Enteropneusts (phylum Hemichordata), although studied extensively because of their close relationship to chordates, have long been considered shallow-water, burrowing animals. The present paper more than doubles the number of enteropneust species recorded in the deep sea based on high-resolution imaging and sampling with remotely operated vehicles. We provide direct evidence that some enteropneusts are highly mobile—using changes in posture and currents to drift between feeding sites—and are prominent members of deep, epibenthic communities. In addition, we provide ecological information for each species. We also show that despite their great morphological diversity, most deep-living enteropneusts form a single clade (the rediagnosed family Torquaratoridae) on the basis of rDNA sequences and morphology of the proboscis skeleton and stomochord. The phylogenetic position of the torquaratorids indicates that the group, after evolving from near-shore ancestors, radiated extensively in the deep sea.

Keywords: deep sea; epibenthic; demersal; Enteropneusta; Hemichordata; Torquaratoridae
(figure 3) using the *Isis* ROV [14]. Density data are not yet available from these transects (D. Jones & C. Alt 2010, unpublished data). Observations and sampling on the Mid-Atlantic Ridge focused on three categories based on sea floor topography: flat plains (0–2'), slopes (8–12') and cliffs (more than 30'). Additional specimen-collecting dives were completed at each of the four main stations on the Mid-Atlantic Ridge.

Specimens were collected with suction samplers mounted on the ROVs. Upon recovery from the ROV, specimens were photographed, a tissue sample fixed in chilled 95 per cent ethanol and the remainder fixed in 4 to 20 per cent formalin.

MBARI’s video archives were searched using the Video Annotation Reference System database [15] for all observations of enteropneusts. Each observation was reviewed, habitat and behaviours were recorded, and representative video frame grabs were taken. Ancillary data linked by video time code provided specific location and depth information for each observation.

(b) Phylogenetics

All available specimens were sequenced to confirm morphospecies designations and for inference of evolutionary relationships of all reliable hemichordate sequences. DNA extraction, amplification and sequencing methods varied in the following ways from those described by Osborn & Rouse [16]. In combination with AmpliTaq Gold Fast Master Mix (Life Technologies Corp., Carlsbad, CA), the following 18S primers were used for amplification and/or sequencing: 18e, 18h [17], 18p and 18qa [18]. All sequencing was carried out with the BigDye Terminator v. 3.1 sequencing kit and analysed on an ABI 3100 capillary sequencer (Life Technologies Corp.). *JModelTest* [19] was used for model choice. The 18S rDNA alignment consisted of 2012 bp and the 16S was 597 bp. Specimen information, GenBank and voucher accession numbers are given in electronic supplementary material, table S1.

3. RESULTS

(a) Ecology

We recorded 498 observations of deep, epibenthic enteropneusts off the west coast of North America, Hawai‘i and in the Gulf of California from 2000 to the present by MBARI ROVs (table 1). In addition to *Torquator bullocki* and

Figure 1. Video frame grabs of representative deep-sea torquatorid enteropneusts in situ. (a) Genus D sp. 1, two shadows created by the lights of the remotely operated vehicle show that the animal is only a few centimetres above the sea floor. (b) Genus D sp. 2, animal shown just off the sea floor surface. (c) Genus D sp. 3 uncollected specimen, observed at least 20 m above the sea floor. (d) Genus A sp. 1. (e,f) Two uncollected species from the Celebes Sea. Images: NOAA Office of Ocean Exploration and Research. (g) Genus B sp. 1. (h) Genus B sp. 2. (i) *Tergivelum* sp. 1. (j) Genus C. (k,l) Two uncollected species from the Gulf of California, Torquatoridae sp. 2 and 3. (a–l) Scale bars: 10 cm.
*Tergivelum baldwinae*, at least nine species were identified from these videos (including those in figure 1a,c,g,h,i–l; electronic supplementary material, figure S2; and [6]). Morphologically distinct specimens recorded at depth but not collected will be designated here simply as *T. orquatoridae* sp. 1, sp. 2 and sp. 3. Pacific specimens were observed on sea floor that ranged from 1642 to 3954 m depth, with individual species ranges spanning as much as 1500 m depth (electronic supplementary material, figure S2; table 1). Atlantic exploration was restricted to sea floor approximately 2500 m deep. Individuals of most of the Atlantic and Pacific species were relatively sparsely distributed, although in certain observations one species (*Saxipendium* sp. 1) reached densities as high as five individuals per square metre. Average density was highly variable (0.1–9.5 individuals per 100 square metres) between locations and animals were patchily distributed along transects. Genus B sp. 1 (figure 1g) and *T. baldwinae* were the most often observed in the Pacific, each with nearly twice the number of observations as any other species.

The eastern Pacific and Gulf of California observation sites for the deep-living enteropneusts are shown in figure 2 and ecological data are provided in table 1. Two species were observed only in the Gulf of California (*Torquaratoridae* sp. 2 and sp. 3; figure 1k and l, respectively), one only off Hawai’i (*Torquaratoridae* sp. 1 [6]), three only off central California (*Saxipendium* sp. 1, shown in figure 1a and e, respectively), and two only off Oregon and southern Washington (*T. bullocki*, and genus C sp. 1, shown in figure 1j). *Tergivelum baldwinae* and genus B sp. 1 and 2 (figure 1g and h, respectively) were observed off central California, Oregon and southern Washington.

We observed and collected three species on the Mid-Atlantic Ridge as part of the MAR-ECO project (figure 1b,d,i and table 1). A single specimen of genus D sp. 2 (figure 1b) was discovered drifting above the sea floor during a survey of flat sediment-covered plain at the SW station (figure 3). Genus A sp. 1 (figure 1d) predominated on flat and slope areas of the SE and SW stations (figure 3), and was observed rarely at the NE station. *Tergivelum* sp. 1 (figure 1i) was found both north and south of the Charlie Gibbs Fracture Zone, but only in low numbers at the SE and SW stations (figure 3). Enteropneusts or their traces were occasionally seen on small sediment-covered ledges in steep rocky areas of the Mid-Atlantic Ridge. They were not seen on vertical rock faces.

Exploration of the Celebes Sea off Indonesia using ROV *Little Hercules* allowed observation of two additional species (figure 1e,f). Gross morphological differences distinguish these specimens from others observed in the Pacific (collar shape, pigmentation of the posterior region, width of posterior lateral folds and plicate nature of posterior gut contents). Genetic differences distinguished morphologically similar Atlantic and Pacific species (1.4% uncorrected genetic distance for approx. 570 bp of 16S).

The extensive in situ observations allowed description of typical behaviour for these animals. Members of genus D were observed drifting in currents from just centimetres above the sea floor (figure 1a,b) to at least 20 m above the sea floor (figure 1c; electronic supplementary material, video S1). Various other *torquaratorids* were observed lifting their anterior bodies off the sea floor, suggesting that they also use the water column for long-distance movement. No drifting enteropneusts had material in
of morphological features uniquely characterizing the group. The diagnosis of Torquaratoridae is here amended to accommodate the increased diversity and to reflect the molecular phylogeny: enteropneusts whose proboscis skeleton is absent or reduced to a small medial plate and whose adult stomochord is either absent or separated from the buccal cavity of the collar.

Monophyly of Hemichordata, Torquaratoridae and Harrimaniidae was recovered with strong support, and that of Ptychoderidae with moderate support (figure 4). Pterobranchs, Harrimaniids and a moderately supported clade consisting of Spengelids, Ptychoderids and torquaratorids formed a basal polytomy (figure 4). Deep-living enteropneusts (excluding the two Saxipendium species) fell within Torquaratoridae, the clade that our analyses strongly suggested was sister to Ptychoderidae.

Within Torquaratoridae, five clades were well supported by molecular data and gross morphology (figure 4; uncorrected genetic distance 3.5–13.5% between and 0–1.4% within generic-level clades for approx. 570 bp of 16S). We found sister taxa in the Atlantic and Pacific within two of the generic-level clades (probably three but no Pacific wide-lipped species were available for molecular analysis; figure 4).

4. DISCUSSION

(a) Ecology and natural history

Our observations of active drifting in deep-sea enteropneusts are the first to show that at least three species use demersal currents to move between locations not by chance, but in a controlled manner through changes in body posture. Further, our observations of empty guts in animals in the water column and recently settled, full guts in animals on the sea floor, and the time-lapse camera observations of Te. baldwinae emptying its gut completely before disappearing from a feeding site [12], suggest that gut contents (consisting of sea floor surface sediments) function in part as ballast. Using gut contents as ballast is also known from swimming sea cucumbers (Echinodermata, Holothuroidea) [20].

Circumstantial evidence suggests several of the other torquaratorids also drift in demersal currents. We observed animals lifting their anterior bodies off the sea floor, their feeding and faecal trails lack tracks leading to or away from them [11,12,21], and they secrete mucus [12] that may increase drag when in the water column (electronic supplementary material, video S1). Enteropneusts or their traces were occasionally seen on small sediment-covered ledges in steep rocky areas (but never on vertical rock faces) of the Mid-Atlantic Ridge, indicating that such topography is not a barrier to their movement. Based on these points, we hypothesize that movement between patches of suitable sediment-covered habitat is achieved by drifting in the water column. At least one shallow-water enteropneust swims seasonally [22–24] and there is one previous report of swimming by a provoked deep-sea specimen [25]. The ability to drift in the water column greatly increases individual ranges in comparison to shallow-water enteropneusts and may be an adaptation to limited or sporadic [26,27] food supply at depth.

While burrowing in the sediment or in crevices around rocks is the typical mode of life for shallow-water enteropneusts, many deep-sea species seem incapable of...
Table 1. Collection, ecological and biogeographic information by species. ESM, electronic supplementary material; n.a., not available.

<table>
<thead>
<tr>
<th>taxa</th>
<th>observed</th>
<th>collected</th>
<th>figure</th>
<th>biogeographic range</th>
<th>depth (m)</th>
<th>habitat</th>
<th>density (number per 100 square metres)</th>
</tr>
</thead>
<tbody>
<tr>
<td>genus A (wide-lipped) sp. 1</td>
<td>272</td>
<td>4</td>
<td>1d</td>
<td>Mid-Atlantic Ridge</td>
<td>2227–2939</td>
<td>sediment</td>
<td>n.a. b</td>
</tr>
<tr>
<td>genus B (extra wide-lipped) sp. 1</td>
<td>155</td>
<td>5</td>
<td>1g</td>
<td>off S. Washington–central California</td>
<td>1712–3287</td>
<td>lava with sediment veneer</td>
<td>0.2–4.7</td>
</tr>
<tr>
<td>genus B (extra wide-lipped) sp. 2</td>
<td>9</td>
<td>3</td>
<td>1h</td>
<td>off S. Washington–central California</td>
<td>1674–2847</td>
<td>lava with sediment veneer</td>
<td>0.3</td>
</tr>
<tr>
<td>Tergivelum baldwinae</td>
<td>155</td>
<td>5</td>
<td></td>
<td>off S. Washington–central California</td>
<td>2712–3954</td>
<td>sediment</td>
<td>0.1–9.5</td>
</tr>
<tr>
<td>Tergivelum sp. 1</td>
<td>92</td>
<td>4</td>
<td>1i</td>
<td>Mid-Atlantic Ridge</td>
<td>2227–2939</td>
<td>sediment</td>
<td>n.a. b</td>
</tr>
<tr>
<td>genus C (narrow-lipped) sp. 1</td>
<td>6</td>
<td>3</td>
<td>1j</td>
<td>off Oregon–Washington</td>
<td>2252–3124</td>
<td>lava with sediment veneer</td>
<td>0.3</td>
</tr>
<tr>
<td>genus D (plain-collared) sp. 1</td>
<td>8</td>
<td>2</td>
<td>1a</td>
<td>off central California</td>
<td>2891–3492</td>
<td>sediment/water column above</td>
<td>n.a.</td>
</tr>
<tr>
<td>genus D (plain-collared) sp. 2</td>
<td>1</td>
<td>1</td>
<td>1b</td>
<td>Mid-Atlantic Ridge</td>
<td>2622</td>
<td>water column above water column</td>
<td>n.a.</td>
</tr>
<tr>
<td>genus D (plain-collared) sp. 3*</td>
<td>2</td>
<td>0</td>
<td>1c</td>
<td>off central California</td>
<td>3460–3488</td>
<td>more than 20 m above sea floor</td>
<td>n.a.</td>
</tr>
<tr>
<td>Torquaratoridae sp. 1*</td>
<td>1</td>
<td>0</td>
<td>3d in [6]</td>
<td>off Hawai‘i</td>
<td>3035</td>
<td>lava with sediment veneer</td>
<td>n.a.</td>
</tr>
<tr>
<td>Torquaratoridae sp. 2*</td>
<td>6</td>
<td>0</td>
<td>1k</td>
<td>Gulf of California</td>
<td>2892–3177</td>
<td>sediment</td>
<td>n.a.</td>
</tr>
<tr>
<td>Torquaratoridae sp. 3*</td>
<td>31</td>
<td>0</td>
<td>1l</td>
<td>Gulf of California</td>
<td>2851–3167</td>
<td>sediment</td>
<td>n.a.</td>
</tr>
<tr>
<td>Saxipendium sp. 1</td>
<td>92</td>
<td>1</td>
<td>S1 (ESM)</td>
<td>off central California</td>
<td>1774–3156</td>
<td>sediment ponds near hard substrate</td>
<td>n.a.</td>
</tr>
<tr>
<td>unidentifiable*</td>
<td>23</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>up to 2.0</td>
</tr>
</tbody>
</table>

*Not included in figure 4 because no tissue was available for genetics.

*D. Jones & C. Alt 2010, unpublished data, National Oceanographic Centre, Southampton UK.
burrowing and instead live on the sea floor surface (figure 1). Because most torquaratorids have minimal musculature, are gelatinous and are extremely fragile (possible adaptations to limited high-quality food in the deep sea and/or use of the water column to move about [28]), it seems unlikely that they are capable of burrowing. Torquaratorids appear to be adapted to feeding on the sea floor surface and these adaptations are particularly obvious in the case of the extra-wide-lipped species (figure 1) that were found almost exclusively on deep-sea lava formations that have only a fine layer of sediment dusting the surface. While these pillow lava formations are near intermittent sediment ponds, animals were not observed feeding in the ponds, but were instead consistently observed on the rocks themselves. On the other hand, broad-collared and wide-lipped species were observed either on mud or hard substrate, but the same species was not observed on both substrates. This partitioning of species between habitats suggests that these intermediate and more common morphologies (wide-lipped and broad-collared) allow animals to survive on either type of habitat.

As with most deep-sea animal distributions, it is not possible to define complete geographical ranges because so little area of the deep-sea floor has been surveyed and the exploration sites are not chosen with systematic coverage in mind. On the Mid-Atlantic Ridge, the zone around the Charlie Gibbs Fracture Zone has been shown to be associated with a faunal divide between

Figure 4. Phylogenetic analysis of phylum Hemichordata based on rDNA; shaded parts of the tree indicate a clade of deep-sea enteropneusts comprising the family Torquaratoridae. Ninety per cent majority rule consensus tree from the Bayesian analysis (50 million generations) of complete 18S and partial 16S concatenated, but unlinked nucleotide sequences. Support is indicated for each branch as posterior probabilities/bootstraps from the parsimony analysis (equally weighted, heuristic search, tree bisection and reconnection branch swapping, 1000 random addition replicates): asterisks on branches indicate 1.0 or 100% support, respectively; unsupported branches were collapsed. Phylogram given in upper left to show relative branch lengths. Drawings show major morphological differences between generic-level clades and the shape of the proboscis skeleton. Asterisks in front of names indicate collection from the Mid-Atlantic Ridge, while bold species names indicate new sequences. IFREMER enteropneust, ‘T ornaria larva’ and ‘Ptychoderid sp. T ampa’ are from Cannon et al. [5]. Scale bar, 0.2.
species north of the sub-polar front, where there is high seasonal surface productivity, and more oligotrophic conditions in the south [29]. Genus A sp. 1 and *Tergivelum* sp. 1 occur both north and south of the sub-polar front; however, the former is more common in the southern oligotrophic conditions and is very rare north of the front, while *Tergivelum* sp. 1 is common below more productive and cooler waters north of the front, and occurs in low numbers in the south. The two most commonly observed Pacific torquatorid species were also the most widespread (*T. baldwinae* and genus B sp. 1; figure 2); however, genus B sp. 2 was also widespread with only eight observations. Genetics confirmed the identity of specimens from the northern and southern reaches of the ranges for both genus B species (electronic supplementary material, table S1). Finding this rare species across a wide geographical range suggests that even biogeographic ranges reported for our rarely observed species are good indicators of how widespread the individual species are within the explored area. Similarly, the even division of the number of observations on hard and soft substrate within the Pacific is probably a factor of the large proportion of time spent exploring areas dominated by hard substrate and may not be a good representation of the typical habitat of deep-sea torquatorid species.

*Tergivelum* is a morphologically distinct genus because of the ‘back veins’, dark pigmentation, proboscis and collar shapes, and thus identification of the genus from still images or video is straightforward. In addition to the observations reported here (figure 2), members of the genus have been reported from the western Pacific [6], both the eastern and western continental margin of Australia [30], and the NE Atlantic directly west of Brest, France (P. Tyler 2011, personal communication). Specimens were not available from the northern portion of the observed Pacific range, so identity could not be genetically confirmed as *T. baldwinae* for the northern observations. However, based on the lack of genetic divergence found within the two other species with similar biogeographic ranges in the Pacific basin (genus B sp. 1 and 2), we would not expect two *Tergivelum* species in the north Pacific. Genetic assessment will be crucial for identification of the diversity within the genus as specimens are eventually collected from the rest of their range.

The four torquatorid species observed only off central California are the most likely to have significantly wider biogeographic ranges than reported here. MBARI’s location within this area means the area is more thoroughly explored than most deep-sea areas and thus we are more likely to have observed animals here than in other parts of their ranges. The number of observations we have accumulated (table 1), coupled with assessment of the genetic diversity within the group, provides a baseline of ecological information and an evolutionary framework for further study of deep-sea enteropneust biology.

The mature eggs of genus D sp. 1 (1.5 mm) are some of the largest invertebrate eggs, surpassed only by various cephalopods whose eggs reach nearly 42 mm in their longest dimension [31]. Large egg size could indicate that these species are direct developers, like many harrimaniid species, which also have large eggs (up to 1.2 mm). Large eggs in torquatorid species contrast with the small eggs of most ptychoderids and spengelids, which pass through a tormaria larva stage. Alternatively, it is tempting to speculate that the exceptionally large eggs of torquatorid species might develop into the exceptionally large planctosphaera larvae (up to 28 mm [32]), which have never been traced to an adult stage and are widespread [32–34]. Hadfield & Young [32] tantalizingly suggested that these enigmatic larvae are part of the life history of an abyssal enteropneust. Appropriate fixation and sequencing of a planctosphaera would almost certainly solve this long-standing mystery now that enteropneust diversity is well represented in public databases.

(b) Evolutionary history

Phylogenetic analyses of the hemichordates as a whole did not recover the monophyly of the pterobranchs, contrasting slightly with two previous analyses that found weak support for monophyly of the group [5,35]. Resolution of pterobranch relationships requires further sampling of spengelids and pterobranchs, and does not pertain directly to the question at hand—that of the relationships of the deep-living enteropneusts to previously known enteropneusts.

Our analyses found Torquaratoridae sister to Ptycho-deridae, not nested within it, as was recently suggested [5]; thus the family is now firmly established within Enteropneusta. The continued inability to collect tissue from the type genus and species for Torquaratoridae is problematic, but it is likely that when finally sequenced *T. bullocki* will fall within the Torquaratoridae as defined here based on the reduced proboscis skeleton and stomo-chord, broad collar and proboscis, and deep-living, epibenthic habit. *Torquator*, *Tergivelum* and the four as yet undescribed genera (A–D in figure 4) show the wide range of morphological diversity within the family. This diversity is fairly unusual within the Enteropneusta, where fine details of internal anatomy are typically required to distinguish species and sometimes even higher taxa [9]. The rediagnosis and phylogenetic framework provided here make it possible to now describe six new species and three new genera for which high-quality specimens are available.

Our phylogenetic analyses further suggested that torquatorid and ptychoderid species do not represent the ancestral hemichordate form, as suggested by preliminary findings based on central nervous system development [36]. This has implications for the evolutionary history and origins of the clade. Many marine invertebrate higher taxa (families and above) originated in shallow water and later spread to deep water, a trend consistently found throughout the Palaeozoic [37]. These findings were based on examination of the fossil records for well-preserved taxa (crinoids, corals, brachiopods, echinoids, sponges, molluscs and bryozoans) compared with poorly preserved ones, which were expected to have random patterns owing to inconsistent preservation. The phylogenetic position of the torquatorid enteropneusts leaves little doubt that they conform to this trend and lends support to this idea from a poorly preserved group. Torquatorid’s oldest reliable fossil suggests their presence in the deep sea since the Early Triassic [38]. The phylogenetic position of the torquatorid enteropneusts suggests they are an example of a deep-water clade that has abandoned its shallow-water beginnings to occur exclusively in the deep sea.

Our findings show that we have probably only scratched the surface of deep-sea enteropneust diversity.
Prior to the present addition of at least 13 undescribed species (seven torquaratorids and one harrimaniid from MBARI observations, three torquaratorids from ISIS ROV observations in the Atlantic and two torquaratorids from Little Hercules observations in the Celebes Sea), only 89 enteropneust species were described. This work shows the value of exploration and direct observation in order to understand the diversity, ecology and evolutionary processes of the deep ocean, particularly the demersal communities that have been missed by historical observing and sampling methods.

We thank Dave Clague, Steve Haddock, Bob Vrijenhoek, Lynne Christianson, Shannon Johnson, Lonny Lundsten, Ken Smith, Antonina Rogacheva, Bruce Robison, Mike Vecchione, Susumu Ohtsuka and Sadaharu Iwasaki for contributions to the project. Steve Hammond, Verena Tunnicliffe and the NOAA Office of Ocean Exploration and Research provided access to images from INDEX 2010: Indonesia–USA Deep-Sea Exploration of the Sangihe Talaud Region expedition. We also thank the pilots and crews of the abovementioned ROVs and RV Western Flyer, RSS James Cook and Okeanos Explorer. The Pacific collections were funded by the David and Lucile Packard Foundation and the North Atlantic collections by the UK Natural Environment Research Council.

REFERENCES


36 Luttrell, S., Bengtsson, B. C. & Swalla, B. J. 2010 Central nervous system development and regeneration in hemichordates. Integr. Comp. Biol. 50(Suppl. 1), e105.


38 Twitchett, R. J. 1996 The resting trace of an acornworm (class: Enteropneusta) from the lower Triassic. J. Paleontol. 70, 128–131.