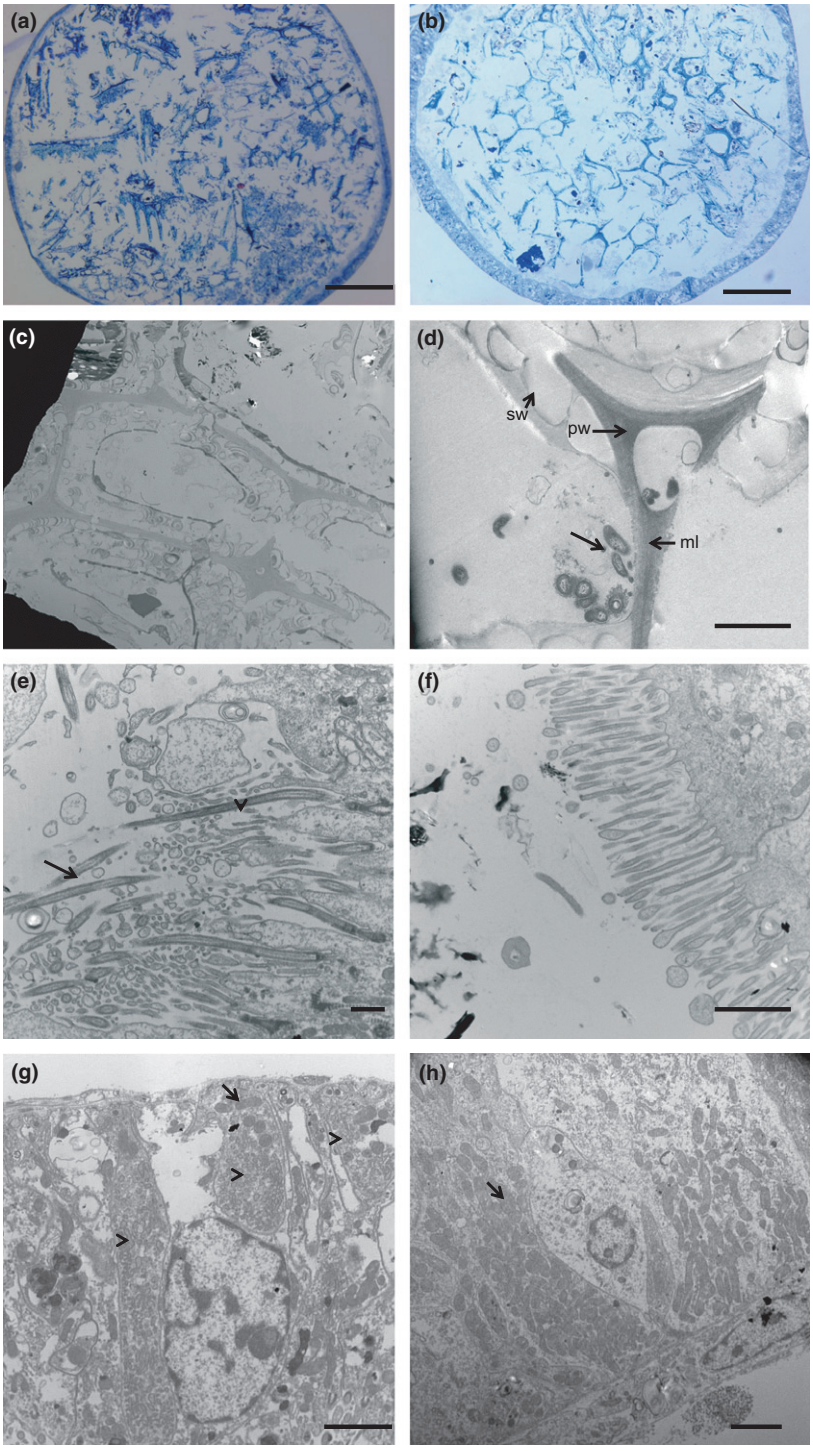

Fig. 4. DT of chitons. Overview of a section through the gut of *Leptochiton boucheti* (a) and *Nierstraszella lineata* (b), scale bars (SB) = 100 μm. TEM micrographs of the gut epithelium (c, d, f–h, SB = 2 μm, e, SB = 1 μm); (c) detail of wood cell debris in the gut of *L. boucheti* with cell wall visible. (d) Detail of wood cells (primary wall: pw, secondary wall: sw, middle lamella: ml) in the gut of *N. lineata* with bacteria associated (arrow). (e) Detail of microvilli (arrowhead) and cilia (arrow) oriented towards the lumen of the gut in *L. boucheti*. (f) Microvilli on gut eipthelial cells of *N. lineata*. (g) *L. boucheti* epithelial cells displaying mitochondria (arrow) and putative prokaryotic structures (arrowheads); (h) *N. lineata*, basal part of the gut epithelial cells devoid of prokaryotic structures but rich in mitochondria (arrow).

microvilli, were seen oriented towards the lumen of the gut epithelium (Fig. 4e). At the basis of epithelial cells, mitochondria were abundant. There were also many small structures, around 0.2 μm diameter, that could be small intracellular prokaryotic cells or vesicles (Fig. 4g).

The gut of *N. lineata* also contained degraded wood fragments (Fig. 4b and d). Microorganisms were seen sparsely within the gut content, associated with the wood debris (Fig. 4d). Epithelial cells displayed microvilli (Fig. 4f) and abundant mitochondria at their basis

(Fig. 4h), but were devoid of the small prokaryote-like structures observed in *L. boucheti*.

Polyplacophoran gills are formed as serial ctenidia, with a vertical axis to which filaments are attached (Fig. 5a and d). The gill epithelium in *L. boucheti* and *N. lineata* displayed microvilli and dense cilia (Fig. 5b and e). No bacteria were seen associated with gills and pallial cavity of *L. boucheti* using TEM (Fig. 5c). By contrast, in *N. lineata*, the gills harboured dense cilia and microvilli among which long coiled filaments resembling bacteria were seen (Fig. 5f).

Fluorescence microscopy observations

DAPI signals were observed on sections of *L. boucheti*. On gut sections, nuclei of epithelial cells were visible (Fig. 6b). A DAPI-labelled layer was also visible in the basal half of gut epithelial cells on some, but not all, sections of the gut (Fig 6c). This DAPI-labelled layer, but not the nuclei, overlaid with the FISH signal obtained using the probe Eub338, indicates the occurrence of a dense layer of bacteria in these sections of the gut epithelium (Fig. 6a and f). For example, on a section where the gut passed twice, only one section displayed the DAPI layer and the FISH signal. The FISH signal was very strong in the gut epithelium and seemed to be localised at the basis of the epithelial cells rather than close to the lumen (Fig. 6b and f). Positive signals were also obtained using probes MolliA-350 (Fig. 6b and d) and Alf968 (Fig. 6b and e), both signals fully overlaid with the Eub338 signal (Fig. 6f). Probe MolliA-350 was designed to hybridise phylotype LBDT0517-A which dominated the gut clone library (Fig. 2) and had no extra hits among *Alphaproteobacteria*. Probe Alf968 was initially designed to target *Alphaproteobacteria*, but a search using Probe Match (Ribosomal Database Project II) indicated that it also targeted many Mollicutes sequences and displayed no mismatch with both LBDT0517-A and LBDT3651-B. The Probes Alf166, targeting a fraction of *Alphaproteobacteria*, and MolliB-350, targeting phylotype LBDT3651-B, did not yield any signal. Altogether, this suggests that the signal observed

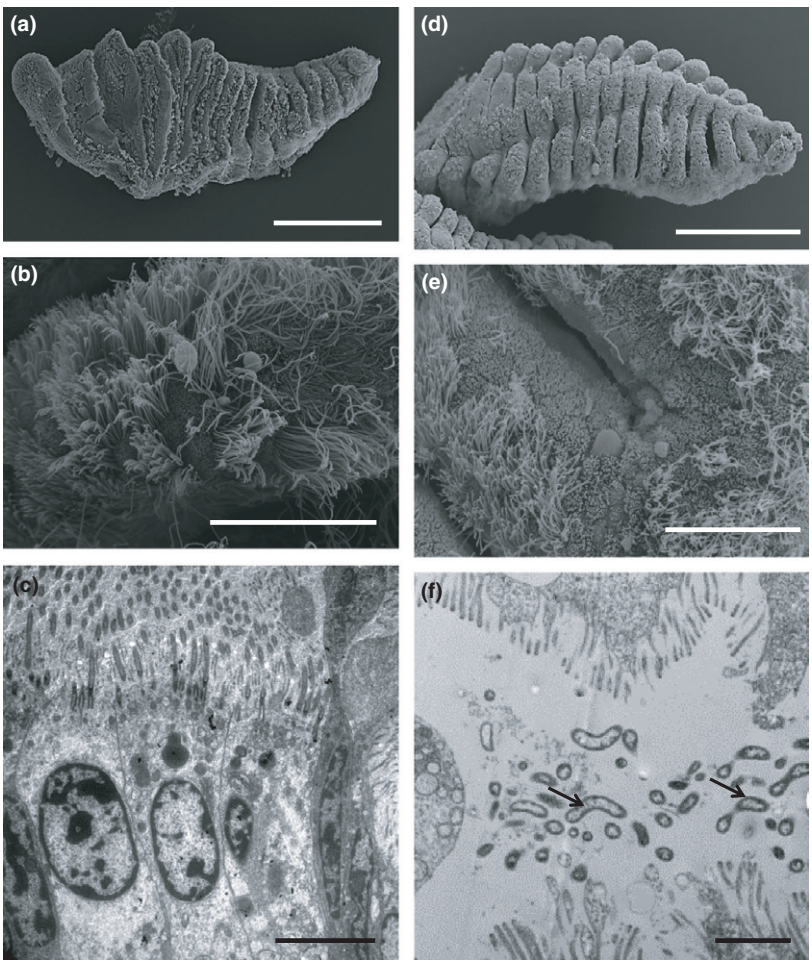

Fig. 5. Electron micrographs of gills. (a–c) *Leptochiton boucheti*; overview of a ctenidium (a, SB = 200 μm), detail of the gill surface displaying densely ciliated areas (b, SB = 20 μm) and TEM micrograph illustrating the gill epithelium with rectangular-shaped cells including a large nucleus and cilia in the upper part of the image (c, SB = 5 μm). (d–f) *Nierstraszella lineata*; overview of a ctenidium (d, SB = 200 μm); detail of the gill surface displaying densely ciliated areas (e, SB = 20 μm) and TEM micrograph illustrating filamentous coiled bacteria (arrows) located between cell microvilli in the gill epithelium (f, SB = 2 μm).

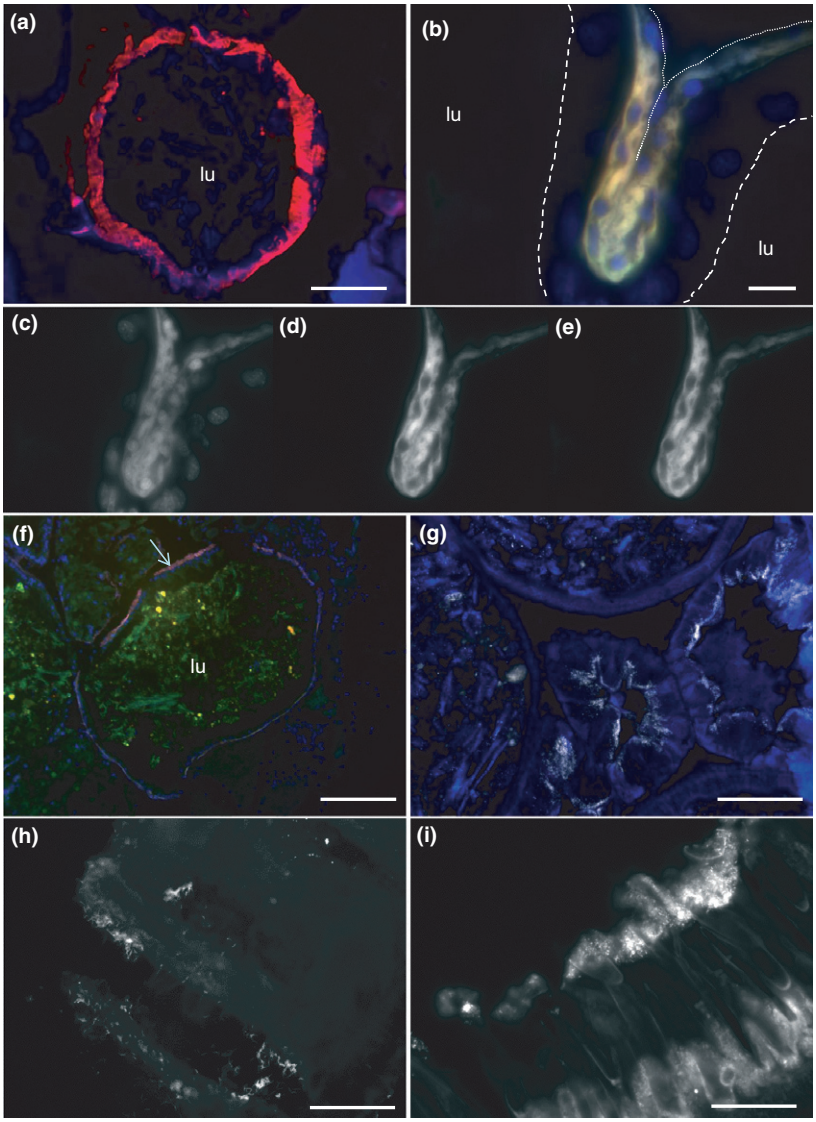

Fig. 6. FISH. (a) Overview of a gut section in *Leptochiton boucheti*, with blue autofluorescence and probe Eub338 in red (Cy-5 labelled). SB = 100 μm. (b) Detail of the gut epithelium, with apical limit of the cells (dashed line), basal lamella (dotted line) and lumen (lu). DAPI staining in blue, signal from probes Alf928 in green (Cy-3) and MolliA-350 in red (Cy-5). Overlying signals result in yellow. SB = 10 μm. (c–e) Individual channels for DAPI (c), probe MolliA-350 (d) and probe Alf968 (e). (f) Overview of a gut section stained with DAPI and hybridised with probes MolliA-350 (Cy-5, in red) and Alf968 (Cy-3, in green). Bacterial signal appears in pink, localised in the basal part of the gut epithelium (arrow). Note the high autofluorescence observed in the gut content. SB = 200 μm. (g) Hybridisation using probe GAM42 (Cy-5, in white) against background fluorescence of the host tissue of *Nierstraszella lineata* (blue). The gut epithelium (upper and left part of the picture) is devoid of any signal, and the digestive gland (middle and right) is lined with small bacteria. SB = 100 μm. (h) Hybridisation using probe Eub338 (white, Cy-5) on a section of the gill of *N. lineata*. Bacterial signals display a typical filament shape. SB = 50 μm. (i) Spicules located on the dorso-lateral region of *N. lineata* and Eub338 signal on top of the spicules (bright white). SB = 50 μm.

with DAPI and FISH actually corresponded to the *Mollicutes* phylotype LBDT0517-A. Some Eub338 and Gam42 signals were also seen sparsely in the *L. boucheti* digestive gland tissue.

Few bacterial FISH signals were seen in the gut lumen of *N. lineata*, associated with wood debris. No FISH signal was detected in the gut epithelium, but strong, unambiguous and abundant signal was seen using probes Eub338 and Gam42 in the digestive gland, with both signals fully overlying (Fig. 6g). Gill tissue of *N. lineata* displayed strong FISH signals using the probe EUB338 (Fig. 6h). Signal was observed on coiled filamentous structures seen under the TEM. They measured approximately 5 μm long and were moderately abundant. Despite the limited resolution, they seemed to be localised on the surface of gill epithelium, confirming TEM observations. No signal was observed using the group-specific probes including Del495a, but a 1-base mismatch occurred between the probe and the NLG1299-A and B sequences. Bacterial signals with similar shapes were also found from the roof of the pallial cavity, proximate to the gill (not shown).

Additional *N. lineata* tissues were associated with bacteria. Indeed, abundant bacterial signals occurred on the ventral surface and on the dorso-lateral regions of the girdle, on the epithelial tissues as well as attached to the spicule armature on both the left and right sides of the specimens (Fig. 6i, topmost part of the picture). Bacterial shapes were diverse, and observations suggested that they occurred within the mucus layer and not directly attached to animal tissue, possibly indicating an opportunistic association.

Stable isotope composition and absence of a cellulase activity

No significant cellulase activity (i.e. above background) was measured in the total protein extracts for any of the samples in either species.

Triplicate wood samples (frozen) yielded $\delta^{13}C$ values respectively of $-26.7 \pm 0.1‰$ and $-25.6 \pm 0.3‰$ and $\delta^{15}N$ values of $-1.2 \pm 0.3‰$ and $-1.0 \pm 0.4‰$. Their C/N ratios were very high (166–228) compared with the normal C/N ratio seen in the two species of chitons (~ 3 and 4). Whole specimens of *L. boucheti* stored in ethanol displayed mean carbon stable isotopes value of $-18.0 \pm 1.2‰$ (-19.4 to $-17.3‰$) and mean $\delta^{15}N$ value of $0.0 \pm 0.3‰$ ($-0.2‰$ to $0.3‰$; $n = 3$); frozen specimens displayed mean $\delta^{13}C$ value of $-19.9 \pm 1.1‰$ ($-21.2‰$ to $-18.1‰$) and mean $\delta^{15}N$ value of $-0.1 \pm 0.6‰$ ($-0.9‰$ to $0.6‰$; $n = 5$). A comparison showed no significant differences between these two sets of samples with different initial preservation (*t*-test, $P > 0.05$). The $\delta^{13}C$ and $\delta^{15}N$ values of the visceral mass ($-21.7‰$ and $0.0‰$, respectively, seven pooled specimens) were depleted compared to the foot ($-18.7‰$ and $0.9‰$; pool of seven specimens).

Two whole specimens of *N. lineata* stored in ethanol were pooled and displayed $\delta^{13}C$ value of $-19.0‰$ and $\delta^{15}N$ value of $2.2‰$; frozen specimens displayed mean $\delta^{13}C$ value of $-21.0 \pm 2.4‰$ ($-22.7‰$ to $-19.4‰$) and mean $\delta^{15}N$ value of $-0.6 \pm 2.4‰$ ($-1.13‰$ to $2.25‰$; $n = 2$).

Discussion

Feeding strategy

Leptochiton boucheti and *N. lineata* both occur on wood substrates (Sigwart & Sirenko, 2012). Molecular analyses confirmed that all the specimens of *L. boucheti* were conspecific, as well as all the specimens of *N. lineata*, even if each species may include several cryptic species in their entire geographical distribution (Sigwart *et al.*, 2011; Yearsley & Sigwart, 2011). Consequently, the results obtained here on specimens from the Vanuatu may not be applicable to other specimens currently identified as *L. boucheti* or *N. lineata*, as they may correspond to different species than the two we analysed, but our results are interesting in terms of the striking difference of bacterial association between two species that are phylogenetically and morphologically generally similar. All of the lines of evidence we examined, radular morphology, cellulolytic activity, gut flora and stable isotope analyses, support a conclusion that the chitons are not feeding directly on the wood, and yet the two species clearly have different associated bacterial communities. To examine the evidence of wood consumption in these polyplacophoran species, we compare each line of evidence to data from the gastropod limpet *Pectinodonta* sp., which is thought to feed directly on wood (Zbinden *et al.*, 2010).

The radular morphology of chitons is constrained, and the radulae of these two species are very different from that of the wood-feeding limpet *Pectinodonta* sp. For example, largest lateral teeth are separated by 150 μm in chitons, vs. only 25 μm in the limpet, and marginal teeth look much more like a carpenter's saw in the later species (Zbinden *et al.*, 2010). The gut is well developed and filled with a mixture of wood and organic material such as diatoms. Wood pieces appear visually more degraded in the chiton foregut compared with *Pectinodonta*, suggesting ingestion of already-degraded wood from the substrate surface in chitons.

No cellulolytic activity was measured in other chiton species where significant activity was reported in the digestive mass of the wood-feeding limpet *Pectinodonta* sp. (Zbinden *et al.*, 2010). The trophic fractionations of ^{13}C between chiton tissues and wood source were between $2.3‰$ and $9.4‰$. Trophic fractionation of ^{13}C between prey and predator (or consumer and food source) is usually around 0–1‰, but recently, Nishimoto *et al.* (2009) suggested that in sunken-wood communities, $\delta^{13}C$ values of direct wood eaters could be 2–3‰ higher than the whole wood matrix, as the cellulose is ^{13}C-enriched compared to the wood itself. This corresponds to the level of fractionation recorded between wood and tissues of the limpet *Pectinodonta* sp., which was shown to digest indirectly the wood using symbionts (Zbinden *et al.*, 2010). In our present study, the trophic fractionation between the wood matrix and chitons themselves is higher, which indicates that although both chiton species belong to the trophic web associated with decaying wood, with *Nierstraszella* endemic to this habitat, they do not digest the wood, even via symbionts.

Carbon signatures support nutrition on intermediates such as biofilms covering the decaying wood, which consist of microorganisms contributing to wood degradation and yield higher $\delta^{13}C$ values. An alternative could be that these two chiton species may partly feed on other food sources such as suspended particulate organic matter (SPOM) derived from the downward flux of detritic material (phytoplankton, plankton exoskeletons, zooplankton and marine snow), which display $\delta^{13}C$ values around $-22‰$ and $-23‰$ (Fanelli *et al.*, 2011). However, this hypothesis is not supported by $\delta^{15}N$ values, as SPOM is usually between $2‰$ and $5‰$ (Nishimoto *et al.*, 2009; Fanelli *et al.*, 2011) and becomes more positive along the food chain. Values measured in chitons ranged between $-1.1‰$ and $2.3‰$, closer to values from the wood (between $-1.4‰$ and $-0.6‰$). As wood contains very

little nitrogen, alternative sources such as ambient nitrogen gas, and its fixation by prokaryotes such as documented for symbionts of wood-boring bivalves, may be important (Lechene *et al.*, 2007; Nishimoto *et al.*, 2009). Marine species able to fix nitrogen gas have $\delta^{15}N$ values ranging from −2.1‰ to 1‰, partially outside the range measured for chitons (Minagawa & Wada, 1986).

Bacterial communities associated with *L. boucheti*

Both *Mollicutes* sequences recovered from the gut and gill of *L. boucheti* are not highly similar to any previously published sequence, suggesting novelty. They are, to our knowledge, the first *Mollicutes* sequences obtained from a deep-sea mollusc. *Mollicutes* are small, wall-less bacteria which have been extensively documented from DTs of various metazoans, mostly chordates (including vertebrates and ascidians) and arthropods (insects, isopods and decapods including the hydrothermal vent species *Rimicaris exoculata*) (Wang *et al.*, 2004; Tait *et al.*, 2007; Demiri *et al.*, 2009; Durand *et al.*, 2010). Recently, *Mollicutes* were also found within the coelomic cavity of terrestrial oligochaetes (Singleton *et al.*, 2003; Nechitaylo *et al.*, 2009). Many pathogenic forms occur, linked with their ability to cross the epithelial barrier. Although no previous work has dealt with any chiton-associated bacterial communities, the presence of *Mollicutes* has been previously documented among other molluscan groups, including the abalones *H. diversicolor* and *Haliotis discus hannai* (representing 35.5% of sequences within a 16S rRNA gut clone library from the former species) and the oyster *Saccostrea glomerata* (44% of clones in the digestive gland) (Green & Barnes, 2010; Huang *et al.*, 2010). FISH-based studies indicate high abundance of low G + C Gram-Positives (which include *Mollicutes*) in *Crassostrea gigas* (Hernández-Zárate & Olmos-Soto, 2006). Some *Mycoplasma*-like bacteria are also reported as potential pathogens (Paillard *et al.*, 2004). In *L. boucheti*, FISH results support that the *Mollicutes* phylotype LBDT0517-A, identified as dominant in the gut clone library, is indeed abundant in some regions of the gut. However, bacteria are not abundant everywhere in the gut. In regions where bacteria are abundant, the aspect of the FISH signal suggests dense populations of very small bacteria, as individual cells cannot be distinguished. The localisation of bacteria is intriguing, as they do not seem to occur on the apical part of epithelial cells, oriented towards the lumen, but rather seem to be basal to the epithelial cells. This suggests localisation either basal within gut epithelial cells or even within the coelomic cavity surrounding the gut, which reminds of the oligochete-associated *Mollicutes* (Singleton *et al.*, 2003). We hypothesise that the FISH signals actually correspond to the small structures located in the basal part of epithelial cells seen under TEM. Although these structures could also be intracellular vesicles, the fact that they, the FISH signals, and the DAPI-labelled layer occurred only in *L. boucheti* and that all have a similar localisation, suggest that these structures are indeed the Mollicutes. They also closely resemble structures described as M-LO (Mollicutes-Like Organisms) in digestive cells of scallops (Hine & Diggles, 2002).

The function of gut-associated *Mollicutes* cannot be inferred with confidence. They could be parasites, yet they do not seem to be associated with tissue degradation, or they might play a role in digestion. In the latter case, this role is probably not cellulose degradation, as this metabolism is not reported in *Mollicutes* (Wang *et al.*, 2004), and isotope results do not suggest wood-derived nutrition in the host. Phylotype LBDT0517-A occurred in all specimens investigated, suggesting an obligate relationship. But because facultative associations involving *Mollicutes* are reported and because the other phylotype, LBDT3651-B, occurred in the gut of only one specimen and was not confirmed using FISH, results from many more than the three specimens sequenced here, and potentially other species of *Leptochiton*, are needed to test whether the association is obligate or not (Wang *et al.*, 2007). Very few bacterial FISH signals were seen in the gill tissue despite several attempts and the application of several probes and conditions. The occurrence of *Mollicutes* sequences in gill clone libraries, despite the scarcity of FISH signals, suggests that they are probably not present in high amounts. If bacteria are rare in the gills, even a small level of contamination from gut tissue could result in the observed discrepancy. Because the gills are located in the posterior region, they are potentially exposed to faecal pellets which could be a source of contamination. Sample manipulation during trawling or dissection could also be a cause.

Other identified phylotypes in both the gut and gill clone libraries were diverse, and no other dominant phylotype was seen, which suggests that these represented a transient rather than resident bacterial community. After *Mollicutes*, *Alphaproteobacteria* are the next most abundant sequences in gut and gill clone libraries, while *Gammaproteobacteria* dominate the wood-associated clone library. Although clone libraries are not quantitative, this strongly suggests that community composition differs between wood substrate and metazoan gut. It is then reasonable to assume that the gut and pallial cavity provide habitats which favour other types of bacteria than the wood. Abundant FISH signal was observed using a *Gammaproteobacteria*-specific probe within the *L. boucheti* digestive gland, and *Gammaproteobacteria* rank third with

regard to abundance in gut clone libraries, but no phylotype can yet be linked with the signals.

Bacterial communities associated with *N. lineata*

No single phylotype dominated clone libraries from the gut of *N. lineata*, and microscopic observations indicate that no dense bacterial community occurs in the DT. Identified phylotypes most likely represent a transient community, a hypothesis supported by the abundance of sequences from diverse *Alphaproteobacteria* and *Gammaproteobacteria*, as observed in the *L. boucheti*-associated transient community. The gut thus seems to be devoid of a resident, dense bacterial community. As in *L. boucheti*, abundant FISH signal was observed using a *Gammaproteobacteria*-specific probe within the digestive gland, but cannot yet be linked to any of the phylotypes recovered.

Clone libraries from the gills were dominated by two distinct deltaproteobacterial phylotypes which form a clade with gill-associated bacteria documented in the wood-eating limpet *Pectinodonta* sp. (Zbinden *et al.*, 2010). They represent a new clade of bacteria associated with molluscs at wood falls, and future studies may reveal additional members. The group is related to free-living bacteria from sulphide-enriched habitats (caves and cold seeps), suggesting a probable sulphur-linked metabolism. Deltaproteobacteria are mostly anaerobic, which seems paradoxical in the case of gill associates, but caution is needed given their low similarity with documented members of the clade. *In situ* hybridizations using the general probe Eub338 confirm the occurrence of bacterial filaments associated with the surface of *N. lineata* gills and more sparsely with the epithelium delimiting the roof of the pallial cavity above gills. No specific probe could be used because of the very limited amount of properly fixed gill material available, but the morphology, shape, size and localisation of bacteria as documented by TEM and FISH images resemble the *Deltaproteobacteria* observed in *Pectinodonta* sp., supporting that these actually correspond to the recovered phylotypes. The bacterial populations appear less dense than observed in *Pectinodonta* sp. Apart from the abundance of *Deltaproteobacteria* sequences, gill-associated clone libraries included representatives of diverse bacterial groups, possibly including bacteria trapped in the epithelial mucus rather than significant symbionts.

Are distinct bacterial communities linked with the distinct habitats or ecology?

In this study, the most striking difference between *L. boucheti* and *N. lineata* is their distinct associations with bacterial partners. *Leptochiton boucheti* harbours a dominant *Mollicutes* phylotype, abundant in certain regions of its gut, and no dense bacteria were seen on their gills. Although *Mollicutes* have not previously been reported in chitons, they are well known as gut-associated bacteria in several metazoan groups and may contribute to digestion through their chemoorganotrophic metabolism. *Nierstraszella lineata* does not harbour *Mollicutes*, but displays filamentous *Deltaproteobacteria* located preferentially on the gill epithelium. Most likely, these have a sulphur-linked metabolism, as postulated for their relatives in *Pectinodonta* sp. (Zbinden *et al.*, 2010). These bacteria potentially help *N. lineata* tolerate or exploit sulphide-rich habitats. Decaying wood indeed produces sulphide and is documented to consist of heterogeneous microhabitats, of which some are rich in reduced sulphur (Leschine, 1995; Laurent *et al.*, 2009; Gaudron *et al.*, 2010). The *N. lineata* gill association might thus be linked with an ability to exploit more anoxic microhabitats compared to *L. boucheti* and thus prevent or limit interspecific competition. Such a slight niche differentiation linked with the occurrence of distinct bacterial communities could explain why both species can occur on wood falls in the same area and even co-occur in a single sample (Sigwart & Sirenko, 2012). Among the 34 chiton species known to inhabit deep sea wood substrates, it is possible that several different novel bacterial associations exist, which allow different species to each exploit a specific niche. A further interesting common point is that the digestive glands of both species seem to shelter *Gammaproteobacteria*, although the phylotypes could unfortunately not be identified and deserve further study.

Conclusion

This study provides the first detailed investigation of bacterial communities associated with chitons, in relation to their nutrition. The two species display striking differences in their associations with bacteria. The wood habitat-endemic species *N. lineata* harbours gill-associated bacteria, related to those documented in the limpet *Pectinodonta* sp., that might help it tolerate higher sulphide concentrations than *L. boucheti* which is devoid of such bacteria. On the other hand, *L. boucheti* harbours Mollicutes in its gut, which might play a role in digestion as documented in various metazoans. The occurrence of *Mollicutes* will probably be confirmed in more deep-sea metazoan species in future studies. Whether these distinct associations with bacteria are cause or consequence of the different levels of habitat endemism of the two chiton families remains to be investigated, using additional species and phylogenetic comparison. Nevertheless, this study confirms that besides nutritional chemosynthetic and xylophagous symbioses, other types of metazoan/bacteria

interactions exist and certainly play a significant role in the functioning of wood fall ecosystems.

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References

Agassiz A (1888) *Three Cruises of the U.S. Coast and Geodetic Survey Steamer "Blake" in the Gulf of Mexico, in the Caribbean Sea, and Along the Atlantic Coast of the United States, from 1877 to 1880*. Houghton, Mifflins and Co, Boston.

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment search tool. *Mol Biol* **215**: 403–410.

Amann R, Binder BJ, Olson RJ, Chisholm SW, Devereux R & Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analysing mixed microbial populations. *Appl Environ Microbiol* **56**: 1919–1925.

Blanchette RA (2000) A review of microbial deterioration found in archaeological wood from different environments. *Int Biodeterior Biodegradation* **46**: 189–204.

Cole JR, Wang Q, Cardenas *et al.* (2009) The ribosomal database project: improved alignments and new toolds for rRNA analysis. *Nucleic Acids Res* **37**: 141–145.

Cragg SM, Piman AJ & Henderson SM (1999) Developments in the understanding of the biology of marine wood-boring crustaceans and in methods of controlling them. *Int Biodeterior Biodegradation* **43**: 197–205.

Dando PR, Southward AJ, Southward EC, Dixon DR, Crawford A & Crawford M (1992) Shipwrecked tube worms. *Nature* **356**: 667–667.

Dayrat B, Tillier A, Lecointre G & Tillier S (2001) New clades of euthyneuran gastropods (Mollusca) from 28S rRNA sequences. *Mol Phylogenet Evol* **19**: 225–235.

Deming JW, Reysenbach AL, Macko SA & Smith CR (1997) Evidence for the microbial basis of a chemoautotrophic invertebrate community at a whale fall on the deep seafloor: bone-colonizing bacteria and invertebrate endosymbionts. *Microsc Res Tech* **37**: 162–170.

Demiri A, Meziti A, Papaspyrou S, Thessalou-Legaki M & Kormas KA (2009) Abdominal setae and midgut bacteria of the mudshrimp *Pestarella tyrrhena* RID H-5022-2011. *Cent Eur J Biol* **4**: 558–566.

Distel DL, Baco A, Chuang E, Morrill W, Cavanaugh C & Smith CR (2000) Do mussels take wooden steps to deep-sea vents? *Nature* **403**: 725–726.

Distel DL, Beaudoin DJ & Morrill W (2002) Coexistence of multiple proteobacterial endosymbionts in the gills of the wood-boring bivalve *Lyrodus pedicellatus* (Bivalvia: Teredinidae). *Appl Environ Microbiol* **68**: 6292–6299.

Dubilier N, Bergin C & Lott C (2008) Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nat Rev Microbiol* **6**: 725–740.

Duperron S, Nadalig T, Caprais JC, Sibuet M, Fiala-Médioni A, Amann R & Dubilier N (2005) Dual symbiosis in a Bathymodiolus mussel from a methane seep on the Gabon continental margin (South East Atlantic): 16S rRNA phylogeny and distribution of the symbionts in the gills. *Appl Environ Microbiol* **71**: 1694–1700.

Duperron S, Laurent MCZ, Gaill F & Gros O (2008) Sulphur-oxidizing extracellular bacteria in the gills of Mytilidae associated with wood falls. *FEMS Microbiol Ecol* **63**: 338–349.

Durand L, Zbinden M, Cueff-Gauchard V, Duperron S, Roussel EG, Shillito B & Cambon-Bonavita MA (2010) Microbial diversity associated with the hydrothermal shrimp *Rimicaris exoculata* gut and occurrence of a resident microbial community. *FEMS Microbiol Ecol* **71**: 291–303.

Fanelli E, Papiol V, Cartes JE, Rumolo P, Brunet C & Sprovieri M (2011) Food web structure of the epibenthic and infaunal invertebrates on the Catalan slope (NW Mediterranean): evidence from delta(13)C and delta(15)N analysis. *Deep Sea Res Part 1 Oceanogr Res Pap* **58**: 98–109.

Felsenstein J (2002) *PHYLIP (Phylogeny Inference Package)*. Department of Genome Sciences, University of Washington, Seattle, Distributed by the author.

Folmer O, Black MB, Hoeh WR, Lutz RA & Vrijenhoek RC (1994) DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from metazoan invertebrates. *Mol Mar Biol Biotechnol* **3**: 294–299.

Gaudron SM, Pradillon F, Pailleret M, Duperron S, Le Bris N & Gaill F (2010) Colonization of organic substrates deployed in deep-sea reducing habitats by symbiotic species and associated fauna. *Mar Environ Res* **70**: 1–12.

Green TJ & Barnes AC (2010) Bacterial diversity of the digestive gland of Sydney rock oysters, *Saccostrea glomerata* infected with the paramyxean parasite, *Marteilia sydneyi*. *J Appl Microbiol* **109**: 613–622.

Gros O & Gaill F (2007) Extracellular bacterial association in gills of "wood mussels". *Cah Biol Mar* **48**: 103–109.

Hernández-Zárate G & Olmos-Soto J (2006) Identification of bacterial diversity in the oyster *Crassostrea gigas* by fluorescent *in situ* hybridization and polymerase chain reaction. *J Appl Microbiol* **100**: 664–672.

Hine P & Diggles B (2002) Prokaryote infections in the New Zealand scallops *Pecten novaezelandiae* and *Chlamys delicatula*. *Dis Aquat Organ* **50**: 137–144.

Holt DM & Jones EB (1983) Bacterial degradation of lignified wood cell walls in anaerobic aquatic habitats. *Appl Environ Microbiol* **46**: 722–727.

Hoyoux C, Zbinden M, Samadi S, Gaill F & Compère P (2009) Wood-based diet and gut microflora of a galatheid crab associated with Pacific deep-sea wood falls. *Mar Biol* **156**: 2421–2439.

Huang Z-B, Guo F, Zhao J, Li W-D & Ke C-H (2010) Molecular analysis of the intestinal bacterial flora in cage-cultured adult small abalone, *Haliotis diversicolor* RID G-4635-2010. *Aquac Res* **41**: e760–e769.

Huelsenbeck JP & Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754–755.

Jovelin R & Justine JL (2001) Phylogenetic relationships within the polyopisthocotylean monogeneans (Platyhelminthes) inferred from partial 28S rDNA sequences. *Int J Parasitol* **31**: 1149–1149.

Jurgens JA, Blanchette RA & Carlon DN (2003) Evaluating the wooden remnants of the Tektas Burnu shipwreck. *Art, Biology and Conservation: Biodeterioration of Works of Art*. The Metropolitan Museum of Art, New York, pp. 390–407.

Kaehler S & Pakhomov E (2001) Effects of storage and preservation on the delta C-13 and delta N-15 signatures of selected marine organisms. *Mar Ecol Prog Ser* **219**: 299–304.

Kohlmeyer J (1968) The first ascomycete from the deep sea. *J Elisha Mitchell Sci Soc Chapel Hill N C* **84**: 239–241.

Laurent MCZ, Gros O, Brulport JP, Gaill F & Le Bris N (2009) Sunken wood habitat for thiotrophic symbiosis in mangrove swamps. *Mar Environ Res* **67**: 83–88.

Lechene CP, Luyten YA, McMahon G & Distel DL (2007) Quantitative imaging of nitrogen fixation by individual bacteria within animal cells. *Science* **317**: 1563–1566.

Leschine SB (1995) Cellulose degradation in anaerobic environments. *Ann Rev Microbiol* **49**: 399–426.

Loy A, Lehner A, Lee N, Adamczyk J, Meier H, Ernst J, Schleifer KH & Wagner M (2002) Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. *Appl Environ Microbiol* **68**: 5064–5081.

Macalady JL, Lyon EH, Koffman B, Albertson LK, Meyer K, Galdenzi S & Mariani S (2006) Dominant microbial populations in limestone-corroding stream biofilms, Frasassi cave system, Italy. *Appl Environ Microbiol* **72**: 5596–5609.

Manz W, Amann R, Ludwig W, Wagner M & Schleifer KH (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. *Syst Appl Microbiol* **15**: 593–600.

Minagawa M & Wada E (1986) Nitrogen isotope ratios of red tide organisms in the East-China Sea - A characterization of bacterial nitrogen fixation. *Mar Chem* **19**: 245–259.

Murray J (1895) A summary of the scientific results obtained at the sounding, dredging and trawling stations of the HMS Challenger. *Rep Sci Results Voyage HMS Challenger* **2**: 817–822.

Nechitaylo TY, Timmis KN & Golyshin PN (2009) "*Candidatus* Lumbricincola", a novel lineage of uncultured Mollicutes from earthworms of family Lumbricidae. *Environ Microbiol* **11**: 1016–1026.

Neef A (1997) *Anwendung der in situ* Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen. Technische Universität München, München, Germany.

Nishimoto A, Mito S & Shirayama Y (2009) Organic carbon and nitrogen source of sunken wood communities on continental shelves around Japan inferred from stable isotope ratios. *Deep-Sea Res II* **56**: 1683–1688.

Paillard C, Le Roux F & Borrego JJ (2004) Bacterial disease in marine bivalves, a review of recent studies: trends and evolution. *Aquat Living Resour* **17**: 477–498.

Post DM, Layman CA, Arrington DA, Takimoto G, Quattrochi J & Montana CG (2007) Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analyses. *Oecologia* **152**: 179–189.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J & Glöckner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35**: 7188–7196.

Sigwart JD (2009) The deep-sea chiton *Nierstraszella* (Mollusca: Polyplacophora: Lepidopleurida) in the Indo-West Pacific: taxonomy, morphology and a bizarre ectosymbiont. *J Nat Hist* **43**: 447–468.

Sigwart JD & Sirenko BI (2012) Deep-sea chitons from sunken wood in the West Pacific (Mollusca: Polyplacophora: Lepidopleurida): taxonomy, distribution, and seven new species. *Zootaxa* **3195**: 1–38.

Sigwart JD, Schwabe E, Saito H, Samadi S & Giribet G (2011) Evolution in the deep sea: a combined analysis of the earliest diverging living chitons (Mollusca: Polyplacophora: Lepidopleurida). *Invertebr Syst* **24**: 560–572.

Singleton D, Hendrix P, Coleman D & Whitman W (2003) Identification of uncultured bacteria tightly associated with the intestine of the earthworm *Lumbricus rubellus* (Lumbricidae; Oligochaeta). *Soil Biol Biochem* **35**: 1547–1555.

Sirenko BI (1992) Nierstraszellidae fam. nov.—a new family of chitons (Polyplacophora, Lepidopleurida) from the bathyal Western Pacific. *Ruthenica* **2**: 81–90.

Sirenko BI (2004) The ancient origin and persistence of chitons (Mollusca, Polyplacophora) that live and feed on deep submerged land plant matter (xylophages). *Bollettino Malacologica* **5**: 111–116.

Tait E, Carman M & Sievert SM (2007) Phylogenetic diversity of bacteria associated with ascidians in Eel Pond (Woods

Hole, Massachusetts, USA). *J Exp Mar Biol Ecol* **342**: 138–146.

Treude T, Smith CR, Wenzhöfer F, Carney E, Bernardino AF, Hannides AK, Krüger M & Boetius A (2009) Biogeochemistry of a deep-sea whale fall: sulfate reduction, sulfide efflux and methanogenesis. *Mar Ecol Prog Ser* **382**: 1–21.

Turner RD (1973) Wood-boring bivalves, opportunistic species in the deep sea. *Science* **180**: 1377–1379.

Turner RD (1978) Wood, mollusks, and deep-sea food chains. *Am Malacol Bull* **1977**: 13–19.

Turner RD (2002) On the subfamily Xylophagainae (Family Pholadidae, Bivalvia, Mollusca). *Bull Mus Comp Zool* **157**: 223–307.

Wang Y, Stingl U, Anton-Erxleben F, Geisler S, Brune A & Zimmer M (2004) "*Candidatus* Hepatoplasma crinochetorum", a new, stalk-forming lineage of Mollicutes colonizing the midgut glands of a terrestrial isopod. *Appl Environ Microbiol* **70**: 6166–6172.

Wang Y, Brune A & Zimmer M (2007) Bacterial symbionts in the hepatopancreas of isopods: diversity and environmental transmission. *FEMS Microbiol Ecol* **61**: 141–152.

Wolff T (1979) Macrofaunal utilization of plant remains in the deep sea. *Sarsia* **64**: 117–136.

Yearsley JM & Sigwart JD (2011) Larval transport modeling of deep-sea invertebrates can aid the search for undiscovered populations. *PLoS ONE* **6**: e23063.

Zbinden M, Pailleret M, Ravaux J, Gaudron S, Hoyoux C, Lorion J, Halary S, Warén A & Duperron S (2010) Bacterial communities associated with the wood-feeding gastropod *Pectinodonta* sp. (Patellogastropoda, Mollusca). *FEMS Microbiol Ecol* **74**: 450–463.

Supporting Information

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

Fig. S1. Bayesian phylogenetic trees obtained for the genera *Leptochiton* (A and B) and *Nierstraszella* (C and D), using the COI (A and C) and 28S (B and D) genes. GenBank accession numbers are provided for previously-published sequences.

Table S1. List of specimens included in metazoan phylogenetic analyses.

Table S2. List of bacterial 16S rRNA phylotypes recovered from clone libraries built from *L. boucheti* and *N. lineata* gut and gill, and wood.

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