https://doi.org/10.5194/egusphere-2025-1794
Preprint. Discussion started: 6 May 2025

Hidden behind the scene – high diversity, low connectivity of deep-sea Amphipoda in the polymetallic nodule fields in the central East Pacific

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Keywords

Clarion-Clipperton Zone, abyssal, DNA barcoding, species richness, species distribution, deep-sea mining

ABSTRACT

The Clarion-Clipperton Zone (CCZ) situated in the central east Pacific holds major portions of manganese nodule deposits and is therefore subject to intense exploration for future deep-sea mining. However, mining ~~rises~~ multiple concerns. Among others about its direct or indirect impact on abyssal environment. ~~The more,~~ proper evaluation of deep-sea protection plans to be applied there is still hindered by insufficient knowledge of ~~the~~ abyssal fauna diversity and their assemblages.

Amphipoda are speciose and abundant in all marine habitats and were proven to be important food source for higher trophic levels. These crustaceans are brooders with no planktonic larval stage, so migration of species depend only on swimming capacities of adult animals. In the CCZ macrofauna-sized and relatively mobile, epibenthic Amphipoda, not collected with commonly used box corers or multicorers, remained unstudied. The present work aims to fill ~~in~~ this gap in knowledge by: 1) characterizing the diversity of amphipods across the CCZ and one Area of

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Particular Environmental Interest (APEI), 2) studying the amphipod species distribution and assemblages as well as 3) inferring ~~the~~ connectivity between eastern and western parts of the CCZ and other deep-sea regions.

The material was collected with epibenthic sledge from three contractor zones (UK, Singapore, Germany) and from APEI-06. Specimens were sorted into families and ~~preferably~~ morphospecies and subjected to cytochrome *c* oxidase subunit I gene (COI) barcoding.

Within total of 708 individuals 23 known and one undescribed families were identified. The barcoding allowed to obtain 581 sequences that were ascribed to 207 Molecular Operational Taxonomic Units (MOTUs). The families that dominated barcoded material in terms of abundance and species richness were: Pardaliscidae (123 ind., 44 MOTUs), Eusiridae (122 ind., 32 MOTUs) and Synopiidae (74 ind., 31 MOTUs). Only 17 known species were identified; another 48 are considered ~~as~~ new to science. Almost 70% of MOTUs were singletons or doubletons and 186 MOTUs were unique for the present study. The remaining 21 taxa appeared to be broadly distributed. Among the MOTUs recorded ~~only~~ in the CCZ majority was distributed within 400 km distance. Just six species reached or exceeded 1000 km distance of occurrence. Additionally only one species was shared between contractor areas and the studied APEI that was supposed to be preserved area representative for the mining zones. The cluster analysis of the fauna collected during three expeditions to the German contractor areas revealed almost no similarity between the ~~West~~ and ~~East~~ part of the CCZ. Additionally, within the eastern part of the studied region geographic proximity appeared to have no influence on station clustering.

The study confirmed low abundance and high species richness of deep-sea amphipod fauna of the CCZ and their highly variable assemblages. Moreover, it has shown weak connectivity between eastern and western parts of the region as well as between the contractor zones, APEIs and other deep-sea regions of the ~~W~~orld. All the above suggest high vulnerability of the assemblages studied and, with the present state of knowledge, weak representativeness of ~~Areas of Particular Environmental Interest~~ for the mining zones. In order to better understand the deep-sea biodiversity and develop Regional Environmental Management Plans for the whole CCZ area two main recommendations are to be made: 1) to conduct more intensive sampling program of APEIs parallel to monitoring studies of contractor zones with the goal to better assess their usefulness as reservoirs of biodiversity for the mining areas, 2) to include the epibenthic sledge

among standard gears used for sampling in order to avoid missing important part of deep-sea fauna in ~~the~~ monitoring studies.

1 INTRODUCTION

The development of high- and green-tech technologies requires growing amount of rare-earth-element metals (Hein et al., 2013). Being finite resource with on-land deposits often limited or not accessible (McLellan et al., 2016) alternative sources of such elements have been investigated. The Clarion-Clipperton Zone (CCZ) situated between the Clarion and the Clipperton Fracture Zones in the central east Pacific and covering approximately 6 million km^2 (Wiklund et al., 2019) holds major portions of manganese nodule deposits and is therefore subject to intense exploration for future deep-sea mining activities (ISA, 2010). At present, the International Seabed Authority (ISA) has issued nineteen nodule exploration licenses within the CCZ (https://www.isa.org.jm/exploration-contracts/polymetallic-nodules/). Promising from the industry point of view, the deep-sea mining rises multiple concerns including technical, environmental, financial, and legal issues (IISD, 2019). The impact on the deep-sea environment may be direct (the removal of nodules~~,~~ and the destruction of the seabed during nodule~~s~~ collection) or indirect (the generation of sediment plumes, the discharge of mine tailings) (Rolinski et al., 2001; Sharma et al., 2001; Washburn et al., 2021b) and has already been proved as having serious consequences on the deep-sea communities (Jones et al., 2017; Miljutin et al., 2011; Lefaible et al. 2023; 2024). To reduce the concerns about the irreversible impact of deep-sea mining activities the conservation planning has been conducted and resulted in CCZ-Environmental Management Plan (CCZ-EMP) (ISA 2012). One of the achievements of that plan was the establishment of the network of nine, Areas of Particular Environmental Interest (APEI) (ISA, 2012) that was later updated by addition of four new APEIs in 2021 (ISA, 2021) (Fig. 1).

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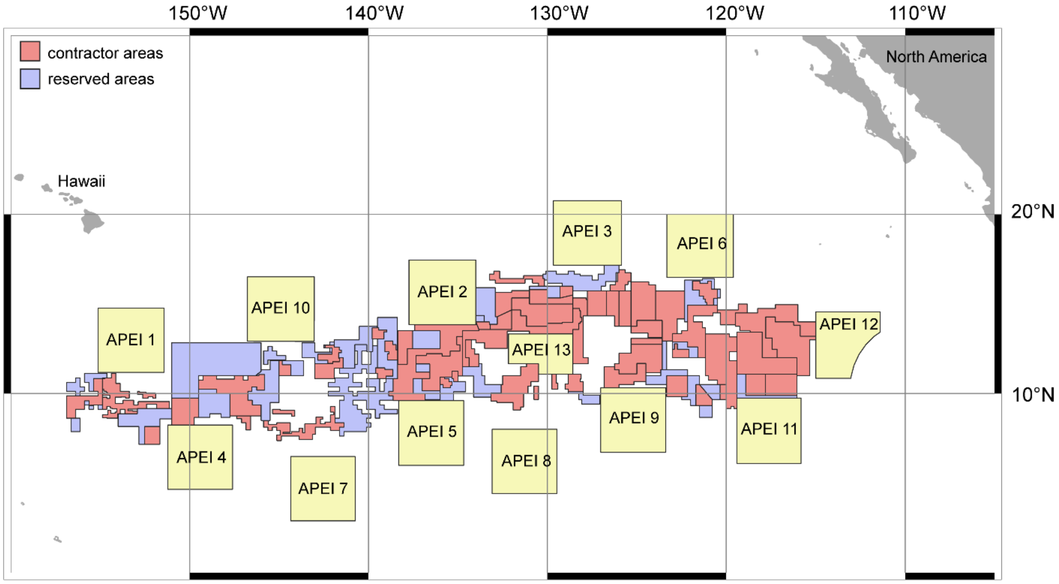

Figure 1. Exploration contract areas for polymetallic nodules in the Clarion-Clipperton Zone, central equatorial Pacific basin, APEI – Area of Particular Environmental Interest.

However, all but one APEIs are situated outside the main area prospected for deep-sea mining and initial studies suggested that the present network of APEIs may not sufficiently reflect environmental conditions as well as faunal communities of the mining zones (Błażewicz et al., 2019; McQuaid et al., 2020; Washburn et al., 2021a, b; Uhlenkott et al., 2023). The more, almost half of the protected areas lack any biodiversity information (see Fig. 1 in Rabone et al., 2023). Among further challenges associated with proper evaluation of deep-sea protection plans is still insufficient knowledge of the abyssal fauna diversity and their assemblages (e.g. Washburn et al., 2021b; Uhlenkott et al., 2023). Numerous studies have been conducted for megafauna (available to be studied also with the use of underwater camera allowing to screen larger parts of the ocean seafloor) (e.g. Uhlenkott et al., 2023) or scavengers easily collected in large numbers with baited traps (e.g. Patel et al., 2020; Bribiesca-Contreras et al., 2021; Mohrbeck et al., 2021). Also meiofauna that is traditionally sampled with multi-corers (MUC) is well represented in the studies of CCZ assemblages (e.g. Hauquier et al., 2019; Tong et al., 2022; Lefaible et al., 2024) as is also the case for sediment dwelling, less mobile macrofaunal taxa like Polychaeta or Tanaidacea collected with box cores (e.g. De Smet et al., 2017; Błażewicz et al., 2019; Pasotti et al., 2021; Washburn et al., 2021b). In contrary, moderately large (macrofauna-sized) and relatively mobile, epibenthic animals, like representatives of the crustacean order Amphipoda, are often not well represented in such studies. They are too small to be recorded with underwater camera, too large to be collected with MUC and too fast or swept away during sampling with box corer. Nevertheless, when sampled with gear designed to collect epi- and suprabenthic taxa, namely epibenthic sledge (EBS), amphipods appear to constitute diverse and sometimes abundant component of deep-sea macrofaunal communities (Frutos et al., 2017; Brandt et al., 2025) so they deserve profound studies.

Amphipoda represented in the present moment by 10,740 described species (Horton et al., 2025a), are speciose and abundant in all marine habitats from coastal waters till the deepest trenches. In the deep sea amphipod diversity and abundance is known to be high in the bathyal, decreasing towards abyssal and hadal depths where they are surpassed in diversity by another order, the Isopoda (e.g. Frutos et al., 2017; Jamieson et al. 2011). Amphipods, belonging to the superorder Peracarida, are brooders with no planktonic larval stage, so migration of species depend only on swimming capacities of adult animals. The order comprises truly pelagic species (mainly representatives of the suborder Hyperiidea) but the majority of amphipods are associated with benthic realm living either in the sediment or ~~being the~~ part of suprabenthic communities (Lowry and Myers, 2017; Brix et al., 2018). The crustaceans of this group were also proven to be important food source for higher trophic levels (Dauby et al., 200; Preciado et al., 2017).

Since the proposal of the DNA barcoding concept by Hebert et al., (2003) the use of molecular methods in species recognition has become broadly applied and often supplements morphological taxonomy (e.g. Hubert and Hanner, 2015; Seefeldt et al., 2017). Within marine and deep sea Peracarida this technique, when associated with morphological studies, often revealed hidden diversity, including the recognition of cryptic or pseudocryptic species (e.g. Bober et al., 2018; Havermans, 2016; Verheye et al., 2016).

The studies of Amphipoda in the CCZ conducted ~~by~~ now concerned mainly the scavengers and gave insights into the diversity and molecular connectivity of representatives of this tropic guild including confirmation of cosmopolitan distribution of some species (e.g. Patel et al., 2020; Bribiesca-Contreras et al., 2021; Jażdżewska et al., 2021; Mohrbeck et al., 2021). Contrariwise, the non-scavenging amphipods were never studied in detail~~s~~.

The present study aims to:

- characterize the morphological and molecular diversity of Amphipoda across three contractor zones of CCZ and one ~~Area of Particular Environmental Interest~~,
- study the amphipod species distribution and assemblages in the area,
- infer the connectivity between eastern and western part of the CCZ as well as with other deep-sea regions.

As such the research fills in the gap in knowledge of important part of the abyssal communities and brings insights into monitoring and protection plans of the zone prospected for deep-sea mining.

2 MATERIAL AND METHODS

The material for present study was collected during five scientific deep-sea cruises (Fig. 2, Table 1). The ABYSSLINE-1 (ABYSSal baseLINE project) expedition, on board the R/V *Melville*, was conducted in 2013 (Smith et al., 2013), while the ABYSSLINE-2 (ABYSSal baseLINE project) expedition, on board the R/V *Thompson*, in 2015 (Smith et al., 2015) and collected samples from the UKSR License Area (UK Seabed Resources Ltd, United Kingdom), OMS License Area (Ocean Mineral Singapore Pte. Ltd.) as well as from APEI-06. The other three expeditions, MANGAN 2016, MANGAN 2018 and MANGAN 2023 sampled the German License Area (BGR—Bundesanstalt für Geowissenschaften und Rohstoffe) on board the R/V *Kilo Moana* (MANGAN 2016 and 2023) and R/V *Sonne* (MANGAN 2018) (Rühlemann et al., 2017, 2023; Rühlemann, 2018).

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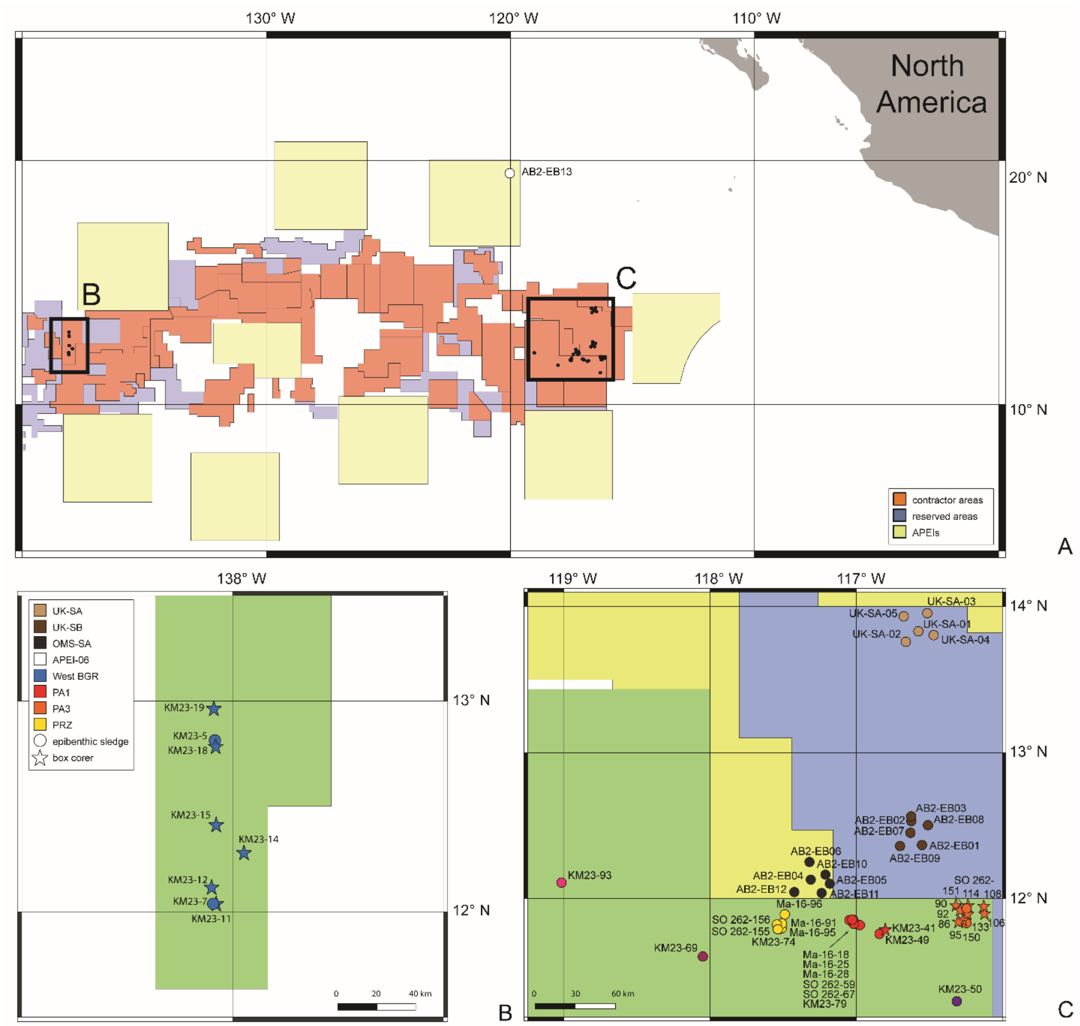

Figure 2. Localization of sampling stations. A – general view of the study area with indication of the locality of station from APEI-06 (APEI – Area of Particular Environmental Interest), B – close up of the West BGR area, C – close up of the eastern part of CCZ (East BGR, OMS and UKSR contractor areas).

Table 1. Station data including sampling gear information (EBS – epibenthic sledge, BC – box corer), total number of individuals collected, the number of individuals used for barcoding as well as number of sequences obtained. NA – no data available.

Station	Area	Latitude (N)	Longitude (W)	Depth [m]	Collection date	Gear	Total no of ind.	No of ind. for barcoding	No of seq. obtained	Sequencing success	Notes
ABYSSLINE-1											
UK-SA-01	UKSR (UK1-Stratum A)	13° 49.86'	116° 34.5'	4597	09-Oct-2013	EBS	NA	18	6	33	No information about the total abundance of Amphipoda in the sample available, no vouchers available, not used for diversity studies.
UK-SA-02	UKSR (UK1-Stratum A)	13° 45.54'	116° 39.66'	4070	12-Oct-2013	EBS	NA	2	2	100	
UK-SA-03	UKSR (UK1-Stratum A)	13° 57.3'	116° 30.84'	4143	13-Oct-2013	EBS	NA	2	1	50	
UK-SA-04	UKSR (UK1-Stratum A)	13° 48.24'	116° 28.2'	4128	17-Oct-2013	EBS	NA	25	11	44	
UK-SA-05	UKSR (UK1-Stratum A)	13° 55.98'	116° 40.5'	4196	19-Oct-2013	EBS	NA	5	3	60	
ABYSSLINE-2											
AB2-EB01	UKSR (UK1-Stratum B)	12° 22.02'	116° 33'	4209	18-Feb-2015	EBS	NA	7	6	86	No information about the total abundance of Amphipoda in the sample available, not used for diversity studies.
AB2-EB02	UKSR (UK1-Stratum B)	12° 31.86'	116° 37.38'	4219	20-Feb-2015	EBS	NA	7	5	71	
AB2-EB03	UKSR (UK1-Stratum B)	12° 33.78'	116° 37.5'	4219	23-Feb-2015	EBS	NA	7	7	100	
AB2-EB04	OMS (OMS-Stratum A)	12° 7.8'	117° 18.66'	4111	25-Feb-2015	EBS	NA	7	6	86	
AB2-EB05	OMS (OMS-Stratum A)	12° 6.12'	117° 10.86'	4100	26-Feb-2015	EBS	NA	7	5	71	
AB2-EB06	OMS (OMS-Stratum A)	12° 15.06'	117° 19.2'	4137	01-Mar-2015	EBS	NA	7	7	100	
AB2-EB07	UKSR (UK1-Stratum B)	12° 27.06'	116° 37.8'	4145	02-Mar-2015	EBS	NA	7	1	14	
AB2-EB08	UKSR (UK1-Stratum B)	12° 30.18'	116° 30.54'	4244	04-Mar-2015	EBS	NA	7	2	29	
AB2-EB09	UKSR (UK1-Stratum B)	12° 21.6'	116° 42'	4170	10-Mar-2015	EBS	NA	8	5	62	
AB2-EB10	OMS (OMS-Stratum A)	12° 9.84'	117° 12.66'	4047	11-Mar-2015	EBS	NA	8	2	25	
AB2-EB11	OMS (OMS-Stratum A)	12° 2.28'	117° 14.22'	4097	14-Mar-2015	EBS	NA	8	4	50	
AB2-EB12	OMS (OMS-Stratum A)	12° 2.7'	117° 25.44'	4223	16-Mar-2015	EBS	NA	8	7	87	
AB2-EB13	APEI-06	19° 27.9'	120° 1.5'	4026	20-Mar-2015	EBS	NA	8	6	75	
MANGAN 2016											
Ma-16-18	BGR-PA1	11° 51.372' - 11° 51.662'	117° 01.535' - 117° 00.482'	4132-4123	28-Apr-2016	EBS	8	8	8	100	
Ma-16-25	BGR-PA1	11° 49.143' - 11° 49.975'	116° 58.492' - 116° 57.797'	4107-4101	29-Apr-2016	EBS	15	15	15	100	
Ma-16-28	BGR-PA1	11° 49.654' - 11° 49.902'	117° 00.299' - 116° 59.174'	4143-4133	01-May-2016	EBS	11	11	11	100	
Ma-16-91	BGR-PRZ	11° 49.792' - 11° 49.842'	117° 30.458' - 117° 29.208'	4344-4344	09-May-2016	EBS	24	24	22	92	

Ma-16-95	BGR-PRZ	11° 47.862' - 11° 47.152'	117° 30.639' - 117° 29.490'	4356-4359	09-May-2016	EBS	28	26	24	86	2 badly damaged individuals not used for barcoding
Ma-16-96	BGR-PRZ	11° 53.492' - 11° 53.791'	117° 29.357' - 117° 28.209'	4193-4256	10-May-2016	EBS	6	6	5	83	
MANGAN 2018											
SO 262-59	BGR-PA1	11° 49.720' - 11° 50.055'	117° 01.080' - 116° 59.530'	4097-4128	22-Apr-2018	EBS	10	10	10	100	
SO 262-67	BGR-PA1	11° 51.190' - 11° 51.621'	117° 02.830' - 117° 00.804 '	4131-4131	24-Apr-2018	EBS	6	6	5	83	
SO 262-150	BGR-PA3	11° 50.009' - 11° 49.978'	116° 14.780' - 116° 13.316'	4074-4095	07-May-2018	EBS	28	27	26	96	1 ind. of Hyperiidea not used for barcoding
SO 262-151	BGR-PA3	11° 55.986' - 11° 55.992'	116° 14.706' - 116° 13.320'	4150-4151	08-May-2018	EBS	5	5	5	100	
SO 262-155	BGR-PRZ	11° 47.436' - 11° 47.677'	117° 32.213' - 117° 30.910'	4352-4351	09-May-2018	EBS	46	45	39	87	1 ind. of Hyperiidea not used for barcoding
SO 262-156	BGR-PRZ	11° 49.381' - 11° 49.752'	117° 32.663' - 117° 30.760'	4340-4340	09-May-2018	EBS	23	22	21	95	1 ind. of Hyperiidea not used for barcoding
SO 262-86	BGR-PA3	11° 50.584'	116° 17.514'	4181	29-Apr-2018	BC, 0-3 cm layer	1	1	1	100	
SO 262-90	BGR-PA3	11° 57.039'	116° 19.096'	4157	29-Apr-2018	BC, 0-3 cm layer	2	2	1	50	
SO 262-92	BGR-PA3	11° 55.449'	116° 17.439'	4172	30-Apr-2018	BC, 0-3 cm layer	2	2	2	100	
SO 262-95	BGR-PA3	11° 50.636'	116° 16.112'	4165	30-Apr-2018	BC, 0-3 cm layer	1	1	1	100	
SO 262-106	BGR-PA3	11° 53.825'	116° 07.305'	4171	02-May-2018	BC, 3-5 cm layer	1	1	1	100	
SO 262-108	BGR-PA3	11° 56.986'	116° 07.628'	4207	02-May-2018	BC, 0-3 cm layer	1	1	1	100	
SO 262-114	BGR-PA3	11° 57.017'	116° 14.157'	4175	03-May-2018	BC, 0-3 cm layer	1	1	1	100	
SO 262-133	BGR-PA3	11° 53.833'	116° 14.262'	4107	05-May-2018	BC, surface water	1	1	1	100	
MANGAN 2023											
KM23-5	West BGR	12°48.7751' - 12°50.0513'	138°05.043' - 138°05.1315'	4996-5020	16-Apr-2023	EBS	14	14	14	100	
KM23-7	West BGR	12°02.2976' - 12°03.5888'	138°05.4932' - 138°05.7092'	5109-5139	17-Apr-2023	EBS	30	29	24	83	Additional 1 badly damaged individual of Oedicerotidae not used for barcoding
KM23-49	BGR WA3 (PA1)	11°45.4904' - 11°46.3035'	116°50.4565' - 116°49.3268'	4150-4173	30-Apr-2023	EBS	52	51	37	72	1 ind. of Hyperiidea not used for barcoding
KM23-50	BGR WA4	11°17.7919' - 11°18.5445'	116°18.8626' - 116°17.6747'	4185-4182	01-May-2023	EBS	111	99	78	79	11 ind. of Hyperiidea and 1 ind. of Oxycephalidae? not used for barcoding
KM23-69	BGR WA5	11°36.252' - 11°37.1050'	118°02.981' - 118°01.2511'	4368-4356	04-May-2023	EBS	83	83	77	93	
KM23-74	BGR WA6 (PRZ)	11°47.6444' - 11°48.0414'	117°30.9650' - 117°29.5413'	4360-4364	06-May-2023	EBS	26	24	24	100	2 ind. of Hyperiidea not used for barcoding
KM23-79	BGR WA2 (PA1)	11°51.3560' - 11°51.7516'	117°01.2662' - 116°59.8924'	4126-4128	07-May-2023	EBS	16	16	14	87	
KM23-93	BGR WA7	12°06.612' - 12°07.3536'	119°01.072' - 118°59.8503'	4381-4430	10-May-2023	EBS	16	16	15	94	
KM23-11	West BGR	12°02.298'	138°05.423'	5105	18-Apr-2023	BC	6	6	6	100	
KM23-12	West BGR	12°06.8609'	138°06.0754'	5173	19-Apr-2023	BC	1	1	1	100	
KM23-14	West BGR	12°16.6825'	137°56.7545'	4976	19-Apr-2023	BC	1	0	x	x	1 ind. of Hyperiidea not used for barcoding

KM23-15	West BGR	12°24.5724'	138°04.6848'	5033	19-Apr-2023	BC	2	0	x	x	2 ind. of Hyperiidea not used for barcoding
KM23-18	West BGR	12°47.3146'	138°04.8688'	4940	20-Apr-2023	BC	2	1	1	100	1 ind. of Hyperiidea not used for barcoding
KM23-19	West BGR	12°57.7304'	138°05.3714'	5009	20-Apr-2023	BC	2	2	2	100	
KM23-41	BGR WA3 (PA1)	11°47.1427'	116°48.0835'	4169	29-Apr-2023	BC	3	3	2	67	

2.1 Sample collection and processing

The samples were collected using a Brenke-type epibenthic sledge (EBS, Brandt and Barthel, 1995; Brenke, 2005). The deployment protocol followed Brenke (2005). Upon recovery, samples were passed through 300 µm and either sorted out immediately and preserved in 80% ethanol kept at -20°C, or immediately transferred into chilled (-20°C) 96% ethanol. In the second case, the sorting by stereomicroscope was carried out after 48 h storage in a -20°C freezer (Riehl et al., 2014). A few individuals collected with box corer during MANGAN 2018 and MANGAN 2023 expeditions were added.

2.2 Morphological study

From ABYSSLINE-1 collection only a set of sequences was available, in case of ABYSSLINE-2 a representation of amphipods (6-7 per station) was given to the authors of the study. In contrast all individuals collected with EBS during MANGAN 2016, 2018 and 2023 were studied. Specimens were initially examined using a Leica M125 stereomicroscope, sorted into families and ~~preferably~~ morphospecies (groups that form phenotypic clusters without overlap between them) and later subjected to cytochrome *c* oxidase subunit I gene (COI) barcoding (details given below). Present analysis considers only non-pelagic species even though the latter are occasionally retrieved using EBS. As a result, the representatives of the suborder Hyperiidea, even if sometimes recorded in the samples were not studied further. After specification of the Molecular Operational Taxonomic Units (MOTUs) representatives of each of them were again studied morphologically (in certain cases including preparation of microscopic slides) to provide as detailed taxonomic identification as possible. The taxa from amphipod suborders other than Hyperiidea that only after the barcoding appeared to belong to pelagic taxa were excluded (although their barcodes were used for delimitation of taxa and are published).

2.3 Molecular investigation

All individuals that possessed at least head were used for the total genomic DNA extraction. The extraction was made from one pleopod (if the posterior part of the body was missing the last remaining leg).

The DNA extraction of individuals collected during all expeditions apart from MANGAN 2023 was performed using 100 µl InstaGene Matrix (BIO-RAD). Digestion was carried out at 56°C for 40 min. The extraction of DNA from individuals from MANGAN 2023 was carried out using a mixture of 112,5 µl pure H_2O with 0.011 g Chelex (Sigma-Aldrich Co.) and 7,5 µl proteinase K. The digestion at 55 °C lasted for 6 h.

The DNA barcoding fragment of COI (658 bp) was amplified using universal LCO1490 (GGTCAACAAATCATAAAGATATTGG) and HCO2198 (TAAACTTCAGGGTGACCAAAAAATCA) primer pair (Folmer et al., 1994; collection from ABYSSLINE-1) or the degenerate LCO1490-JJ (CHACWAAYCATAAAGATATYGG) and HCO2198-JJ (AWACTTCVGGRTGVCCAAARAATCA) primer pair (Astrin and Stüben, 2008; collections from ABYSSLINE-2, MANGAN 2016, MANGAN 2018). The polymerase chain reaction was performed with AccuStart II PCR SuperMix (Quantabio) and the reaction conditions following Hou et al. (2007) (Table 2). Sequencing was done bi-directionally in Macrogen Inc. (the Netherlands) on an Applied Biosystems 3730xl capillary sequencer. Electropherograms were viewed in Geneious 10.2.6 and primer sequences and ambiguous positions were trimmed.

The COI barcodes of Amphipoda from MANGAN 2023 were obtained with Oxford Nanopore Technologies sequencing (ONT). The marker was amplified using a primer mix containing LCO1490-JJ and HCO2198-JJ primers and a tagging sequence specific to each sample, which consisted of a 9 nt (Srivathsan et al., 2023). Samples were amplified in batches, in sets consisting of three 96-well plates. Each sample consisted of 6.5 µl of Dream Taq PCR Mastermix (Thermo Scientific), 2µl of primers mix, 1 µl of matrix DNA and 3.5 µl of H_2O to reach 13 µl of final volume. The PCR conditions were modified from Hou et al. 2007 (Table 2).

Table 2. PCR conditions.

	For Sanger sequencing (Hou et al. 2007)			For ONT sequencing (Hou et al. 2007 modified)		
Step	Temp [°C]	Time [s]	No of cycles	Temp [°C]	Time [s]	No of cycles
Initial denaturation	95	300		95	300	

Denaturation	95	30	5	95	50	5
Annealing	45	90		45	50	
Extension	72	60		72	60	
Denaturation	95	30	35	95	50	35
Annealing	51	90		51	50	
Extension	72	60		72	60	
Final extension	72	300		72	120	

Amplicons in a volume of 5 µl each were pooled and purified according to the Sera-Mag protocol. ~~In the next step, concentration of DNA in the sample using a Qubit 4 fluorometer (Thermo Fisher Scientific) was measured and the Qubit dsDNA BR Assay Kit reagent (Thermo Fisher Scientific)~~. Purified DNA was stored at -20°C until the next steps were initiated. Sequencing was performed according to the Ligation Sequencing Amplicons V14 (SQK-LSK114) protocol (Oxford Nanopore Sequencing 2024). The procedure included editing the ends of barcode sequences, attaching an adapter protein, and loading and running the reaction plate (Flongle). Sequencing took about 24 hours.

The obtained data were then subjected to a decoding process (basecallig). This step involves assigning voltage changes to the corresponding nucleotides to obtain a complete sequence (Wang et al., 2021). It was followed by demultiplexing using the ONTbarcoder tool. This process involves assigning the obtained sequences to a specific sample based on the tagging sequences used earlier. In addition, ONTbarcoder evaluates the quality of the obtained reads, creating a consensus barcode sequence (Srivathsan et al., 2021).

Sequences were blasted using default parameters on NCBI BLASTn and translated into amino acid sequences to confirm that no stop codons were present. All sequences were deposited in GenBank with the accession numbers: PQ734211 – PQ734784. Relevant voucher information, taxonomic classifications and sequences are deposited in the data set "DS-AMPHICCZ" in the Barcode of Life Data System (BOLD) (dx.doi.org/10.5883/DS-AMPHICCZ) (www.boldsystems.org) (Ratnasingham and Hebert, 2007).
All sequences were aligned with the MAFFT v.7.308 algorithm (Katoh et al., 2002; Katoh and Standley, 2013) in Geneious 10.2.6, resulting in a 614 bp alignment and subsequently three molecular species delimitation methods were applied to reveal the Molecular Operational Taxonomic Units (MOTUs). Two methods were distance-based: Barcode Index Number (BIN) System (Ratnasingham and Hebert, 2013), and the Assemble Species by Automatic Partitioning

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(ASAP) (Puillandre et al., 2021) and were supplemented by one tree-based phylogenetic approach using Generalized Mixed Yule Coalescent (GMYC) model-based method (Pons et al., 2006), according to Monaghan et al. (2009).

The Barcode Index Number (BIN) System (Ratnasingham and Hebert, 2013) in BOLD compares newly submitted sequences with the sequences already available. They are clustered according to their molecular divergence using distance-based algorithms (single linkage clustering followed by Markov clustering) that aim at finding discontinuities between Operational Taxonomic Units (OTU). Each OTU receives a unique and specific code (BIN), either already available or new if submitted sequences do not cluster with already-known BINs.

The Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al., 2021) is a method that uses pairwise genetic distances to assemble individuals into groups and proposes species partitioning ranked according to a scoring system. For the purpose of the present study the first two partitions characterized by the lowest ASAP-scores were taken into account.

The GMYC method defines MOTUs through identification of the switch from intraspecific branching patterns (coalescent) to interspecific species branching patterns (Yule process) on a phylogenetic tree. Because for GMYC an ultrametric tree is required, as an input, a Bayesian tree was reconstructed in BEAST 2.6.3 (Bouckaert et al., 2019). The site model was set up with bModelTest (Bouckaert and Drummond, 2017). The tree prior was set to Birth-Death following Bayes factors. Two runs of Markov chain Monte Carlo (MCMC) were performed each 1 M generations-long, sampled every 1,000 generations. Runs were examined for convergence in Tracer 1.7 (Rambaut et al., 2018). The final tree was summarised with TreeAnnotator 2.6.3 with 25% burn-in, all being part of BEAST 2.6.3 package. The Bayesian tree was uploaded into the R 4.0.5 (R Core Team 2021) software package 'SPLITS' (Species Limits by Threshold Statistics) (Ezard et al., 2009) and analyzed using the single threshold model.

To visualize the delimitation results Neighbour-Joining (NJ) trees of all sequences divided into seven different family groups (details of each alignment provided in the Supplementary Material S1) were built based on the uncorrected *p*-distance matrix, with both transitions and transversions included and partial deletion chosen (Saitou and Nei 1987) in MEGA v.11.0.13 (Tamura et al., 2021). Node support was inferred with a bootstrap analysis (1000 replicates) (Felsenstein, 1985). The lineages were collapsed according to their BINs assignment. The COI sequence of

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freshwater isopod from the family Asellidae, *Asellus aquaticus* (Linnaeus, 1758) (GenBank accession number MN810583; Raupach et al., 2022), was used as an outgroup.

In case of taxa represented in our dataset by five or more sequences (and at least two haplotypes) the molecular divergence of COI haplotypes (with all ambiguous positions excluded) was presented as Median Joining Network generated using PopART 1.7 (Bandelt et al., 1999).

2.4 Species distribution

The distribution of each recognized MOTU within the CCZ was checked. Additional check was done to infer which MOTUs were already recorded elsewhere in the World. To infer the range sizes of the MOTUs recorded pairwise distances between all stations were calculated with QGIS 3.36 (QGIS, 2021) using standard distance plugin. For each MOTU that was recorded at more than one station the largest distance between the stations where they occurred was noted (given in values rounded to full kilometres).

Thanks to very high sequencing success associated with samples collected during three MANGAN expeditions that allowed to ascribe more than 80% of individuals caught to separate MOTUs, their composition in each haul was used in community analyses. The similarities between samples were calculated using the Bray–Curtis similarity formula on relative abundances of the MOTUs in each sample. Due to the fact that the difference between the most and the least abundant species was more than 20 times, and to increase the importance of singletons species which constituted 50% of all taxa, square root transformation of data was used. Hierarchical agglomerative clustering was performed using the group average method.

Each assemblage discriminated was characterized using taxon richness (number of BINs), rarefaction (ES(50)), diversity (Shannon index) and evenness (Pielou index) indices (Magurran, 2004).

All figures were adjusted for publication in Adobe Illustrator CS6.

3 RESULTS

Within the collection of 708 individuals 23 known and one undescribed families were identified (Table 3). The latter is presently in the description process (Horton et al., 2025e). Some representatives of the superfamilies Isaeoidea, Photoidea and Aoroidea were not identified further and are presented as “Corophioidea indet.”, whereas unidentified specimens of one of the three families: Lysianassidae, Tryphosidae and Uristidae are left as "Lysianassoidea indet.". The COI gene analysis allowed to obtain 581 sequences that gave overall sequencing success at the level of 82%. The families that dominated barcoded material were Pardaliscidae (123 ind.) and Eusiridae (122 ind.). The other families that contributed largely to the material were: Synopiidae (74 ind.), Phoxocephalidae (70 ind.) and Oedicerotidae (51 ind.).

Table 3. Number of individuals studied (No of ind.), COI sequences, Barcode Index Numbers (BINs), consensus number of Molecular Operational Taxonomic Units (MOTUs) based on different delimitation methods recognized in each family as well as the reference to the figure with NJ tree and MJN. Systematic division based on Myers and Lowry (2017), available online in World Amphipoda Database (Horton et al., 2025a).

	Suborder	**Parvorder**	**Superfamily**	**Taxon**	**COI**	**BINs**	**MOTUs**	**tree & MJN**
1.	Amphilochidea	Amphilochidira	Iphimedioidea	Stilipedidae	2	2	2	Fig. 3
2.			Leucothoidea	Leucothoidae	3	2	2	
3.		Eusiridira	Eusiroidea	Eusiridae	122	32	32	
4.			Liljeborgioidea	Liljeborgiidae	4	4	4	
5.		Haustoriidira	Haustorioidea	Phoxocephalidae	70	14	14	Fig. 4
6.		Lysianassidira	Alicelloidea	Alicellidae	1	1	1	Fig. 5
7.				Valettiopsidae	1	1	1	
8.				Vemanidae	1	1	1	
9.			Lysianassoidea	Cyphocarididae	4	2	2	
10.				Hirondelleidae	2	1	1	
11.				Tryphosidae	23	6	6	
12.				Uristidae	7	6	6	
13.				Lysianassoidea indet.[b]	16	6	6	
14.			Stegocephaloidea	Stegocephalidae	9	5	5	
15.		Oedicerotidira	Oedicerotoidea	Oedicerotidae	51	22	21	Fig. 6
16.		Synopiidira	Dexaminoidea	Lepechinellidae	5	3	3	Fig. 7
17.				Melphidippidae	2	1	1	
18.				Pardaliscidae	123	44	44	
19.			Synopioidea	Ampeliscidae	13	3	3	Fig. 8
20.				Synopiidae	74	31	31	
21.	Hyperiopsidea	Hyperiopsidira	Hyperiopsoidea	Hyperiopsidae	7	4	4	Fig. 9
22.	Senticaudata	Caprellidira	Caprelloidea	Dulichiidae	1	1	1	
23.		Corophiidira	Aorioidea	Unciolidae	2	1	1	
24.		Corophioidea indet.[a]			19	5	5	
25.		Hadziidira	Calliopioidea	Calliopiidae	2	1	1	
26.				Fam. nov.	12	5	5	
27.	Amphipoda indet.				5	4	4	
				Total	**581**	**208**	**207**	

The delimitation methods identified from 184 (ASAP with the lowest ASAP-score, threshold distance – 0.085925) up to 270 (GMYC) MOTUs (Figs 3–9). Although large variation in absolute numbers the differences in the number of recognized MOTUs concerned a small number of taxa and in 17 cases indicated obvious oversplitting of single units. The consensus delimitation identified 207 MOTUs that are congruent with BINs ascription with an exception of one unit within Oedicerotidae, namely two BINs of *Bathymedon* sp. (BOLD:AEA9456 and BOLD:AGC2705) that according to all other methods constitute one MOTU. The *p*-distance between these two BINs is 0.025 but only two sequences are available so it is impossible to infer the intraspecific variation in this case. There are additional six cases where consensus delimitation shall be treated with caution: two within the families: Phoxocephalidae (Fig. 4) and Synopiidae (Fig. 8), one within Pardaliscidae (Fig. 7) as well as within undescribed family of Senticaudata (Fig. 9). In all presented cases additional genes and preferably more individuals shall be studied to help with final delimitation of these taxa.

The family with the largest number of identified taxa was Pardaliscidae (44 MOTUs), followed by Eusiridae (32 MOTUs) and Synopiidae (31 MOTUs). The most species rich genus was *Rhachotropis* (Eusiridae) where 14 MOTUs were identified. It was followed by *Cleonardo* (Eusiridae) represented by 11 MOTUs and *Syrrhoe* (Synopiidae) with 9 MOTUs recognized. *Caleidoscopsis* (8 MOTUs) and *Pardaliscopsis* (6 MOTUs) were the two genera within Pardaliscidae with the highest number of taxa delimited. Other recognized genera were represented by smaller number of species but large part of the material (particularly from MANGAN 2023 expedition) was identified only to the family level.

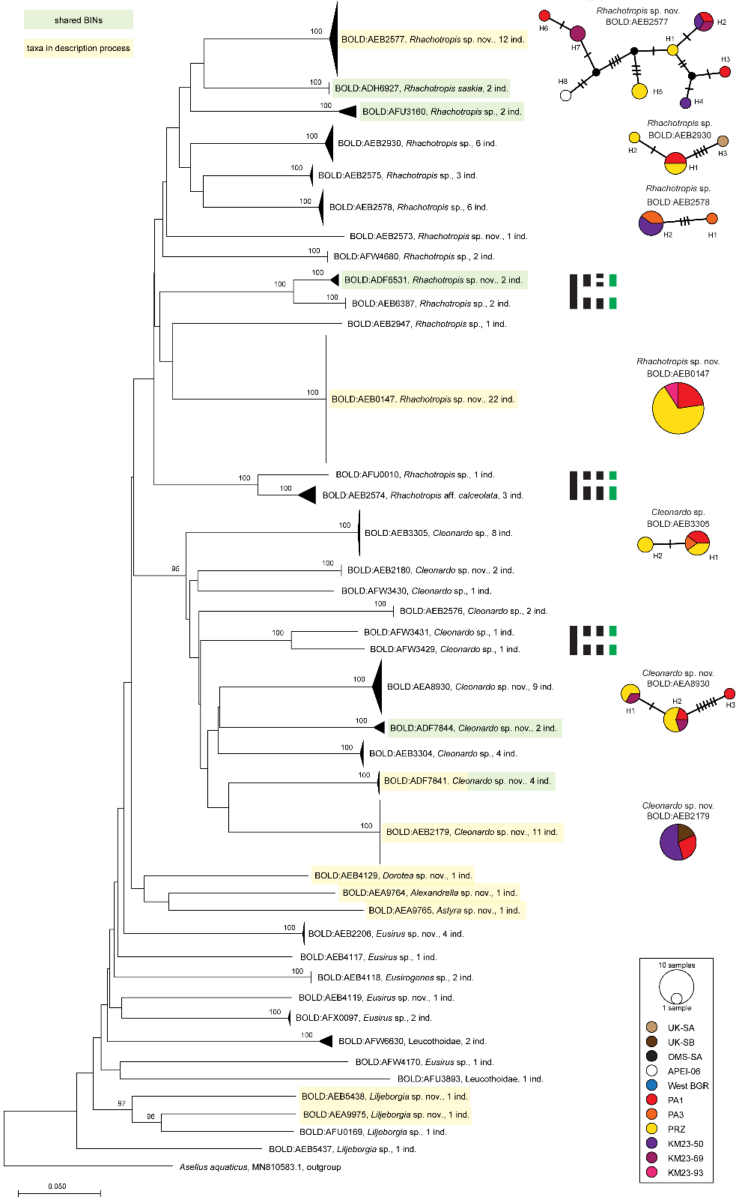

Figure 3. Neighbour-joining (NJ) tree of COI sequences of amphipods belonging to the families Eusiridae, Leucothoidae, Liljeborgiidae, and Stilipedidae (parvorders Amphilochidira and Eusiridira). The distances were calculated with p-distance. Triangles indicate the relative number of individuals studied (height) and sequence divergence (width). The numbers in front of the nodes indicate bootstrap support (1000 replicates, only values higher than 50% are presented). The vertical bars represent species delimitations taxonomies obtained from different species delimitation methods (A1: BIN, A2: ASAP, G: GMYC, C: consensus). Only the cases where incongruence between different delimitation methods were observed are shown. Note that this tree is not the reconstruction of evolutionary history of presented taxa.

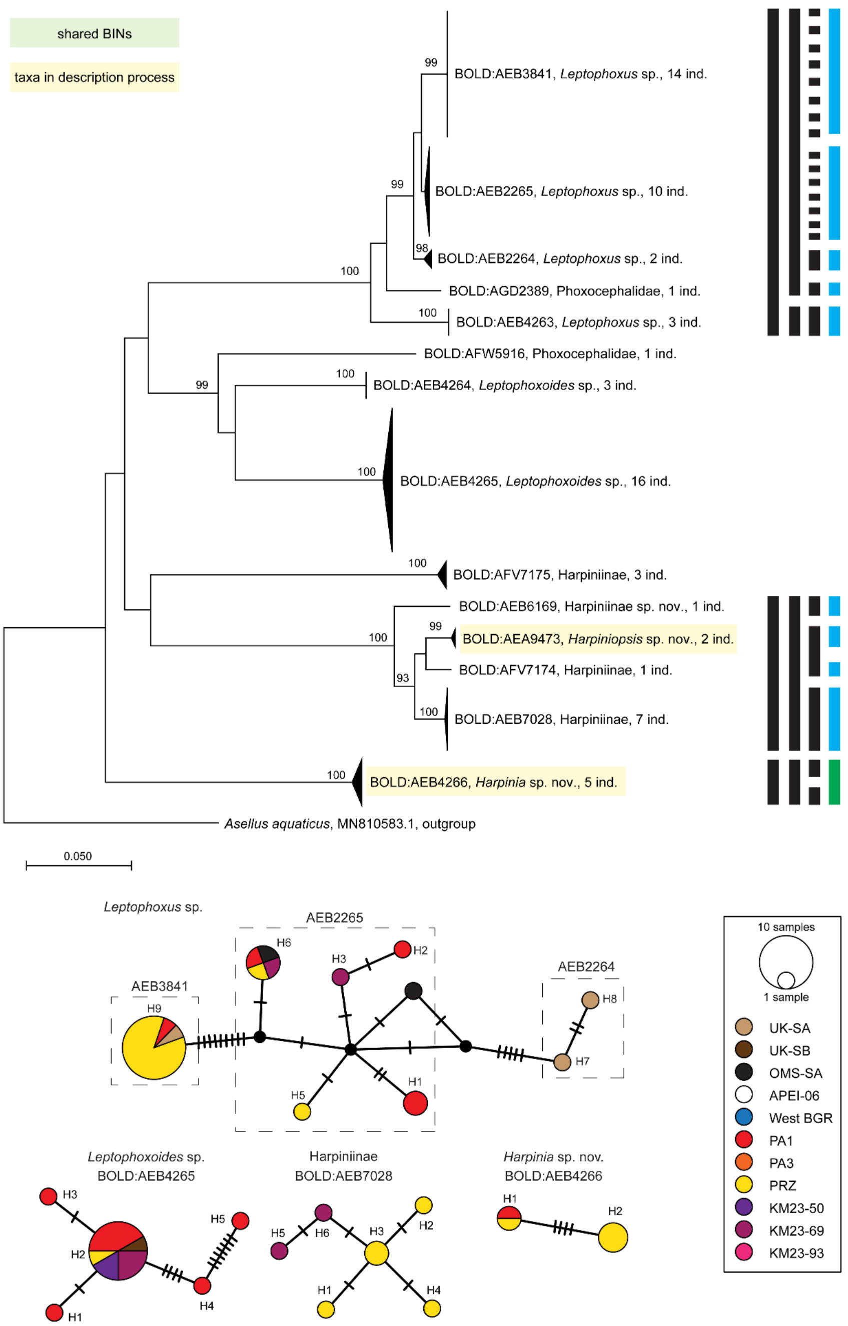

Figure 4. Neighbour-joining (NJ) tree of COI sequences of amphipods belonging to the family Phoxocephalidae. Details about the calculation and presentation of the tree as in Fig. 3

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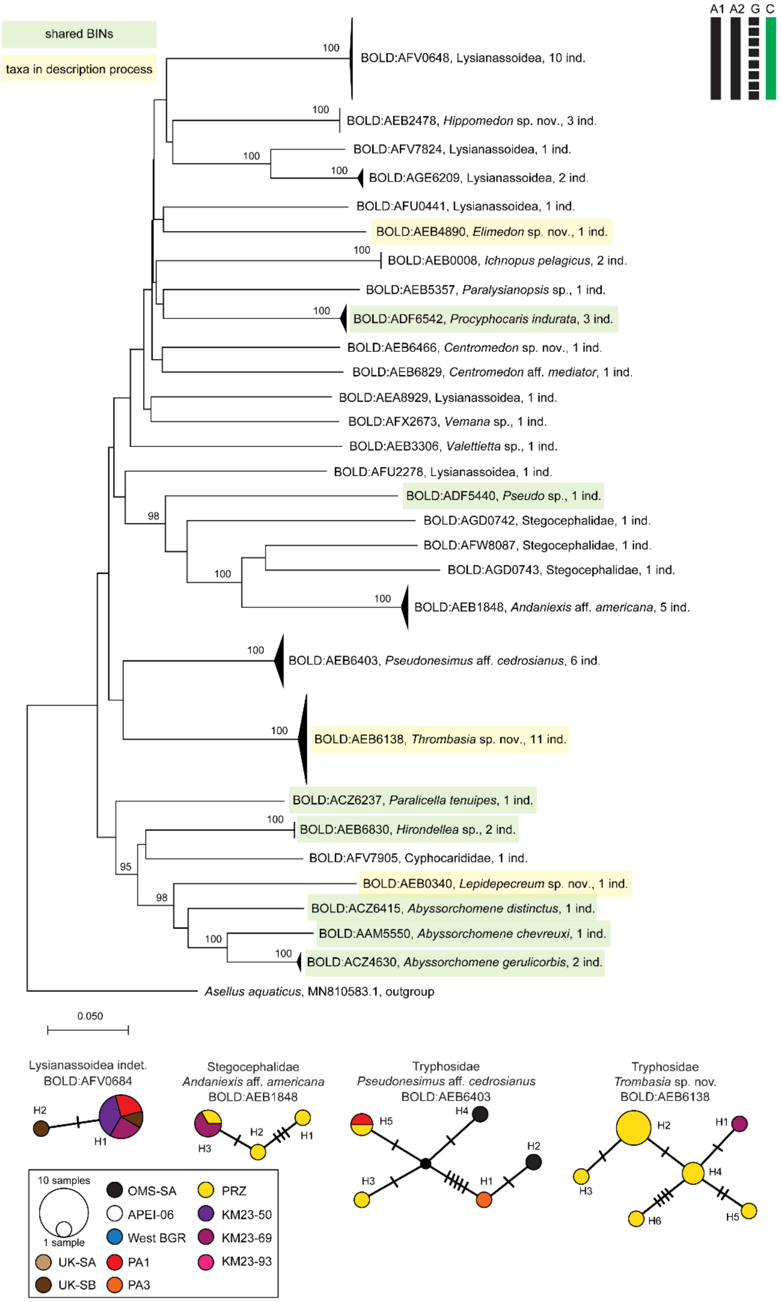

Figure 5. Neighbour-joining (NJ) tree of COI sequences of amphipods belonging to the parvorder Lysianassidira (for families see Table 3). Details about the calculation and presentation of the tree as in Fig. 3.

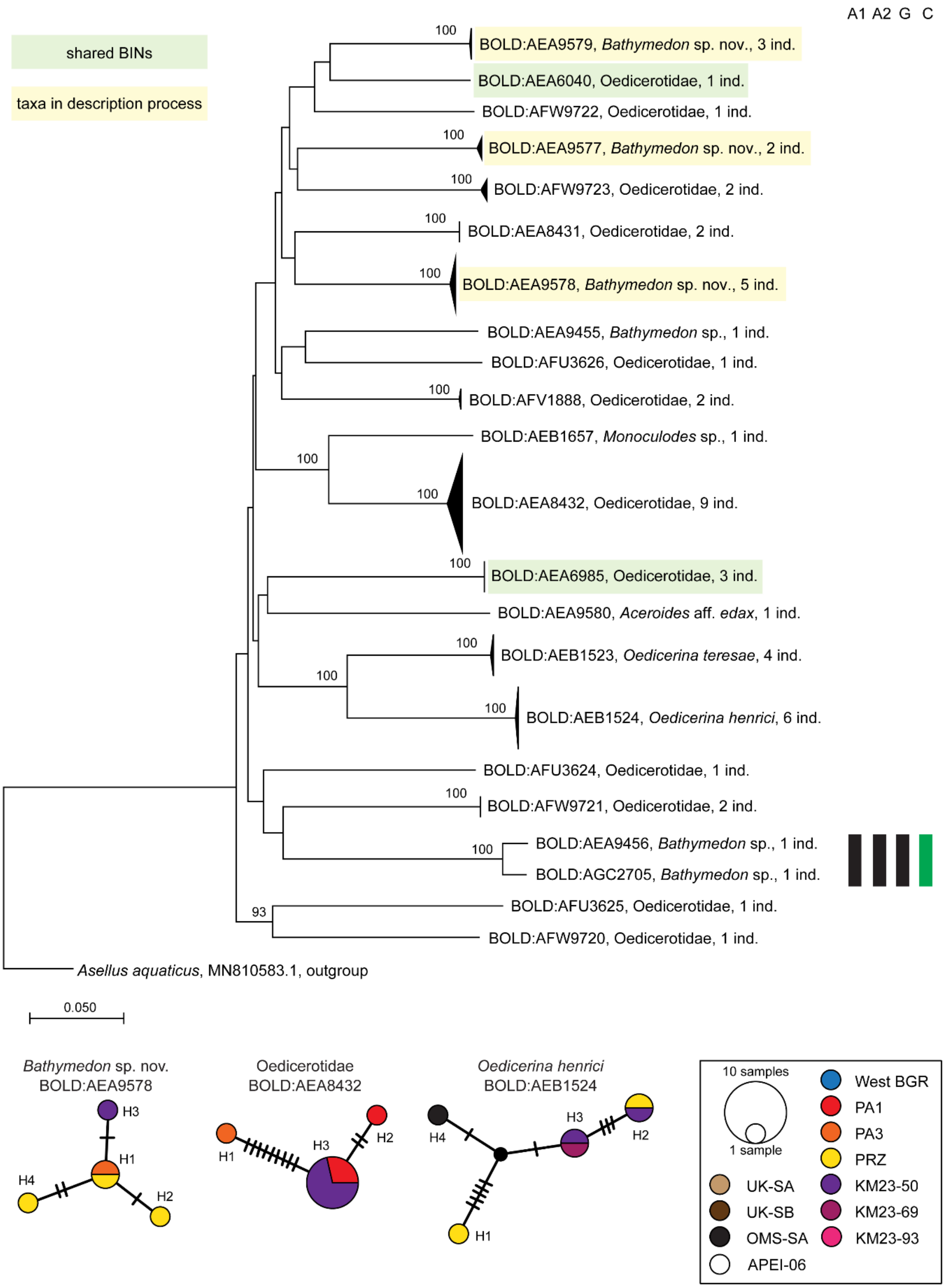

Figure 6. Neighbour-joining (NJ) tree of COI sequences of amphipods belonging to the family Oedicerotidae. Details about the calculation and presentation of the tree as in Fig. 3.

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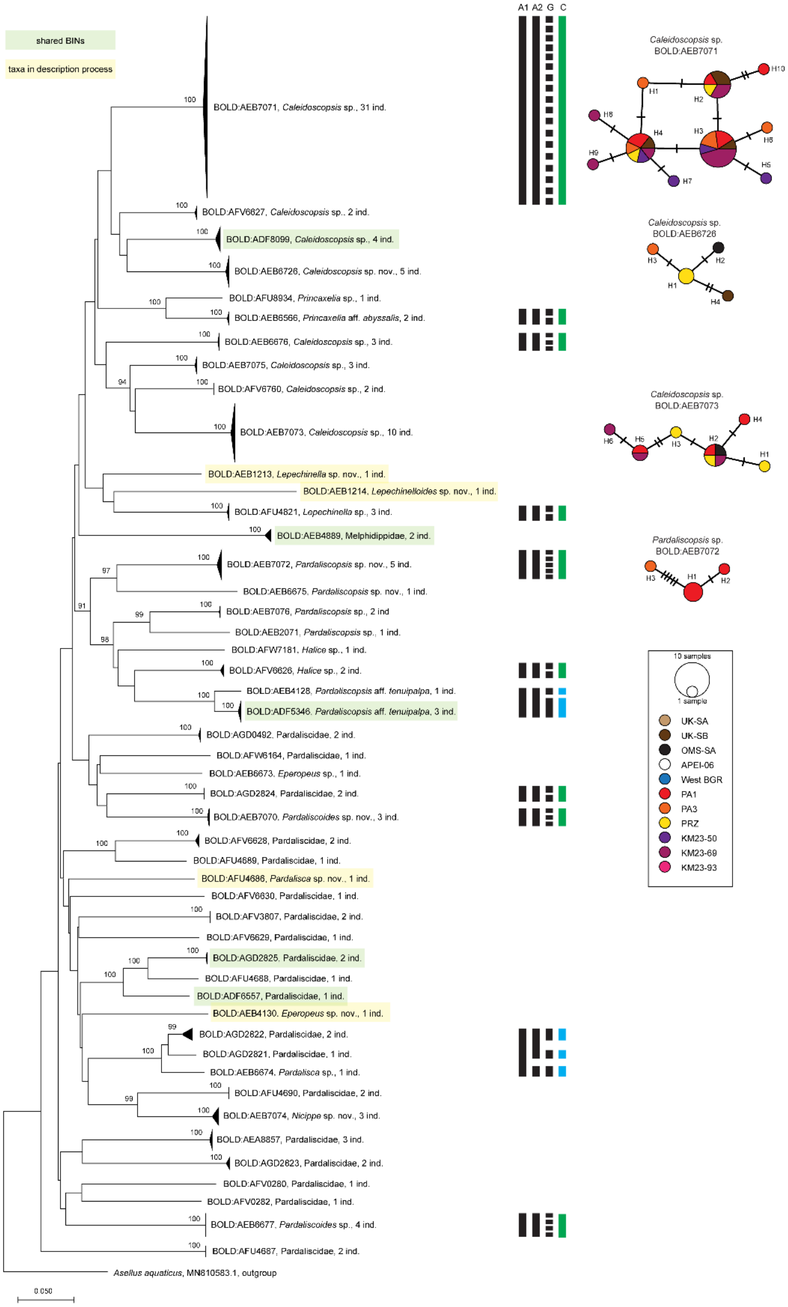

Figure 7. Neighbour-joining (NJ) tree of COI sequences of amphipods belonging to the families Pardaliscidae, Lepechinellidae, Melphidippidae. Details about the calculation and presentation of the tree as in Fig. 3.

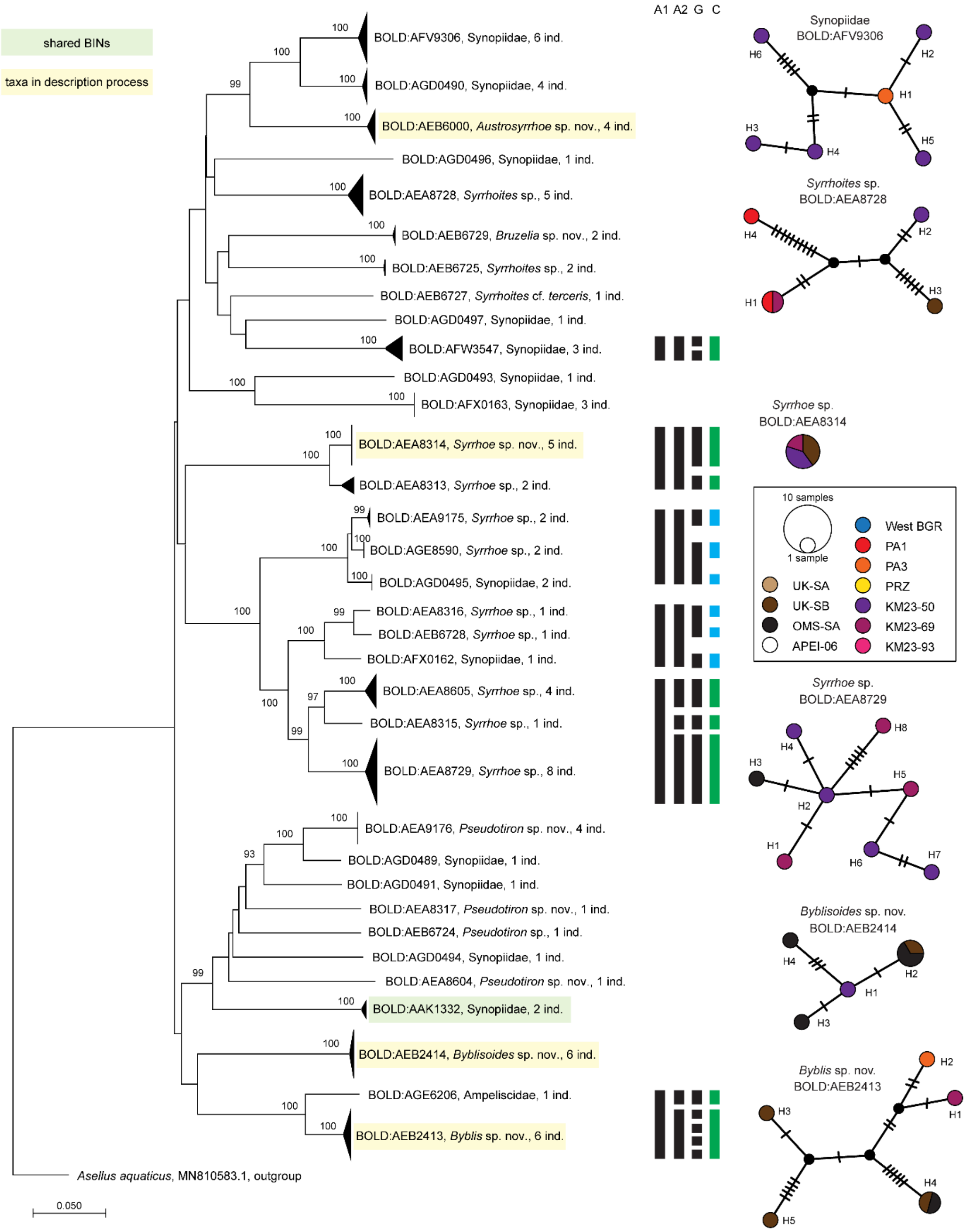

Figure 8. Neighbour-joining (NJ) tree of COI sequences of amphipods belonging to the families Synopiidae, Ampeliscidae. Details about the calculation and presentation of the tree as in Fig. 3.

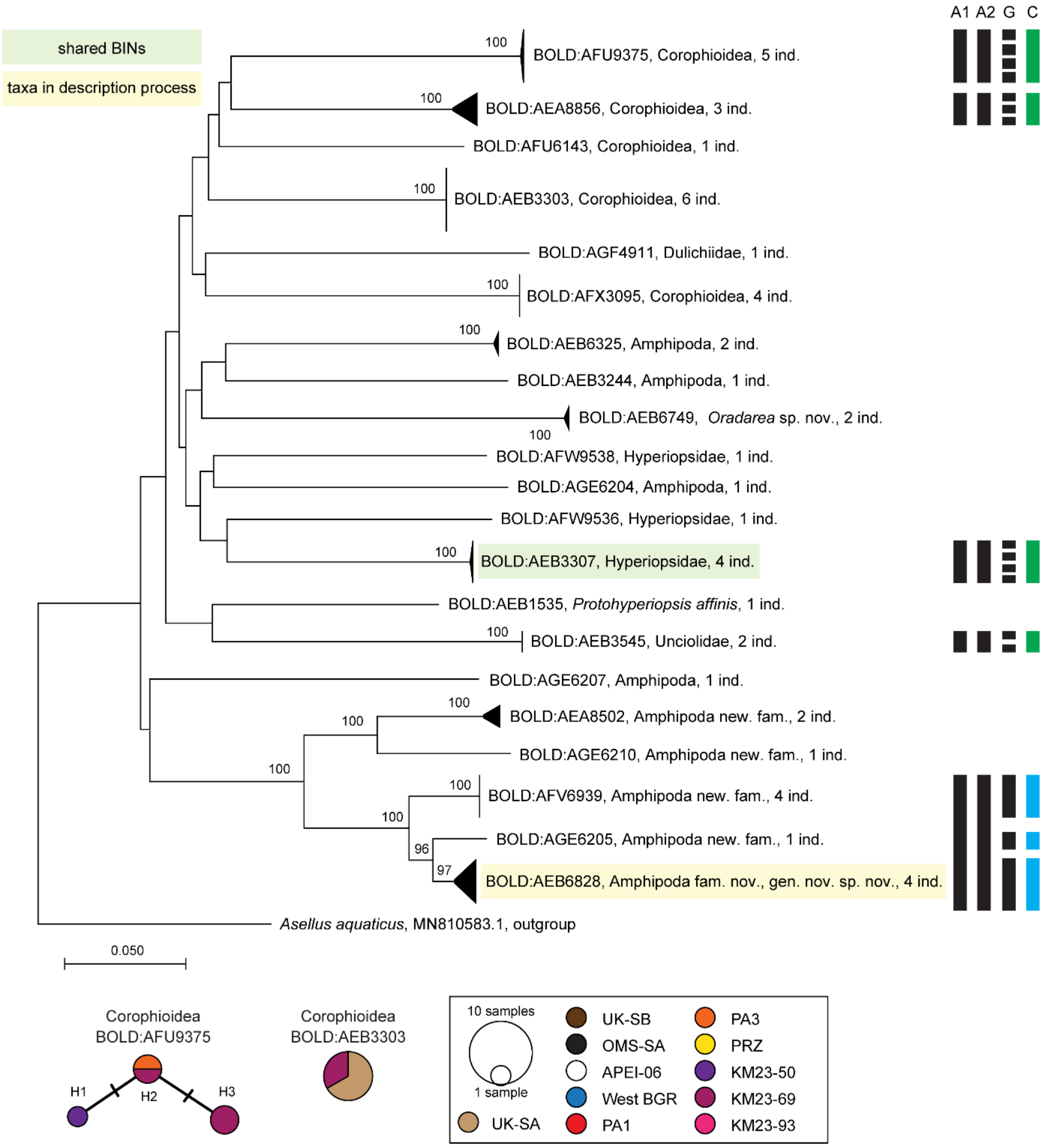

Figure 9. Neighbour-joining (NJ) tree of COI sequences of amphipods belonging to the families Dulichiidae, Calliopiidae, Hyperiopsidae, Unciolidae, Mirabestidae fam. nov. and unidentified taxa. Details about the calculation and presentation of the tree as in Fig. 3.

Combined morphological and molecular identification revealed ten known species: *Rhachotropis saskia* (Eusiridae), *Abyssorchomene chevreuxi, A. distinctus, A. gerulicorbis, Ichnopus pelagicus*

(Uristidae), *Paralicella tenuipes* (Alicellidae), *Procyphocaris indurata* (Cyphocarididae), *Oedicerina henrici, O. teresae* (Oedicerotidae, described on the basis of presently studied material, Jażdżewska et al., 2022), and *Protohyperiopsis affinis* (Hyperiopsidae). Additional seven taxa were identified as closely resembling known species but with some level of uncertainty: *Rhachotropis* aff. *calceolata* (Eusiridae), *Centromedon* aff. *mediator* (Uristidae), *Pseudonesimus* aff. *cedrosianus* (Tryphosidae), *Andaniexis* aff. *americana* (Stegocephalidae), *Aceroides* aff. *edax* (Oedicerotidae), *Princaxelia* aff. *abyssalis, Pardaliscopsis* aff. *tenuipalpa* (Pardaliscidae). Final identification was often hindered by the presence of only single representatives of particular taxon and the damage of the material. Apart from known species the study of the material allowed to recognize one undescribed family (Horton et al., 2025e) and at least 48 undescribed species; two of them were already described based on the present material (Jażdżewska et al., 2022), while 27 are in the description process (Andrade and Jażdżewska, 2025; Biniek et al., 2025; Horton and Lörz, 2025; Horton et al., 2025b-e; Hughes and Tandberg, 2025a, b; Jażdżewska and Horton, in prep, Lörz et al., 2025; Peart and Lörz 2025; Peart and Stewart, 2025; Tandberg and Huges, 2025; Timm et al., 2025; Wróblewski and Jażdżewska, 2025).

Out of 207 MOTUs recognized as many as 94 were singletons (45% of taxa) while additional 49 – doubletons (24% of taxa), only 32 (15%) reached the criterium of being represented by more than five individuals and their haplotypes networks are presented (Figs 3–9). Half of these MOTUs were represented by one to three haplotypes differing in few mutations. Higher diversity of haplotypes was recorded within one MOTU of Eusiridae (*Rhachotropis* sp. nov., BOLD:AEB2577), three MOTUs of Phoxocephalidae (*Leptophoxus* sp., BOLD:AEB2265, *Leptophoxoides* sp., BOLD:AEB4265, Harpiniinae, BOLD:AEB7028), two MOTUs of Tryphosidae (*Pseudonesimus* aff. *cedrosianus*, BOLD:AEB6403, *Thrombasia* sp. nov., BOLD:AEB6138), two MOTUs of Oedicerotidae (*Oedicerina henrici*, BOLD:AEB1524, *Bathymedon* sp. nov., BOLD:AEA9578), three MOTUs of Pardaliscidae (*Caleidoscopsis* sp., BOLD:AEB7071, *Caleidoscopsis* sp., BOLD:AEB6726, *Caleidoscopsis* sp., BOLD:AEB7073), three MOTUs of Synopiidae (*Syrrhoites* sp., BOLD:AEA8728, *Syrrhoe* sp., BOLD:AEA8729, Synopiidae, BOLD:AFV9306), and two of Ampeliscidae (*Byblis* sp. nov., BOLD:AEB2413, *Byblisoides* sp. nov., BOLD:AEB2414) (Figs 3–9).

No MOTUs represented by more than five individuals was shared between ~~East~~ and ~~West~~ regions of the CCZ as well as between ~~West~~ region of CCZ and APEI-06. Only one, *Rhachotropis* sp. nov. (BOLD:AEB2577) was shared between ~~East~~ region and APEI-06. In a broader scale 187 recognized BINs (and subsequently 186 MOTUs) were unique for the present study, while the remaining 21 were shared with other studies and belonged to broadly distributed taxa (Table 4).

Table 4. MOTUs represented by Barcode Index Numbers (BIN) shared between present and former studies with information about their taxonomic affiliation, geographic distribution and ecology. Areas: UKSR – UK contractor area, OMS – Singapore contractor area, BGRE –BGR contractor area East, BGRW – BGR contractor area West, KKT – Kuril-Kamchatka Trench area, NW Pacific – North West Pacific, area not specified, NE Pacific – North East Pacific, area not specified. Asterisk indicates cases where feeding type was confirmed for particular taxon. In case of wide distribution and repeated ecological studies only representative publications cited.

Family	BIN	Taxon name	Present study		Distribution		Ecology		
			No. ind.	Area	Outside CCZ	References	Feeding type	Mobility	References
Alicellidae	BOLD:ACZ6237	*Paralicella tenuipes*	1	UKSR	Cosmopolitan	Jażdżewska et al. 2021	scavenger*	highly mobile swimmer	Jażdżewska et al. 2021
Cyphocarididae	BOLD:ADF6542	*Procyphocaris indurata*	3	BGRE, OMS	KKT	Jażdżewska and Mamos 2019	unknown	abyssopelagic / epibenthic swimmer	Hendrycks and Conlan 2003
Eusiridae	BOLD:ADF7841	*Cleonardo* sp. nov.	4	BGRE, OMS,	KKT	Jażdżewska and Mamos 2019	predator	epibenthic swimmer	Bousfield and Hendrycks 1995
Eusiridae	BOLD:ADF7844	*Cleonardo* sp. nov.	2	BGRE	KKT	Jażdżewska and Mamos 2019	predator	epibenthic swimmer	Bousfield and Hendrycks 1995
Eusiridae	BOLD:ADF6531	*Rhachotropis* sp.	2	BGRE	KKT	Jażdżewska and Mamos 2019	predator	epibenthic swimmer	Bousfield and Hendrycks 1995
Eusiridae	BOLD:ADH6927	*Rhachotropis saskia*	2	UKSR, BGRW	KKT	Lörz et al. 2018, Jażdżewska and Mamos 2019	predator*	epibenthic swimmer	Lörz et al. 2018
Eusiridae	BOLD:AFU3160	*Rhachotropis* sp.	2	BGRW	NE Pacific	Jażdżewska, unpublished	predator	epibenthic swimmer	Lörz et al. 2018
Hirondelleidae	BOLD:AEB6830	*Hirondellea* sp.	2	BGRE	Indian Ocean	Kniesz et al. 2022	scavenger*	highly mobile swimmer	Kniesz et al. 2022
Hyperiopsidae	BOLD:AEB3307	Hyperiopsidae	4	UKSR, BGRE	NE Pacific	Jażdżewska, unpublished	unknown	pelagic	Barnard and Karaman 1991
Melphidippidae	BOLD:AEB4889	Melphidippidae	2	BGRE	NE Pacific	Jażdżewska, unpublished	passive suspension feeder	epibenthic, fast swiming	Enequist 1949, De Broyer et al. 2001
Oedicerotidae	BOLD:AEA6040	Oedicerotidae	1	BGRW	NW Pacific	Jażdżewska, unpublished	deposit feeder or carnivorous	sediment dweller	Brix et al. 2018
Oedicerotidae	BOLD:AEA6985	Oedicerotidae	3	BGRW	NW Pacific	Jażdżewska, unpublished	deposit feeder or carnivorous	sediment dweller	Brix et al. 2018
Pardaliscidae	BOLD:ADF8099	*Caleidoscopsis* sp.	4	BGRW	KKT	Jażdżewska and Mamos 2019	predator	epibenthic or demersal	Hendrycks and Conlan 2003, Brix et al. 2018
Pardaliscidae	BOLD:ADF5346	*Pardaliscopsis aff. tenuipalpa*	3	BGRE	KKT	Jażdżewska and Mamos 2019	predator	epibenthic or demersal	Hendrycks and Conlan 2003, Brix et al. 2018
Pardaliscidae	BOLD:ADF6557	Pardaliscidae	1	BGRW	KKT	Jażdżewska and Mamos 2019	predator	epibenthic or demersal	Hendrycks and Conlan 2003, Brix et al. 2018
Pardaliscidae	BOLD:AGD2825	Pardaliscidae	2	BGRE	NE Pacific	Jażdżewska, unpublished	predator	epibenthic or demersal	Hendrycks and Conlan 2003, Brix et al. 2018
Stegocephalidae	BOLD:ADF5440	*Pseudo* sp.	1	BGRE	KKT	Jażdżewska and Mamos 2019	predator	bentho-pelagic	Brix et al. 2018
Synopiidae	BOLD:AAK1332	Synopiidae	2	BGRE	Belize	Browne et al. 2007	unknown	pelagic	Browne et al. 2007 (collected at 0–10 m in the open waters)
Uristidae	BOLD:AAM5550	*Abyssorchomene chevreuxi*	1	BGRE	widely distributed	Havermans et al. 2010, Fujii et al. 2013, Horton et al. 2020	scavenger*	highly mobile swimmer	Horton et al. 2020

https://doi.org/10.5194/egusphere-2025-1794
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Uristidae	BOLD:ACZ6415	*Abyssorchomene distinctus*	1	BGRE	widely distributed	Kniesz et al. 2022, Dupont et al. 2024,	scavenger*	highly mobile swimmer	Patel et al. 2020
Uristidae	BOLD:ACZ4630	*Abyssorchomene gerulicorbis*	2	BGRE	widely distributed	Barnard and Shulenberger 1976, Horton et al. 2020,	scavenger*	highly mobile swimmer	Barnard and Shulenberger 1976

Of them nine species were shared between CCZ and the region of Kuril-Kamchatka Trench. Two MOTUs were shared with NW Pacific and next four with NE Pacific – the sequences of these taxa from outside CCZ are present in BOLD but they are stored privately, so specific localities are not available. One species found in CCZ was previously recorded from the Indian Ocean, another one was already caught in the Caribbean Sea. The remaining four taxa are known from their wide (sometimes even cosmopolitan) geographic distribution. Among the MOTUs recorded only in CCZ and sampled at two or more stations 32 were found at localities within 100 km distance. The next 35 MOTUs were recorded within the range of 101–200 km, followed by eleven found within 300 km distance and one not exceeding 400 km (its range reached 308 km) (Fig. 10, Supplementary Table S2). Only one species, *Rhachotropis* sp. nov. (BOLD:AEB2577) was present within 1000 km distance between the furthest stations, while five: *Cleonardo* sp. (BOLD:AEB2180), Fam. nov., gen. nov., sp. nov. (BOLD:AEB6828), *Bathymedon* sp. nov. (BOLD:AEA9579), Oedicerotidae (BOLD:AFW9721) and *Austrosyrrhoe* sp. nov. (BOLD:AEB6000) were recorded at the stations within more than 2000 km distance (Table 5).

Table 5. MOTUs represented by Barcode Index Numbers (BIN) found only in CCZ with the largest distribution ranges with information about their taxonomic affiliation, geographic distribution and ecology. Areas: APEI-06 – Area of Particular Environmental Interest No. 6, BGRE –BGR contractor area East, BGRW – BGR contractor area West.

Family	**BIN**	**Taxon name**	**No. ind.**	**Area**	**Distance between furthest stations [km]**	**Ecology**		
						Feeding type	**Mobility**	**References**
Eusiridae	BOLD:AEB2577	*Rhachotropis* sp. nov.	12	APEI-06, BGRE	988	predator	epibenthic swimmer	Bousfield and Hendrycks 1995
Eusiridae	BOLD:AEB2180	*Cleonardo* sp.	2	BGRE, BGRW	2377	predator	epibenthic swimmer	Bousfield and Hendrycks 1995
New family	BOLD:AEB6828	Fam. nov., gen. nov., sp. nov.	4	BGRE, BGRW	2239	unknown	unknown	unavailable
Oedicerotidae	BOLD:AEA9579	*Bathymedon* sp. nov.	3	BGRE, BGRW	2292	deposit feeders or carnivorous	sediment dweller	Brix et al. 2018
Oedicerotidae	BOLD:AFW9721	Oedicerotidae	2	BGRE, BGRW	2293	deposit feeders or carnivorous	sediment dweller	Brix et al. 2018
Synopiidae	BOLD:AEB6000	*Austrosyrrhoe* sp. nov.	4	BGRE, BGRW	2239	unknown	epibenthic?	unavailable

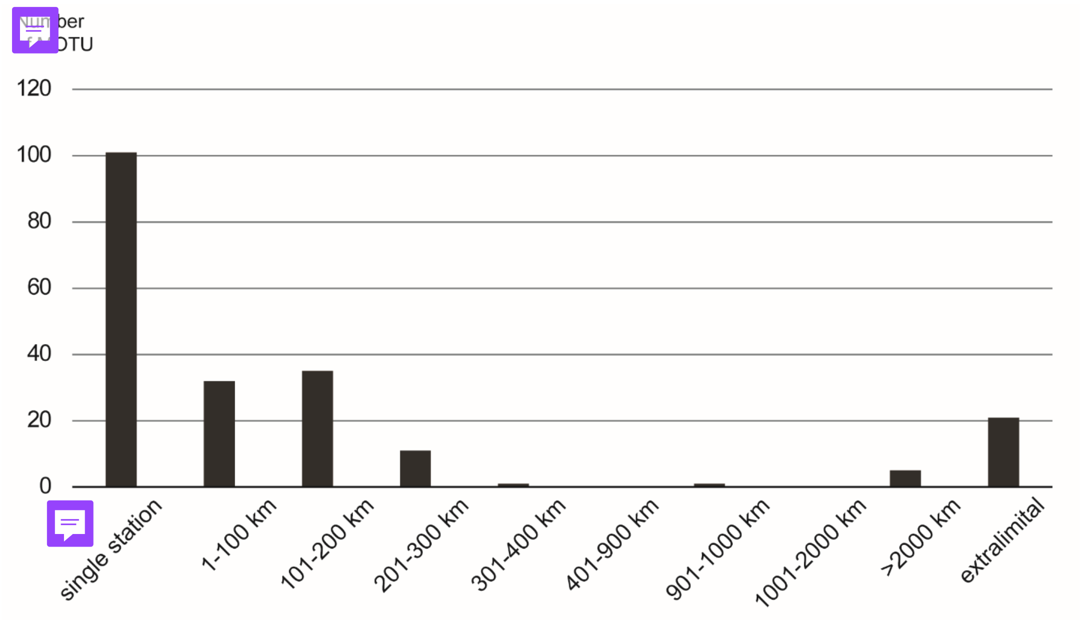

Figure 10. Number of MOTUs recorded at stations within distribution ranges. Extralimital: MOTUs shared with regions outside CCZ.

The cluster analysis of the fauna collected during three MANGAN expeditions at the level of 10% similarity revealed four groups (A–D, of which one is represented by only single station) (Fig. 11A). In general, the similarities between studied stations were very low and oscillated from almost 0% to ca. 40%. There was almost no similarity between the stations situated in the West part of the CCZ area (group A, West BGR) and the ones from the eastern part of the studied region (groups B, C, D, East BGR). In case of stations from East BGR their geographic proximity does not influence clustering. The diversity indices showed that the single station forming group B is characterized by low values of species richness, ES(50) and Shannon indices (Fig. 11B). In contrary the highest values of these indices were characteristic for the group C. The groups A and D were less species rich and diverse and presented similar mean values of diversity indices. The index of a very high value and low variety for all identified clusters was evenness (Pielou index) with the mean value ranging from 0.961 to 0.986; only one station within group D had value of this index lower than 0.850. Due to low similarity within the groups and very high evenness as well as lack of clear geographic grouping the composition of identified assemblages was not studied further.

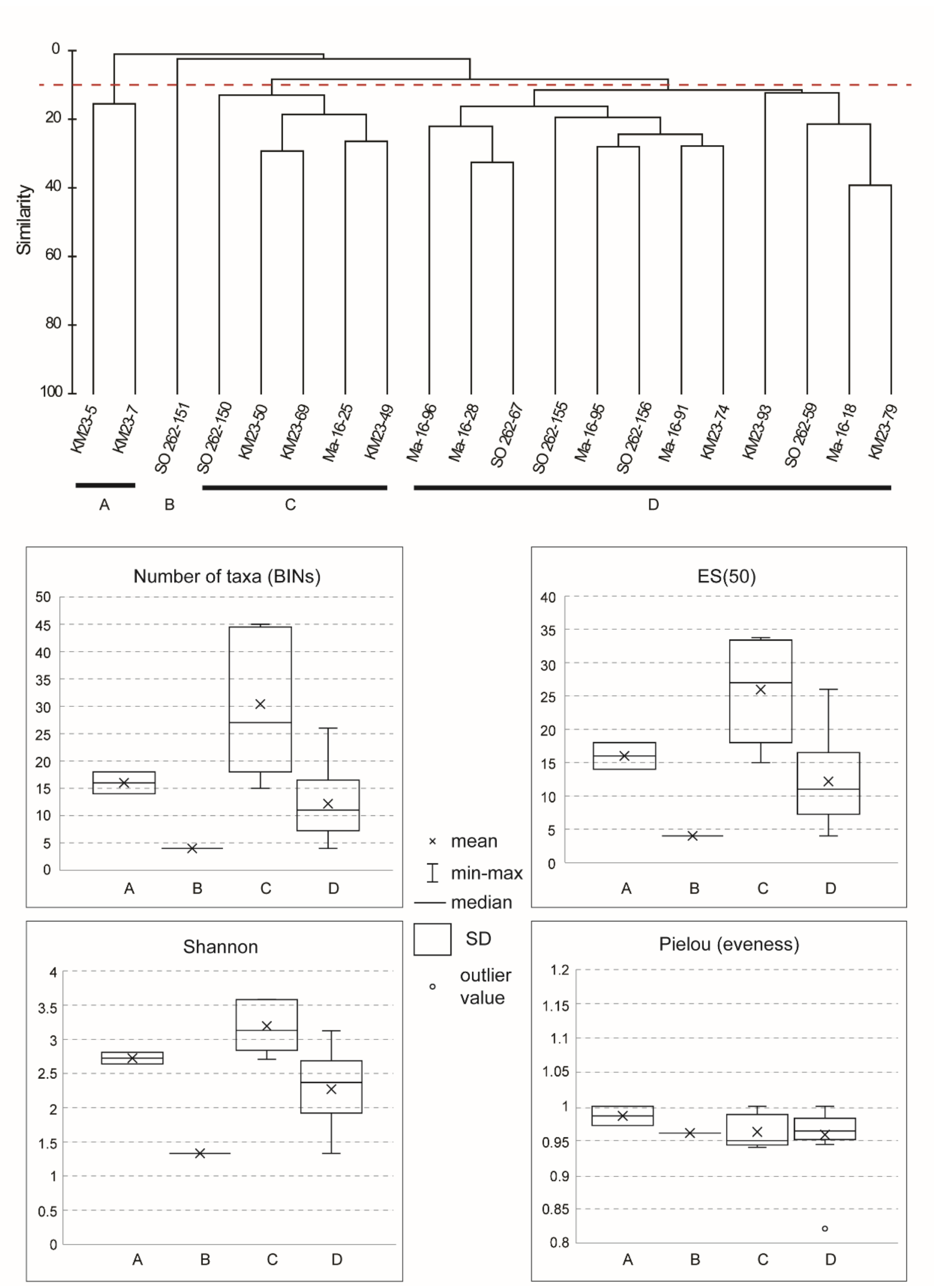

Figure 11. Upper – cluster analysis of stations from BGR contractor area (Bray-Curtis similarity of square-root transformed relative abundances, group average grouping method), dashed line placed at the similarity level of 10%. Lower – comparison of diversity measures for each distinguished group of samples.

4 DISCUSSION

4.1 Low abundance, high species richness

Our study of relatively small amphipod collection (sequences obtained from 581 ind.) revealed high species richness, expressed by identification of as many as 207 MOTUs. This corroborate with studies of Amphipoda conducted in other deep-sea Pacific areas using integrative taxonomy. For example, in the ~~North West~~ Pacific in the Kuril-Kamchatka Trench and adjacent abyssal plain 133 MOTUs were identified from the collection of 510 individuals (Jażdżewska and Mamos, 2019), while in the Aleutian Trench, adjacent abyss and the abyssal Bering Sea over 150 MOTUs were recorded after study of 450 individuals (pers. obs.). Also in the ~~South West~~ Pacific high deep-sea amphipod species richness was observed (263 MOTUs identified from 953 sequences, Knox et al., 2020). The numerically dominant and the most species rich families in the studied material were: Pardaliscidae, Eusiridae, Synopiidae, Phoxocephalidae and Oedicerotidae that is congruent with their diversity and abundance in the deep sea (e.g. Brix et al., 2018; Jażdżewska and Mamos, 2019). It is important to note however that more profound comparisons of species richness between cited regions are challenged due to different depth zones sampled in each of those studies.

High species richness in the CCZ was recorded also for other metazoan groups including, among others, Polychatea, Isopoda, Tanaidacea or Echinodermata (e.g. Janssen et al., 2015; Christodoulou et al., 2020; Kaiser et al., 2023; Bonifácio et al., 2024; Błażewicz et al., in review). Recent summary of the known diversity of Metazoa of CCZ revealed 5,578 spp. recorded in the region of which more than 92% are identified as morphospecies with only temporal name ascribed and may be considered as new to science (Rabone et al., 2023). In our study only 48 spp. were confirmed as belonging to species new to science with two already described and 27 in process of description (Jażdżewska et al., 2022; Jażdżewska and Horton, in prep). This constitutes less than 25% of all MOTUs found. However, this relatively low number derives from the fact that large part of the collection from the most recent expedition (MANGAN 2023) was only identified to the family level and further study of recognized MOTUs has not yet been conducted. The final identification of the recognized MOTUs was also often challenged by the presence of individuals that were severely damaged and lacked taxonomically important characters. Nevertheless, possessing their barcodes will allow to identify them in the future when more individuals of given MOTU are collected.

Kaiser et al. (2023) summarizing the present knowledge of ~~the diversity of~~ Isopoda from CCZ demonstrated that among 361 MOTUs ~~recorded~~ majority (345 MOTUs) were collected with epibenthic sledge, additional 16 were sampled with box corer. In the case of Tanaidacea the comparison of sampling gears (BC vs. EBS) ~~shown~~ their influence on the taxonomic composition and recognized communities but not on the diversity indices (Błażewicz et al., in review). Although our study was focused on the EBS samples, non-pelagic Amphipoda~~,~~ present in BC samples from MANGAN 2018 and MANGAN 2023 expeditions were also considered. Similarly to the study of Kaiser et al. (2023) they constituted only a small portion of all animals processed (23 individuals of which 21 sequences were obtained) and were ascribed to 13 MOTUs. This further reaffirm that only parallel usage of box corer and epibenthic sledge during monitoring studies may assure comprehensive evaluation of the biodiversity of CCZ areas.

4.2 Highly variable amphipod assemblages in the CCZ area

The study of amphipod assemblages from BGR contractor areas based on discriminated MOTUs revealed great variability of all studied stations, high number of singletons and doubletons in the material and no association between the faunal similarity and geographic proximity. ~~The more~~ there is almost complete dissimilarity between East and West BGR areas. Due to unavailability of the full collection of Amphipoda from other contractor zones (UKSLR and OMS) and the APEI-06 we were not able to characterize the amphipod assemblages of these areas. Nevertheless, out of the six individuals from APEI-06 for which the sequences were obtained, each belonged to different species (with two new to science) and only one of them *Rhachotropis* sp. nov. (BOLD:AEB2577) was shared between CCZ and APEI-06. Low similarity between different contractor areas of the CCZ as well as uncertain representativeness of APEIs towards mining zones was already reported for other macrobenthic groups (e.g. Taboada et al., 2018; Błażewicz et al., 2019, in review; Brix et al., 2020; Bonifácio et al., 2020, 2021; Christodoulou et al., 2020; Washburn et al., 2021a, b; Kaiser et al., 2023). It is worth noting that the cited studies used merged results for each contractor zone, here~~with~~ we show that evenness and patchy distribution characteristic for deep-sea fauna (Kaiser et al., 2007) ~~stand at the base of~~ high dissimilarity ~~also~~ within single contractor area. The same phenomenon was recorded for Isopoda and Polychaeta in

French and German contractor areas (Janssen et al., 2015). The variability in the faunal assemblages of the study region both in the smaller (within contractor area) and larger (between eastern and western CCZ) scales may derive from various factors. Locally, it may be associated with nodule density that is very heterogenous and may change within a few tens to hundreds of meters (Peukert et al., 2018). However, by now the influence of this factor was proven only for some taxa, like sessile megafauna (Vanreusel et al., 2016) and in case of another peracarid group, Isopoda it was not confirmed (Kaiser et al., 2023). The presence of nodules may directly support the settlement of low mobile, tube building Amphipoda belonging to the infraorder Corophiida by providing hard substrate for constructing their tubes but even more importantly in a indirect way being the bedding for sessile fauna of other groups (cnidarian, sponges). These sedentary macro- or megabenthic taxa are known to provide the substrate for tube-dwelling amphipods in the deep sea (Brandt et al., 2018) as well as for representatives of other infraorders (family Amathillopsidae) that were found clinging on sessile sponges (Lörz and Horton, 2021; Sigwart et al., 2023). The latter were photographically documented also from the APEI-06 (Lörz and Horton, 2021) but not present in the studied samples, while Corophiida were not numerous. In contrary, majority of Amphipoda collected belonged to either moderately or highly mobile epibenthic (families Eusiridae and Pardaliscidae) or to the sediment-dwelling groups (families Oedicerotidae and Phoxocephalidae) (Brix et al., 2018). For these groups the presence or absence of nodules may not be of direct importance, particularly for the sediment-dwelling amphipods for which the type of sediment may play more important role. This determinant was suggested to shape amphipod assemblages in the Cascadia Abyssal Plain in NE Pacific that appeared to be clearly different between two sites separated by only 100 km (Dickinson and Carey, 1978). Here, within East BGR the stations span over ca. 200 km, while the distance between East and West BGR zones is over 2,000 km. The other factors that may influence diversity and the assemblages of CCZ abyssal fauna in the wider scale are depth and food availability. The potential influence of decreasing primary productivity in the surface waters towards West of the CCZ on the animal densities was observed for the whole macrofauna as well as for isopods, polychaetes separately (De Smet et al., 2017; Bonifácio et al., 2020; Washburn et al., 2021b; Kaiser et al., 2023). In the present case due to the semiquantitative character of EBS the densities between samples were not compared and there was no evidence that the two stations situated in the West BGR contractor zone exhibit lower diversity indexes (ES[50] or Shannon Index, Figure 11B). However, their

https://doi.org/10.5194/egusphere-2025-1794
Preprint. Discussion started: 6 May 2025

faunal composition distinctness may be associated with the primary production changing in the western direction. Another factor that may play a role here is the depth that is well known as a determinant influencing faunal composition in the deep sea (Rex et al., 2006). The high local variability of nodule coverage and sediment type as well as gradual decrease of depth and of primary productivity in CCZ surface waters towards West combined with still insufficient sampling hinder decision which of the cited factors may play the crucial role in shaping the abyssal communities of the region (see also Kaiser et al., 2023).

4.3 Weak connectivity between western and eastern parts of the CCZ as well as other deep-sea regions and its implication to conservation programmes

The start of deep-sea mining in the CCZ will have both direct and indirect impact on the abyssal communities and their recovery (Rolinski et al., 2001; Sharma et al., 2001; Washburn et al., 2021b). The estimations suggest that the recovery of the biochemical fluxes in the upper layer of the sediments may last over 200 years (Haffert et al., 2020), while the results of mining experiment in the Peru Basin already showed very slow faunal recovery and serious changes in the composition of communities in the disturbed sites after 26 years (Miljutin et al., 2011; Simon-Lledo et al., 2019). In order to minimise the severe impact that retrieval of nodules serve to the abyssal environment the system of Areas of Particular Environmental Interest that are put aside of any mining operations was adopted by ISA (ISA, 2012) with later modifications (ISA, 2021). All but one of them are situated outside the main zone prospected for deep-sea mining (Fig. 1) and it has been already suggested that they may not fulfil their role (Błażewicz et al., 2019; McQuaid et al., 2020; Washburn et al., 2021a, b; Uhlenkott et al., 2023). It is partly due to highly heterogenous deep-sea environment and multiple factors shaping the faunal communities making them highly variable within short distances. The important role in hindering fast recovery of impacted sites may also have small population sizes of deep-sea invertebrates expressed by the high number of singletons. In our case almost 50% of all MOTUs were collected only once or at a single station (Fig. 10). ~~The~~ small population sizes mean ~~from~~ one hand that in the event of disturbance there are no individuals available to recolonize impacted site in the near future. ~~From~~ the other hand, it can be expected that such species may also be more prone to extinction due to mining operations. Another issue associated with proposal of APEIs that are mostly situated around the CCZ are the distribution ranges of deep-sea species. Our study revealed that majority of species recorded have distribution spanning to 200 km (Figure 10). That means that effective area planned as recovery source needs to be situated in relatively close vicinity of the mining zone. The distances between presently established APEIs in the latitudinal axis are within 500–750 km that seems sufficient for species with larger ranges, but for those with more local distribution such distances may be too large. Although we realize that our sampling was focused mainly on the eastern part of CCZ (with the station located within 300 km radius), still the distribution of large number of species was more restricted and suggests relatively low connectivity within the region. It is worth noting that some species may exhibit extensive ranges but due to their rarity they are rarely collected. If that is the case the disruption due to mining may not influence such species survival, nevertheless it will not allow recovery of impacted place shortly. Important point to consider is also that the movement of small invertebrates within CCZ may be facilitated or prevented by the deep-water bottom currents and local hydrological conditions. These are not fully recognized for the region. However, if the dominating currents in the bottom zones are directed longitudinally (as is the case for the surface ones), the migration of species may be more intensive in the East-West direction than the North-South. In such case the recolonization may be hindered also by the fact that the residual zones (APEIs) are situated North or South from the mining areas. Further studies based on samples collected both in the claim areas but particularly in the APEIs would help in resolving their usefulness as real conservation zones.

By now the studies of Amphipoda in the CCZ were focused on very specific ecological group – the scavengers – and listed just 23 amphipod taxa generally widely distributed across different contractor zones and APEIs (Patel et al., 2020; Bribiesca-Contreras et al., 2021; Mohrbeck et al., 2021, Supplementary Table S3). Present study revealed that the non-scavengers community is much more diverse and variable. The striking finding is that only 13% of MOTUs recorded appeared to be widely distributed (MOTUs found at stations at the distance of at least 900 km and those with range spanning out of the CCZ). ~~The more~~, many of them belong to taxa considered as highly mobile scavengers or predators (Table 4). For three taxa the knowledge is insufficient to make final conclusions about their ecology. Interestingly, four other species recorded in this group belong to the family Oedicerotidae which representatives are recognized as sediment-dwelling deposit feeders or predators (Brix et al., 2018). The question arise how these brooding and less mobile taxa maintain large distributions. The explanation of this phenomenon may be associated with the specific behaviour observed for some shallow-water oedicerotids who migrate into water column for reproduction (Brix et al., 2018). Nothing is known about the mating behaviour of deep-sea species from this family; however, Hendrycks and Conlan (2003) reported one of the species (*Oedicerina denticulata*) from sediment traps set at 50 m above the seafloor. This support the assumption that species of this family probably spend large part of their life in the sediment, but they may also occur in the water column while mating and migrate at larger distances during this period.

The above widely distributed species may be considered as the first recolonizators of the disturbed areas. It must be underlined however, that this set of taxa is very small and represent only a small portion of deep-sea functional groups. As a result one can expect that after disturbance event the recovering community will be largely impoverished both in terms of species richness and functional groups. It may lead to general uniformization of deep-sea assemblages and irreversible loss of presently observed specificity of CCZ bottom fauna.

5 CONCLUSIONS and RECOMMENDATIONS

Being the first extensive molecular study of the abyssal benthic Amphipoda of Clarion-Clipperton Zone it provides important extension to the barcode library of this group and of the abyssal fauna of the Pacific Ocean, in general. In combination with detailed (although still not finished) morphological identification and description of species new to science (presented in parallel elsewhere) it serves as a baseline for further studies including future monitoring based on eDNA.

It is next study showing high diversity and low connectivity of deep-sea faunal groups in the Clarion-Clipperton Zone underlining high specificity of the benthic assemblages of this region. Additionally, with present extent of sampling and knowledge it further supports previous suggestions of moderate or weak representativeness of Areas of Particular Environmental Interest for the mining zones.

~~In the time of~~ discussions ~~about~~ the development of Regional Environmental Management Plans for the whole CCZ area ~~the~~ two main recommendations are to be made:

1) to conduct more intensive sampling program of APEIs (of which some are virtually unstudied) parallel to monitoring studies of contractor zones with the goal to better assess their usefulness as reservoirs of biodiversity for the mining areas,

2) to include the epibenthic sledge among standard gears used for sampling in order to avoid missing important part of deep-sea fauna in the monitoring studies.

Data availability

Datasets supporting this paper can be accessed online through the Barcode of Life Data Systems (boldsystems.org) in the data set “DS-AMPHICCZ” (dx.doi.org/10.5883/DS-AMPHICCZ). All sequences were also deposited in GenBank with the accession numbers: PQ734211 – PQ734784.

Author contributions (CRediT)

Conceptualization – AMJ, PMA
Data curation – AMJ
Formal analysis – AMJ, KB
Funding acquisition – AMJ, PMA, AV
Investigation – AMJ, KB
Methodology – AMJ
Project administration – AMJ, PMA, AV
Resources – AMJ, PMA, AV
Visualization – AMJ
Writing – original draft – AMJ, KB
Writing – review & editing – AMJ, KB, PMA, AV

Competing interests

authors declare that they have no conflict of interest.

Acknowledgements

The ABYSSLINE cruises were funded by UK Seabed Resources Ltd. The MANGAN 2016, MANGAN 2018 and MANGAN 2023 cruises were funded by the BGR (Bundesanstalt für Geowissenschaften und Rohstoffe - German Federal Institute for Geosciences and Natural Resources). Both institutions are thanked for providing the research material published here. The authors also wish to thank the chief scientists, scientific teams and crews of the sea-going expeditions for their assistance in collecting the material.

The 3rd generation sequencing molecular analysis of material from the MANGAN 2023 cruise was conducted with support from a Polish National Science Centre grant (project no. 2022/45/B/NZ8/02667) to A.M.J. We gratefully acknowledge Polish high-performance computing infrastructure PLGrid (HPC Center:ACK Cyfronet AGH) for providing computer facilities and support within computational grant no. PLG/2024/016952. Furthermore, A.M.J. received financial support to visit the "Deutsches Zentrum für Marine Biodiversitätsforschung" (DZMB) in Wilhelmshaven from the German Academic Exchange Service (Deutscher Akademischer Austauschdienst - [DAAD]) within the program "Research Stays for University Academics and Scientists, 2019" (57440915) to study their collection of Pacific amphipods. This is publication XX of the Senckenberg am Meer Metabarcoding and DNA Laboratory.

Financial support

The ABYSSLINE cruises were funded by UK Seabed Resources Ltd. The MANGAN 2016, MANGAN 2018 and MANGAN 2023 cruises were funded by the BGR (Bundesanstalt für Geowissenschaften und Rohstoffe - German Federal Institute for Geosciences and Natural Resources).

The 3rd generation sequencing molecular analysis of material from the MANGAN 2023 cruise was conducted with support from a Polish National Science Centre grant (project no. 2022/45/B/NZ8/02667) to A.M.J. We gratefully acknowledge Polish high-performance computing infrastructure PLGrid (HPC Center:ACK Cyfronet AGH) for providing computer facilities and support within computational grant no. PLG/2024/016952. Furthermore, A.M.J. received financial support to visit the "Deutsches Zentrum für Marine Biodiversitätsforschung" (DZMB) in Wilhelmshaven from the German Academic Exchange Service (Deutscher Akademischer Austauschdienst - [DAAD]) within the program "Research Stays for University Academics and Scientists, 2019" (57440915) to study their collection of Pacific amphipods.

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