A remarkable diversity of bone-eating worms (Osedax; Siboglinidae; Annelida)

Robert C Vrijenhoek*\(^1\), Shannon B Johnson\(^1\) and Greg W Rouse\(^2\)

Address: \(^1\)Monterey Bay Aquarium Research Institute, Moss Landing, CA, USA and \(^2\)Scripps Institution of Oceanography, UCSD, La Jolla, CA, USA

Email: Robert C Vrijenhoek* - vrijen@mbari.org; Shannon B Johnson - sjo@mbari.org; Greg W Rouse - grouse@ucsd.edu

* Corresponding author

Abstract

**Background:** Bone-eating Osedax worms have proved to be surprisingly diverse and widespread. Including the initial description of this genus in 2004, five species that live at depths between 25 and 3,000 m in the eastern and western Pacific and in the north Atlantic have been named to date. Here, we provide molecular and morphological evidence for 12 additional evolutionary lineages from Monterey Bay, California. To assess their phylogenetic relationships and possible status as new undescribed species, we examined DNA sequences from two mitochondrial (COI and 16S rRNA) and three nuclear genes (H3, 18S and 28S rRNA).

**Results:** Phylogenetic analyses identified 17 distinct evolutionary lineages. Levels of sequence divergence among the undescribed lineages were similar to those found among the named species. The 17 lineages clustered into five well-supported clades that also differed for a number of key morphological traits. Attempts to determine the evolutionary age of Osedax depended on prior assumptions about nucleotide substitution rates. According to one scenario involving a molecular clock calibrated for shallow marine invertebrates, Osedax split from its siboglinid relatives about 45 million years ago when archecete cetaceans first appeared and then diversified during the late Oligocene and early Miocene when toothed and baleen whales appeared. Alternatively, the use of a slower clock calibrated for deep-sea annelids suggested that Osedax split from its siboglinid relatives during the Cretaceous and began to diversify during the Early Paleocene, at least 20 million years before the origin of large marine mammals.

**Conclusion:** To help resolve uncertainties about the evolutionary age of Osedax, we suggest that the fossilized bones from Cretaceous marine reptiles and late Oligocene cetaceans be examined for possible trace fossils left by Osedax roots. Regardless of the outcome, the present molecular evidence for strong phylogenetic concordance across five separate genes suggests that the undescribed Osedax lineages comprise evolutionarily significant units that have been separate from one another for many millions of years. These data coupled with ongoing morphological analyses provide a solid foundation for their future descriptions as new species.
Background

Osedax, a recently discovered genus of bone-eating marine worms, are proving to be far more diverse and geographically widespread than initially realized. The genus was described from two newly discovered species found on whalebones recovered from 2,893 m depth in Monterey Bay, California [1]. Subsequently, three additional species were described from depths between 30 and 3,000 m in the Atlantic and Pacific oceans [2-4]. Now, five additional distinct evolutionary lineages are recognized from Monterey Bay, but these putative species remain to be formally described [5-8]. Here we report genetic evidence for seven additional putative species. Given this unexpected diversity of Osedax worms with distinct morphologies, depth ranges and ecological characteristics, a detailed examination of their evolutionary history is warranted.

The initial description of Osedax [1] included a phylogenetic analysis that placed the new genus in the polychaete annelid family Siboglinidae, which also includes the now obsolete tubeworm phyla Vestimentifera and Pogonophora [9,10]. As adults, all siboglinids lack a functional digestive system and rely entirely on endosymbiotic bacteria for their nutrition. The other siboglinid taxa host chemosynthetic bacteria and live in reducing marine environments such as hydrothermal vents, hydrocarbon seeps and anoxic basins. Osedax, however, are unique because they penetrate and digest bones with the aid of heterotrophic bacteriathat are housed in a complex branching root system [6,11]. Osedax also differ because they exhibit extreme sexual dimorphism involving dwarf (paedomorphic) males that live as harems within a female's tube [1,4,12].

Considering DNA sequence divergence between the only two species known at the time, Rouse et al. [1] suggested that Osedax may have begun to diversify during the late Eocene, around 42 million years ago (MYA), perhaps coinciding with the origin of large oceanic cetaceans. Yet, this hypothesis must be reexamined in view of our current discoveries of far greater morphological and molecular diversity in the genus (Fig. 1). Our present goals were to better characterize the genetic differences among the five named species and to use this information as a foundation for clarifying evolutionary relationships among the 12 undescribed operational taxonomic units, OTUs (Table 1). We examined DNA sequences from five genes. Mitochondrial cytochrome-c-oxidase subunit 1 (COI) was used to assess levels of sequence diversity within and among all 17 OTUs and to provide DNA barcodes that would facilitate the identification of Osedax species in subsequent discoveries. Phylogenetic analyses were conducted independently with mitochondrial COI and 16S rRNA and with three nuclear genes, Histone-H3, 18S and 28S rRNA. A combined analysis involving all five genes provided a robust phylogeny for the genus and identified several well-supported species-groups that diversified over a relatively short time scale, though the timing of these events during either the Mesozoic or Cenozoic remains uncertain. Formal descriptions of the new species from Monterey Bay are currently underway (Rouse, in progress).

Results

We examined DNA sequences from five genes (Table 2). Substitution models were estimated separately for each gene. The percentage GC content was lower in the mitochondrial genes (33.9 - 40.2%) than in the nuclear genes (47.8 - 50.2%). The two protein-coding genes, COI and H3, exhibited the highest sequence divergence. Ratios of transitions to transversions were nearly one, and ratios of synonymous to non-synonymous substitutions were comparable for COI and H3. For each gene, the sequences were partitioned by codon position, and substitution parameters were estimated separately for each position. Indels were found in all three of the rRNA genes. On average, the lengths of indels and numbers of distinct haplotypes were similar. Ratios of transitions to transversions were about one-half for the three ribosomal genes.

Phylogenetic analyses

We initially conducted separate phylogenetic analyses for each gene. Altogether 83 COI sequences from Osedax clustered into 17 evolutionary lineages (Figure 2a). Multiple COI haplotypes were included, when possible, to represent the sequence divergence among (D) versus that found within (\( \pi \)) each lineage (Table 3). Only one Osedax OTUs OTUs (Figures 2b-e). The mean pairwise D values among the Osedax OTUs ranged from 8.4 to 24.3%. The smallest pairwise D value, obtained for O. yellow-collar versus O. orange-collar, was an order-of-magnitude greater than the largest \( \pi \) value observed within these OTUs (0.82%). COI transitions began to saturate after about 12% divergence, but transversions were not saturated and many of them resulted in amino acid substitutions (Table 2).

Four additional genes revealed concordant phylogenetic differences among the Osedax OTUs (Figures 2b-e). The 16S, 28S, and H3 sequences differed among the 15 Monterey Bay OTUs, but sequences were not available for O. japonicus and O. mucrofloris. Although their 18S sequences were identical, O. yellow-collar and O. orange-collar differed from all the other Monterey OTUs and from O. mucrofloris. The nodes leading to O. spiral and O. frankpressi were not stable, but all five gene-trees were broadly congruent in their topologies. Incongruence

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Figure 1
Morphological diversity among Osedax lineages from Monterey Bay, CA. Individual whale-falls are denoted by their depths in meters: (a) O. orange collar from whale-633; (b) O. yellow-collar from whale-385; (c) O. white-collar from whale-1018; (d) O. frankpressi from whale-2893; (e) O. roseus from whale-1018; (f) O. rubiplumus from whale-2893; (g) O. spiral from whale-2893; (h) O. yellow-patch from whale-1018; (i) O. nude-palp C from whale-1018; and (j) O. nude-palp D from whale-1820. Approximate scale bars are provided in each panel.
### Table 1: Characteristics of Osedax OTUs.

| Taxa            | Authority1       | Depth (m) | Approx. Size3 | Palp color | Pinnules3 | Oviduct4 | Roots | Museum Voucher2 |
|-----------------|------------------|-----------|----------------|------------|-----------|-----------|-------|-----------------|
| *O. rubiplumus* | Rouse et al. 2004| 1820--2893| 59            | brilliant red | out       | long      | long, branched | CASIZ 170238 |
| *O. frankpressi*| Rouse et al. 2004| 1820--2893| 23            | red with white lateral stripe | in         | long      | robust, lobate | CASIZ 170235 |
| *O. mucofloris* | Glover et al. 2005| 30--125    | 14            | white to pink | in         | long      | robust, lobate |
| *O. japonicus*  | Fujikura et al. 2006| 224--250  | 16            | pink        | out       | short     | robust, lobate |
| *O. roseus*     | Rouse et al. 2008| 633--1820  | 24            | bright red  | out       | long      | long, branched | SIO-BIC A979-981 |
| *O. spiral*     | Braby et al. 2007| 2893      | 25            | no palps    | none      | absent    | long thin filaments | SIO-BIC A1639 |
| *O. yellow-collar* | Braby et al. 2007| 383       | 18            | red         | both      | long      | robust, lobate | SIO-BIC A1640 |
| *O. orange-collar* | Braby et al. 2007| 383--1018 | 18            | red         | both      | long      | robust, lobate | SIO-BIC A1641 |
| *O. nude-palp-A* | Jones et al. 2008| 1820      | 25            | red         | none      | ?         | ?     | SIO-BIC A1642 |
| *O. nude-palp-B* | Jones et al. 2008| 2893      | 25            | red         | none      | ?         | ?     | SIO-BIC A1643 |
| *O. nude-palp-C* | Rouse et al. 2009| 1018      | 12            | red         | none      | ?         | ?     | SIO-BIC A1644 |
| *O. nude-palp-D* | this report      | 1018-1820 | 12            | red         | none      | ?         | ?     | SIO-BIC A1645 |
| *O. nude-palp-E* | this report      | 1018      | 12            | red         | none      | ?         | ?     | SIO-BIC A1646 |
| *O. nude-palp-F* | this report      | 2892      | 18            | red         | none      | ?         | ?     | SIO-BIC A1647 |
| *O. white-collar* | this report      | 1018      | 6             | red with white stripe | both     | long      | robust, lobate | SIO-BIC A1648 |
| *O. yellow-patch* | this report      | 633--1018 | 5             | pale        | both      | long      | robust, lobate | SIO-BIC A1649 |
| *O. green-palp* | this report      | 1820      | 3             | red/green   | ?         | long      | robust, lobate | SIO-BIC A1650 |
| *S. brattstromi*| Webb 1964        | 600       | 40            | red         | none      | absent   | none     |

1 Authority and date of species description, or reference to first report in literature if species is undescribed.
2 Maximum length of truck and crown (mm) when preserved.
3 Orientation of pinnules if present: in = inward; out = outward; both = inward & outward; none = no pinnules.
4 Extension of oviduct beyond trunk.
5 Abbreviations: CASIZ, California Academy of Sciences Invertebrate Zoology collection; SIO-BIC, The Scripps Institution of Oceanography Benthic Invertebrates Collection.

### Table 2: Characterization of DNA sequences and the substitution models used to correct for saturation in the Bayesian analyses.

| DNA            | Length | Mean % divergence1 | % GC content | Ts:Tv2 | Synonymous3 | Nonsynonymous | Model          |
|----------------|--------|-------------------|--------------|--------|-------------|---------------|----------------|
| Protein-coding |        |                   |              |        |             |               |                |
| mt COI         | 1005   | 17.0              | 41.0         | 1.304  | 259.42      | 94.58         | GTR+SS         |
| nuc H3         | 371    | 13.9              | 48.8         | 1.075  | 222.23      | 71.77         | Sym+SS         |
| Ribosomal      |        |                   |              |        |             |               |                |
| mt 16S         | 506    | 11.1              | 32.6         | 0.527  | 3           | 1.0           | GTR+I+G        |
| nuc 18S        | 1644   | 5.9               | 48.0         | 0.526  | 5           | 1.18          | GTR            |
| nuc 28S        | 400    | 8.6               | 47.4         | 0.584  | 4           | 1.14          | GTR+I+G        |

1 Uncorrected pairwise sequence divergence.
2 Ratio of transitions to transversions.
3 Protein coding genes: synonymous substitutions vs. non-synonymous.
4 Ribosomal genes: the number of distinct haplotypes that differed for indels (insertion/deletion).
5 Average length in base pairs of indels.
Bayesian phylogenetic analyses of *Osedax* OTUs for portions of five genes: (a) mitochondrial COI; (b) 16S rRNA; (c) 18S rRNA; (d) Histone-H3; and (e) 28S rRNA. The black triangles in a represent the maximum depth and breadth of sequence diversity observed among multiple individuals (sample sizes in parentheses following OTU designations). The small numerals represent Bayesian Posterior Probabilities (BPP) expressed as percent, * = 100.

**Figure 2**
Bayesian phylogenetic analyses of *Osedax* OTUs for portions of five genes: (a) mitochondrial COI; (b) 16S rRNA; (c) 18S rRNA; (d) Histone-H3; and (e) 28S rRNA. The black triangles in a represent the maximum depth and breadth of sequence diversity observed among multiple individuals (sample sizes in parentheses following OTU designations). The small numerals represent Bayesian Posterior Probabilities (BPP) expressed as percent, * = 100.
length difference (ILD) tests of homogeneity revealed that four of the five gene partitions were not significantly in conflict (P range: 0.119 - 1.00). Only the H3 tree was incongruent with respect to the 16S and 18S rRNA trees (P = 0.03 and 0.02, respectively). This problem resulted because H3 provided weak resolution among Osedax species that clustered together on a long branch relative to the outgroup S. brattstromi. ILD tests of homogeneity between H3 and the other partitions without the outgroup eliminated all remaining incongruence (P range: 0.125 - 1.00).

Both the individual gene trees and the combined analysis involving concatenated sequences from all five genes identified several well-supported Osedax clades (Roman numerals I - V, Figure 3). Although limited sequence information was available for O. mucofloris (COI and 18S) and O. japonicus (COI), they fell firmly within clade IV. O. frankpressi was also well supported as a member of clade IV in the combined analysis, but its position varied in the COI tree. Estimates of the age of Osedax depended on assumptions about rates of nucleotide substitution for mitochondrial COI. Mitochondrial COI divergence (D) between cognate species of shallow-water marine invertebrates isolated across the Isthmus of Panama grows at a rate of about 1.4% per MY [13]; so, the substitution rate \( r_1 \) equals \( D/2 \) or 0.7% per lineage per My. Assuming \( r_1 = 0.7\% \), Osedax would have split from its moniliferan relatives about 45 MYA (95% HPD bounds: 31 - 47 Mya) (Figure 3). Time \( (T) \) to the most recent common ancestor for the Osedax would be 24 - 29 MY. Alternatively, assuming a slower substitution rate \( r_2 = 0.21\% \) per lineage per MY estimated for deep-sea hydrothermal vent annelids [14], Osedax would have split from its moniliferan relatives about 130 MYA (95% HPD bounds: 104 - 160 Mya). \( T \) for Osedax would be 81 - 97 My.

**Discussion**

**Species diversity**

Genetic and morphological differences among five previously named Osedax species provide a useful reference frame for assessing levels of divergence among the twelve undescribed OTUs considered in this study. Osedax rubiplumus, O. frankpressi and O. roseus live together on whale carcasses at depths greater than 1,000 m in Monterey Bay, CA (Figure 1; Table 1). To date, we have found no evidence for interbreeding among them. For example, an examination of 116 male Osedax sampled from the tubes of 77 O. rubiplumus females found no cases of foreign males in the female's tubes, despite the presence of O. roseus and O. frankpressi on the same carcass at 1820 m depth. Also including O. mucolforis from Sweden and O. japonicus from Japan, the mean sequence divergence (D) for mitochondrial COI between pairs of the named species was 19.6% (range: 15.7 to 23.4%; Table 2). Correspondingly, the mean pairwise D among the undescribed OTUs was 19.9% (range: 8.4 to 23.7%). The smallest value (8.4% between OTUs O. yellow-collar and O. orange-collar) was an order-of-magnitude greater than the largest divergence observed within any of these named or undescribed OTUs considered in this study (O. rubiplumus, O. frankpressi, and O. roseus live together on whale carcasses at depths greater than 1,000 m in Monterey Bay, CA (Figure 1; Table 1). To date, we have found no evidence for interbreeding among them. For example, an examination of 116 male Osedax sampled from the tubes of 77 O. rubiplumus females found no cases of foreign males in the female's tubes, despite the presence of O. roseus and O. frankpressi on the same carcass at 1820 m depth. Also including O. mucolforis from Sweden and O. japonicus from Japan, the mean sequence divergence (D) for mitochondrial COI between pairs of the named species was 19.6% (range: 15.7 to 23.4%; Table 2). Correspondingly, the mean pairwise D among the undescribed OTUs was 19.9% (range: 8.4 to 23.7%). The smallest value (8.4% between OTUs O. yellow-collar and O. orange-collar) was an order-of-magnitude greater than the largest divergence observed within any of these named or undescribed OTUs (\( \pi = 0.8\% \) for O. nude-palp-A). These \( \pi \) values probably are underestimates, however, because each was obtained from a single locality. Isolation-by-distance and population subdivision across oceanic barriers are expected to increase \( \pi \) within broadly distributed species; however, \( \pi \) rarely exceeds 1 - 2%, unless other factors are involved. Global-scale phylogeographic surveys of COI sequence diversity have estimated \( \pi \) values less than 1% within named species of deep-sea hydrothermal vent annelids, mollusks and crustaceans, whereas D values typically are greater than 4% among species [14-22]. Nonetheless, odd cases of accelerated COI substitution rates have been reported. Sex-biased mitochondrial transmission and heteroplasmy are associated with accelerated...
Phylogenetic relationships among Osedax species based on concatenated sequences from two protein-coding genes (COI and H3) and three ribosomal RNA genes (16S, 18S, and 28S).

Figure 3
Phylogenetic relationships among Osedax species based on concatenated sequences from two protein-coding genes (COI and H3) and three ribosomal RNA genes (16S, 18S, and 28S). Roman numerals at the right-hand margin delineate five Osedax species-groups. Three methods were used to denote the support for internal nodes: Bayesian posterior probabilities (BPP), maximum parsimony (MP) jackknife, and RAxML bootstrap values. If all three methods produced values ≥ 95%, the node is marked with a large black dot. Where support values differ, the BPP, RAxML (italics) and MP values are shown in order, and asterisks (*) equal 100%. Nodes that were not recovered with RAxML or MP analyses are indicated by a dash. Support values ≤ 50 are not shown. Based on most parsimonious reconstructions, the white rectangles mark the loss of palps in O. spiral and the loss of pinnules for the nude-palp species group.
divergence in some bivalve mollusks [23], but no evidence exists for this phenomenon in annelids, and we have found no differences in the distribution of mitochondrial haplotypes between males and females for *O. rubiplumus* [12]. High mitochondrial divergence rates have been reported for some marine and freshwater animals [24-26], but for the vast majority of cases mitochondrial COI is relatively conservative in its mutation rate within and among species. It is precisely the tendency of COI to discriminate clearly among the named species in many invertebrate taxa that has made this gene a common reference tool for DNA barcoding and molecular taxonomy [27,28].

Although numerous species concepts have been debated over the years [29,30], genealogical concordance across molecular and morphological characters provides a reliable indicator of longstanding evolutionary independence and consequently provides an operational criterion for species recognition [31]. Our confidence that the 12 presently unnamed OTUs from Monterey Bay represent distinct evolutionary lineages and warrant further consideration for naming as species is bolstered by morphological differences and concordant divergence observed across multiple gene loci. Only 18S rRNA failed to distinguish between members of the closest pair of OTUs, *O.* yellow-collar and *O.* orange-collar. This highly conservative gene barely varies across the bivalve genus *Bathymodiolus*, globally widespread and diverse deep-sea mussels [32], or across a diverse clade siboglinid annelids, the vestimentifers [9]. Consequently the COI to discriminate clearly among the named species in many invertebrate taxa that has made this gene a common reference tool for DNA barcoding and molecular taxonomy [27,28].

**Phylogeny**

Individual gene trees (Figure 2) and the combined phylogenetic analysis (Figure 3) identified several well-supported groupings within *Osedax* (clades I - V). *Osedax* spiral (clade III) stands alone as the most atypical of these worms. Its oviduct does not extend beyond the trunk, and it lacks the vascularized anterior palps that characterize all other *Osedax* (Figure 1g). Unlike all other *Osedax*, O. spiral is a late successional species which lives at the sediment interface and produces long fibrous roots that penetrate the anoxic (black and sulfidic) sediments to exploit buried fragments of bone [5]. The lack of palps in *O.* spiral probably represents a character loss under a most parsi-monious reconstruction, because all other siboglinids bear an anterior crown composed of one or more palps. The nude-palp OTUs (clade II) differ because their palps do not bear the lateral pinnules seen in the other *Osedax* clades (Figures 1i and 1j). Lack of pinnules may represent a character loss, but supporting evidence regarding the homology and distribution of pinnules in other siboglinids is uncertain. Monolifers have two or more palps, with numerous pinnules in the case of Vestimentifera, but pinnules are absent in *Sclerolinum* and in some frenulates [10,35].

The remaining *Osedax* clades (*I*, *IV* and *V*) bear four palps with numerous pinnules that give the crown a feathery appearance (e.g., Figure 1a). The two members of clade *V* have long branched roots that are green in color (Figures 1e-f) and palps that are bright red with outwardly facing pinnules. Clades *II* and *V* share robust lobate roots. The two members of clade *I* have relatively short trunks and palps (Figure 1h), but they have not been found in great numbers because they are small and may have been overlooked in earlier samples. Members of clade *IV*, which have red, pink or even white crowns (Figures 1a-d), were recovered from depths of 1,020 m or less, excepting *O.* *frankpressi*, which has not been found shallower than 1,800 m. Occupation of shallow habitats might be a derived condition for these members of clade *IV*, though support for a *shallow* clade was weak (Figure 3). Addition of comparative sequence data from the other shallow-members of this clade, *O.* *japonicus* and *O.* *mucofloris*, might help to strengthen this relationship (only 18S and COI data are available on GenBank for *O.* *mucofloris* and only COI for *O.* *japonicus*). Otherwise, no clear evolutionary pattern of depth utilization is apparent among the major *Osedax* clades. Several of these OTUs were sampled from a single depth, others were sampled across relatively narrow depth ranges (300 - 600 m for *O.* yellow-patch and *O.* orange-collar), and some were sampled across broad depth ranges (1,000 m for *O.* *frankpressi*, and *O.* *rubiplu- mus* and 1,200 m for *O.* *roseus*).
Table 4: GenBank accession numbers for the DNA sequences used in this study.

| Taxa               | COI       | 16S        | 18S        | 28S        | H3         |
|--------------------|-----------|------------|------------|------------|------------|
| O. rubiplumus      | EU223307  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |
| O. frankpressi     | EU223314  | FJ347658   | FJ347682   | FJ347674   | FJ347705   |
| O. mucorflora      | EU227562  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |
| O. japonicus       | AB295969  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |
| O. roseus          | EU164760  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |
| O. spiral          | DO996622  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |
| O. yellow-collar   | EU223330  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |
| O. orange-collar   | EU223340  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |
| O. nude-palp-A     | EU223356  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |
| O. nude-palp-B     | EU236218  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |
| O. nude-palp-C     | EU267675  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |
| O. nude-palp-D     | EU236218  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |
| O. nude-palp-E     | EU236218  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |
| O. nude-palp-F     | EU236218  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |
| O. white-collar     | EU236218  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |
| O. yellow-patch     | EU236218  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |
| O. green-palp      | EU236218  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |
| S. brattstromi      | EU236218  | FJ347656   | FJ347681   | FJ347671   | FJ347704   |

Age of Osedax

For now, we are unable to confidently delineate a time-frame during which Osedax split from its monoliferan relatives or the age (T) of the most recent common ancestor for this unusual genus. Present evidence indicates that Osedax species live primarily on organic compounds extracted directly from sunken bones. Their Oceanospirillales symbionts are capable of growing on collagen and cholesterol as primary carbon sources [6]. Video evidence suggests that O. japonicus also grows on spermaceti, a wax found in the head of sperm whales [3]. There are arguments to suggest that Osedax may not be nutritionally restricted to living on whale-falls. Experimental deployments of cow bones and observations of sunken possible pig bones reveal that Osedax can grow and reproduce on a range of mammalian tissues including those from terrestrial quadrupeds [7,36]. So, it may be unwarranted to associate the evolution of these bone-eating worms with the origin and spread of oceanic whales, as previously suggested by Rouse et al. [1]. Nonetheless, one of the scenarios that we have considered here is consistent with that hypothesis. If we assumed a divergence rate \( d = 1.4\% \) per MY calibrated for mitochondrial genes from shallow-water marine invertebrates [13], and applied this rate \( r_1 = d/2 = 0.70\% / \text{lineage/MY} \) to COI divergence, we estimated that Osedax split from its monoliferan relatives about 45 Mya, possibly coincident with the origins of large archocete cetaceans during the Eocene [37]. According to this scenario, the most recent common ancestor for the Osedax sampled to date would have lived about 26 MYA, during the Late Oligocene and roughly coincident with the diversification of modern cetaceans [38]. Alternatively, we can assume a slower substitution rate \( r_1 = 0.21\% / \text{lineage/MY} \) calibrated from COI divergence in deep-sea annelids, including Vestimentifera [14], as was used by Rouse et al. [1] for estimating the origin of Osedax when only O. rubiplumus and O. frankpressi were known. Under this rate then Osedax would appear to be much older than previously hypothesized [1]. This result is not surprising given the larger diversity of Osedax shown here. Accordingly, Osedax split from its monoliferan relatives during the Cretaceous, and the most recent common ancestor for the genus would have lived during the Late Cretaceous. Perhaps the calcified cartilage and bones from a variety of large Cretaceous vertebrates supported these worms -- e.g., mosasaurs, plesiosaurs, turtles, and possibly chondrichthyan and teleosts [39-42]. Fossilized snails and bivalves were recently found with plesiosaur bones; so the sunken carcasses of these large marine reptiles appear to be capable of supporting communities much like those found on modern whale-falls [43]. Nevertheless, this scenario is problematic, because the major Osedax clades would have diversified around the Cretaceous-Tertiary (K/T) boundary, after the extinction of most large-bodied reptilians [44]. Although dyrosaurid crocodylomorphs survived the K/T event, they were confined to relatively shallow coastal environments [45] and probably would not have supported Osedax. Large turtles and chondrichthyan also survived the K/T boundary [42], and large teleosts appeared again during the early Paleocene [46]. It is unknown whether Osedax can exploit these resources; so, arguably a 20 MY gap may have existed during the Paleocene when there would have been little in the way of large vertebrate remains for Osedax. Another problem with this scenario is the concern that nucleotide substitution rates may be slower in the deep-
sea vent annelids used to obtain the $r_2^2 = 0.21\%$ calibration rate [9].

Conclusion
The present phylogenetic evidence based on DNA sequences from multiple independent genes provides a solid foundation for future discoveries and taxonomic descriptions of Osedax species. However, our efforts to estimate evolutionary ages for the diversification of this unusual group of worms only allowed the erection of new hypotheses that could be tested with independent evidence from the fossil record. Soft-bodied invertebrates like Osedax do not often leave convincing fossils, but these worms might leave traces of their activity by the distinctive holes they bore into bones. To date, we have found no other animals that create similar borings in bones. Consequently, we have distributed whalebones containing Osedax to several paleontologists who are also examining the taphonomy of fossilized bones from plesiosaurs and cetaceans. It is to be hoped that these efforts will help us to narrow the age of this remarkable genus of bone-eating worms.

Materials and methods
Specimens
Locations of the Monterey Bay whale-falls, except whale-634, are provided elsewhere [5]. Whale-634 is the carcass of a juvenile gray whale that was sunk on 5 October 2004 at a depth of 633 m at 36.802°N and 122.994°W. We used the remotely operated vehicles, ROV Tiburon and ROV Ventana, operated by the Monterey Bay Aquarium Research Institute (MBARI) to collect Osedax-inhabited bones from five whale-fall localities (Table 1). Bones were transported to the surface in closed insulated containers and stored temporarily in cold (4°C) filtered seawater. Worms were dissected from the bones and photographed. Then a palp tip was removed and stored in 95% ethanol or frozen immediately at -80°C. The remainder of each specimen was preserved for anatomical studies and taxonomic descriptions. For the present purpose, we list the approximate sizes (trunk plus crown length) and several morphological characteristics of each OTU (Table 1).

Published DNA sequences from Osedax mucofloris (18S rRNA and COI) and O. japonicus (COI) were recovered from GenBank [2]. A previous phylogenetic analyses [1] placed Osedax in a clade that also includes the Monolifera, which includes Sclerolinum and vestimentiferan tube-worms [10]. The frenulates, a diverse group of slender chemosynthetic worms are basal to the monoliferans and Osedax [10,47]. Ongoing studies of siboglinid phylogeny revealed that Sclerolinum is presently our best choice as outgroup for this study of Osedax phylogeny. The vestimentiferan Lamellibrachia columna was also examined, and its substitution as outgroup did not substantively alter the tree topologies for the ingroup. Other vestimentiferans were not considered, however, because incomplete sequence data are available and because independent evidence from several genes suggests that rates of nucleotide substitution may have slowed down in these deep-sea worms [9,14,48]. Consequently, we have used DNA sequences from the monoliferan Sclerolinum brattstromi, collected near Bergen, Norway. GenBank accession numbers for all the DNA sequences used in this study are listed in Table 4.

DNA methods
Total DNA was extracted using the DNeasy kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. We used primers that amplified approximately 1200 bp of COI [49], approximately 500 bp of 16S rRNA [50], approximately 1000 bp of 28S rRNA [51], approximately 1800 bp of 18S rRNA [52], and approximately 370 bp of H3 [53]. Amplification reactions with AmpliTaq Gold (Applied Biosystems Inc., Foster City, CA, USA) were conducted in a GeneAmp 9700 thermal cycler (Applied Biosystems Inc., Carlsbad, CA, USA) with the following parameters: 95°C/10 min, 35× (94°C/1 min, 55°C/1 min, 72°C/1 min), and 72°C/7 min. If available, at least six individuals of each species were sequenced for each locus. PCR products were diluted in 50 μl sterile water and cleaned with Multiscreen HTS PCR 96 filter plates (Millipore Corp., Billerica, MA, USA). The products were sequenced bidirectionally with the same primers on an ABI 3100 sequencer using BigDye terminator v.3.1 chemistry (Applied Biosystems Inc., Foster City, USA).

Phylogenetic analyses
Sequences were assembled using CodonCode Aligner v. 2.06 (CodonCode Corporation, Dedham, MA, USA), aligned using Muscle [54] and edited by eye using MacClade v. 4.08 [55]. We used MrModelTest [56] and the Akaike information criterion [57] to determine appropriate evolutionary models for each gene (Table 2). COI and H3 were partitioned by codon position, and parameters were estimated separately for each position. RNA secondary structures were predicted with GeneBee and used to partition stems and loops in 16S, 18S, and 28S sequences. The doublet model was used for RNA stems and a standard 4 × 4 nucleotide model was used for RNA loops. The number of indel haplotypes for RNA sequences (total number of indels, number after excluding overlapping indels, and average length of indels) were estimated with DNAsp v. 4.90.1 [58] using the diallelic model. Gaps in the RNA sequences were treated as a fifth character-state in
subsequent Bayesian phylogenetic analyses and as missing data in parsimony and maximum likelihood (ML) analyses. The program DAMBE [59] was used to examine saturation of the mitochondrial COI sequences for the Osedax OTUs and outgroup taxa.

First, each gene was analyzed separately using MrBayes v. 3.1.2 [60,61]. Bayesian analyses were run as six chains for 5·10^6 generations. Print and sample frequencies were 1,000 generations, and the burn-in was the first 100 samples. We used AWTY [62] to assess whether analyses reached convergence and FigTree v. 1.1.2 [63] to display the resulting trees. We then used the incongruence length difference (ILD) function implemented in Paup* v. 4.0 [64] to assess congruence of the tree topologies produced by the individual gene partitions. ILD tests were conducted both with and without the outgroup taxa. The ILD partition homogeneity test was run for 1,000 replicates with 10 random additions of gene sequences.

A combined analysis was conducted with concatenated sequences from the five genes. If available, multiple individuals of each OTU were sequenced for each gene; however, the concatenated multilocus sequences used in the phylogenetic analyses were obtained from a single representative individual for each OTU. The five gene regions were partitioned separately according to the previously determined model parameters. Bayesian phylogenetic analyses were then conducted with MrBayes v. 3.1.2. Maximum parsimony analysis of the combined data set was performed with Paup* v. 4.0 [64] using an equally weighted character matrix, heuristic searches using the tree-bisection-reconnection branch-swapping algorithm, and 100 random addition replicates. The resulting shortest tree included 3481 steps. A parsimony jackknife analysis (with 37% deletion) was run for 100 iterations with the same settings as the parsimony search. ML analysis was conducted using RAxML 7.0.4 (with bootstrapping) using GTR+I+G as the model for each partition on combined data. RAxML analyses were performed with the CIPRES cluster at the San Diego Supercomputer Center.

Relaxed molecular clock
A Bayesian, MCMC method implemented in Beast v. 1.4.8 [65] was used to estimate the evolutionary ages of internal nodes in the tree topology derived from the combined phylogenetic analysis. Estimates of the time to most recent common ancestor (T) were based on two calibrations: the emergence of the Isthmus of Panama [13] and the East Pacific Rise and the northeastern Pacific ridge system about 28.5 mya [14]. Calibrations were not available for the other genes.

We used a relaxed, uncorrelated, lognormal molecular clock with a general time reversible (GTR) substitution model that was unlinked across codon positions. Initial MCMC test runs consisted of 10 million generations to optimize the scale factors of the prior function. Three independent MCMC chains were run for 100 million generations, sampled every 1000 generations. Results were visualized in and FigTree v. 1.1.2 and Tracer v. 1.4 [66].

Abbreviations
COI: mitochondrial large subunit ribosomal RNA; 18S: nuclear small subunit ribosomal RNA; 28S: nuclear large subunit ribosomal RNA; COI: cytochrome oxidase subunit I; GC: guanine-cytosine; GTR: general time reversible; H3: Histone 3; K/T: Cretaceous-Tertiary; MCMC: Monte Carlo Markov chain; MYA: million (10^6) years ago; OTUs: operational taxonomic units; ROV: remotely operated vehicle.

Authors’ contributions
RCV conceived of the project, directed the research, and composed much of the manuscript. SBJ obtained the DNA sequences and conducted most of the phylogenetic analyses. GWR obtained the images of Osedax OTUs, conducted morphological examinations, and contributed to the phylogenetic analyses and writing of the manuscript.

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