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New Species of *Osedax* (Siboglinidae: Annelida) from New Zealand and the Gulf of Mexico

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Abstract

Osedax is now known to be distributed around the world with more than 30 named and undescribed species. Here we report the discovery of four new species from two localities: *Osedax bozoi* n. sp. and *Osedax craigmcclaini* n. sp. from the Gulf of Mexico and *Osedax estcourti* n. sp. and *Osedax traceyae* n. sp. from off New Zealand. *Osedax bozoi* n. sp., *Osedax estcourti* n. sp., and *Osedax traceyae* n. sp. belong to Clade II within *Osedax*, one of the nude palp or apinnulate clades. *Osedax craigmcclaini* n. sp. belongs to the pinnulate palp Clade V. This study relies primarily on phylogenetic analysis, with some morphological analysis. Genetic data clearly show that the four new species are distinctive from their closest *Osedax* relatives. Two of the new species were found from less than 400 m depth, and incidences of shallower water *Osedax* in Clade II are shown here for the first time.

Key words: deep sea, phylogeny, haplotype network, polychaete, whale fall, alligator fall

Introduction

Osedax Rouse *et al.*, 2004 is a clade of marine annelids belonging to Siboglinidae, a family with other well-known taxa such as the Vestimentifera hydrothermal vent worms. *Osedax* is notable for exploiting the organic matrix in the bones of sunken marine carcasses with the aid of symbiotic bacteria (Goffredi *et al.* 2005, 2007). *Osedax* secretes acid that dissolves the inorganic bone matrix so that *Osedax* can root itself in the bone and consume the organic nutrients (collagen) inside (Tresguerres *et al.* 2013). *Osedax* was first described from a whalefall off California and since then has been found in most oceans, with 29 named species and several yet to be named species from many localities (Amon *et al.* 2014; Eilertsen *et al.* 2020; Fujikura *et al.* 2006; Fujiwara *et al.* 2019; Georgieva *et al.* 2023; Glover *et al.* 2005; Rouse *et al*. 2004; Rouse *et al.* 2018). In this study we describe four new species, one of which was previously reported based on DNA data only. Two of the new species were collected from an alligator carcass and cow bones, respectively, that were experimentally sunken in the Gulf of Mexico (McClain *et al.* 2019). The other two new species in the study were collected from a whale skull collected in a trawl off New Zealand. The evidence to support the establishment of the four species is primarily molecular since the available specimens were in poor morphological condition. The descriptions follow precedents set by Amon *et al.* (2014) and Georgieva *et al.* (2023), where minimal morphological data was used. Fragments of two mitochondrial genes (cytochrome c oxidase subunit I and 16S rRNA) and three nuclear genes (18S rRNA and 28S rRNA and Histone-H3) were sequenced to assess the phylogenetic placement of the species.

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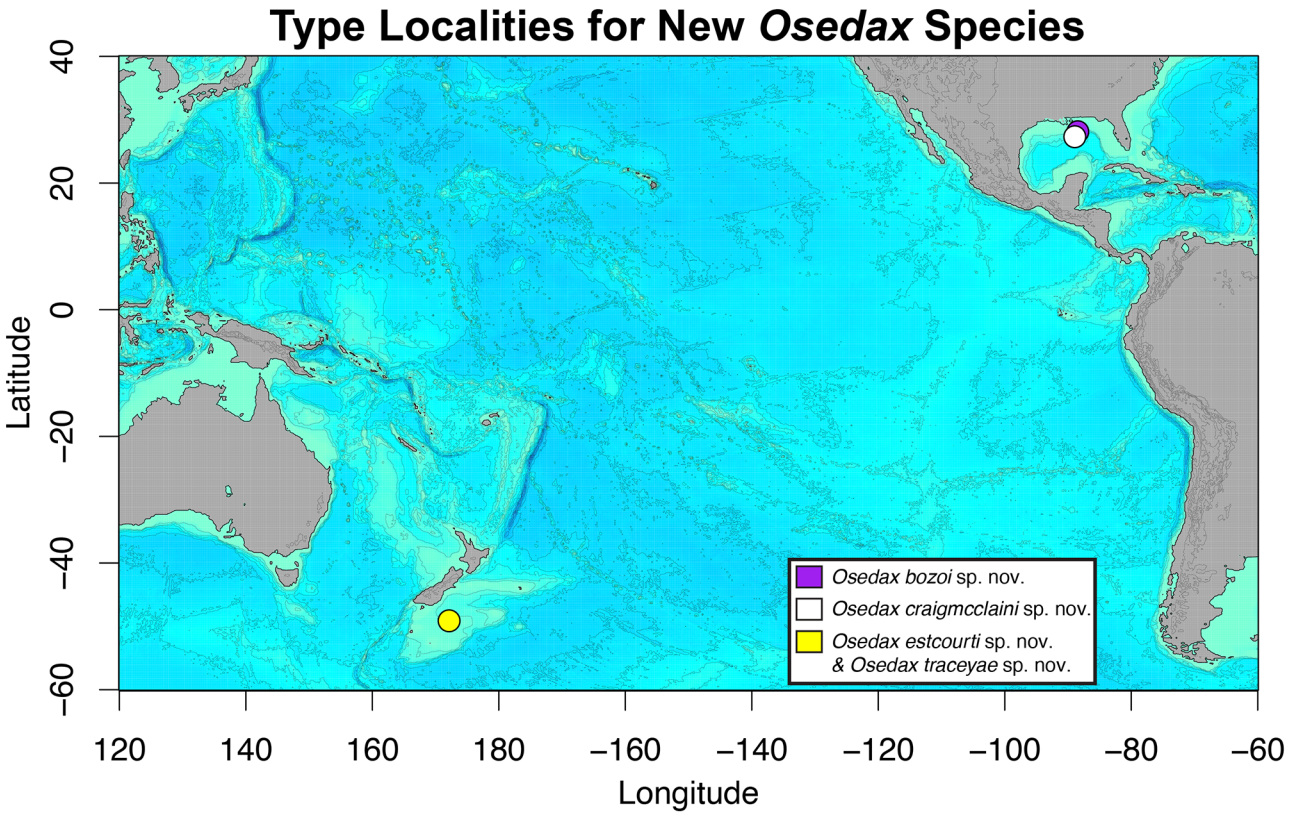

FIGURE 1. Map of geographic distributions of the four new *Osedax* species described here. The map was generated using the R package marmap (Pante & Simon-Bouhet 2013).

Materials and methods

Sample collection, morphology, and vouchering

Specimens were collected from an alligator carcass, *Alligator mississippiensis* Daudin, 1802, and cow, *Bos taurus* Linnaeus, 1758 bones that were experimentally deployed for 51 days at ~2,000 m in the Gulf of Mexico off Mississippi River Delta (Louisiana) by colleagues at the Louisiana Universities Marine Consortium (LUMCON) (McClain *et al.* 2019). Bones were recovered by the Remote Operated Vehicle (ROV) *Global Explorer* operated from the R/V *Pelican*, fixed in 95% ethanol, and sent to Scripps Institution of Oceanography (SIO) where *Osedax* were dissected out and photographed. Samples were also collected from a whale skull recovered from ~390 m in a scientific trawl deployed on the Pukaki Rise off New Zealand by colleagues at the National Institute of Water and Atmospheric Research (NIWA) and then frozen. *Osedax* were scraped off the skull, fixed in 95% and 99% ethanol, and sent to Scripps Institution of Oceanography. Either Leica S8Apo or Leica MZ12.5 stereomicroscopes were used to visualize specimens during dissection and to photograph them with a Canon Rebel T7i camera. *Osedax* specimens and types have been lodged at Benthic Invertebrate Collection at Scripps Institution of Oceanography, La Jolla, California USA (SIO-BIC) and the NIWA Invertebrate Collection (NIC) in Wellington, New Zealand.

DNA extraction, amplification, and sequencing

DNA was extracted from whole *Osedax* specimens or pieces of root tissue using either Zymo Research DNA-Tissue Miniprep Kit or Zymo Research Quick-DNA Microprep Plus Kits (Irvine, California, USA), following the protocols supplied by the manufacturer. Extractions were used to sequence fragments of mitochondrial (cytochrome *c* oxidase subunit I (*COI*) and 16S rRNA (*16S*)) and nuclear (18S rRNA (*18S*), 28S rRNA (*28S*), and Histone H3 (*H3*)) genes.

All specimens were sequenced for *COI* with a single representative of each species sequenced for the other genetic markers. DNA sequencing was completed with the PCR primers and temperature profiles shown in Table 1 and performed with Eppendorf 5345 Epgradient S Mastercycler (Eppendorf, Hamburg, Germany). Amplification was carried out using a PCR mixture of 12.5 µl Apex 2.0x Taq Red DNA Polymerase Master Mix (Genesee Scientific, San Diego, California, USA) or 12.5 µl Conquest PCR 2.0x Master Mix 1 (Lamda Biotech, Ballwin, Missouri, USA), 1 µl each of the appropriate forward and reverse primers (10 µM), 8.5 µl of ddH_2O, and 2 µl of eluted DNA. Final PCR products were purified with the ExoSAP-IT protocol (USB Affymetrix, Ohio, USA), and Sanger sequencing was performed in both directions by Eurofins Genomics (Louisville, Kentucky, USA). Sequences were assembled using Geneious software v11.1 (©Biomatters Ltd.; http://www.geneious.com/, New Zealand) and the new DNA sequences obtained have been deposited in GenBank (Bethesda, Maryland, USA) (Table 2).

TABLE 1. Genes, primers, references, and PCR reaction protocols used in this study.

Gene	Primer set	Source	Reaction protocol
COI	polyLCO/polyHCO	(Carr *et al.* 2011)	95°C/180s–(95°C/40s–42°C/45s–72°C/50s) * 40 cycles–72°C/300s
	COIf/COIr	(Nelson & Fisher 2000)	95°C/300s–(94°C/60s–55°C/60s–72°C/120s) * 35 cycles–72°C/420s
	LCO1490/HCO2198	(Folmer *et al.* 1994)	94°C/180s–(94°C/30s–47°C/45s–72°C/60s) * 5 cycles–(94°C/30s–52°C/45s–72°C/60s) * 30 cycles–72°C/300s
16S	16SarL/16SbrH	(Palumbi 1996)	95°C/180s–(95°C/40s–50°C/40s–72°C/50s) * 35 cycles–72°C/300s
18S	18S-1F/18S-5R	(Giribet *et al.* 1996)	95°C/180s–(95°C/30s–50°C/30s–72°C/90s) * 40 cycles–72°C/480s
	18S-a2.0/18S-9R	(Giribet *et al.* 1996; Whiting *et al.* 1997)	95°C/180s–(95°C/30s–50°C/30s–72°C/90s) * 40 cycles–72°C/480s
	18S-3F/18S-bi	(Giribet *et al.* 1996; Whiting *et al.* 1997)	95°C/180s–(95°C/30s–52°C/30s–72°C/90s) * 40 cycles–72°C/480s
28S	D1F/D3R	(Brown *et al.* 1999)	94°C/180s–(94°C/60s–55°C/30s–72°C/110s) * 35 cycles–72°C/240s
H3	H3F/H3R	(Colgan *et al.* 1998)	95°C/180s–(95°C/30s–53°C/45s–72°C/45s) * 40 cycles–72°C/300s

TABLE 2. Terminals and GenBank numbers for sequences used to generate the phylogeny shown in Figure 1. New species and their sequences are **bold**. Additional *COI* GenBank numbers for the new species described here are listed in the text and the legends for Figures 5 and 7.

Species	Authority	*COI*	*16S*	*18S*	*28S*	*H3*
Lamellibrachia columna	Southward, 1991	DQ996645	FJ347646	FJ347679	MG264417	FJ347696
Riftia pachyptila	Jones, 1985	KP119562	KP119573	KP119591	KP119582	KP119555
Sclerolinum brattstromi	Webb, 1964	FJ347644	FJ347644	FJ347680	FJ347677	FJ347697
Osedax antarcticus	Glover *et al.*, 2013	KF444422	KF444418	KF444420	-	-
Osedax 'BioSuOr-1'	Shimabukuro & Sumida, 2019	MH616036	-	-	-	-
Osedax 'BioSuOr-2'	Shimabukuro & Sumida, 2019	MH616081	-	-	-	-
Osedax 'BioSuOr-3'	Shimabukuro & Sumida, 2019	MH616075	-	-	-	-
Osedax 'BioSuOr-4'	Shimabukuro & Sumida, 2019	MH616012	-	-	-	-
***Osedax bozoi* n. sp.**	**This study**	**ON357627**	**ON261606**	**ON261611**	**ON261610**	**ON254806**

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TABLE 2. (Continued)

Species	Authority	*COI*	*16S*	*18S*	*28S*	*H3*
Osedax braziliensis	Fujiwara *et al.*, 2019	LC381421	-	LC381424	-	-
Osedax bryani	Rouse *et al.*, 2018	KP119563	KP119574	KP119597	KP119584	KP119561
Osedax byronbayensis	Georgieva *et al.*, 2023	OQ801427	OQ820973	OQ803227	-	-
***Osedax craigmcclaini* n. sp.**	**This study, McClain *et al.* 2019**	MN258704	**ON217799**	**ON220153**	**ON226742**	**ON254807**
Osedax crouchi	Amon *et al.*, 2014	KJ598038	KJ598032	KJ598035	-	-
Osedax deceptionensis	Taboada *et al.*, 2015	KF444428	KF444419	KF444421	MG264418	KT860546
Osedax docricketts	Rouse *et al.*, 2018	FJ347626	FJ347650	FJ347688	FJ347666	FJ347710
***Osedax estcourti* n. sp.**	**This study**	**ON211943**	**ON217536**	**ON220129**	**ON220739**	**ON254809**
Osedax fenrisi	Eilertsen *et al.*, 2020	MT556178	-	MT556473	-	-
Osedax frankpressi	Rouse *et al.*, 2004	FJ347607	FJ347658	FJ347682	FJ347674	FJ347705
Osedax jabba	Rouse *et al.*, 2018	FJ347638	FJ347647	FJ347693	FJ347676	FJ347703
Osedax japonicus	Fujikura *et al.*, 2006	FM998111	-	FM995535	-	-
Osedax knutei	Rouse *et al.*, 2018	FJ347635	FJ347648	FJ347692	FJ347664	FJ347700
Osedax lehmani	Rouse *et al.*, 2018	DQ996634	FJ347660	FJ347689	FJ347672	FJ347706
Osedax lonnyi	Rouse *et al.*, 2018	FJ347643	FJ347651	FJ347695	FJ347663	FJ347699
Osedax 'MB16'	Salathé & Vrijenhoek, 2012	JX280613	KP119581	KP119592	KP119588	KP119560
Osedax 'mediterranea'	Taboada *et al.*, 2015	KT860548	KT860551	KT860550	KT860549	KT860547
Osedax mucofloris	Glover *et al.*, 2005	AY827562	-	AY941263	-	-
Osedax nordenskjoeldi	Amon *et al.*, 2014	KJ598039	KJ598033	KJ598036	-	-
Osedax packardorum	Rouse *et al.*, 2018	FJ347629	FJ347661	FJ347690	FJ347673	FJ347707
Osedax priapus	Rouse *et al.*, 2015	KP119564	KP119575	KP119594	KP119585	KP119556
Osedax randyi	Rouse *et al.*, 2018	FJ347615	FJ347659	FJ347684	FJ347675	FJ347712
Osedax rogersi	Amon *et al.*, 2014	KJ598034	KJ598037	KJ598040	-	-
Osedax roseus	Rouse *et al.*, 2008	FJ347609	FJ347657	FJ347683	FJ347670	FJ347709
Osedax rubiplumus	Rouse *et al.*, 2004	MT108936	FJ347656	FJ347681	FJ347671	FJ347704
Osedax ryderi	Rouse *et al.*, 2018	KP119563	KP119574	KP119597	KP119584	KP119561
Osedax 'sagami-3'	Pradillon *et al.* unpublished	FM998081	-	FM995537	-	-
Osedax 'sagami-4'	Pradillon *et al.* unpublished	FM998082	-	FM995541	-	-
Osedax 'sagami-5'	Pradillon *et al.* unpublished	FM998083	-	FM995539	-	-
Osedax sigridae	Rouse *et al.*, 2018	FJ347642	FJ347655	FJ347694	FJ347669	FJ347711
Osedax sp. AM W.52196	Georgieva *et al.*, 2023	OQ801426	-	-	-	-
Osedax talkovici	Rouse *et al.*, 2018	FJ347621	FJ347654	FJ347685	FJ347668	FJ347698
Osedax tiburon	Rouse *et al.*, 2018	FJ347624	FJ347653	FJ347687	FJ347662	FJ347702
***Osedax traceyae* n. sp.**	**This study**	**ON211990**	**ON212680**	**ON210988**	**ON220740**	**ON254808**
Osedax ventana	Rouse *et al.*, 2018	EU236218	FJ347652	FJ347686	FJ347665	FJ347701
Osedax waadjum	Georgieva *et al.*, 2023	OQ801430	OQ820974	OQ803228	-	-
Osedax westernflyer	Rouse *et al.*, 2018	FJ347631	FJ347649	FJ347691	FJ347667	FJ347708

Molecular data analysis

A representative terminal from each of previously published *Osedax* species (named or unnamed) was included in the analysis as well as one terminal from each of the four new species. Some species included in the analysis had only COI or one to two other genetic markers available rather than the full five that we used in our analysis (see Table 2). Outgroups for the analysis were representative siboglinids from Vestimentifera (*Lamellibrachia columna* Southward, 1991 and *Riftia pachyptila* Jones, 1981) and *Sclerolinum* Southward, 1961 that form the sister group to *Osedax* (Li *et al.* 2017). The individual markers were aligned in Mesquite (v3.61) (Maddison & Maddison 2019) using MAFFT (Katoh & Standley 2013) with default settings for *COI* and *H3* and with the G-INS-I option for the rRNA genes. Following concatenation using RAxML GUI v2.0 (Edler *et al.* 2020), a maximum likelihood analysis was conducted with RAxML-NG (Kozlov *et al.* 2019). Optimal models were chosen for each partition using ModelTest-NG v0.1.7 (Darriba *et al.* 2020) as follows (based on AICc): COI= GTR+I+G4, 16S= TIM2+I+G4, 18S= GTR+I+G4, 28S= TIM3+I+G4, H3= TVMef+I+G4. Node support was assessed via thorough bootstrapping (with 1000 pseudo replicates). Interspecific and intraspecific pairwise distances were calculated in PAUP* (v4.0a168) (Swofford 2002) using untrimmed alignments. TCS haplotype networks (Clement *et al.* 2000) were constructed using PopArt (Leigh & Bryant 2015) using trimmed alignments. Redundant diagnostic nucleotide combinations (rDNC) were inferred for each of the new species using the program MolD v1.4 (MOLecular Diagnoses) (Fedosov *et al.* 2022) (https://itaxotools.org/download.html). Fedosov *et al.* (2022) demonstrated that rDNCs allow for more robust diagnoses of species using molecular data. These rDNCs allow for unsampled genetic diversity and were based on the COI alignment of the *Osedax* taxa shown in Table 2 and all the available COI sequences for each of the new species including each holotype (GenBank registrations shown in Fig. 6, 8 legends). The DNA base numbers provided in each diagnosis refer to the complete COI sequence from the mitochondrial genome of the type species of *Osedax*, *O. rubiplumus* (GenBank number MT108936). The alignment was trimmed so that there was no missing data to allow MolD to run correctly. The resulting rDNCs refer to a region of 483 bases corresponding to positions 163 to 645 of the complete COI gene.

Results

Phylogeny

As in previous phylogenetic studies, *Osedax* could be divided into six main clades, I–VI. The four new species of *Osedax* belong to two of these clades (Fig. 2). *Osedax craigmcclaini* n. sp. belongs to Clade V, one of the three clades with pinnulate palps. Clade V was well supported with bootstrap support of 97 but other nodes within the clade showed much less support. *Osedax craigmcclaini* n. sp. was recovered as the sister group to a poorly supported clade of mainly Pacific Ocean species, along with one yet unnamed species (*O*. BioSur-4) from off Brazil and the globally distributed *Osedax rubiplumus* Rouse *et al*., 2004. *Osedax bozoi* n. sp., *O. estcourti* n. sp., and *O. traceyae* n. sp. all belong to Clade II, an apinnulate or nude palp clade. Clade II showed poor support for most nodes (Fig. 2) but showed two main subclades, as well as the outlying *Osedax waadjum* Georgieva *et al.*, 2023. *Osedax bozoi* n. sp. formed one of these poorly supported clades with three species from the North Pacific Ocean. *Osedax estcourti* n. sp. and *O. traceyae* n. sp. fell in the other main clade of Clade II, with members from the Pacific and Antarctic regions.

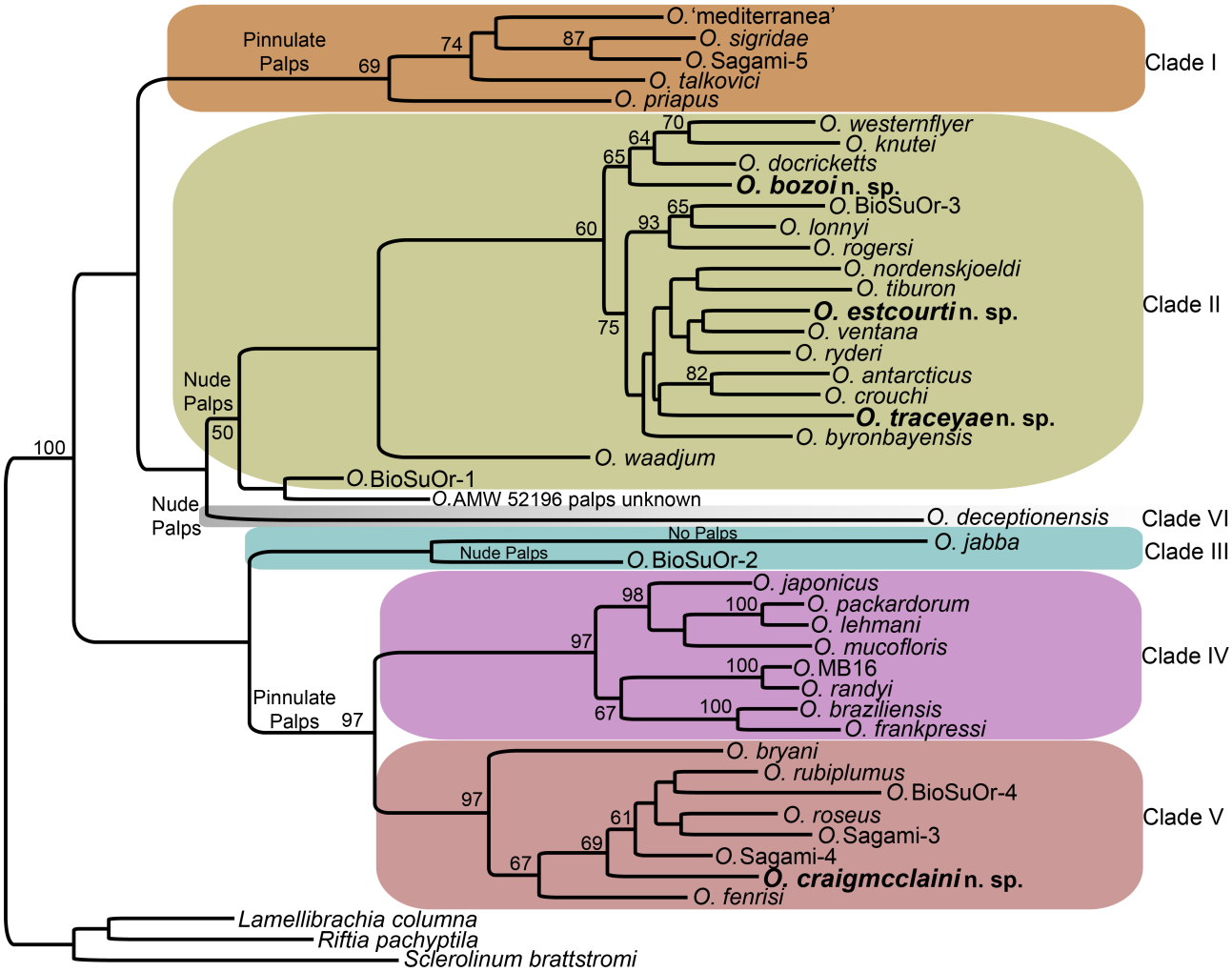

FIGURE 2. Maximum likelihood *Osedax* phylogenetic tree generated with data from five genetic markers as listed in Table 2. Bootstrap support values are on each node; those below 50% not shown. Presence or absence of pinnules is noted for each clade.

Taxonomy

Siboglinidae Caullery, 1914

Osedax Rouse, Goffredi & Vrijenhoek, 2004

Type species: *Osedax rubiplumus* Rouse, Goffredi, & Vrijenhoek, 2004

Osedax bozoi n. sp.

Fig. 3A, 4A–D, 6A

Material examined. Holotype: SIO-BIC A13918, female (GenBank *COI* sequence ON357631), collected from experimentally deployed cow bones deployed at 1,996 m depth in the Gulf of Mexico, offshore of New Orleans, Louisiana (28.103° N; 88.451° W); ROV *Global Explorer* dive number 17, April 15, 2019; fixed and preserved in 95% ethanol. **Paratypes:** SIO-BIC A10278 (destroyed, GenBank numbers in Table 2), A13920 (GenBank *COI* sequence ON357630), A13922 (GenBank *COI* sequences ON357686), females, collection data for paratypes is the same as for the holotype.

Diagnosis and description. Preserved holotype and other specimens white with greenish patches on root/ovisac (Fig. 4A–D). Four apinnulate palps, distally coiled, ~1.5 mm long, ~0.2 mm wide, mainly contained inside transparent tube (Fig. 4A–D). Trunk ~0.5 mm long, 0.2–0.4 mm wide (Fig. 4A, B, D). Clear demarcation between palps and trunk; small 'collar' visible ventrally at truck/palp junction (Fig. 4B). Oviduct visible dorsally along trunk and extends into crown of palps, complete length unknown (Fig. 4A). Roots incomplete in holotype (Fig. 4B–D), though root extensions may be present on either side of the trunk (Fig. 4B, D). Paratype SIO-BIC A13922 with lobed ovisac, lateral root lobes, root extensions present on either side of the trunk (Fig. 4D). No dwarf males observed. The rDNC diagnosis for *Osedax bozoi* n. sp. was recovered as: 'C' at site 465, 'G' at site 468, and 'T' at site 561 of mitochondrial COI.

Distribution. *Osedax bozoi* n. sp. was recovered from cow bones (Fig. 3A) deployed at 1,996 m in the Mississippi River Delta region of the Gulf of Mexico south of New Orleans, Louisiana (Fig. 1).

Etymology. *Osedax bozoi* n. sp. is named for the first author's late cat, Bozo.

Remarks. *Osedax bozoi* n. sp. belongs to Clade II (Fig. 2), an apinnulate 'nude palp' clade. Only associated with deployed cow bones (Fig. 3A). Paratype SIO-BIC A10278 was sequenced for *16S*, *18S*, *28S*, and *H3* as well as *COI* (Table 2), but the specimen was destroyed for DNA extraction. SIO-BIC A13918, which had a close *COI* sequence and was largely intact, has been designated as the holotype (Fig. 4A, B). Specimens SIO-BIC A10276 (ON357629) and SIO-BIC A10277 (ON357628) were also destroyed for sequencing *COI*. *Osedax bozoi* n. sp. had a 1.3% maximum pairwise distance among the six available sequences, which all showed the rDNC diagnostic bases. The haplotype network for *Osedax bozoi* n. sp. had four unique haplotypes (Fig. 6A). One was shared by three of the six sequences, including the holotype. There were three nucleotide substitutions between the most divergent haplotypes, based on a trimmed datafile of 344 bases. *Osedax bozoi* n. sp. was recovered as the sister group to a clade within Clade II that comprised *O. docricketts, O. westernflyer* and *O. knutei* (Fig. 2), though this was poorly supported. These three taxa are all from the Pacific Ocean. In terms of phylogenetic relatedness, the nearest species was *Osedax docricketts*, an apinnulate species known from Monterey Bay (California, USA) and Sagami Bay (Japan) on cow and whale bones (Rouse *et al.* 2018). *Osedax bozoi* n. sp. and *O. docricketts* share some morphological characteristics: both lack pigmentation on the trunk and palps and pinnules, both have a tube containing the palps. However, where *O. bozoi* n. sp. has a distinct demarcation between the palps and the trunk, *O. docricketts* does not, and the ovisac and oviduct are distinctive on *O. bozoi. Osedax docricketts* is suspected to be a cryptic species complex (Berman *et al.* 2023; Rouse *et al.* 2018) and the minimum interspecific distance between the two species was 13.7% based on sequence EU267676, an individual of *Osedax docricketts* from Monterey Bay (Table 3).

TABLE 3. Uncorrected maximum intraspecific distances for the mitochondrial COI gene.

New Species	Intraspecific Distance
Osedax bozoi n. sp.	0.0123
Osedax craigmcclaini n. sp.	0.0122
Osedax estcourti n. sp.	0.0146
Osedax traceyae n. sp.	0.0050

TABLE 4. Minimum interspecific distances (uncorrected) for the mitochondrial COI gene between each new species and its sister species or otherwise proximate species, based on phylogeny shown in Figure 1.

New species	Sister or proximate species	Distance
Osedax bozoi n. sp.	*O. docricketts*	0.136
Osedax craigmcclaini n. sp.	*O. fenrisi*	0.146
Osedax estcourti n. sp.	*O. ventana*	0.136
Osedax traceyae n. sp.	*O. antarcticus*	0.154

***Osedax craigmcclaini* n. sp.**
Fig. 3B, C, 5A, B, 6B

Osedax sp. McClain *et al.*, 2019, p. 7 of 14

Material examined. **Holotype:** SIO-BIC A13910 (GenBank *COI* sequence ON211944), collected from experimentally deployed alligator (*Alligator mississippiensis*) bones deployed at 2,034 m depth in the Gulf of Mexico, offshore of New Orleans, Louisiana, (27.312° N; 88.927° W), ROV *Global Explorer* dive number 16, April 12, 2019. Fixed and preserved in 95% ethanol.

Diagnosis and description. Holotype palps are pinnulated, white in preserved state; less than 1 mm long ~0.33 mm wide (Fig. 5B). No other body parts observed. No dwarf males observed. The rDNC diagnosis for *Osedax craigmcclaini* n. sp. was recovered as: 'C' at site 318, 'T' at site 333, and 'C' at site 462 of mitochondrial COI.

Distribution. *Osedax craigmcclaini* n. sp. was recovered from an alligator skeleton at 2,034 m off the Mississippi River Delta region, Louisiana, in the Gulf of Mexico (Fig. 1).

Etymology. *Osedax craigmcclaini* n. sp. is named for Dr. Craig McClain, an esteemed deep-sea biologist and colleague who led the experimental alligator fall project (McClain *et al.*, 2019) and provided the *Osedax* specimens for this study.

Remarks. *Osedax craigmcclaini* belongs to Clade V, a pinnulate clade (Fig. 2). Evidence for this species was originally published in McClain *et al.* (2019) with *COI* only (GenBank Accession number MN258704), from SIO-BIC A10731. In addition, *16S* (ON217799), *18S* (ON220153), *28S* (ON226742), and *H3* (ON254807) were sequenced from the remaining the SIO-BIC A10731 DNA extraction for this study. Specimen SIO-BIC A13910 has been designated here as the holotype based on its *COI* sequence (ON211944) closely matching MN258704 from McClain *et al.* (2019) (1.2% uncorrected distance). Both sequences showed the three rDNC diagnostic bases. Based on the phylogeny shown in Figure 2, a proximate species is *Osedax fenrisi* Eilertsen *et al.*, 2020, a pinnulate species collected from 2,341 m on the Arctic Mid-Ocean Ridge (Eilertsen *et al.* 2020). The minimum interspecific distance between the two species was 14.6% (Table 3). There are species of *Osedax* with smaller uncorrected *COI* distances, such as *Osedax crouchi* Amon *et al.*, 2014 from Antarctica, which belongs to the nude palp Clade II (Fig. 2), and McClain *et al.* (2019) reported the new species as falling within this clade. However, this proposed placement was based on *COI* data only, which can be misleading (Vrijenhoek *et al.* 2009), and the five gene phylogeny and photographs of the holotype confirm *O. craigmcclaini* n. sp. as actually a member of the pinnulate Clade V. *Osedax craigmcclaini* n. sp. showed two unique haplotypes with seven nucleotide substitutions between them (Fig. 5B). Specimens were not observed alive, however *in situ* images of the alligator corpse from which *O. craigmcclaini* was collected show red *Osedax* coating the jawbone and spine (Fig. 3B, C), suggesting that living *O. craigmcclaini* n. sp. may have red palps.

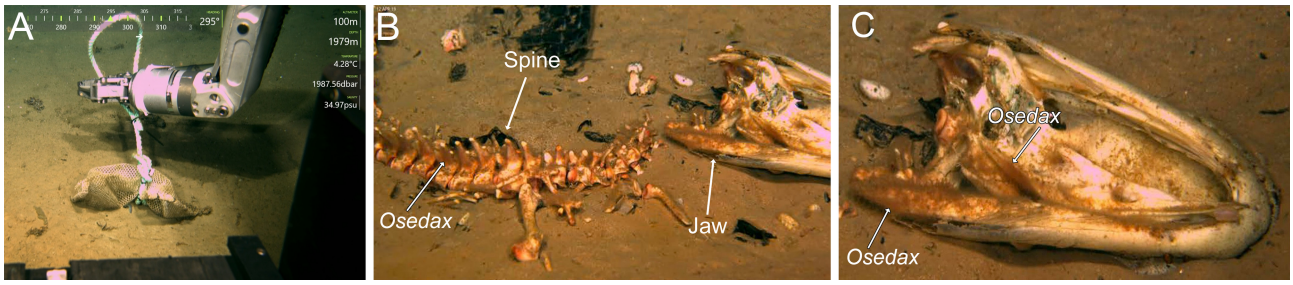

FIGURE 3. A. Cow bones from which *Osedax bozoi* n. sp. were found being recovered by ROV after 51 days at ~2,000 m in the Gulf of Mexico. **B.** Spine and skull of *Alligator mississippiensis* deployed at ~2,000 m in the Gulf of Mexico. *Osedax* are visible on the vertebrae and jaw. *Osedax craigmcclaini* n. sp. was found on these bones. **C.** *Alligator mississippiensis* skull with *Osedax* visible on the jaw. Images courtesy of Craig McClain.

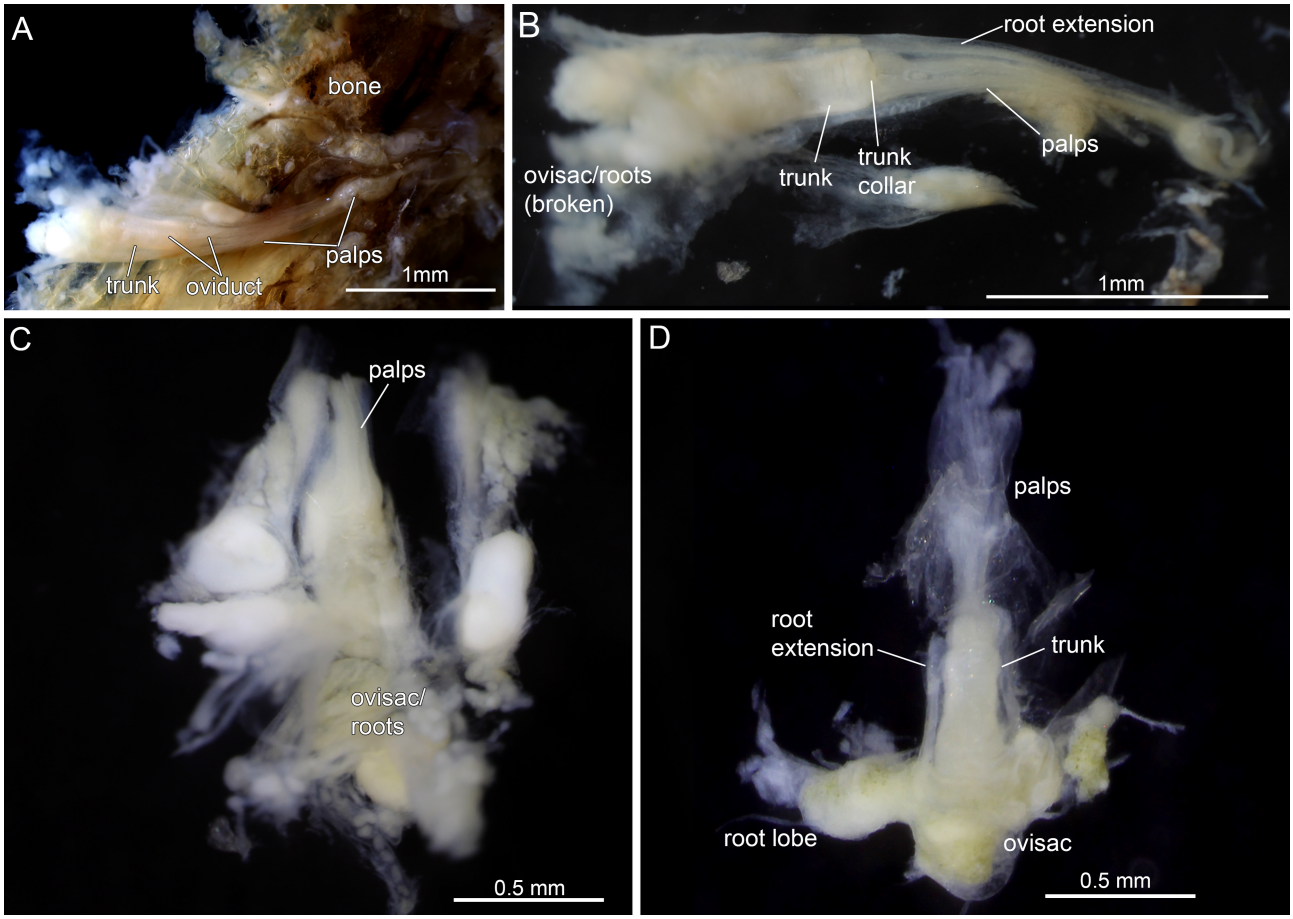

FIGURE 4. A. *Osedax bozoi* n. sp., dorsal view of female holotype (SIO-BIC 13918) still partially in bone. **B.** *Osedax bozoi* n. sp., ventral view of holotype (SIO-BIC 13918) removed from bone. **C.** Paratype female *Osedax bozoi* n. sp. (SIO-BIC 13920) removed from bone showing palps, ovisac, and roots. **D.** Paratype female *Osedax bozoi* n. sp. (SIO-BIC 13922) showing palps, trunk, ovisac, and root system.

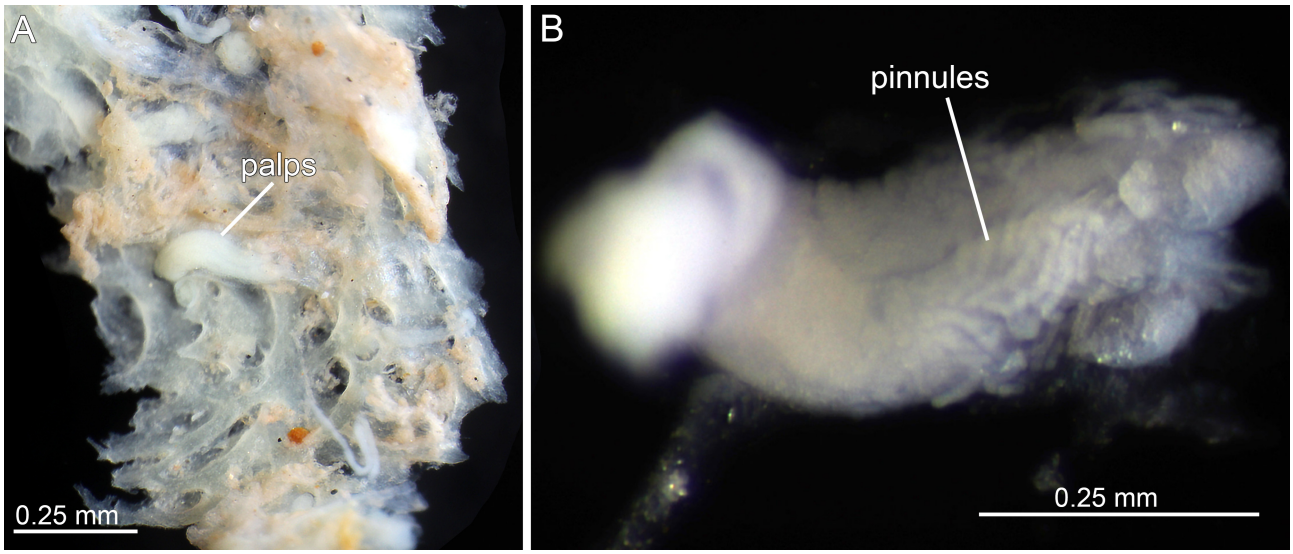

FIGURE 5. A. *Osedax craigmcclaini* n. sp. palps of holotype (SIO-BIC A13910) still in the bone. **B.** *Osedax craigmcclaini* n. sp. pinnulate palps of holotype (SIO-BIC A13910) removed from bone; the remaining body piece was used for DNA extraction.

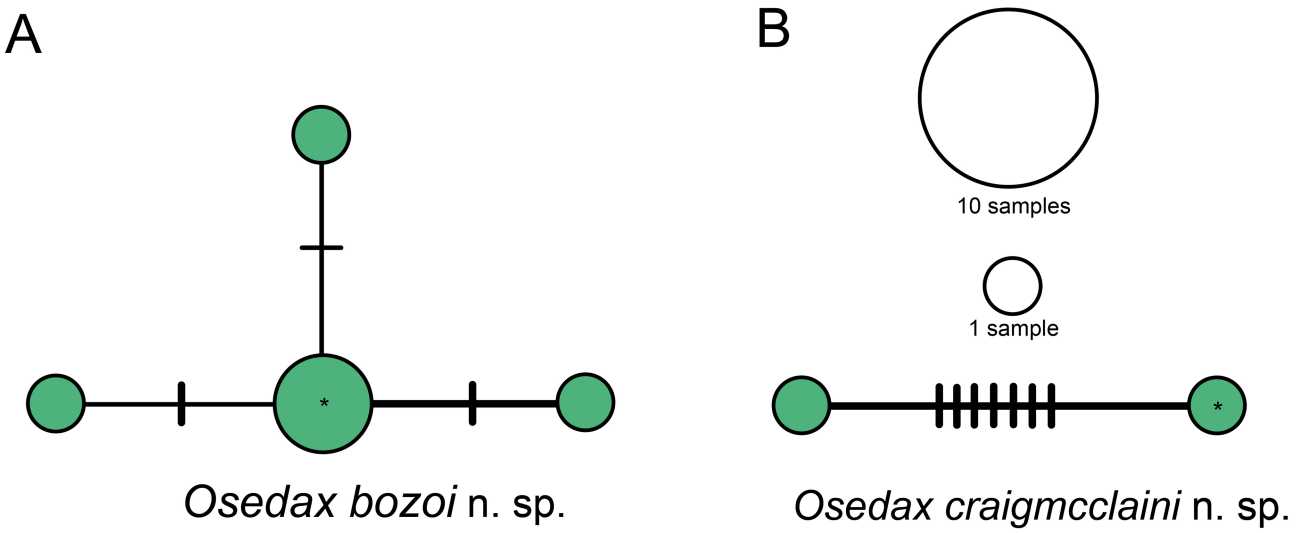

FIGURE 6. Haplotype networks using COI. Circles are haplotypes, green circles and crosshatches are single nucleotide substitutions. * indicates the haplotype of the holotype. **A.** Network for six specimens of *Osedax bozoi* n. sp. GenBank accession numbers: ON357627, ON357628, ON357629, ON357630, ON357631 (Holotype), ON357686. **B.** Network for two specimens of *Osedax craigmcclaini* n. sp. GenBank accession numbers: MN258704, ON211944 (Holotype).

***Osedax estcourti* n. sp.**
Fig. 7A, B, 8A

Material examined. Holotype: NIWA 159436 female (GenBank *COI* sequence ON211943, *18S* = ON220129, *28S* = ON220739, *H3* = ON254809), collected from a whale skull (most likely a southern minke whale *Balaenoptera bonaerensis* Burmeister, 1867 at 390–393 m depth on the Pukaki Rise SE of New Zealand (49.121° S; 172.136° E). Scientific trawl TAN1614, Station 9, R/V *Tangaroa*, December 1, 2016. Fixed and preserved in 95% ethanol.

Diagnosis and description. Live animals red, in transparent tubes on whale skull (Fig. 7A). Fixed roots and palps desiccated (Fig. 7B). Trunk not visible (Fig. 7B). Apinnulate palps are brown, approximately 3 mm long and 1 mm wide (Fig. 7B). Four palps contained inside translucent tube (Fig. 7B). Roots approximately 3 mm long, 1.5 mm wide, brown, still partially embedded in white bone (Fig. 7B). No dwarf males observed. The rDNC diagnosis for *Osedax estcourti* n. sp. was recovered as: 'C' at site 348, 'G' at site 579, and 'G' at site 606 of mitochondrial COI.

Distribution. *Osedax estcourti* n. sp. was recovered from a whale fall on the Pukaki Rise off SE New Zealand at 390–393 m.

Etymology. *Osedax estcourti* n. sp. is named in remembrance of Dr. Ivan Neil Estcourt (1938–1981), benthic ecologist and the first polychaetologist researcher at the former New Zealand Oceanographic Institute (now NIWA).

Remarks. *Osedax estcourti* n. sp. belongs to Clade II, an apinnulate clade. *Osedax estcourti* n. sp. was recovered as the sister species to *Osedax ventana*, known from 2,898 m in Monterey Bay (California, USA), though the relationship was not well supported, with poor bootstrap support (Fig. 2). The minimum interspecific distance between *O. estcourti* and *O. ventana* was 14.6% (Table 3), thus providing ample molecular evidence for it to be a new species. Two other specimens were destroyed for sequencing (ON211941, ON211942). The new species had a 1.5% maximum intraspecific pairwise distance among the three available sequences, though all three sequences showed the rDNC diagnostic bases. The haplotype network for *Osedax estcourti* n. sp. showed three distinct haplotypes, one for each sequence with a maximum of 10 nucleotide substitutions (Fig. 8A). Specimens were not observed alive, however images of the whale skull at the time it was collected show red *Osedax* coating the surface (Fig. 7A), suggesting that living *Osedax estcourti* n. sp. may have red palps.

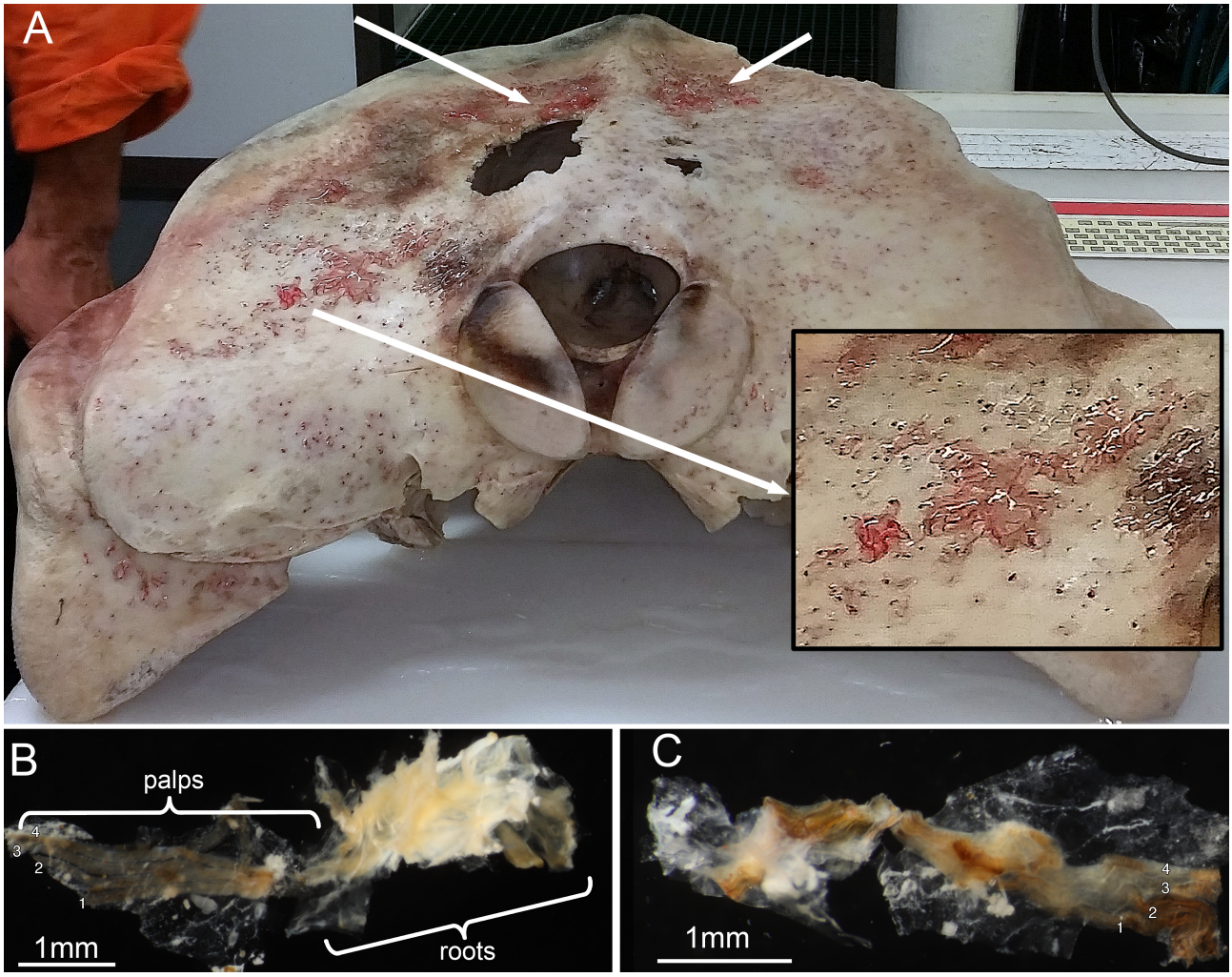

FIGURE 7. A. Whale skull trawled from ~390 m on the Pukaki Rise off New Zealand with an inset close-up of the *Osedax*. Arrows and inset point at *Osedax* patches. **B.** *Osedax estcourti* n. sp. holotype (NIWA 159436), desiccated, with four apinnulate palps (indicated by numbers 1–4) in tube and the ovisac/root region still in bone. **C.** *Osedax traceyae* n. sp. holotype (NIWA 159436) comprising desiccated apinnulate palps (indicated by numbers 1–4) in tube.

***Osedax traceyae* n. sp.**
Fig. 7A, C, 8B

Material examined. Holotype: NIWA 159435 female (GenBank *COI* sequence ON211990, *16S* = ON212680, *18S* = ON210988, *28S* = ON220740, *H3* = ON254808), collected from a whale skull (most likely a southern minke whale *Balaenoptera bonaerensis*) at 390 m depth on the Pukaki Rise SE of New Zealand (49.121° S; 172.136° E) scientific trawl TAN1614 Station 9, from R/V *Tangaroa*, December 1, 2016. Fixed and preserved in 95% ethanol. **Paratypes:** NIWA 159437, SIO-BIC A13927, NIWA 159439, NIWA 159440 (GenBank *COI* ON211991, ON211992, ON211987, ON211988), collection data for paratypes is the same as for the holotype.

Diagnosis and description. Live animals red, in transparent tubes on whale skull (Fig. 7A). Holotype consists of desiccated palps in ethanol (Fig. 7C). Apinnulate palps are brown, approximately 4 mm in length and 1 mm wide (Fig. 7C). Palps contained inside translucent membrane (Fig. 7C). Palp tips curled up inside membrane (Fig. 7C). No dwarf males observed. Paratypes are in a similar state as the holotype, though some have trunk and apparent root tissue. The rDNC diagnosis for *Osedax traceyae* n. sp. was recovered as: 'A' at site 280, 'C' at site 546, and 'G' at site 582 of mitochondrial COI.

Distribution. *Osedax traceyae* n. sp. was recovered from a whale fall on the Pukaki Rise off SE New Zealand at 390–393 m.

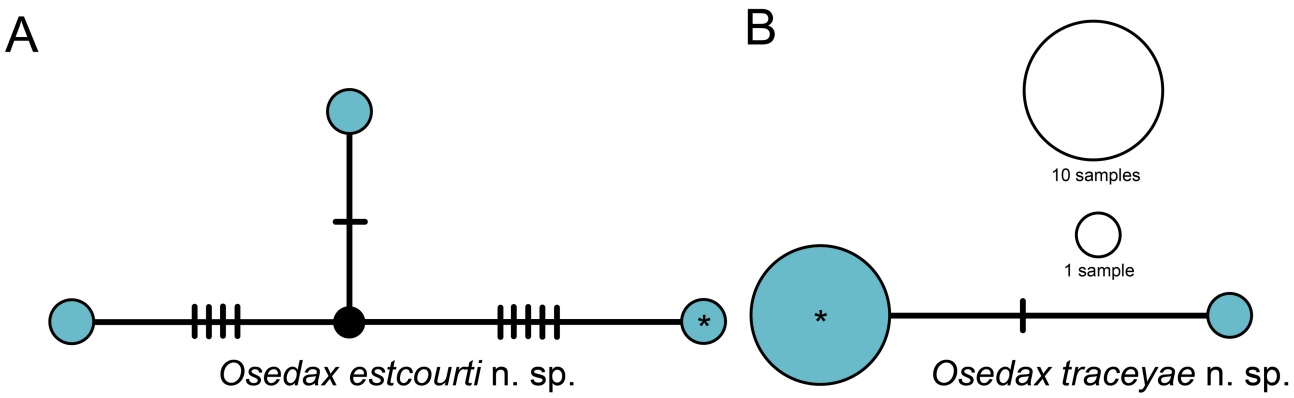

FIGURE 8. Haplotype networks using *COI*. Circles are haplotypes, blue circles and crosshatches are single nucleotide substitutions. * indicates the haplotype of the holotype. **A.** Network for three specimens of *Osedax estcourti* n. sp. GenBank accession numbers: ON211941, ON211942, ON211943 (Holotype). **B.** Network for 11 specimens of *Osedax traceyae* n. sp. GenBank accession numbers: ON211983, ON211984, ON211985, ON211986, ON211987, ON211988, ON211989, ON211990 (Holotype), ON211991, ON211992, ON211993.

Etymology. *Osedax traceyae* n. sp. is named in appreciation of Dianne (Di) M. Tracey of the National Institute of Water and Atmospheric Research (NIWA), Wellington, New Zealand. An outstanding deep-sea fisheries and coral researcher, her shipboard initiatives secured the whale skull and worms for our study.

Remarks. *Osedax traceyae* belongs to Clade II, a nude palp (apinnulate) clade (Fig. 2). The species had a 0.5% maximum intraspecific pairwise distance among the eleven sequences analyzed. The haplotype network for this species revealed two haplotypes, one of which was shared by ten of the eleven sequences (Fig. 8B). All 11 *COI* sequences showed the rDNC diagnostic bases for the species. Specimens of *O. traceyae* n. sp. were not observed alive, however images of the whale skull at the time it was collected show red palps in transparent tubes scattered over the surface (Fig. 7A), suggesting that living *O. traceyae* n. sp. may have red palps. *Osedax traceyae* n. sp. was recovered as the sister group to two Antarctic species, *Osedax antarcticus* Glover *et al.*, 2013 and *O. crouchi*, belonging to Clade II, but the support value for this grouping was very low, as was support for most nodes in that clade (Fig. 2). The minimum interspecific *COI* distance between *O. traceyae* and each of these two Antarctic species was at least 15.4% (Table 3).

Discussion

Since being named in 2004 (Rouse *et al.* 2004), *Osedax* diversity has been steadily growing, and likely contains many more undiscovered taxa. The addition here of four new *Osedax* species from New Zealand and the Gulf of Mexico brings the total number of described species to 33, with a further six yet to be named (Fig. 1). One of these, *O. craigmcclaini* n. sp., has palps with pinnules and falls within Clade V whose members all show this feature. The other three new species all belong Clade II, a nude palp clade. Unfortunately, the generally low support values across the *Osedax* phylogenetic tree (Fig. 1) prevent much in the way of evolutionary and biogeographical conclusions. Although *Osedax* itself is a well-supported clade, as were Clades IV and V, most other nodes were not (Fig. 1). While the terminals in this study were sequenced for all five genes used in the analysis, many of the *Osedax* terminals have had only one to three of the loci sequenced (Table 2). This missing data may be partly responsible for the lack of well supported relationships. We recommend that all five genetic markers be sequenced (i.e., *COI*, *16S*, *18S*, *28S*, and *H3*) for any future new *Osedax* discoveries, and generating data from whole mitochondrial genomes may also eventually result in a more robust phylogenetic hypothesis for the different clades within the genus. We also recommend that when bones are recovered either accidentally or as part of experiments that care be taken to obtain *Osedax* in as good condition as possible. This means careful dissection rather than just scraping the bone surface and that live photos be made to document color and anatomy before fixation.

The four species described were not preserved in a way to allow for detailed morphological description as

performed in other descriptions of *Osedax* (e.g., Rouse *et al*. 2008; Rouse *et al.* 2015), or even for more cursory descriptions (Rouse *et al.* 2018; Eilertsen *et al.* 2020). There are precedents though, with several *Osedax* species being described based on just palps alone, such as *Osedax nordenskjoeldi* Amon *et al.*, 2014, *Osedax rogersi* Amon *et al.*, 2014, *Osedax ventana* Rouse *et al.*, 2018, and *Osedax byronbayensis* Georgieva *et al.*, 2023. All these species were also established based on DNA sequences, such as COI, and this means that future collections will allow for unequivocal identification based on DNA, and better-preserved specimens may allow for more detailed descriptions. This is arguably better than just posting DNA sequences to GenBank with placeholder names as happened with many *Osedax* species in Vrijenhoek *et al*. (2009), though these were all ultimately described (Rouse *et al.* 2018). Fedosov *et al.* (2022) explored the use of rDNCs across a range of datasets and found they could be more than one base long but were rarely more than four bases long. In our results all rDNCs were only one base long and three found for each of the new species. Fedosov *et al.* (2022) argue that their method takes into account unsampled genetic diversity. It will be interesting to see if these remain diagnostic for each species as further *Osedax* species are discovered and sequenced.

Depth has been hypothesized to contribute in part to high species richness of *Osedax* in Monterey Bay Canyon (Rouse *et al.* 2018; Vrijenhoek *et al.* 2009). Many species of *Osedax* have been found across a range of depths while some are known from only a single record (Berman *et al.* 2023; Rouse *et al.* 2018). *Osedax estcourti* n. sp. and *O. traceyae* n. sp. were collected at 390 m from the Pukaki Rise feature on the Chatham Rise, a bathymetric feature that descends to 3,000 m. Only six other species of *Osedax* are known from 400 m or shallower: *O. deceptionensis* (Clade VI), *O. japonicus* (Clade IV), *O.* 'mediterranea' (Clade I), *O. lehmani* (Clade IV), *O. mucofloris* (Clade IV), and *O. packardorum* (Clade IV) (Fujikura *et al.* 2006; Glover *et al.* 2005; Rouse *et al.* 2018; Taboada *et al.* 2015). All other named species and OTUs are known from deeper depths, with the greatest proportion of species known between 1,000 m and 3,000 m (Rouse *et al.* 2018). The four shallower water taxa from Clade IV all formed a well-supported clade (Fig. 1) and are from the Pacific and Atlantic Oceans, suggesting that radiations of *Osedax* diversity may occur at a particular depth profile. However, the incidence of shallower water *Osedax* in Clade II is shown here for the first time with *O. estcourti* n. sp. and *O. traceyae* n. sp., showing that *Osedax* taxa occupy many different depths across the phylogeny of the clade. With the present uncertainty about relationships (Fig. 1), the ancestral depth range of *Osedax* is not resolvable at present.

Fossil evidence demonstrates that ancient *Osedax* were able to exploit fish, bird, reptile, and whale bones and even whale teeth (Danise & Higgs 2015; Kiel *et al.* 2010, 2011, 2013). Modern *Osedax* have been found on whale, dolphin, fish, pig, cow, turtle, turkey, fur seal, elephant seal, and alligator bones and even shark teeth (Jones *et al.* 2008; McClain *et al.* 2019; Rouse *et al.* 2018; Rouse & Goffredi 2023). The discovery of four new species here on three different bone types further highlights the capacity for *Osedax* to utilize a variety of bone substrates.

Acknowledgements

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