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Evolutionary relationships of deep-sea hydrothermal vent and cold-water seep clams (Bivalvia: Vesicomyidae): results from the mitochondrial cytochrome oxidase subunit I

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Abstract Phylogenetic relationships among vesicomyid clams (Bivalvia: Vesicomyidae) and their placement within the order Heterodonta were examined using mitochondrial encoded cytochrome oxidase subunit I (COI) DNA sequences. The presently analyzed vesicomyids represent a recent monophyletic radiation that probably occurred within the Cenozoic. Nucleotide phylogenetic analyses resolved discrete clades that were consistent with currently recognized species: *Calyptogena magnifica*, *C. ponderosa*, *Ectenagena extenta*, *C. phaseoliformis*, *Vesicomya cordata*, *Calyptogena* n. sp. (Gulf of Mexico), *C. kaikoi*, *C. nautilei*, *C. solidissima* and *C. soyoae* (Type-A). However, specimens variously identified as: *V. gigas*, *C. kilmeri*, *C. pacifica*, and *V. lepta* comprised two "species complexes", each composed of multiple evolutionary lineages. Most taxa are limited to hydrothermal-vent or cold-seep habitats, but the "vent" versus "seep" clams do not constitute separate monophyletic groups. Current applications of the generic names *Calyptogena*, *Ectenagena*, and *Vesicomya* are not consistent with phylogenetic inferences.

Introduction

The Vesicomyidae, a family of heterodont clams erected by Dall and Simpson (1901), includes about 50 described species that are associated with sulfide-rich habitats in the vicinity of hydrothermal vents, cold-water sulfide/hydrocarbon seeps, and other reducing environments (e.g. whale-falls). The intense interest in deep-sea vent and seep fauna has led to descriptions of many new species since the family was last revised by Boss (1970). Boss and Turner (1980) noted that "... systematics of the family Vesicomyidae is beset with difficulties because there is at present no satisfactory diagnosis that would exclude the constituent taxa from other heterodonts based on shared derived characters.". Attempts to reconcile vesicomyid relationships with molecular techniques also have proved frustrating. An allozyme analysis of taxa from the eastern Pacific and Gulf of Mexico revealed many morphologically cryptic species; however, the data could not resolve phylogenetic (or taxonomic) relationships above the level of species (Vrijenhoek et al. 1994). An analysis of mitochondrial DNA from western Pacific taxa also identified divergent sequences from morphologically similar individuals (i.e. cryptic taxa) and, to complicate matters, found similar sequences in morphologically distinct individuals (Kojima et al. 1995). This combination of cryptic morphospecies and phenotypic plasticity within species creates a vexing problem for biologists studying evolutionary history and biogeography of vent and seep organisms.

A number of characteristics unite the family Vesicomyidae. All extant members are restricted to habitats that furnish reduced sulfides for chemoautotrophic endosymbiotic bacteria, which in turn provide nutriment for these clams (Cavanaugh 1983; Childress and Fisher 1992). Such environments occur at hydrothermal vents along the mid-ocean ridge system and back-arc basins (German et al. 1995) and at cold-water sulfide/hydrocarbon seeps (Paull et al. 1984; Brooks et al. 1990). Additionally, vesicomyids were found in reducing sediments associated with a decomposing whale carcass (Bennett et al. 1994).

The observation that typical vent species are absent at seeps, and vice versa, led to speculation about fundamental differences between vent and seep ecosystems (Tunnicliffe 1991; Craddock et al. 1995). Eastern Pacific hydrothermal vent fields tend to be ephemeral, lasting

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perhaps a few decades (Fustec et al. 1987), whereas cold-water seeps may be very stable, lasting for many thousands of years (Hecker 1985). Many vent communities live on hard basaltic substrates, although sedimented sites are known. Seeps typically are covered with soft sediments, although some have hard carbonate outcroppings. Differences in sulfide concentration, flux, and accessibility also exist between these habitats (Lowell et al. 1995; Von Damm 1995). What are the evolutionary relationships among taxa found in these ecologically different habitats? Do vent and seep vesicomyids comprise separate evolutionary lineages? How old are the vesicomyids as a group? Evolutionary questions such as these can only be addressed with robust phylogenetic models. In this study, we use DNA sequence information from a 516 base pair (bp) portion of the mitochondrial cytochrome c oxidase subunit I (COI) gene to infer phylogenetic relationships of vesicomyids from the Pacific Ocean and Gulf of Mexico.

Materials and methods

Specimens

Vesicomyid clams were obtained from a variety of locations in the Pacific Ocean and Gulf of Mexico (Table 1). Specimens were collected with the deep submergence vehicles "Alvin", "Nautile", "Shinkai 6500", "Johnson Sea Link" and "Turtle"; the unmanned vehicles "HYSUB" (renamed "ROPOS") and "Ventana", and by ocean surface-dredges. Preliminary identifications of morphospecies (Table 1) were performed by the authors (R.G.G., R.A.L., R.C.V.), R.D. Turner, or the providers of specimens: J. Barry, C. Cavanaugh, J.J. Childress, J. Hashimoto, or A. Fiala-Médioni. Specimens that were of questionable identification were labeled with location designation. (See Vrijenhoek et al. 1994 for an account of sample preparations). In most cases, large specimens were dissected soon after retrieval, while small specimens were frozen whole at −80 °C. Shells were cleaned and dried, and coded for permanent storage. Pending formal taxonomic descriptions of new species, we will deposit representative voucher shell specimens in an appropriate museum collection.

DNA extraction, PCR, and sequencing

Mantle and adductor muscle tissues were dissected from fresh or frozen specimens. Nucleic acids were extracted using CTAB (hexadecyltrimethyl ammonium bromide) (Doyle and Dickson 1987) or proteinase K (Mullenbach et al. 1989) protocols followed by phenol extraction and ethanol precipitation (Sambrook et al. 1989). Purified nucleic acids were hydrated in TE buffer (10 m*M* Tris-HCl, pH 7.5; 1 m*M* EDTA) and stored at −20 °C. A 710 base pair (bp) portion of the mitochondrial cytochrome oxidase subunit I (COI) gene was amplified by the polymerase chain-reaction (PCR) with universal primers designed by Folmer et al. (1994): LCO1490 (5′-ggTCAACAAATCATAAAgATATTgg-3′), and HCO2198 (5′- TAAACTTCAgggTgACCAAAAAATCA-3′). PCR mixtures contained: 1 × Taq DNA polymerase buffer (Promega, Madison, Wisconsin), 2.5 m*M* $MgCl_2$, 200 μ*M* dNTPs (1 μ*M* each primer), 5 to 50 ng template, and 0.5 U Taq DNA polymerase (Promega) in 25 μl final volume. The PCR profile (94 °C min^{-1}; 45 °C min^{-1}; 72 °C min^{-1}) continued for 25 cycles followed by a final extension at 72 °C for 10 min.

Prior to sequencing, the PCR products were separated on agarose gels to determine product size. We designed new vesicomyid-specific sequencing primers that were 20 bases internal to the amplification primers: VesLCO (5′-TTAATAggAACTgCTTTTAg-3′), and VesHCO (5′-TCACCCAAACCAgCAggATC-3′). Sequencing templates were purified by column gel-filtration (Qiagen, Chatsworth, California) or ethanol precipitation. Sequencing reaction mixtures included either 5′-P^{33} end-labeled primers (Perkin-Elmer, Foster City, California), or dye-terminated dideoxy labeling (ABI, Foster City, California) under standard cycle-sequencing conditions.

Phylogenetic analysis

To facilitate alignment of DNA sequences, we inferred amino acid (a.a.) sequences with the *Drosophila yakuba* amino acid code (Clary and Wolstenholme 1985). We used the program ClustalW (Thompson et al. 1994) to provide a preliminary alignment of a.a. sequences, and the GDE 2.2a (Smith) editor to optimize alignments by eye. The a.a. analysis was based on 172 positions. We used the protdist program from PHYLIP 3.57 (see Felsenstein 1989) to assess Kimura protein distances (Nei 1987) among the representative heterodont bivalves. Assuming a JTT (Jones et al. 1992) model of a.a. substitution, we applied the protml program from the MOLPHY 2.2 package (Adachi and Hasegawa 1992) to estimate phylogenetic relationships by the maximum-likelihood method.

Nucleotide sequences were used to examine evolutionary relationships within the family Vesicomyidae. Nucleotide composition of these sequences was estimated with MEGA 1.0 (Kumar et al. 1993). Using the observed nucleotide frequencies and transition:transversion ratio, we applied the fastDNAml 1.0.6 program (Olsen et al. 1994) or the dnaml program from PHYLIP 3.57. Parsimony analysis was performed with PAUP 3.1.1 (Swofford 1993). Alternate phylogenetic hypotheses were constructed with MacClade 3.06 (Maddison and Maddison 1992), and tested with likelihood by the Kishino–Hasegawa method (Kishino and Hasegawa 1989). To test hypotheses based on parsimony methods, we used the Wilcoxon rank-order test to determine whether alternative trees differed significantly in the number of steps (Templeton 1983).

Results

We obtained new COI nucleotide sequences spanning 516 bp for 52 vesicomyid and two other heterodont specimens. These 54 original sequences were deposited with GenBank (accession numbers in Table 1 and Fig. 1 legend). For comparative purposes, we examined published sequences from five additional heterodont bivalves (Baldwin et al. 1996). Subsequent phylogenetic comparisons of these sequences and published COI sequences from eight western Pacific species of vesicomyids (Kojima et al. 1995) used only the 278 positions common to both data sets.

Amino acid (a.a.) sequences

To phylogenetically place the vesicomyids among the heterodont bivalves, we inferred a.a. sequences from all DNA sequences. Among the vesicomyids, we observed no insertions or deletions for the 172 a.a. fragment. However, the glossid *Glossus humanus* was the most divergent heterodont taxon; it contained two unique amino acid insertions and five unique deletions. The venerid *Mercenaria mercenaria* had one unique deletion at the start of this protein segment. Although a.a.

Table 1 GenBank accession numbers, specimen collection sites, and species or identifications of vesicomyid clams (listed in same order as in Fig. 2). Habitat designations are based on presence (vent) or absence (seep) of hydrothermal flow in area of specimen collection; operational taxonomic unit (*OTU*) designations are based upon morphological features of specimens, some OTUs were given multiple designations based upon equivocal morphologies [*Dive* submersibles: *A* "Alvin"; *N* "Nautile" (*ma* "Manon", *na* "Naudur", and *np* "Nautiperc" cruises); *S* "Shinkai 6500"; *J* "Johnson Sea Link"; *T* "Turtle"; *H* "HYSUB"; *V* "Ventana"]

GenBank accession#	Dive No.	Location	Latitude; Longitude	Depth (m)	Habitat	Date	OTU designation
AF008246	A 2796	Oregon Subduction Zone	44°40.53′N;125°07.09′W	765	seep	16 Jul. 1994	Clam Type I #1
AF008247	V 94-11-2	Monterey Canyon	36°N;122°W	1000	seep	11 Jan. 1994	*Vesicomya gigas* revised to *Calyptogena kilmeri*
AF008248	A 2839	Guaymas Transform Fault	27°34.85′N;111°27.00′W	1765	seep	8 Oct. 1994	n.sp.2 #1
AF008249	A 2796	Oregon Subduction Zone	44°40.53′N;125°07.09′W	765	seep	16 Jul. 1994	Clam Type I #6
AF008250	A 2345	Guaymas Transform Fault	27°34.8′N;111°27.6′W	1653	seep	15 Mar. 1991	n.sp. Z-1
AF008251	V 94-11-3	Monterey Canyon	36°N;122°W	1000	seep	11 Jan. 1994	*Vesicomya gigas* revised to *Calyptogena kilmeri*
AF008252	A 2839	Guaymas Transform Fault	27°34.85′N;111°27.0′W	1765	seep	8 Oct. 1994	Clam sp.2 #2
AF008253	A 2345	Guaymas Transform Fault	27°34.8′N;111°27.6′W	1653	seep	15 Mar. 1991	n.sp. Z-10
AF008254	A 2839	Guaymas Transform Fault	27°34.85′N;111°27.0′W	1765	seep	8 Oct. 1994	*Calyptogena kilmeri*
AF008255	A 2839	Guaymas Transform Fault	27°34.85′N;111°27.0′W	1765	seep	8 Oct. 1994	*Calyptogena kilmeri*
AF008256	A 2803	Middle Valley, Juan de Fuca Ridge	48°27.40′N;128°42.52′W	2416	vent	24 Jul. 1994	Clam Type II #1
AF008257	A 2803	Middle Valley, Juan de Fuca Ridge	48°27.40′N;128°42.52′W	2416	vent	24 Jul. 1994	Clam Type II #2
AF008258	H 192	Middle Valley, Juan de Fuca Ridge	48°27.5′N;128°42.5′W	2400	vent	27 Jun. 1992	n.sp. 1/large
AF008259	H 192	Middle Valley, Juan de Fuca Ridge	48°27.5′N;128°42.5′W	2400	vent	27 Jun. 1992	n. sp.1/large
AF008260	A 2798	Oregon Subduction Zone	44°01.11′N;125°17.42′W	2028	seep	18 Jul. 1994	Clam #1
AF008261	A 2340	Guaymas Basin	27°00.4′N;111°24.3′W	2019	vent	10 Mar. 1991	n.sp. 1/*Vesicomya gigas*
AF008262	A 2837	Guaymas Basin	27°00.11′N;111°24.34′W	2008	vent	6 Oct. 1994	Clam #1
AF008263	A 2837	Guaymas Basin	27°00.11′N;111°24.34′W	2008	vent	6 Oct. 1994	Clam #2
AF008264	T 2/26/93	Guaymas Basin	27°N;111°W	2000	vent	26 Feb. 1993	*Vesicomya gigas*
AF008265	A 2287	Monterey Canyon	36°23.2′N;122°53.8′W	3402	seep	30 Sep. 1990	*Ectengena extenta*
AF008266	A 2042	Gorda Ridge	41°00.4′N;127°29.3′W	3271	vent	12 Jun. 1988	*Ectengena extenta*
AF008267	A 2287	Monterey Canyon	36°23.2′N;122°53.8′W	3402	seep	30 Sep. 1990	*Ectengena extenta*
AF008268	A 2801	Gorda Ridge	41°00.2′N;127°29.60′W	3260	vent	21 Jul. 1994	*Ectengena extenta*
AF008269	A 2224	0°N East Pacific Rise, Rosegarden	00°48.2′N;86°13.9′W	2461	vent	29 May 1990	*Calyptogena magnifica*
AF008270	A 2231	21°N East Pacific Rise, Clam Acres	20°49.9′N;109°06.2′W	2618	vent	11 Jun. 1990	*Calyptogena magnifica*
AF008271	N na12	18°S East Pacific Rise	18°36.4′S;113°24.0′W	2700	vent	Dec. 1993	*Calyptogena magnifica*
AF008272	N na12	18°S East Pacific Rise	18°36.4′S;113°24.0′W	2700	vent	Dec. 1993	*Calyptogena magnifica*
AF008273	dredge	Santa Barbara Channel	34°N;120°W	500	seep	Dec. 1988	*Calyptogena elongata*
AF008274	dredge	Santa Barbara Channel	34°N;120°W	500	seep	Dec. 1988	*Calyptogena elongata*
AF008275	dredge	Santa Barbara Channel	34°N;120°W	500	seep	Dec. 1988	*Calyptogena elongata*
AF008276	dredge	Santa Barbara Channel	34°N;120°W	500	seep	Dec. 1988	*Calyptogena elongata*
AF008277	J 3134	Louisiana Continental Slope	27°41.2′N;91°32.3′W	704	seep	17 Sep. 1991	*Vesicomya cordata*
AF008278	J 3133	Louisiana Continental Slope	27°41.3′N;91°32.5′W	737	seep	17 Sep. 1991	*Calyptogena ponderosa*
AF008279	N ma13	Barbados Accretionary Prism	13°50′N;57°45′W	5000	seep	1992	*Calyptogena* n.sp./Clam #2
AF008280	A 2542	West Florida Escarpment	26°01.8′N;84°54.6′W	3313	seep	3 Jun. 1992	*Calyptogena* n.sp.
AF008281	A 2542	West Florida Escarpment	26°01.8′N;84°54.6′W	3313	seep	3 Jun. 1992	*Calyptogena* n.sp.
AF008282	S 272	Japan Trench	40°06.60′N;144°11.10′E	6370	seep	19 Jul. 1995	*Calyptogena phaseoliformis*
AF008283	S 272	Japan Trench	40°06.60′N;144°11.10′E	6370	seep	19 Jul. 1995	*Calyptogena phaseoliformis*

(continued overleaf)

Table 1 (continued)

GenBank accession#	Dive No.	Location	Latitude; Longitude	Depth (m)	Habitat	Date	OTU designation
AF008284	S 272	Japan Trench	40°06.60′N;144°11.10′E	6370	seep	19 Jul. 1995	*Calyptogena phaseoliformis*
AF008285	A 2796	Oregon Subduction Zone	44°40.53′N;125°07.09′W	765	seep	16 Jul. 1994	Clam Type II #3
AF008286	A 2796	Oregon Subduction Zone	44°40.53′N;125°07.09′W	765	seep	16 Jul. 1994	Clam Type III #3
AF008287	V 93-364-3	Monterey Canyon	36°N;122°W	600	seep	30 Dec. 1993	*Calyptogena pacifica*
AF008288	A 2796	Oregon Subduction Zone	44°40.53′N;125°07.09′W	765	seep	16 Jul. 1994	Clam Type III #1
AF008289	A 2797	Oregon Subduction Zone	44°40.53′N;125°17.41′W	2089	seep	17 Jul. 1994	n.sp.1/diagonalis?
AF008290	A 2234	Guyamas Basin	27°00.2′N;111°24.5′W	2020	vent	16 Jun. 1990	n. sp.2/*Vesicomya lepta*/*C.pacifica*
AF008291	A 2235	Guaymas Basin	26°59.9′N;111°24.6′W	2016	vent	17 Jun. 1990	n. sp.2/*Vesicomya lepta*
AF008292	N np6	Peruvian Upper Slope Scarp	5°32′S;81°32′W	2500	vent	1991	Small clam #2
AF008293	A 2251	Middle Valley, Juan de Fuca Ridge	48°27.6′N;128°43.2′W	2437	vent	5 Aug. 1990	*Calyptogena pacifica*/small #3
AF008294	A 2426	Axial Seamount, Juan de Fuca Ridge	45°56.1′N;130°00.8′W	1547	vent	10 Aug. 1991	*Calyptogena pacifica*
AF008295	A 2413	North Endeavour Juan de Fuca Ridge	47°57.4′N;129°05.9′W	2200	vent	20 Jul. 1991	*Calyptogena pacifica*
AF008296	A 2803	Middle Valley, Juan de Fuca Ridge	48°27.40′N;128°42.52′W	2416	vent	24 Jul. 1994	Clam Type I #1
AF008297	A 2803	Middle Valley, Juan de Fuca Ridge	48°27.40′N;128°42.52′W	2416	vent	24 Jul. 1994	Clam Type I #2

substitutions were observed, all other representatives of the superfamilies Dreissenoidea, Corbiculoidea, and Mactroidea shared the 172 a.a positions with the Vesicomyidae.

We used a maximum-likelihood (ML) method to infer evolutionary relationships among the heterodont taxa included in this study. The Vesicomyidae comprised a tight monophyletic group (Fig. 1). Alternative topologies that constrained vesicomyids as polyphyletic were not supported by likelihood analyses. Although vesicomyids are treated as members of the superfamily Glossoidea (Vaught 1989), the present tree placed *Glossus humanus* at the end of a very long branch – a consequence of the unique insertions and deletions mentioned above. Because long branches have a tendency to distort inferred phylogenetic relationships (Felsenstein 1978), we did not use *G. humanus* as an outgroup for further phylogenetic investigations of vesicomyids. Based on its estimated a.a. distance from vesicomyids (Table 2), we chose *Mercenaria mercenaria* as an outgroup for subsequent phylogenetic analyses. The range of a.a. distances among members of the family Vesicomyidae was small (0 to 0.04189). The largest a.a. distance value occurred between putative congeners, *Calyptogena kilmeri* and *C. phaseoliformis*.

Age of the Vesicomyidae

To calibrate a molecular clock based on these a.a. sequences, we referred to the veneroid fossil record. Assuming the vesicomyids and dreissenids separated in the early Triassic, 245 million years ago (mya) (Morton 1996), the maximum a.a. distance between them, 0.47772 ($\pm$0.03692 SD), provides an estimate of 0.00195 Kimura protein distance units per million years. Maximum a.a. divergence among the present vesicomyid taxa is 0.04189, which suggests a last common ancestor only 21.5 mya. On the other hand, if we assume that vesicomyids and dreissenids separated much earlier than their first appearance in the fossil record (e.g. with the origin of the Heterodonta in the Ordovician, 500 mya), we obtain a calibration of 0.00095 Kimura distance units/my. The latter assumption provides a conservative estimate for vesicomyid origins, but still suggests a recent last common ancestor 43.8 mya. It is possible, however, that amino acid evolution in COI has significantly slowed down in these clams. Both estimates for the last common ancestor of the Vesicomyidae are relatively recent, and divergence probably occurred in the Cenozoic era.

Nucleotide sequences of vesicomyids

Although the present amino acid sequences clearly established the Vesicomyidae as a monophyletic group, their analysis provided no phylogenetic resolution within the family. To better assess relationships among

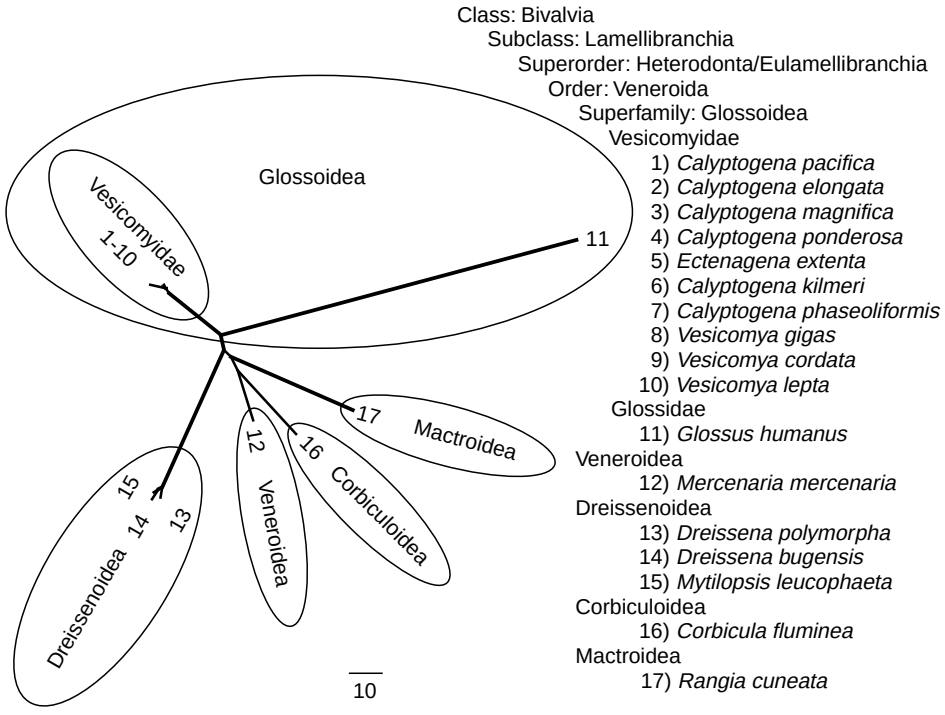

Fig. 1 Unrooted maximum-likelihood phylogeny (natural logarithm of likelihood LnLi −1793.51) based on cytochrome oxidase subunit I amino acid sequence from 17 heterodont bivalves. GenBank accession numbers:
C. pacifica, AF008287;
C. elongata, AF008274;
C. magnifica, AF008271;
C. ponderosa, AF008278;
E. extenta, AF008266;
C. kilmeri, AF008254;
C. phaseoliformis, AF008283;
V. gigas, AF008264;
V. cordata, AF008277;
V. lepta, AF008291;
G. humanus, AF008298;
M. mercenaria, AF008299;
D. polymorpha, U47653;
D. bugensis, U47651;
M. leucophaeta, U47649;
C. fluminea, U47647;
R. cuneata, U47652.
Scale bar is proportional to inferred number of amino acid substitutions

the vesicomyid specimens, we analyzed the original nucleotide sequences for the 516 bp segment of COI. Compositional biases were detected with respect to the nucleotides T and C across all codon positions (Table 3). These biases were greatest at the third position.

The pattern of codon use exhibited no particular biases when overall nucleotide composition was taken into account. Half (19) of the 39 first-position variable sites were silent at the a.a. level. All 13 second-position variable sites resulted in a.a. substitutions. Most variation occurred at third-position sites and was silent at the a.a. level. Transitions greatly exceeded transversions at the 153 phylogenetically informative sites. Most informative substitutions occurred at third-position sites (121) and a high ratio of third-position transitions to transversions (TS:TV = 7.4; Table 3) was observed.

We used maximum-likelihood analysis of the original nucleotide sequences to assess phylogenetic relationships among vesicomyids. *Mercenaria mercenaria* was used as an outgroup. The average nucleotide divergence between vesicomyids and the outgroup was 35%. Comparisons between the outgroup and vesicomyid taxa indicated saturation for third-position transitions. To assess the sensitivity of tree topology to the choice of this outgroup, we performed all phylogenetic analyses with and without *M. mercenaria*. The present topology is robust and independent of *M. mercenaria* as an outgroup.

Four named species (*Calyptogena elongata*, *C. magnifica*, *Ectenagena extenta*, *C. phaseoliformis*) all appeared as discrete monophyletic clades with high bootstrap support (Fig. 2). It is noteworthy that the present data separated the ''bean-shaped'' clams of the western Pacific, *C. phaseoliformis* Metivier et al., from those found in the eastern Pacific, *E. extenta* Krylova and Moskalev. Additionally, an undescribed species (*Calyptogena* n. sp.) from the Florida Escarpment (Gulf of Mexico) constituted a discrete lineage. Although we examined only one specimen each of *C. ponderosa* and *Vesicomya cordata* from the Gulf of Mexico (sequence divergence = 7.9%), they clearly comprised distinct evolutionary lineages, as seen in a previous allozyme study (Vrijenhoek et al. 1994).

The levels of nucleotide variation found within and between these well-defined species also helped to identify species-level divergence in morphospecies complexes. The occurrence of a single taxonomic name in multiple clades or the occurrence of multiple names in a single clade were criteria used to define the species complexes. Each complex was referred to by species names with the complex. For example, the *Vesicomya gigas*/*Calyptogena kilmeri* (hereafter referred to as ''*gigas*/*kilmeri*'') complex contained two clades (A and B) that segregate according to vent versus seep habitats (Fig. 2). The A-clade comprised a genetically homogeneous lineage that lives in relatively shallow seep habitats ranging from the Oregon Subduction Zone and Monterey Bay in the north to the Guaymas Transform Fault in the south. Based on sequence variation within this lineage (1.6%), the A-clade probably comprises a single, geographically widespread species. It is noteworthy that: two specimens within this clade from the Guaymas Transform Fault (GTF) were morphologically identified (by the authors) as *C. kilmeri*, two additional specimens from the same GTF sample were morphologically different enough not to be named, and another two specimens from Monterey Bay were preliminarily identified as *V. gigas* and subsequently assigned to *C. kilmeri* (Barry personal communication). Clearly, the present morphological criteria for genus and species

Table 2 Protein-sequence comparisons between Heterodonta, cytochrome oxidase subunit I (*Above diagonal* Kimura amino acid distances; *below diagonal* standard errors)

		Cpac	*Celo*	*Cmag*	*Cpon*	*Eext*	*Ckil*	*Cpha*	*Vgig*	*Vcor*	*Vlep*	*Ghum*	*Mmer*	*Dpol*	*Dbug*	*Mleu*	*Cflu*	*Rcun*
Calyptogena pacifica	(*Cpac*)		0.01172	0	0	0	0.01766	0.02364	0.01172	0.00584	0.00584	0.83007	0.27053	0.44621	0.42584	0.41585	0.30449	0.38655
C. elongata	(*Celo*)	0.00822		0.01172	0.01172	0.01172	0.02968	0.03576	0.02364	0.01766	0.01766	0.81418	0.26226	0.42584	0.42584	0.39620	0.29586	0.37702
C. magnifica	(*Cmag*)	0	0.00822		0	0	0.01766	0.02364	0.01172	0.00584	0.00584	0.83007	0.27053	0.44621	0.42584	0.41585	0.30449	0.38655
C. ponderosa	(*Cpon*)	0	0.00822	0		0	0.01766	0.02364	0.01172	0.00584	0.00584	0.83007	0.27053	0.44621	0.42584	0.41585	0.30449	0.38655
Ectenagena extenta	(*Eext*)	0	0.00822	0	0		0.01766	0.02364	0.01172	0.00584	0.00584	0.83007	0.27053	0.44621	0.42584	0.41585	0.30449	0.38655
C. kilmeri	(*Ckil*)	0.01005	0.01292	0.01005	0.01005	0.01005		0.04189	0.02967	0.02364	0.02364	0.86279	0.28733	0.47772	0.45658	0.44621	0.32202	0.40597
C. phaseoliformis	(*Cpha*)	0.01158	0.01412	0.01158	0.01158	0.01158	0.01521		0.03576	0.02968	0.02968	0.84628	0.30449	0.47772	0.45658	0.44621	0.33994	0.41585
Vesicomya gigas	(*Vgig*)	0.00822	0.01158	0.00822	0.00822	0.00822	0.01292	0.01412		0.01766	0.01766	0.86279	0.28733	0.45658	0.43597	0.42584	0.30449	0.39620
V. cordata	(*Vcor*)	0.00582	0.01005	0.00582	0.00582	0.00582	0.01158	0.01292	0.01005		0.01172	0.83007	0.27053	0.44621	0.42584	0.41584	0.30447	0.37701
V. lepta	(*Vlep*)	0.00582	0.01005	0.00582	0.00582	0.00582	0.01158	0.01292	0.01005	0.00822		0.83007	0.27053	0.43597	0.41584	0.40597	0.30449	0.38655
Glossus humanus	(*Ghum*)	0.03812	0.03815	0.03812	0.03812	0.03812	0.03804	0.03808	0.03804	0.03812	0.03812		0.76803	0.75318	0.73857	0.76803	0.75318	0.87960
Mercenaria mercenaria	(*Mmer*)	0.03242	0.03212	0.03242	0.03242	0.03242	0.03299	0.03352	0.03299	0.03242	0.03242	0.03821		0.38655	0.39620	0.40597	0.24598	0.33093
Dreissena polymorpha	(*Dpol*)	0.03651	0.03620	0.03651	0.03651	0.03651	0.03692	0.03692	0.03665	0.03651	0.03636	0.03822	0.03551		0.03576	0.04189	0.39620	0.47772
D. bugensis	(*Dbug*)	0.03620	0.03620	0.03620	0.03620	0.03620	0.03665	0.03665	0.03636	0.03620	0.03604	0.03823	0.03569	0.01412		0.05432	0.39620	0.46709
Mytilopsis leucophaeta	(*Mleu*)	0.03604	0.03569	0.03604	0.03604	0.03604	0.03651	0.03651	0.03620	0.03604	0.03587	0.03821	0.03587	0.01521	0.01716		0.42584	0.46709
Corbicula fluminea	(*Cflu*)	0.03352	0.03326	0.03352	0.03352	0.03352	0.03401	0.03448	0.03352	0.03352	0.03352	0.03822	0.03150	0.03569	0.03569	0.03620		0.37702
Rangia cuneata	(*Rcun*)	0.03551	0.03532	0.03551	0.03551	0.03551	0.03587	0.03604	0.03569	0.03532	0.03551	0.03799	0.03425	0.03692	0.03679	0.03679	0.03532	

Table 3 Nucleotide substitutions and composition by codon position for cytochrome oxidase subunit I

Characteristic	Position in codon			All sites
	1st	2nd	3rd	
Percentage nucleotide composition				
A	25.3	11.7	25.7	20.9
C	13.4	22.3	5.7	13.8
G	30.7	19.3	19.5	23.1
T	30.6	46.7	49.2	42.2
No. of variable sites	39	13	132	184
2-fold synonymous	19	0	62	81
4-fold synonymous	0	0	70	70
Non-synonymous	20	13	0	33
Phylogenetically informative sites	24	8	121	153
Transitions (TS)	16	3	74	93
Transversions (TV)	5	5	10	20
TS:TV	3.2	0.6	7.4	4.7

identifications are not consistent with the tight genetic cohesiveness of these phenotypically variable clams.

The B-clade of the *gigas*/*kilmeri* complex was much more diverse than the A-clade, and also contained specimens tentatively identified as *Vesicomya gigas*. Nucleotide variation among the lineages of the B-clade was 7.7%, which suggests this clade may contain several discrete species. Again, the specimens contained within the B-clade were sampled from habitats in the vicinity of hydrothermal venting with the exception of one individual from the Oregon Subduction Zone. Other genetic markers and larger sample sizes are needed to resolve evolutionary relationships among members of this diverse lineage.

A second species complex involved specimens identified as *Calyptogena pacifica* and *Vesicomya lepta* (hereafter "*pacifica*/*lepta*"). The *pacifica*/*lepta* complex also contained two well-supported clades (C and D) that segregate according to vent versus seep habitats. The C-clade comprised a genetically homogeneous lineage (1.8% divergence) that lives in seep habitats from the Oregon Subduction Zone and Monterey Bay. Specimens tentatively identified as *C. pacifica* and two unnamed morphotypes (II and III) occur within the genetically cohesive C-clade, again suggesting phenotypic plasticity in the genetically cohesive seep clams. The vent associated D-clade was genetically more diverse (7.4%), and contained individuals from the Juan de Fuca Ridge, Guaymas Basin and Peru Upper Slope Scarp. The high diversity of the D-clade suggests it may contain several cryptic morphospecies that warrant further investigation with additional genetic markers.

Phylogenetic-hypothesis testing

Alternative phylogenetic hypotheses can be tested with parsimony criteria by comparing the number of steps required to produce the alternative trees (the hypotheses). An analogous method tests alternative phylogenetic hypotheses by comparing the likelihood values for the trees. Maximum parsimony and likelihood methods produced congruent phylogenetic trees when compared by alternative methods – i.e. the Wilcoxon rank-order test (Templeton 1983) or likelihood criteria (Kishino and Hasegawa 1989). Subsequent tests between alternative phylogenetic trees also were consistent with both parsimony and likelihood methods, but only the likelihood values are stated.

First, we asked: do the vesicomyid genera *Calyptogena* (*Ectenagena*) and *Vesicomya* comprise natural phylogenetic groups? This hypothesis of monophyletic origins for generic level designations was not supported (LnLi = −4172.99, 46.85 SD) when contrasted to the maximum likelihood tree (Fig. 2; LnLi = −3714.20). Secondly, we asked: do vent versus seep vesicomyids comprise natural phylogenetic groups? This hypothesis used the habitat of the collection sites as the criterion for constraining the alternative topology. Monophyletic origins of vent versus seep taxa also were not supported (LnLi = −4006.42, 31.52 SD).

Eastern vs western Pacific vesicomyids

To compare the present data with published COI sequences from western Pacific vesicomyids (Kojima et al. 1995), we analyzed a subset including 278 nucleotides that were shared between these studies. We found no differences between the present and the published sequences for this region of COI in *Calyptogena phaseoliformis*. Based on this smaller fragment, we attempted to place several western Pacific taxa within the present phylogenetic context. *C. kaikoi* clustered with the clade comprised of *Calyptogena* n. sp. Gulf of Mexico (GofM) and *C. phaseoliformis* in the present study. *C. fausta* clustered with the *pacifica*/*lepta* D-clade. *C. nautilei* clustered with the *gigas*/*kilmeri* B-clade. *C. soyoae*-Type B (from the two types of *C. soyoae* described by Kojima et al.) clustered with the *gigas*/*kilmeri* A-clade. Specimens identified as *C. solidissima*, *C. soyoae*-type A, and *Calyptogena* n. sp. (Iheya Ridge) were grouped with a clade that comprised the *gigas*/*kilmeri* complex plus *Ectenagena extenta*. Based upon these limited sequence-data, attempts to phylogenetically place these western Pacific taxa should be considered tentative. However, *C. soyoae*-Type B may be conspecific with the eastern Pacific *gigas*/*kilmeri* A-clade. Also, *C. fausta* clustered within the eastern Pacific *pacifica*/*lepta* D-clade lineages.

Discussion

The present molecular data revealed that extant species of clams in the family Vesicomyidae constitute a natural (i.e. monophyletic) group that had a relatively recent

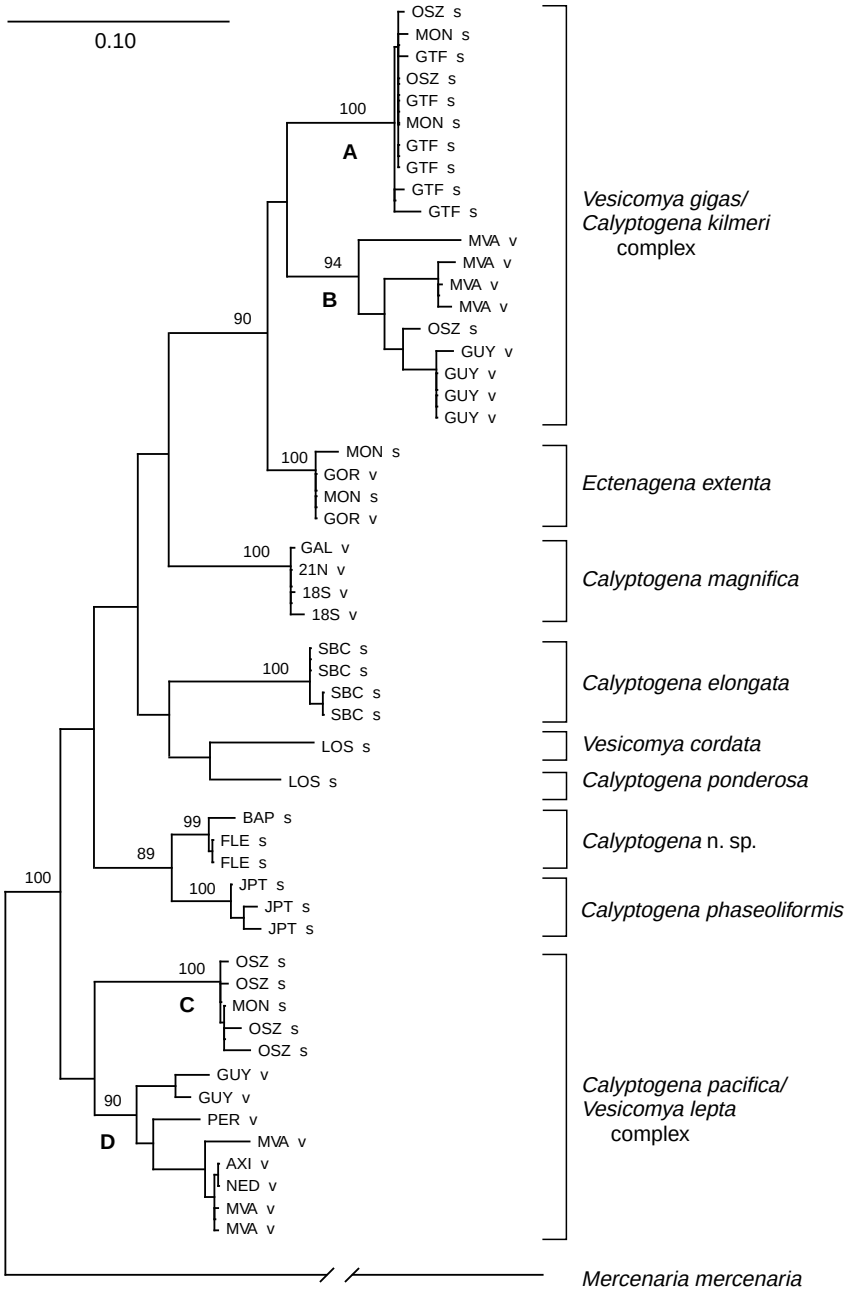

Fig. 2 DNA maximum-likelihood tree (LnLi - 3714.20) based on COI nucleotide sequences and rooted with *Mercenaria mercenaria*. Bootstrap values (≥60% from 200 replicates) are given above branches and clade labels below. Morphological species designations are on far right, and collection sites at ends of tree branches with following abbreviations (alphabetical order): *18S* 18° South, East Pacific Rise; *21N* 21° North, East Pacific Rise; *AXI* Axial Seamount, Juan de Fuca Ridge; *BAP* Barbados Accretionary Prism; *FLE* West Florida Escarpment ; *GAL* 0°N-Galápagos Rift, East Pacific Rise; *GOR* Gorda Ridge; *GTF* Guaymas Transform Fault; *GUY* Guaymas Basin; *JPT* Japan Trench; *LOS* Louisiana Continental Slope; *MON* Monterey Canyon; *MVA* Middle Valley, Juan de Fuca Ridge; *NED* North Endeavour, Juan de Fuca Ridge; *OSZ* Oregon Subduction Zone; *PER* Peruvian Upper Slope Scarp; *SBC* Santa Barbara Channel (*Lowercase v and s* indicate hydrothermal-vent or cold-seep habitat collection sites, respectively; *scale bar* is proportional to inferred nucleotide divergence)

origin, probably less than 50 million years ago (mya). However, this estimate may seem young given the fossil record of bivalve mollusks. For example, the order Veneroida first appeared in the early Ordovician 500 mya, and underwent extensive diversification during the Triassic and Jurassic 245 to 140 mya (Allen 1985). However, most extant veneroid morphotypes cannot be traced past the Jurassic, 195 mya (Lehmann and Hillmer 1983). Reports exist for "vesicomyid-like" bivalves from the Devonian 400 mya (Kuznetsov et al. 1990, 1993), but these specimens appear to be an undescribed species of acrotretacean inarticulate brachiopod (Little et al. 1997). Less controversial vesicomyids, identified as *Calyptogena chinookensis*, first appeared in a fossilized "cold-methane-seep" community from the late middle Eocene (≃50 mya) deposits in the state of Washington (Squires and Goedert 1991). Oligocene deposits (≃30 mya) from subduction-related limestone deposits also contain *C. chinookensis* (Goedert and Squires 1993). Kanno et al. (1989) identified vesicomyid fossils from Miocene (≃24 mya) deposits as *C. pacifica*. More recent Pliocene (≃5 mya) deposits also contained *Calyptogena* specimens (Niitsuma et al. 1989). Thus, the present molecular evidence for a recent radiation of the extant Vesicomyidae is consistent with the fossil record and supports Newman's (1985) hypothesis for recent invasion of hydrothermal environments by these clams.

Genetic studies (Vrijenhoek et al. 1994; Kojima et al. 1995; and present results) clearly warrant caution in the application of morphologically-based genus and species

names to extant taxa, much less to fossilized forms. Two problems are clear from the genetic studies. First, numerous morphologically-cryptic species exist within the Vesicomyidae. Second, field identifications often place specimens with highly divergent conchological features in distinct genera, whereas genetic analyses may reveal they are closely related. Additionally, the divergent body plans, hinge structures, and ligament sizes that have been associated with the application of different generic names may be phenotypically plastic. Morphological plasticity appears to be a common feature of vent- and seep-endemic organisms (Southward et al. 1996).

As with an allozyme study that included many of the same specimens (Vrijenhoek et al. 1994), we found no correspondence between phylogenetic relationships and the current application of generic names. In his revision of the family, Boss (1969) used the name *Calyptogena* (type: *pacifica* Dall, 1891) to encompass species with elongate shells (length to height ratios ≥1.5), a highly variable escutcheon, and absent lunule. *Ectenagena* is considered a distinct genus (type: *elongata* Dall, 1916) (Woodring 1938; Boss 1968; Krylova and Moskalev 1996), or alternatively, a subgenus of *Calyptogena* (Keen 1969; Boss and Turner 1980; Metivier et al. 1986). The name *Vesicomya* (type: *Callocardia atlantica* E.A. Smith, 1885) has been used to encompass species with more ovate shells and a developed lunule. However, it is clear from the present analysis that recognized genera are not reciprocally monophyletic, and generic assignments of new species should proceed with caution until a thorough revision of the family considers additional characters including soft-part anatomy.

Unfortunately, the present COI segment does not adequately resolve the deeper nodes in the tree (Fig. 2). These deep nodes are at an evolutionary depth that is saturated for third-position substitutions. In an attempt to better resolve these nodes, we are currently examining additional genes. We anticipate that other heterodonts may serve as better outgroups for subsequent phylogenetic analyses.

Genus-level problems notwithstanding, the present data clearly recognized discrete genealogical lineages that correspond to several named species (Fig. 2: *Ectenagena extenta*; *Calyptogena magnifica*; *C. elognata*; *Vesicomya cordata*; *C. ponderosa*; and *C. phaseoliformis*). These named species also differed for allozyme patterns at nearly all the 16 gene loci examined previously (Vrijenhoek et al. 1994). An undescribed ''ovate'' species from the Barbados Accretionary Prism and West Florida Escarpment was phylogenetically related to *C. phaseoliformis* Metivier et al., the ''bean-shaped'' clam from the Japan Trough. Although closely resembling *C. phaseoliformis* in its body proportions, another ''bean-shaped'' species from Monterey Bay was recently described as *E. extenta*. Krylova and Moskalev (1996) based on its distinct hinge dentition. The present COI data clearly supports its separation as a distinct species. *E. extenta* clusters tightly with other eastern Pacific species in the *gigas*/*kilmeri* complex (ML bootstrap value = 90%). Given the present difficulties with generic names, however, we attach no phylogenetic significance to the assignment of this species to *Ectenagena*. Also, the ''bean-shaped'' clams from the Gorda Ridge should be recognized as *E. extenta*. The allozyme study (Vrijenhoek et al. 1994) identified differences between Monterey Bay and Gorda Ridge ''bean-shaped'' clams, but enzymes from the single Gorda Ridge specimen examined in that study may have degraded (J.F. Grassle personal communication) prior to electrophoretic analysis.

Considerable nucleotide-sequence variation was found within several of these ''well-defined'' species. For example, nucleotide-sequence variation in *Calyptogena magnifica* was 0.78%. All four nucleotide substitutions in this species represented autapomorphic changes – two synonymous and two nonsynonymous. Examination of allozymes and nuclear DNA markers in *C. magnifica* (Karl et al. 1996) found a relatively homogenous species, with high levels of gene flow throughout its known range from 21°N to 18°S on the East Pacific Rise. Intraspecific levels of nucleotide variation in *Ectenagena extenta*, *C. magnifica*, *C. elongata*, *Calyptogena* n. sp. and *C. phaseoliformis* (Gulf of Mexico) averaged 1.04% (range = 0.59% to 1.38%). Sequence divergence among these species averaged 11.30% (range = 5.42 to 15.37%). These levels of within- and between-species genetic diversity can be used to gauge divergence in the more poorly-resolved cryptic species complexes.

Specimens variously identified as *Calyptogena pacifica* and *Vesicomya gigas* in an allozyme study (Vrijenhoek et al. 1994) also were problematic in the present study. The COI data clearly identify two cryptic species complexes (*gigas*/*kilmeri* and *pacifica*/*lepta,* Fig. 2) that each contained divergent evolutionary lineages which segregated according to hydrothermal vent versus cold-water seep habitats. For example, the A-clade in the *gigas*/*kilmeri* complex probably represents a single species that is widely distributed in relatively shallow seeps (< 2000 m) from Gulf of California in the south to the Oregon Subduction Zone in the north, and most likely is *C. kilmeri* (Barry personal communication). Individuals with the A-clade sequence also occur in Sagami Bay, Japan, where they are named *C. soyoae*-Type B. The cohesive nature of this lineage suggests the existence of a single transPacific species; however this hypothesis should be tested with additional genetic markers and population samples. In contrast, the *gigas*/*kilmeri* B-clade occurred at greater depths and predominantly in vent habitats. The B-clade encompasses several divergent lineages and may contain several distinct species. Additional genetic and morphological studies of type specimens are needed to relate the lineages to the names presently used for members of this complex.

The C- and D-clades of the *pacifica*/*lepta* complex provide a remarkably parallel pattern (Fig. 2). Again, the seep-associated lineage (C-clade) had lower genetic diversity than the vent-associated lineage (D-clade). Population genetic studies of the two complexes are

warranted, as the contrasts between vent and seep lineages may provide useful insights into the ecological and evolutionary dynamic processes that produce these differences in mitochondrial diversity.

The distribution and abundance of vesicomyid species are associated with water depth and sulfide concentration (Olu et al. 1996; Barry et al. 1997). Several species differ in their abilities to concentrate environmental sulfide (Childress et al. 1991, 1993), and the divergent body shapes seen in these clams (e.g. ovate vs elongate forms) may reflect adaptations to substrate conditions and the steepness of gradients in sulfide and oxygen concentration. Habitat distributions for vesicomyids also appear to be influenced by their phylogenetic relationships. However, these are not simple relationships consistent with singular cladogenic events. Relationships between vesicomyid morphology and phylogeny are also not simple. Similar elongate body plans have occurred in at least two independent phylogenetic lineages. Extreme body plans undoubtedly reflect fixed evolutionary adaptations in some species (e.g. the ''bean-shaped'' clams *Calyptogena phaseoliformis* and *Ectenagena extenta*). However, some of the more subtle differences that result in confused identifications within and between members of the two cryptic species complexes probably reflect phenotypically plastic responses to local environmental conditions, convergent evolution, or both. These matters clearly warrant further investigation.

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