Giant protists (xenophyophores, Foraminifera) are exceptionally diverse in parts of the abyssal eastern Pacific licensed for polymetallic nodule exploration

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ABSTRACT

Xenophyophores, giant, fragile, agglutinated foraminifera (protists), are major constituents of the abyssal megafauna in the equatorial Pacific Clarion-Clipperton Zone (CCZ), a region where seabed mining of polymetallic nodules may occur in the future. As part of a baseline study of benthic communities we made extensive collections of xenophyophores in two areas (UK-1 and OMS) licensed for exploration by the International Seabed Authority. Based on test morphology, we distinguished 36 morphospecies (34 new to science) among 130 specimens. Twenty of these morphospecies yielded 184 DNA sequences, a 14-fold increase in genetic data for xenophyophores that confirms their high diversity in the eastern CCZ. A further 15 morphospecies (8 new to science) were recognised in samples from two other areas (APEI-6 and Russian exploration license area) within or adjacent to the CCZ. This large number of species confirms that the CCZ is a focal area for xenophyophore diversity. More broadly, it represents an unprecedented increase in the known global diversity of xenophyophores and suggests that many species remain undiscovered in the World's oceans. Xenophyophores are often sessile on nodules in the CCZ, making these delicate organisms particularly vulnerable to mining impacts. They can also play a crucial role in deep-sea ecosystems, providing habitat structures for meiofaunal and macrofaunal organisms and enhancing the organic content of sediments surrounding their tests. The loss of xenophyophores due to seabed mining may therefore have wider implications for the recovery of benthic communities following major human disturbances on the abyssal seafloor.

1. Introduction

Although protected by several kilometres of water, to an increasing extent the deep ocean floor is becoming an arena for human activities, including fishing, hydrocarbon extraction, waste disposal and, at least potentially, seabed mining (Glover and Smith, 2003; Thiel, 2003; Thurber et al., 2014; Levin and Le Bris, 2015). The possible environmental consequences for the deep-sea biota of this suite of developing and emerging industries has prompting environmental concerns and calls for a concerted research effort aimed at conserving fragile deep-sea ecosystems (Wedding et al., 2013, 2015; Mengerink et al., 2014). The mineral deposits of commercial interest include massive hydrothermal sulphides, cobalt-rich ferromanganese crusts on seamounts, together with the polymetallic nodules ('manganese nodules') that are abundant in some abyssal regions of the deep sea. There was a wave of interest in nodule mining during the 1970s, when a substantial research effort was directed towards evaluating the impact of pre-pilot mining (Thiel, 2003; Radziejewska, 2014). These activities waned along with falling metal prices but the new millennium has seen a considerable upsurge of interest in nodules as potentially major sources of certain commercially important metals, notably nickel, cobalt, manganese, copper and rare earths that have numerous applications in modern technologies (Morgan, 2012; Hein et al., 2012).

Polymetallic nodules grow very slowly over millions of years in areas where relatively low sedimentation rates are balanced by levels of surface ocean productivity that are sufficient to supply enough sinking

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particles to carry the metals necessary for nodule formation to the seafloor (Morgan, 2012). The most extensive deposits of high-grade nodules are found in the Clarion-Clipperton Zone (CCZ) (Hein et al., 2012), a vast east-west tract of abyssal seafloor in the eastern equatorial Pacific that is a focus of attention for countries and contractors with interests in deep-seabed mining. The CCZ lies in international waters beyond national jurisdictions and the exploitation of its resources is regulated by the International Seabed Authority (ISA), a body established within the framework of the 1982 United Nations Convention on the Law of the Sea. Blocks of seabed within the CCZ are licenced to contractors by the ISA for prospecting and exploration prior to possible mining (Lodge et al., 2014; Wedding et al., 2015). These licences require contractors to conduct detailed environmental surveys, including baseline assessments of the benthic fauna within the exploration area. The present study is a contribution to ABYSSLINE (ABYSSal baseLINE), a baseline survey in the UK-1 exploration license area, a region encompassing ~50,000 km^2 of seafloor licensed by the ISA to UK Seabed Resources Development Ltd (Glover et al., 2016).

We focus here on xenophyophores, a group of deep-sea foraminifera that attract interest because of their spectacularly large size compared to the vast majority of other protists (Tendal, 1972; Gooday and Tendal, 2002) and their often high abundance compared to megabenthic animals living in the same seafloor settings (Kamenskaya et al., 2013; Amon et al., 2016). These single-celled organisms use sediment grains to build delicate agglutinated tests that are typically several centimetres in size and may reach maximum dimensions of 10–15 cm or more (Tendal, 1972). The first species were described towards the end of the 19th Century, either as foraminifera (Brady, 1879; Brady, 1883; Goës, 1892), sponges (Haeckel, 1889), or a distinct group of rhizopod protozoans (Schultze, 1907). However, they remained little-known curiosities until the publication of Tendal's landmark 1972 monograph, where xenophyophores were classified as a subclass within the class Rhizopodea. Tendal recognised two main groupings of ordinal rank, the psamminids in which the test is rigid, and the stannomids in which the test is more or less flexible. Since then, molecular genetic studies have shown that several psamminid species branch within the radiation of single-chambered foraminifera (monothalamids) (Pawlowski et al., 2003; Lecroq et al.,

2009; Gooday et al., 2011). Morphology-based classification systems for monothalamous foraminifera do not reflect their phylogenetic relationships. Species have therefore been organised into a series of clades based on DNA sequences (e.g., Pawlowski, 2000; Voltski and Pawlowski, 2015). However, genetic data for xenophyophores are scarce (13 sequences are currently in GenBank) and are completely lacking for the stannomids. More sequences are needed in order to clarify their position with respect to other monothalamids as well as phylogenetic relationships between different xenophyophore taxa.

Knowledge of the distribution and ecology of xenophyophores has advanced considerably in recent years (Gooday et al., 1993; Levin, 1994; Laureillard et al., 2004), together with a steady increase in the number of described species (Tendal, 1996; Gooday et al., 2011), and they are now established as a major component of deep-sea communities in all oceans. Xenophyophores dominate the megabenthos in some settings, notably continental slopes (Bett, 2001), seamounts (Levin and Thomas, 1988), submarine canyons (Gooday et al., 2011) as well as abyssal plains, including the CCZ nodule fields where they are abundant and diverse (Schultze, 1907; Kamenskaya et al., 2013; Amon et al., 2016). They live on hard substrates as well as soft sediments, and limited evidence suggests that they grow quickly (Gooday et al., 1993) and can colonise new substrates (Hess and Kuhnt, 1996, Hess et al., 2001). Where they are abundant, these giant protists are believed to play the role of keystone species in deep-seafloor communities. In particular, they create structures that can increase seafloor diversity by hosting assemblages of macrofaunal, meiofaunal and microbial organisms (Gooday, 1984; Levin et al., 1986; Levin and Thomas, 1988; Hughes and Gooday, 2004), as well as enhancing the deposition of organic-rich particles, which benefits sediment communities (Levin, 1991, 1994; Levin and Gooday, 1992).

During two cruises as part of the ABYSSLINE project, we collected numerous xenophyophores within the UK-1 exploration license area and the adjacent Ocean Mineral Singapore (OMS) area. Material was preserved for both morphological and molecular genetic study. It was supplemented by samples and published information from two other areas within the CCZ, one located near the northern border of the Zone and the other in the central part to the west of the UK-1 and
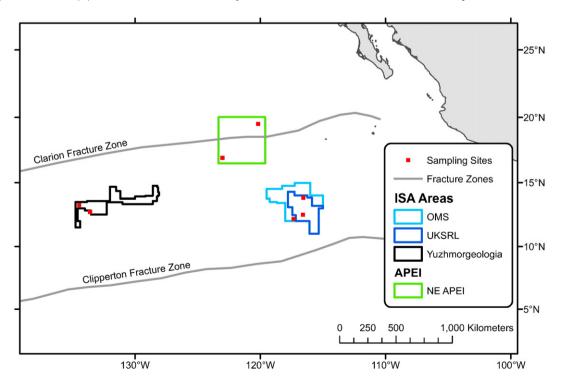

Fig. 1. Map of Clarion-Clipperton Zone in the eastern Equatorial Pacific showing location of sampling sites in the UK-1, OMS and Russian exploration license areas and APEI-6. UKSRL = UK-1 area; OMS = OMS area; NE APEI = Area of Particular Environmental Interest number 6; Yuzhmorgeologia = Russian contract area.

OMS areas (Fig. 1). We used this extensive collection to address two overall goals. The first was to assess the abundance, diversity and distribution of xenophyophores, and their affinity for nodules, within our survey areas. Diversity was evaluated using a combination of morphological and molecular data. Xenophyophores are particularly vulnerable to mining activities in the CCZ by virtue of their fragility and frequent attachment to nodules. This information will therefore be crucial for understanding the possible impact of deep-sea mining on these important components of CCZ benthic communities, as well as greatly enhancing our knowledge of their global diversity. The second major objective was to use the large amount of new molecular genetic data generated by this study to clarify phylogenetic relationships within the xenophyophores and between them and other monothalamids.

2. Methods

2.1. Sampling

The majority of xenophyophores were obtained in the UK-1 and adjacent OMS exploration areas during the two ABYSSLINE cruises (AB01 and AB02) (Fig. 1). The AB01 cruise (R/V *Melville* cruise MV1313; October 3 to 27, 2013) sampled in 'UK-1 Stratum A', a 30 × 30 km area centred around 13°49′ N, 116°36′ W in the northern part of UK-1 (Smith et al., 2013). The AB02 cruise (R/V *Thomas G Thompson* cruise TN319; February 12 to March 25, 2015) sampled in 'UK-1 Stratum B', centred around 12° 28.9′ N, 116° 36.3′ W, and in part of the OMS area, centred around 12° 8.2′ N, 117° 17.7′ W (Smith et al., 2015). They were collected using either an USNEL box corer or an OSIL Bowers & Connelly Megacorer equipped with 10-cm-diameter core tubes, at depths between 4054 and 4258 m. There is no evidence that xenophyophores were displaced by the bow wave effects that disturb box core surfaces (Bett et al., 1994). A few were picked from epibenthic (Brencke) sled samples collected in the OMS area. Some additional xenophyophores were obtained during the AB02 cruise in the northern part (19° 28′ N, 120° 00′ W) of Area of Particular Environmental Interest number 6 (APEI-6), which straddles the northern boundary of the CCZ.

Where possible, specimens were photographed *in situ* on the surfaces of core samples before being carefully removed, placed in a bowl of chilled seawater on ice, and stored in a refrigerator or cold room (3 °C) on the ship until they could be examined in more detail. All specimens and fragments of xenophyophores were documented photographically in the shipboard laboratory. In species (notably *Psammina limbata* and *Aschemonella* sp. nov. 1) where the cell body forms prominent strands, pieces of cytoplasm were removed from the test and preserved separately for molecular analysis. Otherwise, complete or partial specimens were preserved intact for this purpose. In all cases, RNAlater® solution was used for preservation and samples were stored at −20 °C. In the laboratory, preserved xenophyophores were dissected and pieces of cytoplasm removed for molecular analysis.

To supplement the ABYSSLINE material, we included xenophyophores obtained in the southwestern corner of the APEI-6 area (~17°N, 123°W; Fig. 1) during RRS *James Cook* Cruise 120 (JC120; 15 April to 19 May 2015; Jones et al., 2015). Specimens were picked from box corer or Megacorer samples and dried without fixation. We compared ABYSSLINE and JC120 material with xenophyophores recovered in box cores during cruises of the RV *Yuzhmorgeologia* in the Russian exploration area in the central CCZ (13-14°N, 130-135°W; Fig. 1) (Kamenskaya et al., 2015, 2016).

Data for all samples yielding xenophyophores from the UK-1, OMS and APEI-6 areas are summarised in Table 1.

2.2. DNA extraction, amplification, cloning and sequencing

DNA was extracted from isolated cytoplasm using the DNeasy® Plant Mini Kit (Qiagen). A total of 184 sequences was generated across 38 individuals and 20 morphotypes from various sampling locations

Table 1
Station data for samples yielding analysed xenophyophores. BC = Box corer; MC = Megacorer; EB = Epibenthic sledge.

Cruise	Site	Deployment	Latitude N	Longitude W	Depth (m)
UK-1 Stratum A					
AB01	B	BC04	13°50.994′	116°38.697′	4182
AB01	F	BC08	13°48.700′	116°42.600′	4076
AB01	I	BC10	13°45.001′	116°30.799′	4036
AB01	H	BC11	13°53.299′	116°41.399′	4219
AB01	K	BC12	13°51.801′	116°32.800′	4118
AB01	J	BC13	13°54.100′	116°35.400′	4229
AB01	D	EB03	13°50.172′	116°33.679′	4143
AB01	G	MC07	13°45.706′	116°27.601′	4170
AB01	I	MC08	13°45.699′	116°27.600′	4169
UK-1 Stratum B					
AB02	U02	MC02	12°22.024′	116°31.020′	4150
ABO2	U03	MC03	12°24.086′	116°29.086′	4152
AB02	U05	MC04	12°22.264′	116°36.818′	4163
AB02	U04	MC05	12°37.741′	116°43.424′	4236
AB02	U09	MC14	12°27.125′	116°30.736′	4199
AB02	U12	MC16	12°25.196′	116°37.474′	4137
AB02	U05	BC04	12°22.259′	116°36.819′	4160
AB02	U07	BC13	12°27.066′	117°35.661′	4130
AB02	U11	BC16	12°30.382′	116°29.073′	4224
AB02	U10	BC17	12°34.190′	116°32.333′	4228
AB02	U12	BC18	12°25.195′	116°37.477′	4136
AB02	U14	BC19	12°31.273′	116°41.889′	4237
AB02	U13	BC20	12°35.813′	116°29.614′	4258
OMS Area					
AB02	S03	MC06	12°34.743′	116°41.221′	4234
AB02	S01	MC07	12°07.074′	117°20.604′	4185
AB02	S03	MC08	12°10.872′	117°15.654′	4116
AB02	S02	MC10	12°04.910′	117°10.695′	4072
AB02	S06	MC12	12°08.696′	117°19.526′	4040
AB02	S10	MC21	12°03.279′	117°15.095′	4096
AB02	S08	MC22	12°11.417′	117°22.284′	4182
AB02	S12	MC24	12°01.644′	117°19.509′	4138
AB02	S02	BC09	12°04.912′	117°10.691′	4070
AB02	S04	BC10	12°00.567′	117°10.687′	4144
AB02	S05	BC11	12°13.042′	117°19.523′	4090
AB02	S06	BC21	12°08.156′	117°12.900′	4054
AB02	SO2	EB05	~12°06.52′	~117°10.36′	4072–4100
AB02	S11	EB12	~12°02.87′	~117°24.80′	4223–4235
APEI-6					
AB02	A01	BC28	19°27.998′	120°00.172′	4141
JC120	049	MC11	17°21.5641′	122°54.1816′	4015
JC120	050	BC07	17°18.851′	122°54.053′	4045
JC120	077	BC16	17°19.6855′	122°53.2744′	4017
JC120	082	BC17	16°54.1681′	123°0.9585′	4290
JC120	092	MC24	16°54.1516′	123°0.9716′	4291

(Table 2, Supplementary Table 1). Between 2 and 15 sequences were obtained from individual xenophyophores in order to ensure that they were consistent and therefore not derived from contaminating foraminifera. PCR amplifications of the 3′ end fragment of SSU rDNA were performed using foraminifera-specific primer pairs (Supplementary Table 2). The amplified PCR products were purified using the High Pure PCR Purification Kit (Roche Diagnostics) cloned with the TOPO TA Cloning Kit (Invitrogen) following the manufacturer's instructions and transformed into competent *E. coli*. Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analysed on a 3130XL Genetic Analyser (Applied Biosystems). The newly obtained sequences of xenophyophores were deposited in the EMBL/GenBank database (accession numbers LT576118-LT576138).

2.3. Phylogenetic analyses

The sequences obtained were added to an existing foraminiferal database using the Muscle automatic alignment option as implemented in Seaview vs. 4.3.3. (Gouy et al., 2010). The alignment of xenophyophores and three monothalamid clades (A, B and C) contains 1766 sites of which 1059 were used for analyses. The GC content ranged from

Table 2
Summary of sequencing details for xenophyophores from UK-1 and OMS areas. See Supplementary Table 1 for further information.

Species	Number of individuals analysed	Sequences per individual	Total no. of sequences per species
Aschemonella sp. nov. 1	8	2–12	46
Aschemonella sp. 2	1	9	9
Aschemonella sp. 3	1	3	3
Xenophyophore sp. nov. B	1	15	15
Galatheammina sp. 2	1	2	2
Galatheammina sp. 3	1	4	4
Galatheammina sp. 4	1	4	4
Galatheammina sp. 6	1	5	5
Galatheammina sp. 6	1	8	8
Psammina limbata	6	3–6	25
Psammina sp. 1	1	5	5
Psammina sp. 2	1	6	6
Psammina sp. 3	1	3	3
Rhizammina sp. 1	5	2–5	19
?*Rhizammina* sp. 2	1	2	2
Semipsammina sp. nov. 1	3	2	6
Stannophyllum zonarium	1	7	7
Xenophyophore sp. 1	1	6	6
Xenophyophore sp. 2	1	2	2
Xenophyophore sp. nov. A	1	7	7
TOTAL	38		184

30.7% to 44.7 % (*Galatheammina* sp. 2 and *Semipsammina* sp. 1, respectively). The alignment of xenophyophores and sixteen monothalamid clades (A, B, BM, C, CON, D, E, F, G, I, J, M, M1, O, V, Y) contained 1899 sites of which 1426 were used for analyses. The GC content ranged from 30.7% to 56 % (*Galatheammina* sp. 2 and undetermined allogromiid_17394 respectively).

Phylogenetic trees were constructed using PhyML 3.0 with automatic model selection as implemented in ATGC:PhyML (Guindon et al., 2010). A GTR substitution model was selected for both analyses and 100 Bootstrap replicates were performed.

3. Results

3.1. Abundance and relationship to nodules in UK-1 and OMS areas

The AB01 cruise yielded 31 complete and damaged xenophyophores from UK-1 Stratum A; the AB02 cruise yielded 114 complete and damaged specimens from UK-1 Stratum B and the OMS area, combined. Xenophyophores were recovered from 23 of the 26 successful box corer deployments and 5 of the 25 Megacorer deployments during AB02 compared with 9 out of 12 box corer and 5 out of 11 Megacorer deployments during AB01. Density estimates (including indeterminate specimens), based on box cores since these provided a greater sampled area than megacores, were 7.7 individuals.m^{-2} for UK-1 Stratum A, 6.9 individuals.m^{-2} for UK-1 Stratum B and 9.3 individuals.m^{-2} for OMS Stratum A.

More than half of the complete and fragmentary xenophyophores recovered in cores were found attached to polymetallic nodules (Fig. 2): 13 (48%) during AB01 and 65 (62%) during AB02 (59% overall). Only 6 (22%) specimens from AB01 and 10 (9.5%) from AB02 (12% overall), were considered to have been free living based on their morphology, occurrence in cores, and lack of signs of breakage. These unattached xenophyophores belonged to the following species: *Galatheammina* sp. 4, *Psammina* aff. *limbata*, *Psammina* spp. 4 and 5, *Occultammina* sp., *Rhizammina* sp. 2, Xenophyophore sp. nov. A and Xenophyophores spp. 1 and 4 (Supplementary material). It is likely that at least some of the remaining 38 specimens (29%) from ABYSSLINE cores were detached from nodules during sample recovery and processing.

3.2. Morphological and genetic diversity in UK-1 and OMS areas

Fifteen fragmentary xenophyophores were considered indeterminate, the remaining 130 specimens were assigned to 36 morphospecies (Table 3), all of which are briefly described and illustrated in the Supplementary material. A few stannomids were obtained by the epibenthic sledge, but the majority of xenophyophores, almost all originating from core samples, were psamminids. Only two (*Psammina limbata* and *Stannophyllum zonarium*) are described scientifically; the other 34 were previously unknown, including 7 that could not be assigned with confidence to existing genera. Together, these morphospecies span a bewildering range of morphologies (Supplementary Figs. 1–12). Many have laterally compressed, basically plate-like tests, which are flat, undulating, contorted or branched in overall form (Fig. 2a–c,f; Supplementary Figs. 1a–c, 2b–g, 3, 4a–f, 5a-c, 6a). Some are attached to nodules by a basal stalk or trunk (Fig. 2a–c). These forms are placed in either *Galatheammina* or *Psammina* based on the presence or absence, respectively, of internal agglutinated particles. In 4 morphotypes assigned to the genus *Aschemonella*, the test is basically tubular and either segmented or forming an anastomosing system of tubular elements. The most common species in our samples, *Aschemonella* sp. nov. 1, has a test typically comprising a sequence of more or less globular elements that break readily between the 'segments' (Fig. 2d; Supplementary Fig. 1e). Two samples yielded numerous fragments of a very delicate species (probably representing a new genus) in which the tubular test forms a polygonal network (Supplementary Fig. 1d). Other tubular xenophyophores are placed in the genera *Rhizammina* and *Occultammina*.

Some morphospecies form flat-lying structures that encrust nodule surfaces (Fig. 2e). In *Semipsammina* sp. nov. 1, a rounded or reticulated test gives rise to several long, tubular processes that may help it to colonise adjacent nodules (Supplementary Fig. 1f). Another species assigned to *Semipsammina* forms a thin, single-layered crust growing across the nodule surface (Supplementary Fig. 1g). In places, it rises up as a free-standing double-layered plate (Supplementary Fig. 5e, f), thereby bridging the distinction between the genera *Psammina* and *Semipsammina*. Other xenophyophores build complex reticulated structures that spread across the nodule surface (Supplementary Fig. 6b). A single specimen that probably represents a new genus forms a complex mass of soft, inter-connecting organic-walled branches extending vertically from the surface of the host nodule to form a structure resembling a patch of moss (Xenophyophore sp. nov. B; Supplementary Fig. 1h).

The 184 DNA sequences that we obtained from 20 of the 36 xenophyophore species collected in the UK-1 and OMS areas (2-46 sequences per species; Table 2) confirm that these species are genetically distinct entities. The intra-specific sequence divergence ranges from 0.0 to 0.8% (Supplementary Table 4). No evidence of intra-genomic polymorphism was found in any of the sequenced species. The lowest inter-specific sequence divergence (2.3 %) was found in following species pairs: *Aschemonella* sp. 2/*Psammina limbata*, *Aschemonella ramuliformis*/*Aschemonella* sp. 1, and *Galatheammina* sp.5/sp. 6. The minimum divergence in all other closely related species averaged 4.4%. The consistency between the morphological and molecular genetic diversity supports the conclusion that xenophyophore diversity is very high in the eastern CCZ.

3.3. Distribution of morphospecies within the CCZ

Three species (*Aschemonella* sp. nov. 1, *Psammina limbata* and *Rhizammina* sp. 1) were found in samples from all three 30 × 30 km ABYSSLINE study areas (UK-1 Strata A and B, OMS Stratum A), and five others (*Aschemonella* sp. 3, *Galatheammina* spp. 5 and 7, *Homogammina* sp. 4, *Semipsammina* sp. 4) occur in two of these areas, but the remaining 28 species were confined to one area (Table 3). More widely, *Aschemonella* sp nov.1 is also recorded in both APEI-6 and the Russian license area while *P. limbata* was first described from
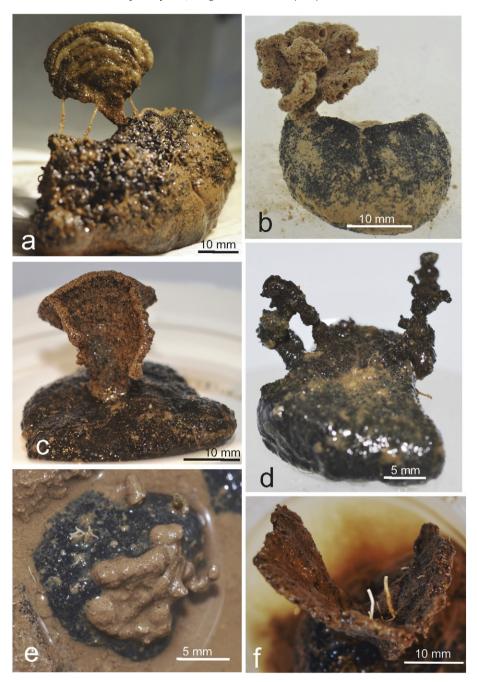
Fig. 2. Selected xenophyophores associated with nodules. (a) *Psammina limbata*; Cruise AB01, UK-1 area, Stratum A, Site F. (b) *Galatheammina* sp. 8; Cruise AB02, OMS area, Site S02. (c) *Galatheammina* sp. 5; Cruise AB02, UK-1 area, Stratum B, Site U12. (d) *Aschemonella* sp. nov. 1; Cruise AB02, UK-1 area, Stratum B, Site U01. (e) Xenophyophore sp. 3; Cruise AB02, UK-1 area, Stratum B, Site U13. (f) *Galatheammina* sp.5 with attached polychaete (serpulid) tubes; Cruise AB01, UK-1 area Stratum A, Site I.

the Russian area. *Psammina* aff. *limbata*, *P.* sp. 4 and *Homogammina* sp. occur in both ABYSSLINE and APEI-6 samples. Five species are presently known only from APEI-6, while at least 10 species are only recorded in the Russian license area (Table 3). However, distributions that currently appear restricted may expand as new samples are collected, while wider distributions need to be confirmed by genetic data, which are currently unavailable for xenophyophores from the APEI-6 and the Russian area.

3.4. Phylogenetic significance of the genetic data

The large amount of genetic data enables us to clarify phylogenetic relationships within the xenophyophores and between xenophyophores and other monothalamids. The PhyML analysis shows that xenophyophores (including *Rhizammina*) build a monophyletic group that is strongly supported with a 100% Bootstrap value (BV) (Figs. 3 and 4). The xenophyophores branch within Clade C, which comprises a heterogeneous assemblage of monothalamous species. Clades A and B form a highly supported group (100% BV) branching as a sister group to xenophyophores and Clade C species but with only moderate support (68% BV). Within the xenophyophores, some taxa cluster together with strong bootstrap support: Xenophyophore sp. 1 and *S. zonarium* (100% BV); *Galatheammina* sp. 4 and *Shinkaiya lindsayi* (100% BV); *Galatheammina* spp. 5 and 6 with *Psammina* sp. 3 (92 % BV); *Aschemonella* spp. 2 and 3 (83 % BV). Some of these genetic groupings, notably Xenophyophore sp. 1 and *S. zonarium*, combine species with very different test morphologies (Fig. 3).

In order to test the monophyly of xenophyophores further we conducted a PhyML analysis that also included sixteen monothalamid clades

Table 3

Distribution of xenophyophore species at different locations in the eastern and central Clarion-Clipperton Zone. Species indicated as 'sp. nov.' will be described and named elsewhere (Gooday et al., in prep). 'A' and 'B' indicate sampling 'strata' within the UK-1 area and APEI-6. The asterisks indicate the availability of genetic data. Suppl. = Supplementary. Please note that the figure references refer to Supplementary figures, not to figures in the main text

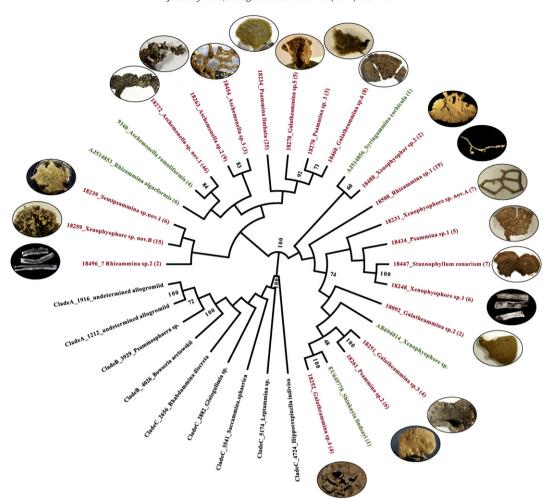| Species	Suppl. Figure	UK-1 A	UK-1 B	OMS-1	APEI-6 A	APEI-6 B	Russian	Reference
Aschemonella sp. nov. 1	Fig.1e	*	*	*		▦	▦	This study; Kamenskaya (unpub.)
Aschemonella sp.2	Fig.8a-d		*					This study
Aschemonella sp.3	Fig.7a,b	▦		*				This study
Aschemonella sp.4	Fig.6c			▦				This study
Psammina limbata	Fig.4b	▦	*	*			▦	This study; Kamenskaya et al. (2015)
Psammina aff. *limbata*	Fig.4a			▦		▦		This study
Psammina sp. 1	Fig.4b		*					This study
Psammina sp. 2	Fig.1c			*				This study
Psammina sp. 3	Fig.4d		*					This study
Psammina sp. 4	Fig.5a,b			▦		▦		This study
Psammina sp. 5	Fig.6a,b		▦					This study
Galatheammina sp. 1A	Fig.2a			▦				This study
Galatheammina sp. 1B	Fig.2b			▦				This study
Galatheammina sp. 2	Fig.1q	*						This study
Galatheammina sp. 3	Fig.2e		*					This study
Galatheammina sp. 4	Fig.1b		*					This study
Galatheammina sp. 5	Fig.2d	▦	*					This study
Galatheammina sp. 6	Fig.2c			*				This study
Galatheammina sp. 7	Fig.3a-d	▦	▦					This study
Galatheammina sp. 8	Fig.2f,g			▦				This study
Homogammina sp.	Fig.7c	▦	▦		▦			This study
Occultammina sp.	Fig.7e-g			▦				This study
Rhizammina sp. 1	Fig.11b,c	▦	*	*				This study
?Rhizammina sp. 2	Fig.9a,b	*						This study
Semipsammina sp. nov.1	Fig.1f		*					This study
Semipsammina sp. 4	Fig.1h;5e,f		▦	▦				This study
Semipsammina sp. 5	Fig.5c,d		▦					This study
Stannophyllum zonarium	Fig.11a			*				This study
?Syringammina sp.	Fig.8e,f			▦				This study
Xenophyophore sp. nov. A	Fig.1d			*				This study
Xenophyophore sp. nov. B	Fig.1i			*				This study
Xenophyophore sp. 1	Fig.9c,d		*					This study
Xenophyophore sp. 2	Fig.4f,g	*						This study
Xenophyophore sp. 3	Fig.7d		▦					This study
Xenophyophore sp. 4	Fig.10,a,b			▦				This study
Anastomosing encrusting species	Fig.6b			▦				This study
Aschemonella sp.5	Fig.12f,g					▦		This study
Psammina aff. *multiloculata*	Fig.12c-e					▦		This study
?Shinkaiya sp.	Fig.12a,b					▦		This study
Pale *Aschemonella*-like domes	Fig.10c,d				▦	▦		This study
Large pale patches	Fig.10e,f				▦			This study
Psammina multiloculata							▦	Kamenskaya et al. (2015)
Semipsammina licheniformis							▦	Kamenskaya et al. (2015)
Spiculammina delicata							▦	Kamenskaya (2005)
Stannophyllum radiolarium							▦	Kamenskaya et al. (2015)
Stannophyllum sp							▦	Kamenskaya et al. (2015)
Aschemonella tubulosa							▦	Kamenskaya et al. (2016)
Cerelasma implicata							▦	Kamenskaya et al. (2016)
Stannophyllum paucilinellatum							▦	Kamenskaya et al. (2016)
Maudammina sp. nov.							▦	Kamenskaya (unpublished)
Psammina sp. nov							▦	Kamenskaya (unpublished)

Fig. 3. PhyML phylogenetic tree showing the evolutionary relationships of 25 xenophyophore species (20 from ABYSSLINE material in red, 5 previously published sequences in green) and 9 monothalamid sequences belonging to Clades A, B and C. Numbers at nodes indicate bootstrap values for 100 bootstrap replicates. The tree is based on a single representative sequence for each species (identified by numbers beginning with 18 for ABYSSLINE species and listed in Supplementary Table 1). Numbers in brackets indicate the total number of sequences obtained for each xenophyophore species. The tree was rooted with clade A and clade B specimens (4 sequences).

(Fig. 5). This clearly confirms that xenophyophores constitute a monophyletic group (100% BV) that is distinct from other monothalamous foraminifera. The tree contains 64 sequences and is divided in two subgroups, one containing the xenophyophores together with species from clades C, A, B and BM, the other including species from clades F, V, G, D, E, CON, M, M1, I, J, Y and O. Except for clade C, most clades are supported by strong bootstrap values. Within the xenophyophores, the same groupings as in Figs. 3 and 4 are strongly supported with BV values of 96% and above.

The sequenced xenophyophores seem to exhibit different evolutionary rates, with one moderately supported (74% BV) group displaying long branches, while the other group, which includes all of the species assigned to the genus *Aschemonella*, has very short branches (Figs. 4,5). Notably, species of morphologically-defined genera (e.g. *Psammina*, *Galatheammina* and *Rhizammina*) do not cluster together based on genetic data, suggesting they have converged morphologically. Finally, phylogenetic relationships among different xenophyophore taxa (Fig. 3) often lack bootstrap support and therefore caution should be exercised when inferring evolutionary relationships between them.

4. Discussion

4.1. High xenophyophore diversity in the CCZ

The 36 xenophyophore species in the ABYSSLINE material, all collected from the 3 sampled 'strata' (total area 2700 km²) in the eastern CCZ, were recognised based on clear differences in their test morphology and internal structure (Supplementary material). Only two of these species, *Psammina limbata* and *Stannophyllum zonarium*, have been formally described. Previous taxonomic studies of xenophyophores in the wider equatorial Pacific, also based on test morphology, have recognised 27 described species (13 stannomids and 14 psamminids) and 31 undescribed forms (4 stannomids and 27 psamminids) at 91 localities (Supplementary Table 3). Within the CCZ itself, however, only 9 species (*Aschemonella tubulosa*, *Cerelasma implicata*, *Psammina limbata*, *P. multiloculata*, *Semipsammina licheniformis*, *Spiculammina delicata*, *Stannophyllum paucilinellatum*, *S. radiolarium* and *S. setosum*) have been described in earlier studies with another 7 undescribed species recorded, 7 of the described species being confined to a relatively small part of the Russian license area (Kamenskaya, 2005; Kamenskaya et al., 2015, 2016). Our new records therefore increase the number of species (described and undescribed) reported from the CCZ from 16 to 51 (Table 3).

The morphological evidence for high xenophyophore diversity in the eastern CCZ is strongly supported by our molecular results, which confirm that the 20 morphospecies from which sequences were obtained are genetically distinct entities (Supplementary Table 4). These 20 morphospecies yielded 184 xenophyophore DNA sequences, raising the global total of such sequences from 13 to 197, a remarkable 14-fold increase in genetic data for this group of foraminifera. They also increase the number of morphospecies that have been successfully sequenced from 5 (Gooday et al., 2011; Hori et al., 2013) to 25
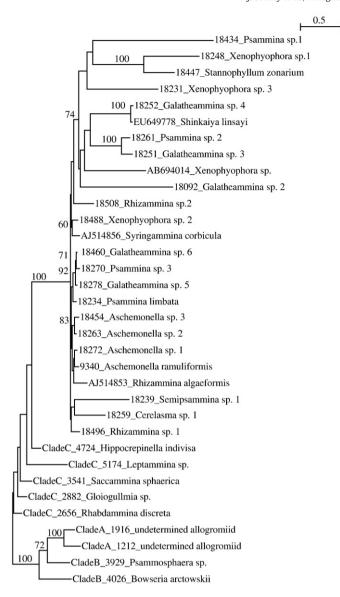
0.5

18434_Psammina sp.1
100 — 18248_Xenophyophora sp.1
18447_Stannophyllum zonarium
18231_Xenophyophora sp. 3
100 — 18252_Galatheammina sp. 4
EU649778_Shinkaiya linsayi
100 — 18261_Psammina sp. 2
18251_Galatheammina sp. 3
AB694014_Xenophyophora sp.
18092_Galatheammina sp. 2
18508_Rhizammina sp.2
60 — 18488_Xenophyophora sp. 2
AJ514856_Syringammina corbicula
71 — 18460_Galatheammina sp. 6
92 — 18270_Psammina sp. 3
18278_Galatheammina sp. 5
18234_Psammina limbata
83 — 18454_Aschemonella sp. 3
18263_Aschemonella sp. 2
18272_Aschemonella sp. 1
9340_Aschemonella ramuliformis
AJ514853_Rhizammina algaeformis
18239_Semipsammina sp. 1
18259_Cerelasma sp. 1
18496_Rhizammina sp. 1
CladeC_4724_Hippocrepinella indivisa
CladeC_5174_Leptammina sp.
CladeC_3541_Saccammina sphaerica
CladeC_2882_Gloiogullmia sp.
CladeC_2656_Rhabdammina discreta
100 — CladeA_1916_undetermined allogromiid
72 — CladeA_1212_undetermined allogromiid
100 — CladeB_3929_Psammosphaera sp.
CladeB_4026_Bowseria arctowskii

74, 100, 60, 71, 92, 100, 83

Fig. 4. PhyML phylogenetic tree showing evolutionary relationships of 25 xenophyophore species (20 from ABYSSLINE material) and 9 monothalamid sequences belonging to clade A, B and C. Numbers at nodes indicate bootstrap values. The tree was rooted with clade A and clade B specimens (4 sequences).

(Supplementary Table 1), revealing an unprecedented level of genetic diversity among xenophyophores in this part of the Pacific.

Morphological data suggest that xenophyophores may be much richer in species at regional scales within the CCZ. Five undescribed morphospecies are recorded only from APEI-6 and 10 species (3 undescribed) are recorded only from the Russian license area (Table 3). *Spiculammina delicata*, a very distinctive species and the most common xenophyophore in the Russian area (Supplementary Table 3; Kamenskaya et al., 2013, 2015, 2016), is not represented at all in ABYSSLINE samples. On the other hand, only 5 species are shared between our samples and either one or both of the Russian and APEI-6 areas (Table 3) and only one species (*Stannophyllum zonarium* from the OMS stratum) is recorded, based on test morphology, from regions outside the equatorial Pacific (Tendal, 1972, 1996). These patterns may change with improvements in our knowledge of xenophyophore distributions in the CCZ and other parts of the Pacific. In particular, it will be important to confirm and refine distributions using molecular data. Nevertheless, the current morphology-based records suggest that the turnover of xenophyophore species, and hence regional

diversity, is quite high in relation to the west to east gradients in depth and organic matter flux (food supply) to the seafloor across the eastern equatorial Pacific (Morgan, 2012). High regional diversity is inconsistent with a global-scale modelling study predicting moderate to low levels of habitat suitability for xenophyophores in the CCZ (Ashford et al., 2014). This discrepancy underlines the need for improved knowledge of the abundance and biogeography of xenophyophores in many parts of the ocean. The likelihood that xenophyophores have more restricted distributions than some small multichambered abyssal foraminiferal species (e.g. Gooday and Jorissen, 2012) may reflect differences in their mode of reproduction. The wide dispersal of tiny propagules appears to underlie the wide dispersal of smaller species (Alve and Goldstein, 2010). Unfortunately, very little is known about reproduction in xenophyophores (Tendal, 1972).

The numerous undescribed species in our ABYSSLINE material, combined with likely high levels of species turnover across the CCZ, confirms earlier evidence (Schulze, 1907; Tendal, 1972, 1996) that the eastern Equatorial Pacific hosts an unusually large concentration of xenophyophore species. These observations have implications for the diversity of this group of megafaunal protists at a global scale. In his synoptic checklist of xenophyophore species, Tendal (1996) recognised a total of 58 species described from the World's oceans together with another 8 that are known but not named. Subsequent publications have increased the number of described species to 68 and undescribed species to 37 (total 105). Our new records from the UK-1, OMS and Russian areas and the APEI-6 increase this total from 105 to 141. This number already exceeds Tendal's (1996) estimate that around 100 xenophyophore species may be present in the World's oceans. Global estimates of diversity are subject to considerable uncertainty. However, our results, together with the fact that xenophyophores appear to exhibit distinct biogeographic patterns (for example, there is little overlap between the fairly well-known North Atlantic and Pacific faunas; Tendal, 1996), suggest that the total number of species could be of the order of 1000 or more.

4.2. Ecology and vulnerability to seafloor disturbance

The 36 xenophyophore species recognised in the UK-1 and OMS areas compare with only 7 (2 undescribed) recorded at the intensively studied Porcupine Abyssal Plain Sustained Observatory site (PAPSO, ~4850 m depth) in the NE Atlantic (Gooday, 1996). This may be related to the presence in the CCZ of nodules, which create a higher degree of habitat heterogeneity, compared to the absence of nodules at the PAPSO site. More than half (59%) of the xenophyophores collected during the ABYSSLINE cruises were attached to nodules (Fig. 2) while only 12% were judged to be free-living. A sessile mode of life is also common among xenophyophores in the Russian license area (Kamenskaya et al., 2013, 2015) and probably the CCZ generally. Nodule coverage on individual box cores ranged from 0.2 to 50% during the AB01 cruise (Amon et al., 2016), suggesting that xenophyophores have a positive association with nodules. Apart from providing a firm surface to live on, attachment to these slightly elevated substrates exposes suspension-feeding species to a somewhat enhanced supply of organic particles suspended in the near-bottom water (Levin and Thomas, 1988). Mullineaux (1989) found that sessile organisms (mainly foraminifera) inferred to be suspension feeders were concentrated near the summits of nodules. Xenophyophores that stand upright on nodule surfaces (Fig. 2) are most likely suspension feeders; for example, the fan-shaped test of *Psammina limbata* (Fig. 2a) seems ideally suited to intercepting suspended particles. Species that form flat-lying crusts or canopies on nodules (e.g., *Semipsammina* sp. nov. 1; Supplementary Fig. 1f) might possibly be feeding on biofilms or bacteria associated with the nodule surface, while those that live on or within surficial sediments (e.g. Xenophyophore sp. nov. A; Supplementary Fig. 1d) are more likely to be deposit feeders. Xenophyophore may therefore encompass several different trophic traits. However, the accumulation of stercomata,
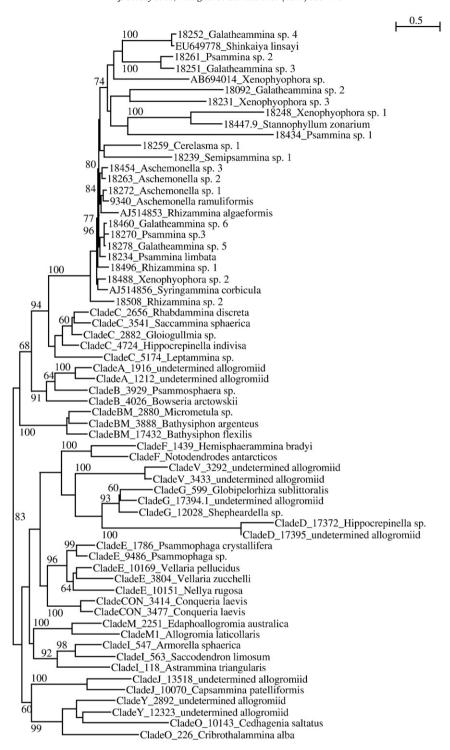
Fig. 5. PhyML phylogenetic tree showing evolutionary relationships of 25 xenophyophore species (20 from ABYSSLINE material) and 39 monothalamid sequences belonging to clades C, A, B, BM, F, V, G, D, E, CON, M, M1, I, J, Y and O. The tree is unrooted. Numbers at nodes indicate bootstrap values.

which consist mainly of small mineral grains, suggests that all xenophyophores collect fine sediment particles, either from the water column, from nodule surfaces, or directly from the sediment.

Indications that xenophyophores grow rapidly (Gooday et al., 1993) and are pioneer recolonisers of new substrates (Hess and Kuhnt, 1996, Hess et al., 2001) suggest that they may recover from mining impacts fairly quickly. Nevertheless, if attachment to a hard substrate is obligatory for some species, the removal of nodules by mining may substantially limit their recovery, possibly leading to local extinctions. Xenophyophores often play a keystone role in abyssal ecosystems.

Their tests, both living and dead, provide a range of potential services to the meiofaunal and macrofaunal organisms (e.g., polychaetes, nematodes, sipunculans, crustaceans, foraminifera) that live on or within their often complex test structures (Fig. 2f). These services include the provision of a settlement substrate, a refuge from predation, locations for reproduction, and access to an enhanced food supply (Levin and Thomas, 1988; Levin, 1991). There is evidence from gut content analyses and possible feeding traces that xenophyophores provide a food source for some metazoans (Tendal, 1985; Sokolova, 2000). Shallow pits visible on the test surfaces of a few ABYSSLINE specimens (e.g.

Supplementary Fig. 7C) resemble the traces illustrated by Tendal (1985). Large xenophyophore tests that stand erect above the sediment surface may promote the deposition of organic-rich particles (Levin and Gooday, 1992). This probably explains the common occurrence of ophiuroids near the base of these structures (Levin and Thomas, 1988), as well as the enhanced meiofaunal and macrofaunal abundances observed in sediments beneath them (Levin et al., 1986). The removal of xenophyophores as a result of mining may therefore have implications for the recovery of the wider benthic community, as well as for the xenophyophores themselves. More detailed information than is currently available regarding the distribution and diversity within the CCZ of xenophyophores would provide a better basis for evaluating these risks.

4.3. Xenophyophore origin and phylogeny

The massive increase in genetic data obtained during this study supports the monophyly of xenophyophores and their position within the radiation of Clade C monothalamous foraminifera. Our molecular results are consistent with earlier evidence for a close relationship between the well-known tubular genus *Rhizammina* and xenophyophores (Pawlowski et al., 2003; Lecroq et al., 2009; Gooday et al., 2011). The sequence data confirms that another atypical species (Xenophyophore sp. nov. B; Supplementary Fig. 1h), in which an organic-walled test encloses separate accumulations of sediment and stercomata, is also a xenophyophore. However, molecular data for *Stannophyllum zonarium*, the only representative in our material of the Stannomida, provides no support for the division of xenophyophores into two orders. Molecular data suggest that plate-like morphotypes, assigned on morphological grounds to *Galatheammina* and *Psammina*, are not closely related. The only genetically coherent group to emerge from our molecular analyses comprises species placed in the genus *Aschemonella* (Fig. 3). These results indicate that xenophyophores are genetically very variable and that their current morpho-taxonomy requires considerable revision. The multinucleate organisation of xenophyophores (Schultze, 1907; Tendal, 1972; Lecroq et al., 2009) makes them well suited for molecular analyses. We hope that future collections will help to clarify phylogenetic relationships within this important group of deep-sea megabenthic protists, as well as improving our knowledge of their biogeography.

Our molecular data have possible implications for the geological antiquity of xenophyophores. Although they have no proven fossil record, xenophyophores have featured in the palaeontological literature because of suggestions that they are modern analogues of some late Precambrian (Vendian) members of the Ediacaran biota (Seilacher et al., 2003). However, a recent study found no support for these ideas and suggested that xenophyophores probably originated more recently (Antcliffe et al., 2011). Our DNA data support this interpretation by anchoring them firmly within Clade C (Fig. 3), which forms part of the monothalamid 'crown group' (Habura et al., 2008).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.biocon.2017.01.006.

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