The phylogenetic relationships of whale-fall vesicomyid clams based on mitochondrial COI DNA sequences

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ABSTRACT- Whale skeletons on the deep-sea floor provide sulfide-rich habitats that may act as stepping stones for the dispersal of animals dependent on chemoautotrophic production. However, the phylogenetic relationships between the faunas of whale falls, hydrothermal vents and cold seeps are not fully evaluated. To examine vesicomyid phylogenetic relationships, we collected 10 vesicomyid clams from 2 whale falls on the California margin, one at 1240 m in the Santa Catalina Basin and one at 960 m on the slope west of San Nicolas Island. We then compared DNA sequences for a portion of the mitochondrial cytochrome c oxidase subunit I gene from the whale-skeleton clams to those from other clam populations in this taxonomically difficult family. Seven adult whale-fall vesicomyids clustered with clams identified as Vesicomya gigas, a species also found near hydrothermal vents in Guaymas Basin (Gulf of California) and Middle Valley (Juan de Fuca Ridge). A single small whale-fall individual clustered with clams identified as Calyptogena kilmeri, a species found at cold seeps in Guaymas Basin, Monterey Bay, and along the Oregon Subduction Zone. A single small whale-fall clam clustered with Calyptogena elongata, a species found in anoxic California basins. Finally, a single adult clam was difficult to assign to any previously examined species group and could represent a new species in the 'gigas/kilmeri' cryptic species complex. With the inclusion of these vesicomyids, whale falls are known to share a total of 16 species with the faunas of deep-sea hydrothermal vents and cold seeps.

KEY WORDS: Vesicomyid • Whale bones • Deep sea • Phylogeny • Mitochondrial DNA • COI • Hydrothermal vent • Cold seep

INTRODUCTION

In 1987, a faunal community utilizing sulfide-based chemoautotrophy was discovered on a whale skeleton in the Santa Catalina Basin (SCB). This community exhibited similarities to assemblages found at other deep-sea reducing habitats, such as hydrothermal vents and cold seeps (Smith et al. 1989, McLean 1992, Smith 1992, Pettibone 1993, Bennett et al. 1994, Deming et al. 1997). Since the discovery of the SCB skeleton, additional whale-skeleton communities have been observed in situ, including 1 on the Torishima Seamount (Fujisaka et al. 1993, Wada et al. 1994, Nagahama et al. 1996), 2 in Monterey Bay (J. Barry pers. comm.), and 8 on the California Slope west of San Nicolas Island (C. Smith unpubl. data). Whale bones with attached fauna have been trawled up from several other deep-sea sites including 4 skulls from the California Slope (McLean 1992, W. Wakefield pers. comm., A. Baco & C. Smith unpubl. data), numerous skulls and bones from the Chatham Rise off New Zealand (Gibbs 1987, Marshall 1987, 1994, B. Marshall pers. comm.), and a skull from the deep-sea near Iceland (Waren 1989). Fossil chemoautotrophic communities have also been found associated with 8 fossil
whale skeletons that are up to 30 million yr old (Goedert et al. 1995). Before the existence of whales, it is possible that large marine reptiles supported similar chemoautotrophy-based communities (Martill et al. 1991, Hogler 1994, Marshall 1994).

Sunken whale carcasses may play several roles in the deep sea. First, they may provide habitats for an endemic fauna. For example, the sipunculan Phascolosoma saprophagicum, the limpet Pyropelta wakefieldi, and the polychaete Harmothoe craigslisti have been found only in association with deep-sea whale skeletons (Gibbs 1987, McLean 1992, Pettibone 1993). In addition, whale falls may serve as refugia for organisms dependent on sulfide-based chemoautotrophy, acting as dispersal stepping stones on the generally organic- and sulfide-poor deep-sea floor (Smith et al. 1989). Both roles may be important in maintaining deep-sea biodiversity (Butman et al. 1995).

A stepping-stone role for the whale falls is supported by faunal overlap among whale carcasses and other deep-sea reducing habitats. At least 12 of the animal species living on whale falls have also been reported at hydrothermal vents, cold seeps, and/or anoxic basins (Smith et al. 1989, McLean 1992, Smith 1992, Pettibone 1993, Bennett et al. 1994, Bac0 et al. 1996, Deming et al. 1997). In addition, 2 species of vesicomyid clams from a whale carcass (Calyptogena pacifica and Vesicomya gigas) are thought to occur at vents and seeps (Smith et al. 1989). However, this conclusion should be treated with caution as subsequent studies of vesicomyids revealed that species identifications based on morphological criteria (i.e. 'morphospecies') can be problematic. Discoveries of genetically distinct, yet morphologically indistinguishable, 'cryptic' species, and the existence of extensive morphological plasticity within genetically well-defined species have raised doubts about prior identifications of many vesicomyid 'species' (Vrijenhoek et al. 1994, Kojima et al. 1995).

Vesicomyids appear to be restricted to deep-sea reducing habitats, including hydrothermal vents (Southward 1985, reviewed in Tunnuclife 1991), cold seeps (reviewed in Sibuet & Olu 1998), and anoxic sediments (Felbeck et al. 1983, Vetter 1985, Distel et al. 1988). Members of this family studied to date depend entirely on sulfur-oxidizing chemoautotrophic endosymbionts for their nutrition (Cavanaugh 1983, Felbeck et al. 1983, Childress et al. 1987, Fiala-Medioni & Le Pennec 1988, Fisher 1990). Despite the fragmented distribution of these sulfide-rich habitats, some vesicomyids (e.g. the vent clam Calyptogena magnifica) show essentially no genetic differentiation across vast distances (21°N to 18°S on the East Pacific Rise), indicating effective long-distance dispersal (Karl et al. 1996). The large, yolk-filled eggs of C. magnifica are buoyant at ambient temperatures and pressures, and they transmit the obligatory sulfur-oxidizing endosymbionts to zygotes (Berg 1985, Endow & Ohta 1990, Cary & Giovannoni 1993). Successful establishment of a vesicomyid population depends on a suitable source of reduced sulfur compounds.

We recently collected new vesicomyid specimens from 2 whale-fall sites on the California margin to determine whether whale falls contained clams that also occur at hydrothermal vents and cold seeps. We compared the DNA sequences of a 710 base pair (bp) region of the mitochondrial cytochrome c oxidase subunit I gene (COI) from the whale-fall clams to previously published vesicomyid sequences (Peek et al. 1997). Our analysis indicates that the whale-fall clams represent at least 3 vesicomyid species that occur at other reducing habitats in the northeast Pacific, supporting the hypothesis that whale-falls provide alternative habitats for a variety of sulfophilic deep-sea species.

**METHODS**

**Study site and collection of specimens.** We collected live clams from 2 whale-skeleton sites on the California margin (Fig. 1, Table 1). The first skeleton lies on the floor of the SCB (33° 12' N, 118° 30' W) at a water depth of 1240 m. Characteristics of the SCB and the whale community are summarized in Bennett et al. (1994), and Smith et al. (1989, 1998). Three adult clams were collected from this site with the submersible ALVIN (November 1991), and 6 were collected with the remotely operated vehicle ATV and the submersible TURTLE (May 1995). Collections were made with a scoop net from sediments surrounding the whale bones. On shipboard, clams were immediately placed on ice and dissected to remove gill and adductor-muscle tissues. Gill and adductor tissues were frozen on dry ice, and transported on dry ice to Rutgers University where they were stored at -80°C. Adductor muscle tissues were minced and DNA was extracted by a standard CTAB protocol (Doylce & Dickinson 1987).

The second skeleton lies on the slope 37 km west of San Nicolas Island (SNI, 33° 20.3477' N, 119° 58.8414' W) at a water depth of 960 m. The SNI whale-fall community was similar to that in SCB with the exception that adult vesicomyids were not abundant (Baco et al. 1996). The single small vesicomyid collected at the SNI site in May 1995 was found attached to a whale vertebra recovered by the ATV. The entire specimen was preserved in 95% non-denatured ethanol. Subsequently, soft tissues were removed from the shell, symbiont-containing gills were removed, and all remaining tissues were used for DNA extraction, as above.

**FIGURE 1**
Genetic analyses. Using the polymerase chain reaction (PCR; see Saiki et al. 1988), and the HCO2198 and LCO1492 primers of Folmer et al. (1994), we amplified a 710 bp region of the mitochondrial COI gene from the 10 new specimens. The 25 μL PCR reaction mixtures contained: 1× Taq DNA Polymerase buffer (Promega, Madison, WI), 2.5 mM MgCl₂, 200 μM dNTP’s, 1 mM of each primer, 5 to 50 ng of template DNA, and 0.5 U Taq DNA Polymerase (Promega). The amplification reaction profile was 1 min at 94°C, 1 min at 40°C, 1 min at 72°C for 30 cycles with a 10 min extension at 72°C. Amplification products were purified using Qiagen purification (Qiagen, Chatsworth, CA). Cycle sequencing reactions were dye-terminated dideoxy labeling reactions (ABI, Foster City, CA) following manufacturer’s protocols, using vesicomyid primers VesLCO and VesHCO (see Peek et al. 1997 for primer sequences), followed by running on an ABI373 automated sequencer (ABI, Foster City, CA). Sequences were edited and aligned using ‘Auto Assembler’ and ‘Sequence Navigator’ (ABI, Foster City, CA) and GDE programs (Smith et al. 1994). The number of nucleotide transitions and transversions was estimated with MEGA (Kumar et al. 1993). The 10 new vesicomyid sequences are deposited in GenBank with accession numbers AF1114391-392 to AF114296-303.

Whale-fall clam sequences were aligned with COI sequences of vesicomyids from hydrothermal vents, cold seeps, and anoxic basins (data from Peek et al. 1997, Table 1). As in the study by Peek et al. (1997), we used maximum likelihood (fastDNAm1 1.0.6; Olsen et al. 1994) and parsimony methods (PAUP 3.1.1; Swofford 1993) for phylogenetic analyses. The veneroid clam Mercenaria mercenaria was used as an outgroup. Alternate phylogenetic hypotheses were constructed with MacClade 3.06 (Maddison & Maddison 1992) and tested for likelihood by the Kishino-Hasegawa method in DNAml (Kishino & Hasegawa 1989).

RESULTS

Unambiguous sequences were obtained for a >700 bp region of the mitochondrial COI gene in 10 whale-fall vesicomyids. We considered these new sequences in the context of the 516 bp region of the 52 vesicomyid sequences published by Peek et al. (1997). Altogether, the observed number of transitions (Ts) and transversions (Tv) for the 62 sequences increased linearly with genetic distance, providing no evidence for saturation within the vesicomyids. Consequently, all substitutions were used in the subsequent phylogenetic analyses. Because the Ts:Tv ratio was approximately 2.1 aver-
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aged across all codon positions, we used a Kimura 2-Parameter estimate in further analyses.

The present phylogenetic analysis of the combined 62 vesicomyid sequences and outgroup produced essentially the same tree topology reported by Peek et al. (1997) (Fig. 2). The present whale-fall clams appear to represent at least 3 discrete species. A single clam, W10 from the SCB whale site, clustered with specimens of *Calyptogena elongata* from the Santa Barbara Channel. The other 9 SCB individuals clustered within the ‘gigas/kilmeri’ cryptic species complex. A single small specimen, W9, from the SNI site clustered with vesicomyids from seeps along the Guaymas Basin Transform Fault, the Oregon Subduction Zone, and Monterey Bay (Group A, Fig. 2), which are thought to represent *Calyptogena kilmeri* (J. Barry pers. comm.). Seven large individuals, W1 to W7, from the SCB site were essentially identical to clams from the Guaymas Basin hydrothermal vents, and related to similar clams from Middle Valley on the Juan de Fuca Ridge and from seeps on the Oregon Subduction Zone. Taken together, this diverse monophyletic group of clams (Group B, Fig. 2) is currently thought to represent *Vesicomya gigas* (J. Barry pers. comm.).

It proved more difficult to assign the SCB clam specimen W8 to an existing taxon. The present maximum likelihood analysis placed it basal to the Group B clade; however, the bootstrap support for this arrangement was weak (Fig. 2). We tested the likelihood of 4 different tree topologies regarding the placement of W8 under the ‘user trees’ option of DNAmI (Felsenstein 1981). The tests showed that placement of W8 within the Group B clade log-likelihood (lnLi = -4127.44, SD 12.26) was not significantly different from the maximum likelihood topology (lnLi = -4106.77) (p = 0.093). However, placement of W8 within the Group A clade (lnLi = -4171.92, SD 14.47) or
Fig. 2. Maximum likelihood phylogeny of vesicomyids based on a 516 base pair region of the mitochondrial cytochrome c oxidase subunit I gene, log likelihood (lnL) of -4106.77. Bootstrap values (numbers at nodes) are expressed as the percent of 100 replications (values less than 50 not shown). The 10 whale-fall sequences (W1 to W10) and the location abbreviations SCB (Santa Catalina Basin) and SNI (San Nicolas Island slope) are in bold type. The remaining sequences retain the OTU designations of Peek et al. (1997). A: specimens collected from hydrothermal vent sites. GenBank accession numbers and collection information for specimens are listed in Table 1 in the order they appear in the figure.
within the *Ectenagena extenta* clade (lnLi -4172.65, SD 13.62) were both significantly worse (p < 0.00001) than the maximum likelihood topology. Thus, it remains unclear whether W8 represents a highly divergent form of *Vesicomya gigas* or a new species in this cryptic species complex.

The distribution of Kimura 2-parameter distances between all pairs of these 62 specimens revealed a bimodal distribution that helped to infer current species assignments (Fig. 3). Intraspecific distances found among specimens of the well-defined species (i.e. not including members from the 'gigas/kilmeri' and 'pacificalepta' cryptic species complexes) could be used to assess the relationships of new specimens. Intraspecific distances were all less than 0.0177, contributing to the notable lower peak of the present bimodal distribution. All interspecific distances for the well-defined species were 0.054 or greater, yielding the second higher peak. The genetic distances between SCB clams W1 to 7 and other group B specimens (putatively *Vesicomya gigas*) were less than 0.0177 and consistent with conspecificity. Similarly, genetic distances between SNI W9 and other Group A specimens (putatively *Calyptogena kilmeri*) were all less than 0.0177 and consistent with conspecificity.

Species assignments of the remaining 2 specimens were less clear, however. The SCB W10 specimen was closest to specimens identified as *Calyptogena elongata* (genetic distances ≤0.054) and more divergent from all other taxa (distance ≥0.080). Maximum likelihood bootstrapping strongly supported its clustering with *C. elongata* and we have tentatively considered it a highly divergent variant of this species. Similarly, the SCB W8 specimen was highly divergent from all other taxa. Its closest relationship was with Group B specimens (≤0.083); thus, we have tentatively included it within a diverse complex that probably contains additional cryptic species. Clearly, more specimens of these 2 taxa must be examined with additional genetic markers before confident species assignments can be made.

**DISCUSSION**

The present analysis identified at least 3 distinct species of vesicomyid clams sampled from whale falls along the California margin, increasing the number of invertebrate species shared between whale falls and vents or seeps to 16. Of the 10 whale-fall vesicomyid specimens examined here, 7 clustered within the Group B clade (putatively, *Vesicomya gigas*), 1 clustered within the Group A clade (putatively, *Calyptogena kilmeri*), 1 was closely related to *C. elongata*, and the final specimen clustered within the 'gigas/kilmeri' cryptic species complex, and may represent a novel species.

Problems remain with the present species assignments, however. Sequence divergence (d) between the single SCB W10 specimen and individuals of *Calyptogena elongata* from the Santa Barbara Channel (d = 0.058) was greater than that typically found within well-defined species of vesicomyids. In general,
intraspécific divergence for mitochondrial COI was less than 0.018 and interspecific divergence exceeded 0.054. Similarly, divergence between SCB W8 and other members of the "gigas/kilmeri" cryptic species complex was 0.08. High $d$ levels notwithstanding, we cannot be confident that these specimens represent novel taxa. Cases are known in bivalve mollusks and other invertebrates where intraspecific divergence of mitochondrial sequences can be high. For example, divergent mitochondrial haplotypes found in Mytilus edulis populations exhibit $d$ values of 0.10 for the same COI region as examined in this study (Hoeh et al. 1996). However, these mitochondrial haplotypes exhibit sexually biased inheritance in M. edulis, which has not been examined in vesicomyids. Although we cannot exclude the hypothesis that divergent mitochondrial haplotypes are similarly maintained in vesicomyid species, we found no evidence for heteroplasmy (2 or more mitochondrial haplotypes in a single individual), one indicator of sexually biased mitochondrial transmission. In other invertebrates, intraspecific mitochondrial COI $d$ values are generally about 0.01 to 0.02 (reviewed in Avise et al. 1987, Juan et al. 1996). In some beetles however, $d$ values for intraspecific divergence between populations with disjunct geographic locations may be as high as 0.07 to 0.10 (Juan et al. 1996). Perhaps vesicomyid populations follow a pattern similar to beetles.

Clearly, more individuals of the ambiguous vesicomyid taxa must be sampled from a broader geographical range to assess the extent of mitochondrial polymorphism within species. Furthermore, examination of independent nuclear gene loci (e.g. allozymes) could help to resolve relationships among these poorly resolved groups of clams. Better morphological criteria for species assignments may be helpful, but the present criteria based mostly on conchology have been problematic (Boss & Turner 1980). Future sampling should focus on specimens representative of the size and developmental variation seen in these clams. Larger sample sizes (e.g. >30 individuals site$^{-1}$) can help address other questions, including the rates of gene flow and modes of dispersal between populations at whale falls and other reducing habitats.

Despite the present taxonomic ambiguities, whale falls clearly provide additional habitat for several vesicomyid species. However, we do not know whether whale falls are ecological sinks that support non-reproductive individuals produced at other sites, or if they contribute significantly to the vesicomyid reproduction and dispersal in the deep-sea. Bennett et al. (1994) estimated a population size of 400 to 800 large vesicomyids at the SCB skeleton. If these clams primarily represent a single species (putatively Vesi- comya gigas in the current study) this skeleton may support a population size as great as has been seen near Guaymas Basin or Middle Valley hydrothermal vents (R. Vrijenhoek pers. obs.). Comparative studies of reproductive condition in whale-fall clams and their vent and seep counterparts are warranted to assess potential contributions to the vesicomyid larval population.

![Fig. 4. Two models for the habitat distributions of vesicomyid clams.](image-url)
Peek et al. (1997) suggested that most vesicomyid lineages are restricted to a single type of reducing habitat (i.e., vents, seeps, or anoxic basins). However, whale fall assemblages deviate from this pattern, containing vesicomyid lineages also found at soft-sediment hydrothermal vents, cold seeps, and anoxic basins. This suggests that whale falls may offer habitat conditions that are intermediate to, or broader than, those found in other reducing habitats (Fig. 4). If we assume that vents, whales and seeps fall along a gradient of environmental factors required for vesicomyid survival, then there are 2 possible explanations for the habitat distribution of vesicomyid clams (Fig. 4). In the first case (Fig. 4A), vents, whales and seeps have narrow non-overlapping habitat conditions. In this instance, the vesicomyid species found on whales would need relatively broad tolerance ranges, allowing these species to colonize both whales and vents, or whales and seeps. Alternatively, whale habitats may provide a broad range of the habitat gradient (Fig. 4B), overlapping with the divergent conditions at both vents and seeps. In this case, vesicomyids with narrow tolerance ranges in the regions of overlap could colonize whale falls from vents and seeps. In either case, Fig. 4A or B, whale falls appear to represent an intermediate habitat type between soft-sediment vents and seeps, with the potential to provide evolutionary stepping stones between divergent soft-sediment reducing habitats at the deep-sea floor.

Fig. 4B is consistent with the findings of Barry et al. (1997) that 2 vesicomyid seep species have narrow sulfide tolerance ranges. Whale falls may offer a broader range of habitat conditions than many other reducing habitats because they offer both hard and soft substrates (e.g., bones and sediments) that are rich in sulfides. However, while sulfide levels at the SCB whale fall in 1988 and 1991 (<20 μM; Smith et al. 1998) were similar to those measured in the Santa Barbara Channel sediments (<10 μM), they were relatively low compared to levels measured at hydrothermal vents (6 to 95 μM) and cold seeps (up to 180 μM) (reviewed by Scott & Fisher 1995). This suggests that the clam species found at whale falls may be restricted to the periphery of more sulfide-rich habitats such as vents and seeps. Furthermore, whale fall species may prefer to burrow, since all the whale fall adult clams were collected from sediments. In fact, all the sites that share vesicomyid species with whale-falls (the Guaymas Basin vents and transform fault seeps, Santa Barbara Channel anoxic basin, Monterey Canyon seeps, Oregon Subduction Zone seeps, and Middle Valley vents) contain soft sediments. The fossil record for the vesicomyids extends to the early Cretaceous, 106 million yr ago (Kanie & Sakai 1997), but based on a very rough protein molecular clock, Peek et al. (1997) suggested that extant vesicomyids diversified during the Cenozoic Era, less than 50 million yr ago. Inclusion of the present whale-fall specimens does not alter this estimate. The earliest recognized ancestors of modern cetaceans evolved in the Paleocene (about 55 to 65 million yr ago) and rapidly diversified during the Eocene (37 to 55 million yr ago) (Gaskin 1982). The first large representatives of cetaceans were the basilosaurs (21 m in length), which evolved in the Late Eocene about 40 million yr ago (Gaskin 1982, Briggs & Crowther 1990). Fossil evidence suggests that chemosynthetic communities including vesicomyid clams have been associated with whale skeletons for at least 30 million yr (Goedert et al. 1995). We note that, based on very rough estimates, the diversification of vesicomyid clams may have been synchronous with the diversification of large cetaceans, suggesting that the relationship between whale and vesicomyid evolution merits further scrutiny.

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