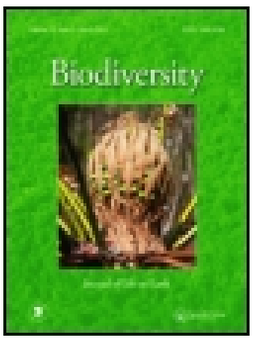

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Have we so far only seen the tip of the iceberg? Exploring species diversity and distribution of the giant amphipod *Eurythenes*

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ABSTRACT

Additional material of the iconic giant amphipod *Eurythenes* was investigated. Recently, the species *E. gryllus* has been separated into 12 distinct species-level lineages of which several have been described as distinct species, based on both morphology and genetics. This study revealed three additional species-level lineages from unique sampling localities, showing that with minimal sampling effort, species diversity within *Eurythenes* can still increase. One species-level lineage was found in the Indian Ocean and another one in the Pacific, which was subsequently identified as *E. thurstoni*. In addition to the three species already reported from the Southern Ocean (*E. maldoror*, *E. gryllus s.s.* and *E. andhakarae*), a supplementary bathyal species was found in the Weddell Sea. *E. gryllus* was confirmed to be amphitropical including newly sampled localities around the Kerguelen Islands and additional samples from the Svalbard Archipelago. Building on new and earlier data, geographic and bathymetric distributions of the different species that have been discovered so far are presented here and several factors are evaluated for their likelihood of having triggered past speciation events in this scavenger. Topographic and hydrographical features are discussed but rejected as sufficient reasons for the distributional patterns observed. Bathymetric segregation is interpreted with regard to what is known about the ecology of the species. The previously reported genetic break around 3000 m persists in this new data-set for all species but one. This study underlines the need of processing all individuals sampled, since two or more sympatric species are found in different proportions, and that conclusions regarding diversity and distribution may drastically change when increasing sampling intensity and coverage. Finally, I suggest here that only a mere fraction of all *Eurythenes* species has yet been discovered and that a more complete knowledge of the ecology of the species is of paramount importance for interpreting their evolution.

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Introduction

The giant lysianassoid amphipod *Eurythenes gryllus* (Lichtenstein in Mandt, 1822) is one of the icons of the deep-sea fauna, owing to its large size and conspicuous colour, high abundances and rapid appearance at baited traps. It has been extensively sampled and various aspects, e.g. basic life history characteristics (Baldwin and Smith 1987; Thurston and Bett 1995), metabolism (Premke and Graeve 2009), feeding strategy (Hargrave 1985; Hargrave et al. 1995), pigments (Thoen, Johnsen, and Berge 2011) and vertical distribution (e.g. Ingram and Hessler 1983), investigated. However, none of these studies tackled the question of whether the investigated specimens belonged to the same species occurring throughout the world's deep ocean. Despite the studies of France and Kocher (1996a,b) two decades ago, which raised suspicion about the cosmopolitan nature of its distribution, the genetic connectivity of *E. gryllus* has only recently been addressed with large-scale sampling and modern molecular methods. An integrative study based on detailed morphological examinations and various molecular species-delimitation methods confirmed that *E. gryllus* represents a species complex (Havermans et al. 2013). The previously overlooked *Eurythenes* species clearly differ in their genes, morphology and distribution patterns, the latter being geographically partly overlapping but clearly separated on the bathymetric scale. A genetic break was observed around 3000 m, since there was no lineage that comprised both specimens sampled below and above this depth. So far, based on morphological and molecular analyses,

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five different species have been (re-)described based on a reverse-taxonomy approach (Markmann and Tautz 2005): *E. andhakarae* d'Udekem d'Acoz and Havermans 2015; *E. maldoror* d'Udekem d'Acoz and Havermans 2015; *E. sigmiferus* d'Udekem d'Acoz and Havermans 2015; *E. magellanicus* (H. Milne Edwards 1848) and *E. gryllus* (Lichtenstein in Mandt, 1822) s.s. (d'Udekem d'Acoz and Havermans 2015). A more extensive data-set of 16S rDNA sequences, complemented with sequences from previous studies, unveiled another four supplementary species-level lineages, which could not be investigated morphologically. In the Southern Ocean alone, three distinct *Eurythenes* species were found, with the two abyssal ones (sampled at depths below 3000 m) occurring in sympatry and the bathyal species (from depths above 3000 m) representing a bipolar species (Havermans et al. 2013). The study by Ritchie, Jamieson, and Piertney (2015) revealed four more species-level lineages of *Eurythenes* in a single trench, i.e. the Peru–Chile Trench: one hadal, two abyssal occurring in sympatry, and one bathyal lineage. One of the abyssal ones corresponded genetically to *E. magellanicus*, extending its distribution previously limited to the Southwest Atlantic to the Southeast Pacific Ocean, but apparently occurring only at abyssal depths. However, a specimen from the Peru–Chile Trench identified as *E. magellanicus* was illustrated by Eustace et al. (2016). If at all corresponding to any of the described species based on its morphology, it would rather be *E. sigmiferus* due to the crested pleonites, instead of *magellanicus* from which it is visibly distinct. Crested individuals have also been reported from other localities such as the Mid-Atlantic Ridge and the Indian Ocean at abyssal depths (for an exhaustive list see d'Udekem d'Acoz and Havermans 2015) – whether these and the abyssal ones represent the same species needs to be further verified.

The genus *Eurythenes* was, until recently, considered to be composed of only three species: *E. gryllus*, *E. obesus* (Chevreux 1905) and *E. thurstoni* (Stoddart and Lowry 2004) but with the aforementioned studies, it now exceeds a dozen. All species but *E. obesus* have been found in baited traps on the seafloor, and hence are at least facultative scavengers. *E. obesus* has never been found in baited traps but has been sampled with midwater trawls, but very little is known about its ecology. Its feeding regime covers a wide array of prey: from soft-bodied zooplankton (e.g. pelagic cnidarians and tunicates) to sponges as well as predation on pelagic fish (d'Udekem d'Acoz and Havermans 2015). *E. thurstoni* often enters baited traps but is also frequently caught in midwater trawls (Stoddart and Lowry 2004) and hence, a more pelagic lifestyle has been attributed to this species. *Eurythenes* species previously identified as *E. gryllus* are believed to be rather benthopelagic since the majority has been caught by means of baited traps, and more mature stages including ovigerous females migrate far above the seafloor, where they have been caught with midwater traps or trawls (e.g. Charmasson and Calmet 1987; Smith et al. 1979). A summary of the current knowledge on the biology of the different species can be found in d'Udekem d'Acoz and Havermans (2015). Nonetheless, despite the significant research effort dating as far back as the seventies, the ecology and life history of these giant amphipods remain elusive: questions related to their distribution between the seafloor and the sea surface, their feeding habits when not feeding on artificial food falls and their social structure are yet unsolved. Moreover, due to the taxonomic splitting initiated with molecular evidence, studies on the biology of '*E. gryllus*' should actually be scattered across the complex of species which will now render generalisations of their results more difficult.

With an aim to increase sampling intensity and coverage for a better understanding of the distributional ranges of *Eurythenes* species and the factors determining them, I examined here additional specimens from museum collections, from previously studied material and from new samples gathered during recent expeditions. This material for molecular analyses originates from several high-Antarctic and sub-Antarctic localities in the Southern Ocean, localities in the northern and southern Pacific Ocean and the previously unsampled Indian Ocean. Building on new and earlier data, a review of the different species that have been discovered so far is presented and different factors are evaluated for their likelihood of having triggered past speciation events in this giant scavenging amphipod.

Material and methods

Material identified as *E. gryllus* or *Eurythenes* spp. was obtained from museum or institute collections and was previously sampled during expeditions of RV *Jan Mayen*, RV *Polarstern*, RV *Vizconde de Eza* and RV *Coriolis*, using baited traps or trawls. Sampling details are listed in Table 1. The newly obtained material originated from depths ranging from 750 m to 4625 m covering both the bathyal (<3000 m) and abyssal (>3000 m) zone and included geographic localities off Taiwan, Samoa, in the Mozambique Channel, in the Weddell Sea and along the Kerguelen Islands and the Svalbard archipelago.

Genomic DNA was isolated from pereopod 6 using the Nucleospin Tissue kit® (Macherey-Nagel) according to the manufacturer's protocol. Polymerase chain reaction (PCR) amplifications of a fragment of the mitochondrial cytochrome oxidase subunit 1 (COI) gene were carried out using the LCO1490 and HCO2198 primers (Folmer et al. 1994). Since some of the material was old (the specimens from Samoa were collected in 1977) and/or preserved in diluted ethanol, only the COI gene fragment

Table 1. Data on *Eurythenes* specimens sampled for this study: locality, geographic coordinates, depth (m), supplementary information on sampling and collection numbers, as well as GenBank accession numbers. Abbreviations: n.d. – no data, BT – baited traps.

Abbreviation	Code	Locality	Coordinates	Depth	Suppl. information	Acc Number
Arctic-c8	EgrC182	Svalbard	82°16′N 20°52′E	1660	RV *Jan Mayen* TSZCr13640	KX078265
Arctic-c9	EgrC183	Svalbard	82°16′N 20°52′E	1660	RV *Jan Mayen* TSZCr13640	KX078264
Arctic-c10	EgrC184	Svalbard	82°16′N 20°52′E	1660	RV *Jan Mayen* TSZCr13640	KX078263
Arctic-c11	EgrC185	Svalbard	82°16′N 20°52′E	1660	RV *Jan Mayen* TSZCr13640	KX078262
Arctic-c12	EgrC186	Svalbard	82°16′N 20°52′E	1660	RV *Jan Mayen* TSZCr13640	KX078261
WDL-a4	EgrC169	Weddell Sea	67°30′S 00°00′W	4625	RV *Polarstern* ANDEEP III 59BT	KX078270
WDL-a5	EgrC170	Weddell Sea	67°30′S 00°00′W	4625	RV *Polarstern* ANDEEP III 59BT	KX078269
WDL-a6	EgrC171	Weddell Sea	67°30′S 00°00′W	4625	RV *Polarstern* ANDEEP III 59BT	KX078268
WDL-a7	EgrC172	Weddell Sea	67°30′S 00°00′W	4625	RV *Polarstern* ANDEEP III 59BT	KX078267
WDL-a8	EgrC173	Weddell Sea	67°30′S 00°00′W	4625	RV *Polarstern* ANDEEP III 59BT	KX078266
WDL-a9	EgrC174	Weddell Sea	67°30′S 00°00′W	4625	RV *Polarstern* ANDEEP III 59BT	KX078260
WDL-a10	EgrC175	Weddell Sea	67°30′S 00°00′W	4625	RV *Polarstern* ANDEEP III 59BT	KX078259
WDL-a11	EgrC176	Weddell Sea	67°30′S 00°00′W	4625	RV *Polarstern* ANDEEP III 59BT	KX078258
WDL-a12	EgrC177	Weddell Sea	67°30′S 00°00′W	4625	RV *Polarstern* ANDEEP III 59BT	KX078257
WDL-a13	EgrC178	Weddell Sea	67°30′S 00°00′W	4625	RV *Polarstern* ANDEEP III 59BT	KX078256
WDL-a14	EgrC179	Weddell Sea	67°30′S 00°00′W	4625	RV *Polarstern* ANDEEP III 59BT	KX078255
WDL-d1	EgrC099	Weddell Sea	n. d.	<1200	RV *Polarstern* EASIZ II Trap	KX078273
KERG-a1	EspC201	Kerguelen Isl.	50°14′S 65°25′E	1476	ALCP377.4	KX078254
KERG-a2	EspC202	Kerguelen Isl.	50°14′S 65°25′E	1476	ALCP377.4	KX078253
KERG-a3	EspC203	Kerguelen Isl.	50°14′S 65°25′E	1476	ALCP377.4	KX078252
KERG-a4	EspC204	Kerguelen Isl.	50°14′S 65°25′E	1476	ALCP377.4	KX078251
KERG-a5	EspC205	Kerguelen Isl.	50°14′S 65°25′E	1476	ALCP377.4	KX078250
KERG-b1	EspC206	Kerguelen Isl.	48°29′S 65°09′E	1732	ALCP 324.08	KX078249
MOZ-1	EspC194	Mozambique Channel	21°38′S 36°07′E	1161	RV *Vizconde de Eza* MAINBAZA CP3146 MNHN-IU-2009–2501	KX078271
SAM-1	EspC197	Apolima Strait, Samoa	n.d.	750	RV *Coriolis*, SAMOA-I, 20.11.1977 MNHN-IU-2009–2512	KX078272
TAI-1	EspC226	Off Taiwan	22°22′N 119°48′E	1342	Sta C22T2	KX078274

could be successfully amplified and sequenced and sometimes only for one specimen per locality. The 25 µl PCR reactions consisted of 0.02U/µl Hotmaster Taq® (5Prime GmbH), 0.2 mM dNTPs, 0.5 µM of forward and reverse primers, 1x PCR-buffer and 1 µl (about 30 ng) of template DNA. PCR conditions were: initial denaturation at 94 °C for 2 min, followed by 36 cycles of 94 °C for 20 s, annealing at 42 °C for 20 s, extension at 65 °C for 1 min and a final extension at 65 °C for 15 min. Amplified products were purified using the Exo-SAP-IT kit® (Affymetrix, Santa Clara, Canada). Both forward and reverse strands of the gene were sequenced on an ABI 3130xl sequencer after cycle sequencing with the BigDye Terminator Kit® (Applied Biosystems, Foster City, Canada). Sequences were checked for ambiguities and aligned using the software CodonCode Aligner v.3.7.1.1®. (CodonCode Corporation, Deham, MA, USA). In order to prevent inclusion of pseudogenes in the analyses, electropherograms were checked for ambiguous base calls and sequences were translated into amino acids and checked for stop codons. The COI data-set was composed of 44 sequences generated by Havermans et al. (2013) and 26 sequences from newly provided specimens for this study (Table 1), which have been deposited in GenBank (KX078249-KX078274). A first Bayesian analysis was carried out based on 70 COI sequences of *E. gryllus sensu lato*, two sequences of *E. obesus* (from d'Udekem d'Acoz and Havermans 2015) and one sequence of *Abyssorchomene chevreuxi* (Acc. No. GU109229) as outgroup, using MrBayes 3.1.2. (Ronquist and Huelsenbeck 2003). The best-fit substitution model was selected using jModeltest 0.1.1. (Posada 2008) based on the Bayesian Information Criterion (BIC, Schwarz 1978). TPM1+I+G was selected for position 1, F81 for position 2 and TPM3+G for position 3. Two parallel runs with four chains each were run for 10 million generations; every 1000th generation was sampled, resulting in 10,000 trees. Convergence of runs was monitored using Tracer v1.5 and the first 50% of the trees were discarded as burn-in, while the last 5000 trees were used to reconstruct the consensus tree and estimate Bayesian posterior probabilities. Relationships between COI haplotypes and their geographic distribution were investigated by generating haplotype networks using TCS 1.21 (Clement, Posada, and Crandall 2000), with gaps considered as a fifth state and a 95% probability threshold. Finally, a second Bayesian analysis was carried out with the same parameters on the aforementioned data-set now also including the five COI sequences available for *Eurythenes* from Ritchie, Jamieson, and Piertney (2015) (Acc. Nos. KP713954–KP713958), in order to verify whether the new specimens investigated for this study belong to one of the species-level lineages recently discovered in the Peru–Chile Trench. MrEnt (Zuccon and Zuccon 2014) has been used for the graphical representation of the tree and network analysis. Genetic divergences were compared within and between the different clades using the Kimura two-parameter (K2P) distance model (Kimura 1980), with MEGA version 6 (Tamura et al. 2013). Finally, all distributional data from

species and lineages, confirmed to be genetically homogeneous, were illustrated on a map and plotted according to their bathymetric records.

Results

The COI data-set used for the first Bayesian analysis and for the haplotype network analysis comprised 73 sequences (of which 30 unique) consisting of 658 bases, 192 of which were parsimony-informative. The second data-set comprised 78 (34 unique) sequences of 658 bases, of which 200 were parsimony-informative. Translation revealed a higher mutation rate at third codon positions as well as the absence of stop codons, typical for a functional protein-coding gene as opposed to a pseudogene.

For the first Bayesian analysis, similar clades were recovered as in previous molecular analyses (Havermans et al. 2013; d'Udekem d'Acoz and Havermans 2015) based on the COI gene and the results of the statistical parsimony network analysis showed identical unconnected networks Eg1–5 (Figure 1). Three additional lineages were identified: (1) SAM-1 representing a specimen sampled at bathyal depth off Samoa, (2) MOZ-1 representing a specimen from bathyal depth in the Mozambique Channel and (3) WDL-d from bathyal depth in the Weddell Sea. Each of these highly divergent lineages (Figure 1) consisted of only one sequence, and corresponded to a separate unconnected singleton in the network analysis. The divergences between these three lineages, as well as between each of them and the described species of *Eurythenes* are much higher than previously observed interspecific divergences. Indeed, the smallest genetic distance by which the MOZ-1, the WLD-d and the SAM-1 sequences were separated from known *Eurythenes* species were 22.9, 25 and 23.6%, respectively (Table 2). Upon a detailed morphological examination, the specimen sampled in the Apolima Strait, off Samoa (South Pacific), corresponded to the description of *E. thurstoni*. France and Kocher (1996a) published a 16S sequence of *E. thurstoni* but since both sequences cannot be compared I decided to refer to the SAM-1 specimen as *E.* cf. *thurstoni*, until evidence of genetic homogeneity can confirm their status as a single species.

Additional specimens sampled around Svalbard (Arctic-c), as well as newly sampled specimens from the Kerguelen Islands were recovered in the tree and network described as *E. gryllus sensu stricto* (Eg1) (Figure 1). The Kerguelen specimens represented three haplotypes that were scattered across the network and one of them was connected, by a few mutational steps, with the most common haplotype observed for the specimens originating from the Arctic region. The 11 additional sequences from specimens sampled at abyssal depths in the Weddell Sea (locality WDL-a) were recovered in two distinct clades and networks, one corresponding to *E. andhakarae* (Eg2; 5 sequences) and one to *E. maldoror* (Eg3; 6 sequences). COI haplotypes were shared between the Antarctic Peninsula and Weddell Sea both for *E. andhakarae* as well as for *E. maldoror*, whereas for the latter species, the most abundant haplotype was shared between the Argentine Abyssal Basin and these localities. The single, small-sized (±4 cm) specimen sampled at bathyal depth in the Weddell Sea was recovered as a highly divergent lineage, clearly separated from all other specimens sampled in the Southern Ocean. Unfortunately, precise information on depth and coordinates was lost; however, it was known that the specimen was sampled with traps during the expedition EASIZ at a site situated in the eastern Weddell Sea (De Broyer, Rauschert, and Scailteur 1999). Trap sampling was only carried out between 200 and 1450 m so the specimen is definitely a bathyal one. Hence, four species-level lineages of *E. gryllus sensu lato*, of which two are bathyal and two abyssal, are now found to occur within the Southern Ocean, several of which are in sympatry.

For specimens belonging to the clade representing the re-described species *E. magellanicus*, three unconnected networks were recovered: Eg4, Eg5 as in Havermans et al. (2013) and a third one (singleton), representing the sequence (TAI-1) from the specimen sampled at bathyal depth off Taiwan. This sequence was recovered in the Bayesian tree as embedded in the *E. magellanicus* clade. Finally, one unique haplotype was recovered for the two specimens of *E. obesus*, forming a distinct clade in the tree.

The second analysis included data from specimens sampled in the Peru–Chile Trench at bathyal, abyssal and hadal depths by Ritchie, Jamieson, and Piertney (2015) and aimed to elucidate whether any of these sequences would be recovered in a clade representing a known or newly detected *Eurythenes* species. Previously, the authors demonstrated, with various species-delimitation methods, the presence of four distinct species-level lineages along the Peru–Chile Trench, of which three were distinct from the nine other lineages so far uncovered based on 16S rDNA divergences by Havermans et al. (2013). According to their results, a fourth group comprising several abyssal specimens (Abyssal-minor) was recovered within the clade composed of *E. magellanicus* sequences. However, this could not be verified here since no COI sequences were available. The results of the Bayesian analysis (Figure 2) confirm the findings of Ritchie, Jamieson, and Piertney (2015) that the specimens from abyssal depth (Abyssal-major) and those from the hadal group (Hadal) form distinct clades. However, the sequence of the specimen sampled at shallower depth (Bathyal) clustered here together with *E. obesus* and was only separated from these sequences by a genetic K2P distance of 1.4% (Table 2). Since *E. obesus* is easily distinguished from the

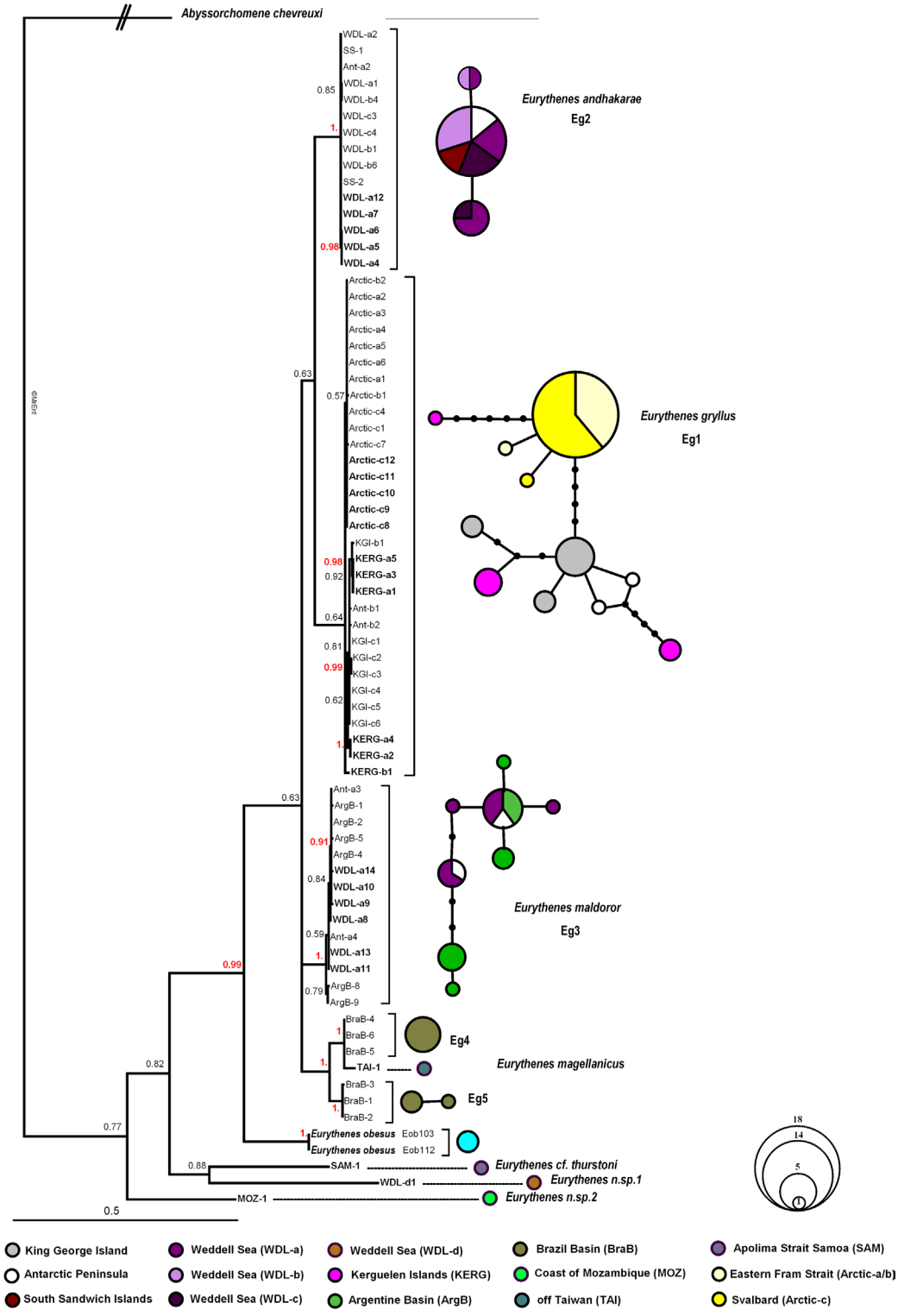

Figure 1. Bayesian tree inferred for the first COI data-set including previously described species (d'Udekem d'Acoz and Havermans 2015) and the newly obtained sequences in this study. Posterior probabilities above 0.50 are shown with values > 0.95 indicated in red. Results of the statistical parsimony network analysis (95% probability threshold) based on the COI data-set are represented next to the tree. Colours refer to the different sampling localities and the area of each circle is proportionate to the frequency of the haplotype in our sampling (a scale is presented at the right). Black nodes represent hypothetical haplotypes and each line a single substitution. Unconnected networks representing the different clades found in Havermans et al. (2013) are indicated with Eg1–5. Five additional unconnected networks/singletons were found, of which one for the sequence TAI-1, embedded in the clade representing *E. magellanicus*, all others represent distinct species (*E. obesus*, *E.* cf. *thurstoni*, *E.* n. sp. 1 and *E.* n. sp. 2).

Table 2. Range and mean of pairwise K2P intra- and interclade distances for each clade or species identified with the COI data-set of *Eurythenes* sequences from d'Udekem d'Acoz and Havermans (2015), Ritchie, Jamieson, and Piertney (2015) as well as this study. The highest intraspecific value and the lowest interspecific value observed are indicated in bold, highlighting the presence of a barcoding gap between them.

	Intraclade divergences		Interclade divergences	
	Min. – Max.	Mean	Min. – Max.	Mean
E. gryllus	0.0 – 0.022	0.008	**0.085** – 0.281	0.121
E. andhakarae	0.0 – 0.003	0.001	**0.085** – 0.283	0.113
E. maldoror	0.0 – 0.013	0.005	0.094 – 0.299	0.117
E. magellanicus	0.0 – **0.064**	0.033	0.094 – 0.305	0.122
E. obesus	0.0 – 0.014	0.002	0.142 – 0.279	0.181
E. cf. *thurstoni* (SAM-1)	/	/	0.236 – 0.292	0.267
E. n. sp. 1 (WDL-d)	/	/	0.250 – 0.305	0.276
E. n. sp. 2 (MOZ-1)	/	/	0.229 – 0.288	0.253
E. Hadal	0.002	/	0.158 – 0.257	0.175
E. Abyssal-major	0.002	/	0.111 – 0.276	0.135

other *Eurythenes* species by its morphology (e.g. narrow linear eyes and extremely long dactyls), species identity should be verified for these bathyal specimens. *E. obesus* is presumably cosmopolitan; here a genetic homogeneity between the southeast Pacific and localities in the Atlantic sector of the Southern Ocean (near South Georgia, Eob112 and around the Polar Frontal Zone in the mid-Atlantic sector, Eob103) could be observed. This is the first record of *E. obesus* being caught with a baited trap. To summarise, two lineages have been found so far only in the Peru-Chile Trench, whilst the two others extend the distribution of previously described species. With these and the findings of Ritchie, Jamieson, and Piertney (2015), *E. magellanicus* is now no longer confined to the southwest Atlantic, but also distributed in the North and South Pacific (TAI-1 and A-Minor). The two other groups (Abyssal-major and Hadal) might be endemic to the Peru–Chile Trench.

Discussion

An up-to-date overview of the distributional ranges of Eurythenes

The geographic ranges of the different *Eurythenes* species and their bathymetric recordings are illustrated in Figure 3 and 4, respectively. Concerning the geographic ranges, different patterns are observed. The species *E. maldoror*, *E. gryllus*, *E. magellanicus*, *E. sigmiferus* and the lineage Eg8 are characterised by distributions that span several oceanic basins, whilst *E. andhakarae* has only been found in a single basin, i.e. throughout the Southern Ocean at several distinct localities (e.g. off the Antarctic Peninsula and in the eastern Weddell Sea). Other lineages have so far only been reported from single localities, such as the two lineages discovered on the seamount Horizon Guyot (Eg7, Eg9), the hadal and abyssal lineages each found at a distinct locality along the Peru–Chile Trench, as well as the newly uncovered lineages *E.* n. sp. 1 and *E.* n. sp. 2, found in the eastern Weddell Sea and the Mozambique Channel, respectively. The specimen identified as *E.* cf. *thurstoni* found off Samoa would, if genetically homogeneous, have been found within the currently known distribution of *E. thurstoni*, encompassing the south Pacific and Atlantic oceans. A previous record of *E. thurstoni*, used in the molecular analysis of France and Kocher (1996a), was situated around the Bahamas. Despite the geographically extended sampling effort, *E. gryllus* has not been reported yet from localities outside the Southern and Arctic oceans. The fact that the three haplotypes from the Kerguelen Islands were spread over the network and one of them was connected, although by a few mutational steps, with the most common haplotype in the Arctic region, confirms the validity of the bipolar – or rather amphitropical – *E. gryllus s.s.*, representing a single species distributed in both (sub-)polar regions, with its distribution now also including the sub-Antarctic waters. However, the presence of populations in other oceans cannot be ruled out because of the clear patchiness with both more abundant and rare species observed in a particular site, which is further discussed in a next section. The distribution of *E. maldoror* in the Southern Ocean, previously only found in the Argentine abyssal basin and around the Antarctic Peninsula, now also comprises the eastern Weddell Sea; hence, distributions of the two abyssal species *E. andhakarae* and *E. maldoror* are now characterised by a substantial overlap. Finally, five cases of true sympatry, representing specimens caught in the same traps, could be observed: (1) the lineage Abyssal-major and *E. magellanicus* off Ecuador in the Peru-Chile Trench, (2) the species *E. sigmiferus* and *E. magellanicus* in the Brazil Abyssal Basin, (3) the lineage Eg7 and *E. maldoror* on the Horizon Guyot seamount in the North Pacific and (4,5) *E. andhakarae* and *E. maldoror* in the

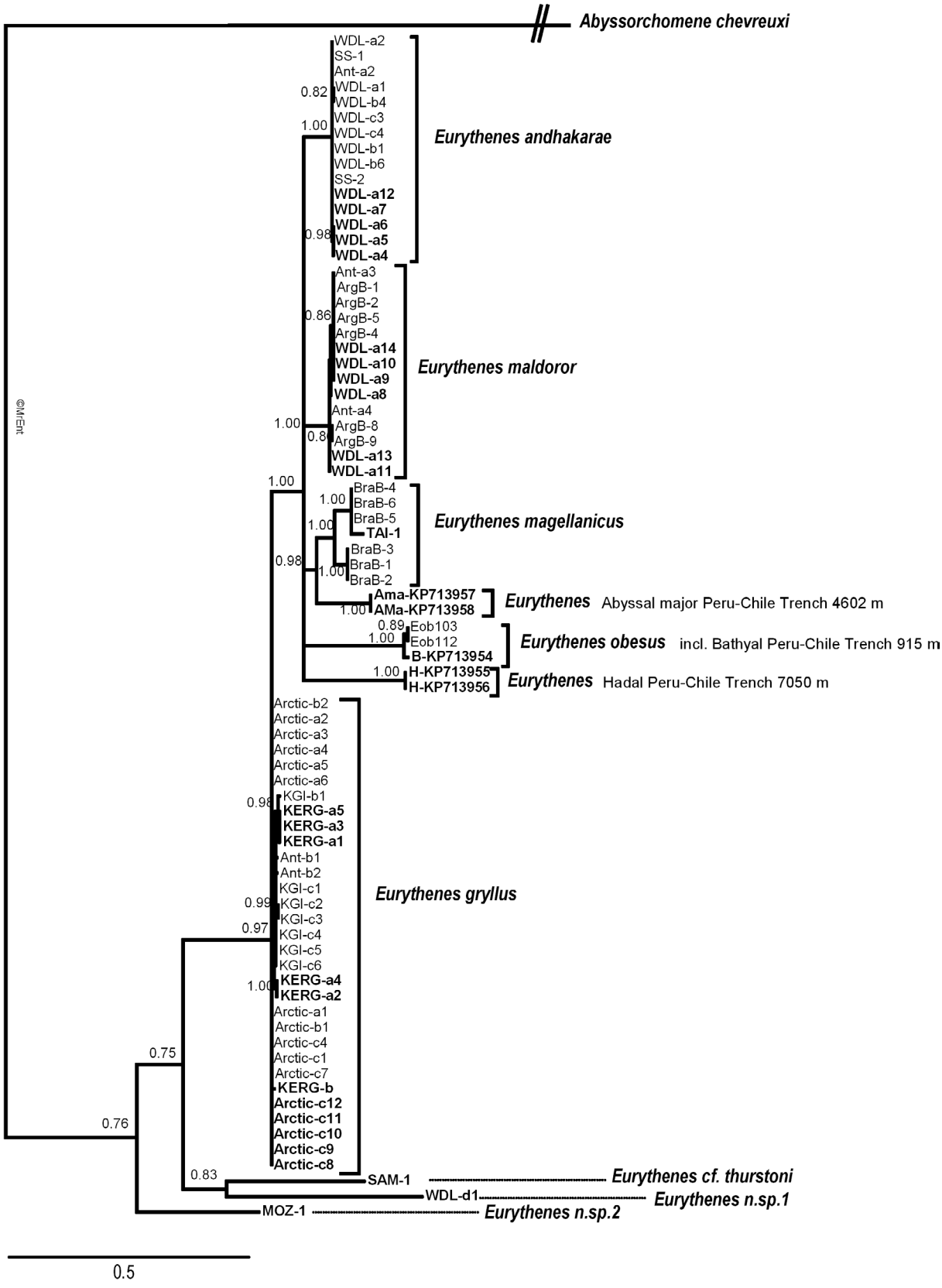

Figure 2. Bayesian tree inferred for the second COI data-set including previously described species (d'Udekem d'Acoz and Havermans 2015), the newly obtained sequences in this study and the COI sequences from Ritchie, Jamieson, and Piertney (2015). Posterior probabilities above 0.50 are shown.

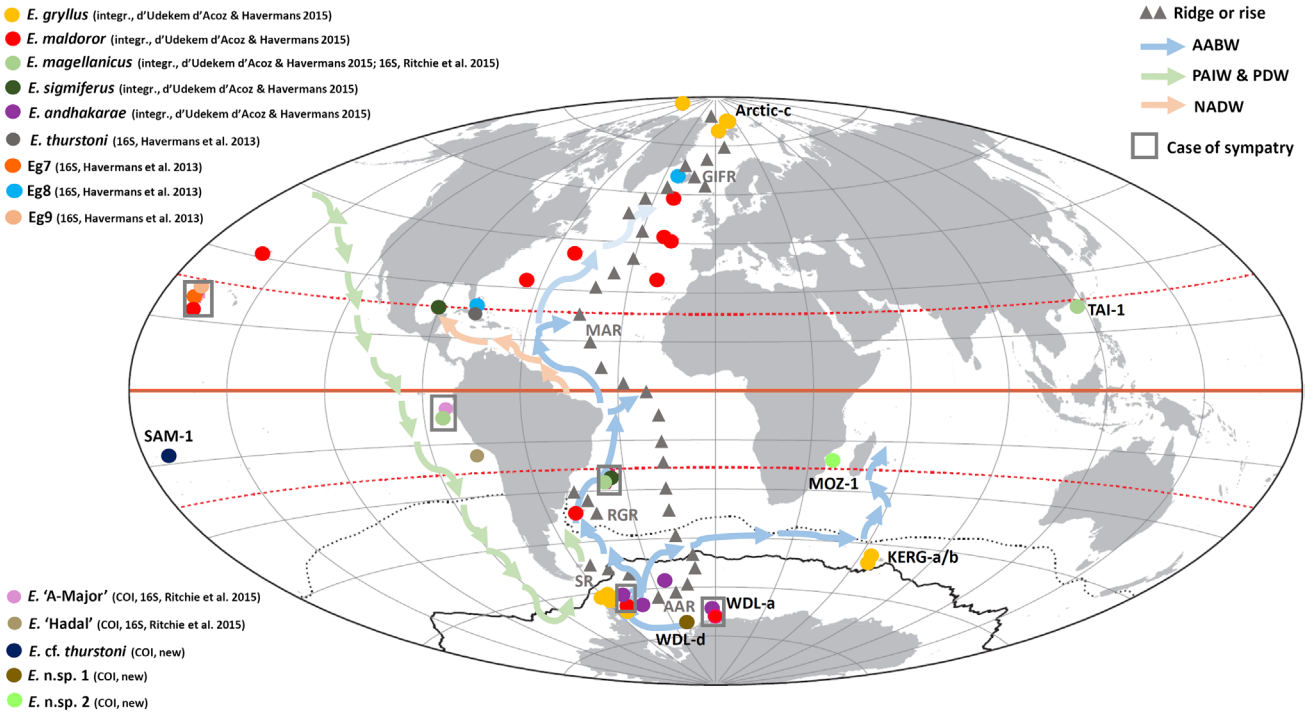

Figure 3. Geographic distributional ranges of the different *Eurythenes* species that have so far been confirmed with molecular or integrative (morphology and genes) methods. All described species or molecular species-level lineages of *Eurythenes*, excluding the pelagic species *E. obesus*, are represented here by different colours. Data include sequences from specimens obtained from France and Kocher (1996a), Escobar-Briones, Nájera-Hillman, and Alvarez (2010), Havermans et al. (2013), Ritchie, Jamieson, and Piertney (2015) and d'Udekem d'Acoz and Havermans (2015). Topographic and hydrographical features, hypothesised to limit or enhance dispersal, respectively, of the different species are indicated. The abbreviations of the new sampling sites for this study are added (e.g. Arctic-c). Localities where species have been found to occur in geographic and bathymetric sympatry (i.e. recovered in the same trap) are highlighted with a rectangle. Abbreviations: AABW – Antarctic Bottom Water, AAR – American Antarctic Ridge, GIFR- Greenland–Iceland–Faeroe Ridge, MAR – Mid-Atlantic Ridge, NADW – North Atlantic Deep Water, PAIW – Pacific Arctic Intermediate Water, PDW – Pacific Deep Water, RGR - Rio Grande Rise, SR – Scotia Ridge.

eastern Weddell Sea (WDL-a) and off the Antarctic Peninsula.

Nevertheless, wherever species are co-occurring geographically they are often separated on the bathymetric scale (Figure 4). There are examples at the scale of the ocean basin: the North Atlantic comprises three different species with *E. maldoror* only found at abyssal depths whilst *E. thurstoni* and Eg8 occur at bathyal depths, the Southern Ocean contains four species, with *E.* sp. 1 (WDL-d) and *E. gryllus* only found above 3000 m whilst *E. maldoror* and *E. andhakarae* have only been sampled below 3000 m. This is also true at the more local scale: three species, *Eurythenes maldoror* as well as the two species-level lineages Eg7 and Eg9, were recorded at four distinct depths (between ca. 3000 and 5000 m) on the seamount Horizon Guyot in the North Pacific. However, in the South Atlantic three species occur, that were all sampled at abyssal depths: *E. magellanicus*, *E. sigmiferus* and *E. maldoror*.

So far the highest species richness was recorded in the Pacific with seven species-level lineages compared with five in the Atlantic, four in the Southern Ocean and only one in the Arctic, however, this picture is certainly biased due to an uneven sampling effort. As an example, this is the first time material from the Indian Ocean was included and the single specimen investigated represented a distinct species-level lineage. However, sampling effort in the Arctic was high compared to elsewhere, although limited to bathyal depths, but only one species was found so far. The newly uncovered species-level lineages *E.* n. sp. 1 and *E.* n. sp. 2 were sampled at bathyal depths and hence, the species richness of *Eurythenes* is now equal between abyssal and bathyal depths with seven species recorded for each zone (not considering the midwater species *E. obesus*) (Figure 4). This alters the earlier view of a comparatively lower (species) diversity at bathyal compared with abyssal depths (Havermans et al. 2013). Nevertheless, 'true' species richness between different regions or depths will only be comparable with a higher and non-biased sampling effort, both at a geographic and bathymetric scale.

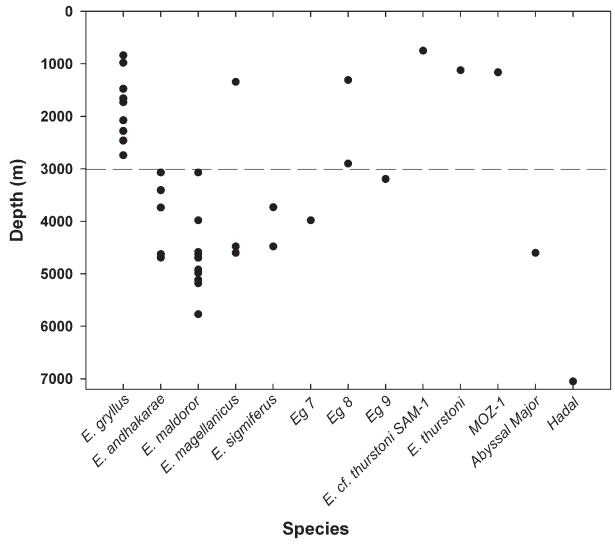

Figure 4. Depth distributions of the different *Eurythenes* species, except *E. obesus*, which is an entirely pelagic species. All records plotted here have been confirmed with molecular or integrative (morphology and genes) methods. Data included specimens investigated by France and Kocher (1996a), Escobar-Briones, Nájera-Hillman, and Alvarez (2010), Havermans et al. (2013), Ritchie, Jamieson, and Piertney (2015) and d'Udekem d'Acoz and Havermans (2015).

Horizontal and vertical segregation: identifying true barriers

Topographic and hydrographical features

It is known that topography can play a role as barriers for dispersal, reflected by a high genetic differentiation observed between populations situated at each side of an undersea mountain ridge. For example, the Mid-Atlantic Ridge, one of the largest and most distinctive topographic features in the deep sea, can restrict transoceanic gene flow for strictly abyssal organisms, which has been shown e.g. for molluscs (Etter et al. 2011). However, the distribution of *Eurythenes* species does not seem to be restricted by geological features, since several species have distributions encompassing mountain ridges or rises, indicating that recent or ongoing gene flow has occurred, if not on ecological, then at least on evolutionary timescales. This is the case for the abyssal species *E. maldoror*, distributed on each side of the Mid-Atlantic Ridge (MAR), which shows that at some point, there has been dispersal over the ridge at less elevated, bathyal, depths or across deep-water corridors (Figure 3). The Greenland–Iceland–Faeroe Ridge (GIFR) extends across the Atlantic in an east-west direction, separating the deep water basins of the Arctic Ocean and the northernmost Atlantic Ocean. The average sill depth ranges only from 480 to 600 m (Brix and Svavarsson 2010). Hence, the bathyal amphitropical species *E. gryllus* must be capable of, at least, carrying out jump-dispersal across this ridge. The same is true for the American Antarctic Ridge (AAR) and the Scotia Ridge (SR), which do not limit the distributions of the species *E. andhakarae* and *E. maldoror*, and the species *E. maldoror* and *E. gryllus*, respectively (Figure 3). Distinct species are present at each side of the Rio Grande Rise (RGR), which only allows a restricted exchange of abyssal waters (AABW) through the Vema and Hunter channels (Zenk et al. 1999). Indeed, *E. maldoror* was sampled in the Argentine Basin whilst *E. sigmiferus* and *E. magellanicus* occurred in the Brazil Basin, however, on a larger scale, this feature did not seem an obstacle for dispersal and gene flow since *E. maldoror* was also sampled in the North Atlantic.

Hydrographical features such as the Antarctic Bottom Water, the North Atlantic Deep Water, the Pacific Arctic Intermediate Water and the Pacific Deep Water (Figure 3) were proposed as conduits for dispersal (Havermans 2012; Havermans et al. 2013). COI haplotypes were shared between the Antarctic Peninsula and Weddell Sea both for *E. andhakarae* and *E. maldoror*, whereas for *E. maldoror*, the most abundant haplotype was shared between localities as far apart as the Argentine abyssal Basin and the eastern Weddell Sea. This could indicate that dispersal events are common within the Southern Ocean and between the Southern Ocean and the South Atlantic at abyssal depths. Hence, it was previously hypothesised that dispersal between these regions could be facilitated by the presence of the Antarctic Bottom Water, formed in the Weddell Sea and of which the main pathway flows via the Argentine abyssal Basin (Murray and Reason 1999). Another hypothesis implies that the connectivity between the North Pacific, the Southern and southwest Atlantic (for *E. maldoror* and *E. magellanicus*, Figure 3) is facilitated by southward spreading of the Pacific Arctic Intermediate Water and mixing with the Pacific Deep Water (PDW) that crosses the equator and moves further south along South America. Ultimately, this water mass mixes with the Circumpolar Deep Water and passes the Drake Passage to the Southwest Atlantic (Arkhipkin, Laptikhovsky, and Brickle 2010). For *E. sigmiferus*, the connectivity between the Brazil Basin and the Gulf of Mexico, at abyssal depths, may be facilitated by the North Atlantic Deep Water (NADW, only partly illustrated on Figure 3) through the western South Atlantic, by deep passages permitting the inflow of NADW into the Venezuelan and Columbian basins and further through the Yucatán Channel into the Gulf of Mexico (Fratantoni et al. 1997; Sheinbaum et al. 2002).

Whether these species are linked to particular water masses cannot be ruled out, however, for a strong swimmer such as *Eurythenes*, passive dispersal with currents is no longer tenable. Drifting with currents may be a valuable hypothesis for less mobile amphipods that display

swimming behaviour towards the surface which enables them to disperse with the strong surface currents (e.g. epibenthic amphipods in the North Sea, Havermans et al. 2007). However, scavenger species such as *Eurythenes* are not only able to easily swim against currents (Laver et al. 1985) but are also supposed to do so in order to detect chemical signatures of food falls (Premke et al. 2003). Scavenger amphipods passively drifting with water currents above the seafloor, just like vultures hovering with air currents (Ruxton and Houston 2004), may not be a suitable analogy when bearing in mind the sluggish currents typical for the deep sea (e.g. Schmitz and McCartney 1993) and the unlikelihood to detect food falls in the surroundings when swimming with the current.

It becomes clear from these findings regarding *Eurythenes* species' distributions but also from logical reasoning that hydrographical and topographic features are not influencing dispersal of this and other lysianassoid amphipods to such an extent that they represent true barriers or conduits. Cosmopolitan or widespread distributions have yet been confirmed with molecular methods in a vast array of deep-sea organisms. The vent-associated tubeworm *Sclerolinum contortum* was shown to be cosmopolitan, characterised by a genetic homogeneity between the two poles and in the Gulf of Mexico (Georgieva et al. 2015) and the genetically investigated coral species *Paragorgia arborea* was observed to have a distribution that spans several oceanic basins (Herrera, Shank, and Sánchez 2012). However, these species can accomplish longer distance dispersal via larval propagules, whilst peracarid crustaceans do not have larval stages. Nonetheless, an undersea ridge did not represent a barrier for a small non-dispersive isopod species (Brix, Svavarsson, and Leese 2014). Smaller lysianassoid amphipod species also showed a genetic connectivity between the Southern Ocean, the South and North Atlantic (Havermans 2014). Thus, the large and mobile *Eurythenes* species should definitely be capable of dispersing across ridges, they are not restricted to particular water masses characterised by certain temperatures and do not rely on drifting with large current systems to accomplish wide-ranging dispersal, hence, other yet unknown factors should be responsible for the restricted distributions observed for some species and the widespread occurrences observed in others.

The genetic break at 3000 m: a persistent sampling artefact?

The genetic break observed around 3000 m is still apparent for all species but one (Figure 4). Indeed, the specimen sampled off Taiwan at a bathyal depth of around 1300 m clustered within the abyssal clade representing *E. magellanicus*, and hence this is the first species – corroborated with molecular data – that has been reported from both below and above 3000 m. Previously, this limit was interpreted as an ubiquitous barophysical tolerance boundary (Rex and Etter 2010) – below which selection would have induced enzymatic adaptations – observed throughout all oceans for *Eurythenes* and for other organisms such as the bivalve *Deminucula atacellana* (Zardus et al. 2006) and *Neilonella salicensis* (Glazier and Etter 2014). However, the effect of pressure on chemical reactions is not as straightforward and predictable as that of temperature, affecting some whilst not others; it is believed to be the most pronounced on the permeability of membranes and enzyme stability (Hochachka 1971). The latter is true particularly for enzymes that are involved in energy-yielding reactions (e.g. (per)oxidases, hydrogenases) opposite to hydrolytic enzymes (e.g. amylase) that are pressure-tolerant (Kim and Zobell 1972). Furthermore, enzymatic sensitivity to hydrostatic pressure is in many cases temperature dependent, and since distinct temperatures might be encountered across the different oceanic basins, this break at 3000 m seems to be rather a sampling artefact than an effect from hydrostatic pressure as a selective factor. George (1979) collected what is likely to be *E. gryllus* s.s. from the central Arctic basin at a depth of ca. 1800 m and maintained the specimens alive for three months in an aquarium. Their metabolism, measured by respiration rate and pleopod activity, did not vary whilst exposed experimentally to the *in situ* and *in vitro* pressures. Data on abyssal *Eurythenes* specimens are not available, however, in the case of the lysianassoid *Paralicella caperesca*, abrupt decompression after recovery at almost 6000 m only temporarily inactivated locomotion which was reversible, suggesting that vertical migrations of 3000 m are physiologically possible (Yayanos 1981). Moreover, *Eurythenes* species can easily cope with hydrostatic pressure gradients (Yayanos 1978); distinct life stages occupy different depths above the seafloor (e.g. Eustace et al. 2016) and individuals have also been reported from the sea surface (Templeman 1967). How bathymetric segregation can be maintained for a vertically migrating species remains a question to be further explored. Various other variables than hydrostatic pressure itself, embedded within the 'factor depth' are more likely to play a significant role in promoting population differentiation and eventually speciation, as outlined in Brix, Svavarsson, and Leese (2014). These environmental factors that vary across wide depth ranges include temperature, dissolved oxygen concentration, nutrient flux, topographic complexity and sediment characteristics (reviewed in Gage and Tyler 1991). The synergistic effect of temperature and hydrostatic pressure seems to be an important factor determining the physiological limits and hence the distributional range of a species, which has been shown for the lysianassoid bathyal amphipod *Stephonyx biscayensis* (Brown and Thatje 2011). Thus, selection

might have favoured different enzymes tuned to particular a combination of temperature and pressure gradients.

A patchwork of rare but widespread species

These and previous results corroborate the 'patchwork theory' on deep-sea brooding crustaceans, stating that these are often composed of several distinct species occurring in sympatry (Raupach et al. 2007). Several *Eurythenes* species were characterised by a widespread, presumably cosmopolitan, distribution, whilst others seemed to be restricted to single ocean basins or topographic features (e.g. trench, seamount), with partly overlapping horizontal and vertical distributions and in some cases, a segregation along distinct depth ranges. The same mixture of overlapping horizontal and vertical species' distributions, of which some are widespread and others restricted, was observed within the gastropod genus *Scaphander* (Eilertsen and Malaquias 2015). Such findings highlight the need for processing a high number of specimens per locality, since several species-level lineages often occur in the same sample but in different proportions. As an example, of all specimens sampled in the Brazil Abyssal Basin, only one specimen belonged to *E. sigmiferus*, the remaining (around twenty) were *E. magellanicus*. Results also demonstrate that these locally less abundant species are not necessarily endemic but can be widely distributed, which seems to be the case for *E. sigmiferus*, supporting the theory that local rarity does not always equal a small geographic range (McClain and Hardy 2010; Rex 2002). After investigating a small number of additional samples, the diversity within *E. gryllus sensu lato* continues to increase, clearly suggesting that only a fraction of the species has yet been discovered. Morphological investigations also pointed out the presence of a distinct species in the Atacama Trench (Thurston, Petrillo, and Della Croce 2002) and Bowman and Manning (1972) also report morphological variations for specimens collected in the bathyal Caribbean. Finally, a higher (molecular) sampling effort is also needed to confirm whether some of the uncovered *Eurythenes* species are truly confined to a certain region or depth range or are actually more widespread.

Ecological differentiation as a trigger for diversification

It is believed that, particularly for the abyssal plains, few obvious barriers exist in the deep sea that would impede dispersal of organisms and this is even more the case for the mobile *Eurythenes*. The abyss is characterised as a uniform, homogeneous environment with few variable abiotic factors that could allow population differentiation and new species to form (e.g. Etter and Grassle 1992). However, in this argumentation, the variability of biotic factors, and their role in speciation and diversification, is often neglected since little is known about biotic interactions and the variability of food supply at local and regional scales in the deep ocean. It has been stated before that selection pressures may not differ over large distances in the deep sea (e.g. Bucklin, Wilson, and Smith 1987), however, in the case of scavenging amphipods, biotic factors such as food supply, in the shape of animal carcasses sinking down to the seafloor, and predation pressure, might vary across the different ocean basins and even at much more localised scales. The different *Eurythenes* species, and in particular those occurring in bathymetric and geographic sympatry, might occupy distinct ecological niches, being specialised on particular types of food falls or by displaying a distinct feeding behaviour, e.g. scavenging complemented by predation on organisms in the water column or feeding on detritus on the seafloor. DNA analyses of the gut content of abyssal specimens of *E. gryllus* showed a diverse diet composed of some invertebrates unlikely to be fed upon as carrion, suggesting predation as a feeding mode (Blankenship and Yayanos 2005). Moreover, using molecular and stable isotope analyses, hadal *Eurythenes* specimens were shown to use other sources of nutrition than large carrion alone, e.g. predation and feeding on detritus (Blankenship and Levin 2007). Some information on the specimens investigated here can be deduced from the sampling methods: one specimen of *E. gryllus*, from the Kerguelen Islands was caught with a trawl and was found clinging onto a Patagonian toothfish (*Dissostichus*), and the species found in the Mozambique Channel was sampled with a midwater trawl, possibly indicating feeding by predation or on immobilised or dead fish in the trawls, near the surface. In areas of high productivity, *Eurythenes* species could display a more preferential or specialised feeding on a particular type of food falls, such as whale (Smith and Baco 2003) or large fish falls (Higgs et al. 2014). Particular deep-sea habitats that are often characterised by a higher food supply, such as trenches, canyons or seamounts, might have favoured species diversification, which could explain the co-occurrence of several distinct species-level lineages of *Eurythenes* on a single seamount (Horizon Guyot) or in a single trench (the Peru-Chile Trench).

Conclusions

It has to be noted that assumptions on taxon diversity and distribution drastically change when increasing sampling in the deep sea but are of crucial importance for impact studies in the context of planned deep-sea exploitation activities. The systematic processing of all samples with cost-effective molecular tools will help to unravel the patterns and vulnerability of the extremely diverse, patchy

and underexplored fauna that the deep sea harbours. Future studies combining fast and slowly evolving genetic markers will allow us to test dispersal on ecological and evolutionary timescales and help to interpret the current distributional patterns. Nevertheless, getting a better picture of a species' ecology is of paramount importance for grasping how the organisms partition their environment and their feeding resources and hence, for understanding what is key in promoting allo- or sympatric speciation events in the deep ocean.

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