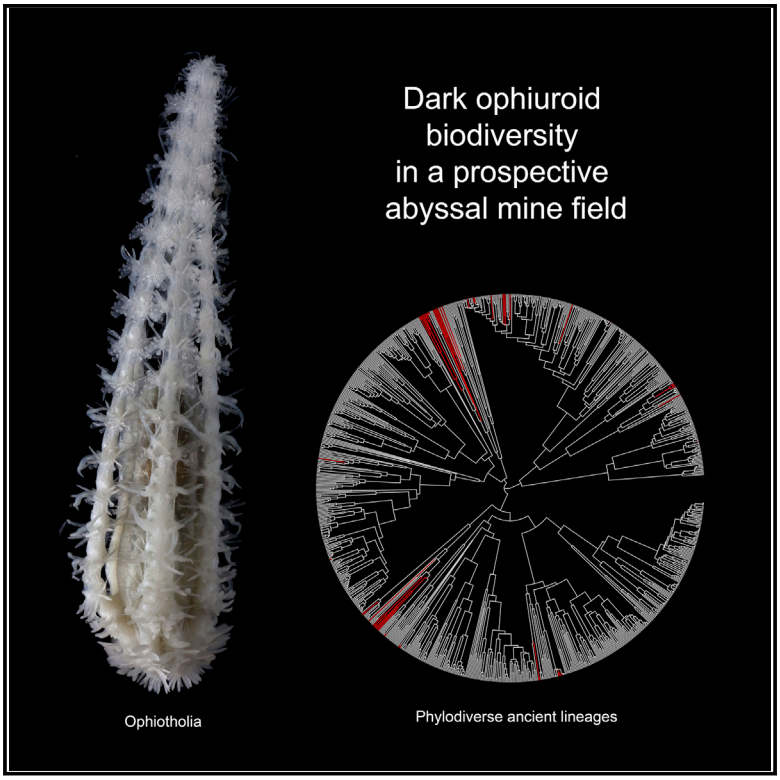
Report

Current Biology

Dark Ophiuroid Biodiversity in a Prospective Abyssal Mine Field

Graphical Abstract



Highlights

- Unprecedented levels of abyssal ophiuroid biodiversity in the Eastern Pacific Ocean
- The new lineages occur in polymetallic nodule mining license areas
- Three of the new lineages are greater than 70 million years in age

Authors

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In Brief

Christodoulou et al. report unprecedented abyssal phylodiversity of ophiuroids from areas licensed for polymetallic nodule mining exploration in the Eastern Pacific Ocean. The new data suggest that this fauna is composed of ancient lineages, some *in situ* radiations, and representatives of species complexes that occur throughout the abyssal oceans.

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CellPress

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Dark Ophiuroid Biodiversity in a Prospective Abyssal Mine Field

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SUMMARY

The seafloor contains valuable mineral resources, including polymetallic (or manganese) nodules that form on offshore abyssal plains. The largest and most commercially attractive deposits are located in the Clarion Clipperton Fracture Zone (CCZ), in the eastern Pacific Ocean (EP) between Hawaii and Mexico, where testing of a mineral collection system is set to start soon [1]. The requirement to establish pre-mining environmental management plans has prompted numerous recent biodiversity and DNA barcoding surveys across these remote regions. Here we map DNA sequences from sampled ophiuroids (brittle stars, including post-larvae) of the CCZ and Peru Basin onto a substantial tree of life to show unprecedented levels of abyssal ophiuroid phylogenetic diversity including at least three ancient (>70 Ma), previously unknown clades. While substantial dark (unobserved) biodiversity has been reported from various microbial meta-barcoding projects [2, 3], our data show that we have considerably under-estimated the biodiversity of even the most conspicuous mega-faunal invertebrates [4] of the EP abyssal plain.

RESULTS AND DISCUSSION

Trees of life are greatly increasing our understanding of large-scale phylodiversity patterns [5]. Here, we use a super-matrix approach to combine a dataset of two DNA barcodes of ophiuroids (42 operational taxonomic units [OTU], 1,035 bp 28S rDNA + 658 bp cytochrome *c* oxidase subunit [COI] mtDNA) collected from 7 expeditions to the abyssal plains of the eastern Pacific Ocean (EP) with our existing, exceptional global phylogenomic tree-of-life dataset (945 species, 50 kbp nDNA + 28S + COI; Table S1; Data S1A) to produce a robust, time-calibrated [6] phylogeny illustrating the relationships and phylogenetic diversity of the abyssal EP fauna (Figure 1).

Similar to other abyssal faunas [5], the Clarion Clipperton Fracture Zone (CCZ)-Peru lineages are spread across the phylogeny, indicating multiple separate adaptations to this habitat. The lineages form three categories based on branch length. The first category with short branches includes regional representatives of known widespread abyssal species (e.g., *Amphioplus daleus*, *Ophiotypa simplex*, *Ophiacantha cosmica*, and *Amphiophiura bullata*) or species-complexes (e.g., *Ophiuroglypha* "irrorata"). The second category consists of abyssal EP endemics, mostly undescribed, with branches subtending nodes of 5–60 Ma (Figures 1 and S2) in age (*Amphilepis* sp., *Ophioleuce gracilis*, *Ophiernus* sp., *Ophiotoma* sp., *Ophiotholia* sp., *Ophiomyces* sp., *Ophiosphalma glabrum*, *Perlophiura cf.* "profundissima," *Asteroschema* sp., and various Ophiopyrgidae). The third group contains three new multi-species clades, within the Ophioleucidae, Ophiohelidae, and putatively a new family within the order Ophioscolecida. There are also four species (*Ophioleuce gracilis*, *Ophiuroglypha* sp 11, *Anophiura* sp., and *Asteroschema* sp.) that were only recorded from bathyal depths (1,667–2,882 m) on seamounts in the CCZ. Thus, the DNA barcoding data reveal an ancient and previously unknown ophiuroid fauna on the EP abyssal plain (Figure 2). This fauna has relatively high phylogenetic diversity compared to other abyssal faunas that have been sequenced to date and even some shallow (0–2,000 m) water faunas (Figure 3A). The tip branches are on average 50% longer than that of the global fauna (Figure 3B).

These new findings are not solely the result of increased sampling effort. The ophiuroid fauna of the abyssal EP had been historically described from collections obtained by the US steamer *Albatross* (1891–1904), numerous Soviet oceanic expeditions (1958–1978), and early US exploration for manganese nodules (1958–1980) [8]. However, what has changed is the development of new collection devices (e.g., Brenke sleds) that preserve fragile organisms and new DNA barcoding approaches that enable the identification of post-larvae and juveniles that lack adult morphological characters (Figure 1). Dark marine biodiversity can be discovered not only from meta-barcoding of eDNA samples [3], but also from meticulous collection of juvenile and delicate organisms (Figure 1) [9].

The choice of genetic loci is critical for a barcoding-based biodiversity project [10]. The COI mtDNA fragment alone facilitates the resolution of species-complexes, but sequence divergence is generally too high to robustly determine the relationships of the unknown lineages. Meta-barcoding projects frequently target the 18S ribosomal subunit for eukaryotes, which has highly conserved regions suitable for universal primers. However, its conserved nature precludes its usefulness as a "barcode" in ophiuroids, and we find that it reliably discriminates lineages only at family to suborder levels of classification. Conversely, we find that an ~1,000 bp fragment

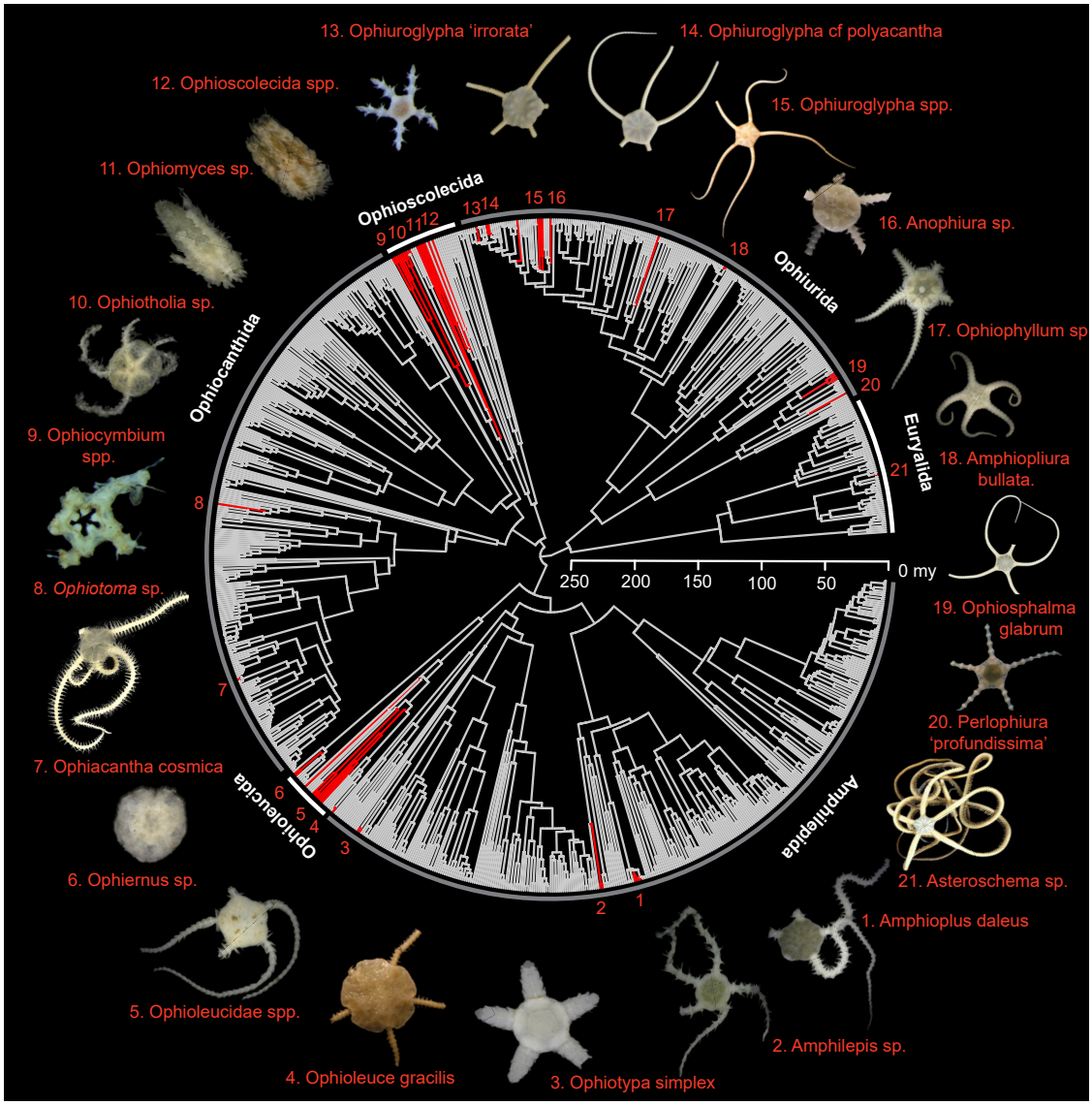

Figure 1. Simplified Fossil-Calibrated Phylogenomic Tree for the Ophiuroidea (Brittle Stars)

The relationships of 42 operational taxonomic units (in red, 49 samples; see Table S1 and Data S1) are shown from the abyssal seafloors of the Eastern Pacific Ocean. Selected tips are labeled with the corresponding image number. Order-level taxa are labeled in white. The images show the range of species sequenced, including tiny post-larva that are unidentifiable using conventional morphological techniques.

of the more variable 28S ribosomal subunit is adequate for lineage identification, better bridging the gap between variable COI sequences and our exon-based phylogenetic backbone. Moreover, although 28S was not targeted in our exon-capture protocol [6], it occurs in high copy number in eukaryote genomes [11], and we were able to retrospectively assemble long fragments from our transcriptome samples [12] or from off-target exon-capture reads, and hence build a large reference database. The implication for environmental assessment protocols is that multi-locus DNA data are desirable for a comprehensive assessment of target biodiversity [10, 13]. Traditional collection and identification techniques can fail to detect entire branches of invertebrate biodiversity.

The few bathyal species in the EP dataset are all phylogenetically distinct from the surrounding abyssal fauna. This confirms the findings from the southern hemisphere that abyssal and bathyal ophiuroid clades are generally distinct [5] and does not support the hypothesis of Rex et al. [14] that the abyssal fauna is an ecological "sink," where assemblages are only sustained by larval dispersal from bathyal habitats on continental margins. The new multi-species lineages detected across the abyssal EP also indicate that the abyss is not always an evolutionary "sink" either, composed solely of lineages originating from shallower depths. Instead the new data suggest that the abyssal EP fauna is composed of ancient lineages, some *in situ* radiations, and representatives of global species complexes that dominate abyssal

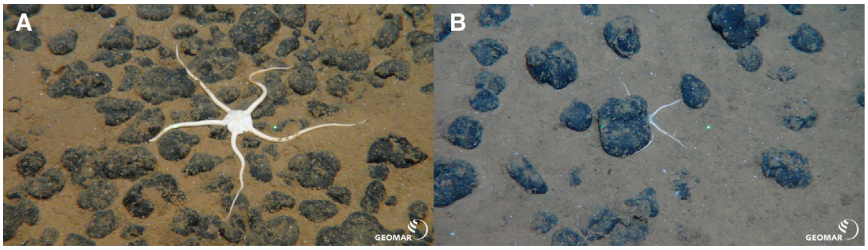

Figure 2. *In Situ* Photographs of Ophiuroids among Manganese Nodules on the Clarion Clipperton Abyssal Plain
(A) *Ophiosphalma glabrum*.
(B) *Ophiuroglypha* sp.
Photos: ROV KIEL 6000 Team/GEOMAR Kiel.

assemblages throughout the Atlantic, Indian, and Atlantic Oceans.

Most of the Ophiuroid fauna of the CCZ and Peru Basin lack formal Linnaean scientific names. This widens the gap between described species and actual biodiversity, which, as also shown here, appears to be far greater than previously estimated [15]. Our limited knowledge of these dark taxa ensures that they remain in the shadows of research and conservation policies. The lack of adequate baseline information at the onset of commercial-scale mining may result in serious species declines before they are described or even discovered. Given the very slow natural rates of deep-sea ecosystems' recovery, mining-induced loss of biodiversity is likely to last for hundreds of years [16]. The range of ecological and evolutionary services performed by deep-sea species is important to a productive, sustainable ecosystem and thus beneficial to humankind [17].

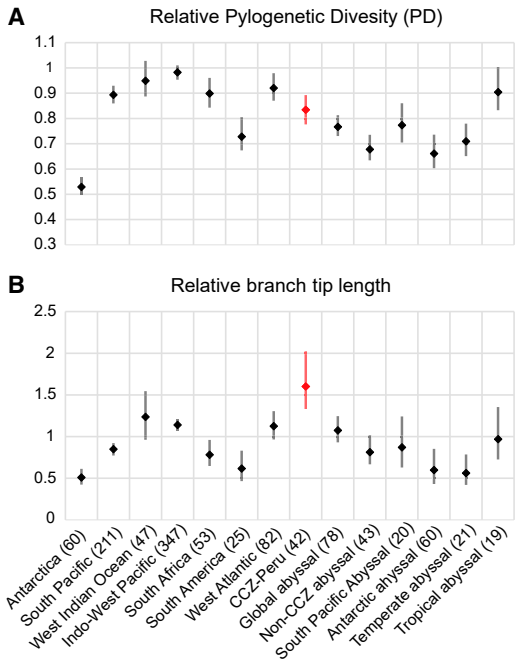

Figure 3. East Pacific (EP) Relative Phylogenetic Diversity Patterns Compared with Other Brittle Star Regional Assemblages
(A) Relative phylogenetic diversity of the EP abyssal fauna and comparative bioregions [7]; the last three refer to deep-sea biomes within the greater Southern Hemisphere region analyzed by O'Hara et al. [5]. Labels include sample size (= number of tips).
(B) Relative tip branch length. Based on the RAxML/PLRS Figure 1 tree (pruned to a single representative per species).
Graphs show median and decile confidence interval null resampling statistics [5].

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.09.012.

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AUTHOR CONTRIBUTIONS

P.M.A. and M.C. designed and performed the EP sampling and sequencing program. A.F.H. performed the phylogenomic analyses. T.D.O'H. interpreted the data and took the lead in writing the manuscript, to which all authors contributed.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
CCZ/Peru ophiuroid specimens	German Centre for Marine Biodiversity Research (DZMB) Senckenberg am Meer	See Table S1, this work
Exon capture ophiuroid specimens	Various museum collections	See Table S1, this work
Deposited Data		
Eastern Pacific COI sequences and trace files	BOLD systems	EP Deep Sea Ophiuroidea
Eastern Pacific COI and 28S sequences	NCBI nucleotide	COI: MN088035-MN088083; 28S: MN170900-MN170941
Phylodiversity scripts	[5], Dryad	https://doi.org/10.5061/dryad.9jk90f6
Exon capture pipeline	[18], Dryad	https://doi.org/10.5061/dryad.f5g2482
RAxML and BEAST files	This paper, Dryad	https://doi.org/10.5061/dryad.h77450k
Oligonucleotides		
COI primers	LCOech1aF1 and HCO2198, tailed with M13F and M13R-pUc	[19, 20]
28S primers	28S_F1a and 28S_R1a, tailed with M13F and M13R-pUc	[21]
Software and Algorithms		
RAxML v8.1.20	[22]	https://cme.h-its.org/exelixis/web/software/raxml/index.html
BEAST v2.4	[23]	https://www.beast2.org
PLRS r8s v7.3	[24]	http://ceiba.biosci.arizona.edu/r8s/index.html
MAFFT	[25]	https://mafft.cbrc.jp/alignment/software/
Geneious v.9.1.7	[26]	https://www.geneious.com/

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, T. O'Hara (tohara@museum.vic.gov.au) for the tree-of-life phylogenetics and M. Christodoulou (magdalini.christodoulou@senckenberg.de) for the Eastern Pacific samples and sequences. Specimens collected or used in this study are held in various natural history museums (see Table S1 for details). This study did not generate new reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Eastern pacific (EP) samples

Ophiuroid specimens were sorted from epibenthic (Brenke) sled [27] samples from six recent expeditions to the eastern CCZ (11 to 19°N, 116 to 130°W, 2013-2015), including to five different mining exploration license areas and one of the international Seabed Authority's designated "Area of Particular Environmental Interest" (APEI-3) [16, 28], and two expeditions to the Peru Basin (7°S, 88°W, 2015). Specifically, the CCZ expeditions include (1) the BIONOD cruise with R/V L'Atalante (29 March to 10 May 2012) to the areas licensed by France, through the Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER) and by Germany through the Federal Institute for Geosciences and Natural Resources, Germany (BGR); (2) two ABYSSLINE research cruises, AB01 with R/V Melville (3 to 27 October 2013) and the AB02 cruise with R/V Thompson (12 February to 25 March 2015) to the area licensed by the United Kingdom (UK-1) via the company UK Seabed Resources Ltd. (UKSRL); (3) two MANGAN exploration cruises with R/V Kilo Moana, MANGAN 2013 (1 April to 13 May) and MANGAN 2014 (15 April to 3 June) from the area licensed by Germany; and (4) the scientific cruise 'EcoResponce' with R/V Sonne, SO239 (11 March to 30 April 2015) from the areas licensed by Germany, Belgium and the Interoceanmetal Joint Organization (IOM. Ophiuroids were also collected from a 10.8 km2 circular area (DISturbance and reCOLonisation experimental area, DEA) of the nodule-rich seafloor in the Peru Basin during two cruises with the R/V Sonne, S0241/1 (28 July to 25 August 2015) and S0241/2 (28 August to 1 October 2015) under the framework of the JPIO Pilot Action 'Ecological Aspects of Deep-Sea Mining'. Shipboard, cod ends of the supra- and epi-net of the epibenthic sleds were sieved through a 500 μm- and 300 μm-mesh with cold (+10°C) sea water and immediately transferred to pre-cooled (−20°C) 96% EtOH. Large-sized

ophiuroid samples were also collected with the remotely operated vehicle (ROV 6000, Kiel, GEOMAR) using either the ROV's suction sampler or the ROV's manipulator arm by direct picking, manipulating scoops, shovels and nets. Voucher specimens are stored in Senckenberg am Meer, DZMB, Wilhelmshaven, Germany.

METHOD DETAILS

Overall approach

We used a super-matrix approach to combine a dataset of two DNA barcodes of ophiuroids (42 Operational Taxonomic Units, 1035 bp 28S rDNA + 658 bp COI mtDNA) collected from the EP with an existing global phylogenomic tree-of-life dataset (945 species, 50kbp nDNA + 28S + COI, Data S1A; Table S1) to produce a robust, time-calibrated [6] phylogeny illustrating the relationships of the EP fauna (Figure 1) and determining the relative phylogenetic diversity of this fauna compared to other regions (Figure 3).

DNA extraction, PCR amplification, and DNA sequencing of EP samples

DNA was extracted from arm tissue in individuals larger than 1-2 mm or from whole individuals when smaller than 1-2 mm. DNA extractions were carried out using 30 μL Chelex (InstaGene Matrix, Bio-Rad) according to the protocol of Estoup et al. [29] and directly used as DNA template for PCR. Fragments of the mitochondrial cytochrome *c* oxidase subunit (COI, 658bp) and nuclear 28S rRNA (~1038bp) genes from 49 specimens were amplified by polymerase chain reaction (PCR). Amplifications were performed using Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare) in a 25 μL volume containing 22 μL ddH2O, 0.5 μL of each primer (10 pmol μL^{-1}) and 2 μL of DNA template or AccuStart PCR SuperMix (ThermoFisher Scientific) in a 25 μL volume containing PCR SuperMix (9.5 μL ddH2O, 12.5 μL AccuStart), 0.5 μL of each primer (10 pmol μL^{-1}) and 2 μL of DNA template. For the COI amplification, the forward primer LCOech1aF1 and the reverse primer HCO2198, tailed with M13F and M13R-pUc were used respectively [19, 20]. For the 28S amplification the forward primer 28S_F1a and the reverse primer 28S_R1a were used, tailed again with M13F and M13R-pUc respectively [21]. The amplification conditions consisted of an initial denaturation step of 3min at 94°C, 35 cycles of 30 s at 94°C, 60 s at 42–52°C and 1min at 72°C were performed, followed by a final extension step of 5min at 72°C. The amplified fragments were sequenced in both directions at Macrogen Europe Laboratory (Amsterdam, the Netherlands).

Forward and reverse sequences for each individual were assembled and edited using Geneious v.9.1.7 (https://www.geneious.com/ [26]) and aligned using MAFFT v7.308 under E-INS-i and G-INS-I algorithms [25]. The dataset of 223 COI and 49 28S sequences were supplemented with 22 COI sequences from the study of Glover et al. [4]. From the COI dataset, we identified 42 species-level OTUs. However, we selected 49 COI+28S sequences to represent divergent lineages from all regions.

Exon-capture dataset

The multi-locus dataset used here is an extension of that used in the phylogenetic analyses of O'Hara et al. [5], [30] and built using the same methodologies and dating calibrations. Briefly, we used 52 transcriptomes to design probes to capture 1500 exons (285 kbp) from 418 single-copy genes [12]. With these probes, we used a target-enrichment or 'exon-capture' methodology [6] to sequence our target from 945 ophiuroid species across the globe, including 5 species from the CCZ. The full dataset (Table S1) represents almost 46% of described ophiuroid species-level diversity and has been used to completely restructure the higher-level taxonomy of the group [30].

Bioinformatic recovery of barcode sequences from exon-capture samples

Of possible barcode loci [31, 32], only the mitochondrial COI gene was targeted as part of our exon-capture protocol [6]. However, we managed to obtain ribosomal sequences (28S and 18S) of varying length from our assembled transcriptomes and off-target exon-capture reads using much the same procedure. The exon-capture reads were *de novo* assembled and searched for the longest contig > = 750bp per sample best matching an ophiuroid reference. Together with GenBank ophiuroid data, the candidate sequences were then aligned with MAFFT [25], trimmed of ambiguous sites [33] and a RAxML (v8.1.20) [22] tree compared to our exon data tree to check for anomalous sequences.

QUANTIFICATION AND STATISTICAL ANALYSIS

Choice of barcode loci

In order to produce a combined tree of the abyssal EP OTUs and the exon-capture samples, we used a super-matrix approach [34] to experiment with adding one or more typical meta-barcode sequences as separate partitions. We started with adding the 658 bp 'barcode' section of the Cytochrome Oxidase I (COI) mitochondrial gene, as we typically capture this gene as part of our exon-capture protocol [6]. However, this sequence could only robustly place 15 EP OTUs in trial phylogenies, specifically those species that were fortuitously < 12% p-distance to one of our tree-of-life samples. Consequently, we experimented with adding a section of nuclear rDNA as another partition. We found that the 400 bp of 18S (small ribosomal subunit) typically used in meta-barcode studies was too conserved to resolve relationships within family-level clades. However, adding a 1035 bp section of 28S (large subunit) robustly placed the majority of the abyssal EP OTUs while not perturbing the underlying exon capture framework. The only exception was within the *Ophiuroglypha*-like clade of the Ophiopyrgidae, where 28S+COI data was insufficient to robustly place several un-related species (Data S1A). In total we obtained 354 28S rDNA sequences from the transcriptome/exon capture samples that had at least

400bp overlap with the 1035bp PCR fragment (see Eastern Pacific Samples above). Full details of the 994 taxa exon, COI and 28S super-matrix are in Table S1.

Phylogenetic methods

We used two methods to produce ultrametric phylogenies. First we used Penalized Likelihood Rate Smoothing (PLRS; r8s v7.3 [24]) on a RAxML (v8.1.20) tree. Second, we used BEAST v2.4 [23] to infer a Bayesian phylogeny. Both of these approaches used exon data codon position model and calibration information drawn from previous analyses of our phylogenomic data [5, 6, 30]. To facilitate computationally demanding methods, we determined that a 46kbp subset of our exon data (219 exons, one per gene) essentially produced the same phylogeny as the full 275kb exon data, with only slightly reduced bootstrap support (BS) (85% versus 90% of nodes having > 95% BS). We then added the 28S and COI 'barcode' data, including the 49 EP taxa, to this phylogenomic framework and inferred a RAxML bootstrap consensus tree using GTRGAMMA 5 partition model (codon positions plus 28S and COI) and 200 BS replicates. This tree (Data S1A) recovered 80% of nodes with > 95% BS (and 90% of nodes > 70% BS) while retaining the underlying phylogenomic framework. Finally it was converted to an ultrametric chronogram (Figure 1) using PLRS with 12 fossil-based minimum-maximum calibrations [30], ADD penalty function and smoothing factor = 2.

A combination of a subset of taxa with this reduced sequence dataset then allowed us to infer a Bayesian dated phylogeny, using matching sequence model and calibrations (here invoked as offset gamma priors). We restricted the taxa to the 49 CCZ/EP samples plus 200 exon capture samples framing the family lineages [30] including those closest to the EP taxa. Two BEAST v2.4 [23] analyses using lognormal relaxed clock and Yule speciation prior were run for 100 million steps (1/20,000 sampling), and post burnin (25%) samples combined (ESS > 150), to yield a consensus tree (Data S1B) that is well correlated to the full taxon set PLRS tree dating (Data S1C). Full details of the model and calibrations are in the xml file in the Dryad depository. The BEAST analyses recovered 94% of nodes with posterior probability > 0.95 (Data S1B), confirming the general robustness of the supermatrix and placement of the abyssal EP taxa.

Phylodiversity methods

To provide some additional context for the pattern of phylogenetic diversity in the EP taxa, we assessed phylogenetic diversity (PD) and average tip age (terminal branch length) for a range of regions where sampling effort permitted (Figure 3). These were computed using the methodology in [5], based on the general community PD concepts [35, 36]. Relative PD is the PD relative to the species diversity and gives an indication of how clustered or dispersed the lineages are across the whole tree. Relative tip age is the mean tip age relative to the overall tree average. These indices were calculated using the RAxML/PLRS Figure 1 tree, pruned back to a single representative for each taxon (846 tips in total). PD and tip age were then expressed as values relative to the null expectation from 1000 random draws of matching species diversity (number of tips) from the whole tree. Median relative tip age and decile confidence limits for each region are shown on Figure 3. The biogeographic regions and depth layers are based on those in Stöhr et al. [7]. Taxa were assigned to bioregions based on the distributional data in [5, 8, 30] and Table S1.

DATA AND SOFTWARE AVAILABILITY

Information on all taxa are provided in Table S1, and phylogenomic data, calibrations and trees are available in the Dryad depository https://doi.org/10.5061/dryad.h77450k. Abyssal East Pacific COI Sequences and trace files are available from the project ''Deep Sea Biodiversity'' of the Barcode of Life (BOLD) online database as a separated dataset (EP Deep Sea Ophiuroidea). Sequences are also deposited in NCBI Nucleotide (GenBank) with accession numbers (COI: MN088035-MN088083, 28S: MN170900-MN170941). Scripts for the phylodiversity procedures are in a previous Dryad depository https://doi.org/10.5061/dryad.9jk90f6 [5] and exon capture pipelines in https://doi.org/10.5061/dryad.f5g2482 [18].