

# Phylogenetic Position of Vesicomyid Clams from a Methane Seep off Central Chile (~36°S) with a Molecular Timescale for the Diversification of the Vesicomyidae

Francisco Valdés<sup>1,\*</sup>, Javier Sellanes<sup>1,2</sup>, and Guillermo D'Elía<sup>3</sup>

<sup>1</sup>Departamento de Biología Marina, Universidad Católica del Norte, Larrondo 1281, Coquimbo, Chile

<sup>2</sup>Centro de Investigación Oceanográfica en el Pacífico Sur-Oriental (COPAS), Universidad de Concepción, Casilla 160-C, Concepción, Chile. E-mail:sellanes@ucn.cl

<sup>3</sup>Instituto de Ciencias Ambientales y Evolutivas, Universidad Austral de Chile, campus Isla Teja s/n, Valdivia, Chile. E-mail:guille.delia@gmail.com

(Accepted March 30, 2012)

Francisco Valdés, Javier Sellanes, and Guillermo D'Elía (2012) Phylogenetic position of vesicomvid clams from a methane seep off central Chile (~36°S) with a molecular timescale for diversification of the Vesicomyidae. Zoological Studies 51(7): 1154-1164. Bivalves of the family Vesicomyidae are conspicuous inhabitants of sulfide-rich settings associated with both hydrothermal vents and methane seeps. The taxonomy of the family has been the subject of recent reviews, and currently, 2 subfamilies, the Vesicomyinae and Pliocardiinae, are recognized. Although the family has a worldwide distribution, material from the Southeast Pacific available so far for study has been scarce. In the present study, we consider 2 species from the Concepción Methane Seep Area (CMSA; 36°27.87'S, 73°43.25'W), Calyptogena gallardoi Sellanes and Krylova, 2005 and a larger species, which based in its morphology was tentatively assigned to the genus Archivesica (Archivesica sp. 1). We include new mitochondrial DNA sequences of the cytochrome c oxidase subunit I (COI) gene of these species, together with previously available sequences, in a phylogenetic analysis using maximum parsimony and Bayesian inference. In addition, a relaxed molecular clock was calibrated to estimate the divergence times of the lineages from which these species originated. The topologies recovered generally agree with a recent classificatory scheme of the family. Calyptogena gallardoi falls within the clade of Calyptogena sensu stricto, corroborating the morphologically based generic assignation. Similarly, Archivesica sp. 1, as expected from its morphology, falls within the clade corresponding to the genus Archivesica. According to results of the molecular clock, lineages leading to both CMSA species date back to the Miocene: C. gallardoi to ca. 11.2 Mya and Archivesica sp. 1 to ca. 6.18 Mya. Two independent vesicomyid invasions of the southeastern Pacific Ocean are proposed to account for the diversity seen at the CMSA. In general, the chronogram shows that lineages of pliocardiine vesicomyds have accumulated at a regular pace since the Eocene with no signs of diversification pulses. http://zoolstud.sinica.edu.tw/Journals/51.7/1154.pdf

Key words: Methane seep, Pacific Ocean Phylogenetic analysis, Central Chile.

he family Vesicomyidae comprises a diverse group of heterodont bivalves, which are generally associated with sulfide-rich reducing environments, including cold seeps at continental margins, hydrothermal vents at mid-ocean ridges, and large organic remains such as whale and wood falls. Their dominance in this kind of habitat is

explained by the fact that except for a single genus, they host endosymbiont chemosynthetic bacteria, from which they derive their nutrition (Sibuet and Olu 1998).

Based in the oldest reliable fossil record, *Archivesica* cf. *tschudi*, the origin of the family dates back to the late middle Eocene (Amano

<sup>\*</sup>To whom correspondence and reprint requests should be addressed. E-mail:fco.valds@gmail.com

and Kiel 2007). A recent taxonomic review of the family conducted by Krylova and Sahling (2010) proposed the division of Vesicomyidae into 2 subfamilies: i) the Vesicomyinae, formed solely by the genus Vesicomya Dall, 1886; and ii) the Pliocardiinae, composed of the genera Abyssogena Krylova Sahling and Janssen, 2010; Akebiconcha Kuroda, 1943; Archivesica, Dall 1908; Callogonia Dall, 1889; Calvptogena Dall, 1891; Ectenagena Woodring, 1938; Elenaconcha Cosel and Olu, 2009; Hubertschenckia Takeda, 1954; Isorropodon Sturany, 1896; Laubiericoncha Cosel and Olu, 2008; Phreagena Woodring, 1938; Pliocardia Woodring, 1925; Waisiuconcha Beets, 1942; and Wareniconcha Cosel and Olu, 2009. This classification system, based mainly on morphology, is partially congruent with previously published molecular-based phylogenies (Peek et al. 1997 1998 2000, Baco et al. 1999, Goffredi et al. 2003, Kojima et al. 2004, Stewart et al. 2008). Molecular phylogenetics of the family retrieve several main lineages, which probably could be regarded as genera (Peek et al. 1997, Goffredi et al. 2003, Kojima et al. 2004). For instance, the so-called pacifica/lepta complex, a wellsupported monophyletic group, corresponds to the genus Calyptogena sensu stricto (s.s.); as such, the species *lepta*, which did not fall in this clade (E. Krylova, pers. commun.), is now tentatively allocated to Wareniconcha (Krylova and Sahling

2010). However, further morphologic and genetic analyses of specimens with unequivocal identity are needed to reveal the correct taxonomic status of this species. Similarly, the so-called gigas/kilmeri complex may be formed by the genera Archivesica, Phreagena, Akebiconcha, and Ectenagena and some other lineages the phylogenetic affinities of which within the complex are still unresolved (Peek et al. 1997, Kojima et al. 2004). Other evolutionary lineages place the species within the genus Abyssogena s.s., which were formerly defined as the phaseoliformis complex (Krylova et al. 2010). Finally, the distinctiveness of Pliocardia has been much discussed, because the type species of the genus corresponds to a fossil, and so far, no living representative of the genus has been found (Krylova and Sahling 2010, Martin and Goffredi 2011). Even with knowledge of the composition and evolutionary history of marine biota greatly improving in recent years (Donrung et al. 2011, Mantelatto et al. 2011), large areas remain unclear.

Vesicomyids reported from a recently discovered methane seep site off central Chile (Sellanes et al. 2004), known as the Concepción Methane Seep Area (CMSA), include *Calyptogena gallardoi*, Sellanes and Krylova 2005, and at least 1 species of a larger form, tentatively assigned to the genus *Archivesica* (Sellanes et al. 2008; Fig. 1). The historical biogeography of CMSA vesicomyids is obscure. For instance,



Fig. 1. Archivesica sp. 1. (A) Exterior of left valve; (B) exterior of right valve; (C) interior of left valve; (D) interior of right valve; (E) left hinge plate; (F) right hinge plate.

it is unclear if these lineages of vesicomyids colonized the CMSA from a single source, and the times of these colonizations are unknown. The main reason for this gap in our knowledge is that CMSA vesicomyids have so far not been included in any phylogenetic analysis. Given these antecedents, the objective of the present study was to generate a phylogenetic hypothesis, based on DNA sequences of the mitochondrial cytochrome c oxidase subunit I (COI) gene, for 2 CMSA vesicomyids, Cal. gallardoi and Archivesica sp. 1. In addition, a chronology of the divergence times for the main lineages of the family is presented. Results are discussed in the context of the taxonomy, biogeography, and evolution of the family, with special emphasis on the 2 CMSA species analyzed.

# MATERIALS AND METHODS

Specimens of *Cal. gallardoi* and *Archivesica* sp. 1 were collected at the CMSA (36°27.87'S, 73°43.25'W) at about 750-800 m in depth. Samples were collected onboard the *RV Vidal Gormáz* (during the VG-04 cruise in Oct. 2004 and

the VG-07 cruise in Sept. 2007), using a modified Agassiz trawl. Freshly collected specimens were dissected, and portions of branchial and foot tissues were immediately preserved in 95% ethanol. Four specimens of *Cal. gallardoi* were obtained, while only 1 specimen of *Archivesica* sp. 1 was available for the study. Dry shells of *Cal. gallardoi* were deposited in the Museum of Zoology, Univ. of Concepción (MZUC 35955, 35956, 35957, and 35958; Concepción, Chile) (Table 1). The *Archivesica* sp. 1 specimen is currently under study and will be the basis of a new taxon.

DNA was extracted using the Wizard SV DNA purification system (Promega, Madison, WI, USA). A portion of the COI gene (~657 bp) was amplified using the universal primers, LCO1490 and HCO2198 (Folmer et al. 1994). Amplicons were purified using a Jetquick polymerase chain reaction (PCR, Genomed, Löhne, Germany) and sequenced at Macrogen (Kumchum, Korea). DNA sequences were edited using SeqMan II (DNASTAR 2003) and then aligned with other sequences of vesicomyids obtained from GenBank (Table 1). The outgroup was formed of the veneroid bivalves *Corbicula japonica*, *Dreissena* 

**Table 1.** Geographical distribution, collection depth, and type of habitat of vesicomyid species included in this study. GenBank sequence accession numbers and bibliographic references are indicated

No.	OTU designation	Location	Geographic coordinates	Depth (m)	Habitat	GenBank or MZUC no.	Reference
1	Abyssogena kaikoi	Nankai Trough, Japan	33°39'N, 137°55'E	4600	Seep	AB110761	Kojima et al. (2004)
2a	Abyssogena phaseoliformis	Nankai Trough, Japan	33°39'N, 137°55'E	4600	Seep	AB110765	Kojima et al. (2004)
2b		Japan Trench	40°6.60'N, 144°11.10'E	6370	Seep	AF008283	Peek et al. (1997)
3a	Abyssogena southwardae	West Florida Escarpment, USA	26°1.8'N, 84°54.6'W	3313	Seep	AF008280	Peek et al. (1997)
3b		West Florida Escarpment, USA	26°1.8'N, 84°54.6'W	3313	Seep	AF008281	Peek et al. (1997)
4a	Abyssogena sp. 1	Logatchev, Mid-Atlantic Ridge	14°45.189'N, 44°58.829'W	30387	Vent	AF114402	Peek et al. (2000)
4b		Logatchev, Mid-Atlantic Ridge	14°45.189'N, 44°58.829'W	30387	Vent	AF114405	Peek et al. (2000)
5a	Akebiconcha kawamurai	Nankai Trough, Japan	34°4'N, 137°48'E	300-900	Seep	AB479089	Okutani et al. (2009)
5b		Nankai Trough, Japan	34°4'N, 137°48'E	600	Seep	AB191403	Kojima et al. (2006)
6a	Archivesica gigas	Santa Catalina Basin, USA	33°11.7'N, 118°29.5'W	1240	Whale	AF114392	Baco et al. (1999)
6b		Oregon Subduction Zone, USA	44°1.11'N, 125°17.42'W	2028	Seep	AF008260	Peek et al. (1997)
6c		Guaymas basin, Mexico	27°N, 111°W	2000	Vent	AF008264	Peek et al. (1997)
7a	Calyptogena fausta	Suruga Bay, Japan	34°55'N, 138°39'E	1500	Seep	AB110742	Kojima et al. (2004)
7b		Suruga Bay, Japan	34°55'N, 138°39'E	1900-2200	Seep	AB110743	Kojima et al. (2004)
8a	Calyptogena pacifica	Eel River, USA	40°47.4'N, 124°35.4'W	512	Seep	AY143294	Goffredi et al. (2003)
8b		Oregon Subduction Zone, USA	44°40'N, 125°7'W	765	Seep	AF008286	Peek et al. (1997)
9a	Calyptogena sp. mt-ll	Juan de Fuca Ridge, USA	48°27.4'N, 128°42.5'W	2416	Vent	AF008296	Peek et al. (1997)
9b		Monterey Bay, USA	36°21.6'N, 122°6.6'W	1575	Seep	AY143318	Goffredi et al. (2003)
10a	Calyptogena sp. mt-III	Peru Upper Slope Scarp, Peru	5°32'S, 81°32'W	2500	Vent	AF008292	Peek et al. (1997)
10b		Monterey Bay, USA	36°37.79'N, 122°19.92'W	2200	Seep	AY143325	Goffredi et al. (2003)
11	Calyptogena sp. mt-V	Acretionary Prism, Costa Rica	9°42.67'N, 86°4.66'W	3096	Vent	AF114395	Peek et al. (2000)
12a	crenulomarginata	Nankai Trough, Japan	33°39'N, 136°34'E	2000	Seep	AB110740	Kojima et al. (2004)
12b		Nankai Trough, Japan	33°39'N, 136°34'E	2000	Seep	AB110741	Kojima et al. (2004)

# Table 1. (continued)

No.	OTU designation	Location	Geographic coordinates	Depth (m)	Habitat	GenBank or MZUC no.	Reference
13a	cordata	Green Canyon, Gulf of Mexico	27°41'N, 91°32'W	700	Seep	AF114397	Peek et al. (2000)
13b		Louisiana Continental Slope, USA	27°41.2'N, 91°32.3'W	704	Seep	AF008277	Peek et al. (1997)
14a	elongata	Santa Catalina Basin, USA	33°11.7'N, 118°29.5'W	1240	Whale	AF141303	Baco et al. (1999)
14b		Santa Barbara Channel, USA	34°N, 120°W	500	Seep	AF008275	Peek et al. (1997)
15a	extenta	Gorda Ridge, USA	41°0.4'N, 127°29.3'W	3271	Vent	AF114389	Peek et al. (2000)
15b		Gorda Ridge, USA	41°0.2'N, 127°29.60'W	3260	Vent	AF008268	Peek et al. (1997)
16	fossajaponica	Japan Trench	39°06'N, 143°53'E	5400-6400	Seep	AB110766	Kojima et al. (2004)
17	kaikoae	Nankai Trough, Japan	32°33'N, 134°42'E	3600-3800	Seep	AB110739	Kojima et al. (2004)
18a	kilmeri	Sagami Knoll, Japan	35°N, 139°E	1400-1500	Seep	AB110744	Kojima et al. (2004)
18b		Monterey Canyon, USA	36°47.1'N, 122°2.6'W	635	Seep	AF114387	Peek et al. (2000)
18c		San Nicolas Island, USA	33°20.3'N, 119°58.8'W	1000	Whale	AF141302	Baco et al. (1999)
19a	krylovata	Jaco Scarp seep, Costa Rica	9°10.29'N, 84° 47.92'W	740	Seep	JF784422	Martin and Gofferdi (2011)
19b		Jaco Scarp seep, Costa Rica	9°10.29'N, 84°47.92'W	740	Seep	JF784421	Martin and Gofferdi (2011)
20a	kuroshimana	Kuroshima Knoll, Japan	24°07'N, 124°12-13'E	700-810	Seep	AB110736	Kojima et al. (2004)
20b		Kuroshima Knoll, Japan	24°07'N, 124°12-13'E	700-810	Seep	AB110738	Kojima et al. (2004)
21a	laubieri	Nankai Trough, Japan	33°39'N, 137°55'E	3800	Seep	AB110747	Kojima et al. (2004)
21b		Juan de Fuca Ridge, USA	48°27.40'N, 128°42.52'W	2416	Vent	AF008256	Peek et al. (1997)
22a	lepta	Guaymas basin, Mexico	27° 0.2' N ; 111° 24.6'W	2020	Vent	AY143335	Goffredi et al. (2003)
22b		Guaymas basin, Mexico	26°59.9'N, 111°24.6'W	2016	Vent	AF008291	Peek et al. (1997)
23a	magnifica	East Pacific Rise, Pacific ocean	18°36'S, 113°24'W	2600	Vent	AF120665	Giribet and Wheeler (2002)
23b		East Pacific Rise, Pacific ocean	18°36.4'S, 113°24.0'W	2700	Vent	AF008272	Peek et al. (1997)
24a	magnocultellus	Nankai Trough, Japan	33°50'N, 137°54'E	1900-2200	Seep	AB110757	Kojima et al. (2004)
24b		Juan de Fuca Ridge, USA	48°27.5'N, 128°42.5'W	2400	Vent	AF008258,	Peek et al. (1997)
25a	nankaiensis	Nankai Trough, Japan	34°12'N, 137°46'E	1100	Seep	AB110748	Kojima et al. (2004)
25b		Nankai Trough, Japan	34°12'N, 137°46'E	1100	Seep	AB110750	Kojima et al. (2004)
26a	nautilei	Zenisu Ridge, Japan	33°33'N, 138°26'E	3300	Seep	AB110758	Kojima et al. (2004)
26b		Zenisu Ridge, Japan	33°33'N, 138°26'E	3300	Seep	AB110759	Kojima et al. (2004)
27	okutanii	Sagami Bay, Japan	34°57'N, 139°12'E	800-1200	Seep	AB110745	Kojima et al. (2004)
28	packardana	Monterey Canyon, USA	36°47.1'N, 122°2.6'W	635	Seep	AF114396	Peek et al. (2000)
29a	ponderosa	Louisiana Continental Slope, USA	27°41.3'N, 91°32.5'W	737	Seep	AF008278	Peek et al. (1997)
29b		Gulf of Mexico, USA	27°40.88'N, 91°32.10'W	720	Seep	EU403473	Stewart et al. (2008)
30a	similaris	Nankai Trough, Japan	34°06'N, 138°7'E	2100	Seep	AB110751	Kojima et al. (2004)
30b		Nankai Trough, Japan	34°06'N, 138°7'E	2100	Seep	AB110752	Kojima et al. (2004)
31	stearnsii	Chishima Trench, Japan	36°77'N, 122°4'W	659-683	Seep	AB479086	Okutani et al. (2009)
32	tsubasa	Nankai Trough, Japan	33°39'N, 137°55'E	3800	Seep	AB110753	Kojima et al. (2004)
33	undesc sp. 1	Costa Rica Accetionary Wedge	09°42.67'N, 86°4.66'W	3096	Low-temp vent	AF114390	Peek et al. (2000)
34	undesc sp. 2	Santa Catalina Basin, USA	33°11.7'N, 118°29.5'W	1240	Whale	AF141301	Baco et al. (1999)
35	undesc sp. 3	Off Sunda Strait, Thailand	7°25'N, 105°47'E	2100	Seep	AB110778	Kojima et al. (2004)
36a	aff. venusta	Blake Ridge, USA	32°29.623'N, 76°11.467'W	2155	Seep	AY163387	Van Dover et al. (2003)
36b		Blake Ridge, USA	32°29.623'N, 76°11.467'W	2155	Seep	AY163386	Van Dover et al. (2003)
37a	Calyptogena gallardoi	Concepción Metane Seep Area, Chile	36°22'S, 73°43'W	750-1000	Seep	KC164255, MZUC 35955	This study
37b		Concepción Metane Seep Area, Chile	36°22'S, 73°43'W	750-1000	Seep	KC164256, MZUC 35956	This study
37c		Concepción Metane Seep Area, Chile	36°22'S, 73°43'W	750-1000	Seep	KC164257, MZUC 35957	This study
37b		Concepción Metane Seep Area, Chile	36°22'S, 73°43'W	750-1000	Seep	KC164258, MZUC 35958	This study
38	Archivesica sp. 1	Concepción Metane Seep Area, Chile	36°22'S, 73°43'W	750-1000	Seep	KC164259, AGT-9	This study

*blanci*, *Mercenaria mercenaria*, and *Venus rosalina*. Sequences were aligned with Clustal W (Thompson et al. 1994) using default values for all alignment parameters.

Reported genetic distances corresponded to observed (p) values calculated with MEGA 5 (Tamura et al. 2011). Phylogenetic hypotheses were obtained using a maximum parsimony (MP) analysis (Farris 1983, Kluge and Farris 1969) and Bayesian inference (reviewed in Huelsenbeck et al. 2001). The MP analysis was performed using PAUP\* (Swofford 2002), by executing 2000 replicates of a heuristic search, with the random addition of sequences and tree bisection and reconnection rearrangement of branches. The degree of support of the recovered clades was estimated by a bootstrap (BS) analysis with 10<sup>4</sup> replicates, each with 20 replicates of random additions of sequences. A Bayesian analysis was implemented in BEAST vers. 1.4.3 (Drummond and Rambaut 2003). The molecular evolution model used, GTR+G+I, was selected using Modelgenerator 0.85 (Keane et al. 2007). Four Markov chain Monte Carlo runs of 6 × 10<sup>6</sup> generations, with a sampling frequency of 100 generations, were carried out. To establish the amount of samples to discard as burn-in, logs were examined with Tracer vers. 1.3 (Rambaut and Drummond 2003). Trees and parameters were combined with LogCombiner vers. 1.4.1 (Rambaut and Drummond 2006). In addition, BEAST was used to calibrate a relaxed molecular clock (Drummond et al. 2006) to estimate divergence times of some vesicomyid cladogenetic events. Two internal nodes were calibrated using the fossil record of the family Vesicomyidae in the form of lognormal prior distributions. The node at the base of the vesicomyid clade was calibrated with the oldest (45 Mya) available fossil record for the family, corresponding to the species, Archivesica cf. tschudi (Amano and Kiel 2007). Similarly, the basal node of the pacifica/lepta clade was calibrated with the oldest fossil (30 Mya) known for the genus Calyptogena s.s., Cal. katallaensis (Kiel and Amano 2010).

# RESULTS

New COI sequences were obtained for 4 specimens of *Cal. gallardoi* and 1 for *Archivesica* sp. 1. Sequences were deposited in GenBank (accession numbers given in Table 1). Although the sample of *Cal. gallardoi* (n = 4) showed minimal

genetic variation, 2 haplotypes were found. One haplotype was present in 3 specimens and differed from the other in only two of 513 sites (p = 0.004); one of these changes was synonymous, while the other was not. The matrix analyzed had a length of 516 base pairs (bp) of which 3 bp corresponded to an indel that accounted for an extra codon in the outgroup), and presented 284 variable sites, of which 232 were phylogenetically informative under the MP criterion. The MP analysis recovered 76 shortest trees (with lengths of 1302 steps, a consistency index of 0.3955, and a retention index of 0.7183). The strict consensus of those trees (Fig. 2) revealed the subfamily Pliocardiinae to be a monophyletic group (with BS support of 100%). The pliocardiine clade was mostly resolved, although basal relationships and some more-recent divergences were weakly supported. In addition, the phylogenetic positions of both CMSA forms were unambiguous. The basal-most vesicomyid dichotomy led to fossajaponica on 1 hand and to the remaining vesicomyids on the other hand; however, this later clade was weakly supported. Calyptogena s.s. (i.e., the pacifica/ lepta complex) was found to be being monophyletic with moderate support (with BS support of 73%). Calyptogena pacifica was sister to all other forms of Calyptogena s.s. Calyptogena gallardoi fell in this clade and appeared sister to a clade formed by Calyptogena sp. mt II, Calyptogena sp. mt III, and Cal. fausta. The Abyssogena s.s. clade was well supported (with BS support of 99%), and was composed of Abyssogena sp. 1, Aby. southwardae, Aby. kaikoi, and Aby. phaseoliformis.

The gigas/kilmeri complex was weakly supported (with BS support of 57%) and was composed of various subclades, some of which may correspond to distinct genera. The Archivesica clade was moderately supported (with BS support of 77%), and was formed by Archivesica sp. 1 from the CMSA together with haplotypes of Arc. gigas, Arc. laubieri, Arc. magnocultellus, and undescribed sp. 1 from the Costa Rican margin. The clade corresponding to the putative genus 'Phreagena' (with BS support of 75%) was formed from haplotypes of kilmeri and okutanii. Other haplotypes that comprised the gigas/kilmeri complex were Akebiconcha kawamurai, Ake. extenta, Ake. nankaiensis, Ake. tsubasa, Ake. similaris, undescribed sp. 2 (whale habitats), and undescribed sp. 3 (seep habitats). Within the gigas/kilmeri complex, elongata, possibly a representative of the genus *Ectenagena*, was sister to the clade formed by the



**Fig. 2.** Strict consensus of 42 shortest trees (length of 1145 steps, with a consistency index (CI) of 0.425 and a retention index (RI) of 0.662), recovered in a maximum-parsimony analysis of the family Vesicomyidae, based on DNA sequences of the mitochondrial cytochrome c oxidase subunit I (COI) gene. *Corbicula japonica, Dreissena blanci, Mercenaria mercenaria,* and *Venus rosalina* were used as the outgroup. Tentative genera are indicated with quotation marks. Numbers to their right of the nodes depict bootstrap values (values of < 50 not shown).

remaining forms of the clade.

Haplotypes of species previously assigned to "*Pliocardia*" fell into non-sister clades: '*Pliocardia*' I (with BS support of 96%), formed by haplotypes of *krylovata* and *ponderosa*; and '*Pliocardia*' II (with BS support of 79%) was formed by haplotypes of *crenulomarginata* and *kuroshimana*.

The topology found in the Bayesian analysis had several differences with that of the MP analysis; as expected, these differences involved weakly supported relationships such as those at the base of the vesicomyid clade (Fig. 3). Importantly for the objectives of this work, the Bayesian analysis placed the CMSA forms, *Cal. gallardoi* and *Archivesica* sp. 1, in the same position as did the MP analysis; i.e., respectively within *Calyptogena* s.s. and *Archivesica* clades. Similarly, the Bayesian analysis strongly supported (with a posterior probability (PP) of 1) the monophyly of the subfamily Pliocardiinae.

The molecular clock (Fig. 3) suggested that the vesicomvid lineages of the CMSA originated in the Miocene. The lineage leading of Cal. gallardoi split from the other Calyptogena at about 9.3 Mya, and the lineage leading to Archivesica sp. 1 split from the other Archivesica at about 10.9 Mya. The crown group of Cal. gallardoi had an age of 1.5 Mya. The age estimated for the crown groups of the main clades recovered in the analysis are as follows: Abyssogena 9.7 Mya (with a PP of 1), Calyptogena s.s. 21.1 Mya (with a PP of 1), the gigas/kilmeri complex 33.4 Mya (with a PP of 0.9), Archivesica 12.4 Mya (with a PP of 0.99), 'Ectenagena' 12.8 Mya (with a PP of 1), 'Phreagena' 8.2 Mya (with a PP of 1), 'Pliocardia' I 6.0 Mya (with a PP of 1), and 'Pliocardia' II 20.3 Mya (with a PP of 1).



**Fig. 3.** Chronogram resulting from a Bayesian analysis of the family Vesicomyidae based on DNA sequences of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene. Molecular clock-based estimates for the timing of the cladogenetic events are shown above the branches, and posterior probability values are shown below the branches (summary statistics were computed only for those nodes that appear in the majority of sampled trees; i.e., the posterior probability limit was set to 0.5). Tentative genera are indicated with quotation marks. The outgroup was as described in figure 2.

#### DISCUSSION

## **Taxonomic considerations**

In the most recent taxonomic review of the family Vesicomyidae, Krylova and Sahling (2010) recognized 2 subfamilies: the Vesicomyinae, which is monogeneric for *Vesicomya*, and the Pliocardiinae, which is composed of several genera. Due to a lack of genetic data available in GenBank for the Vesicomyinae, this phylogenetic study, as well as previous ones, focused on the Pliocardiinae.

Phylogenetic studies of the subfamily Pliocardiinae based on different molecular markers inter-transcribed spacer (ITS), COI, COIII, 16s, and 28s), allowed the recognition of consistent evolutionary lineages (Peek et al. 1997 1998 2000, Baco et al. 1999, Goffredi et al. 2003, Kojima et al. 2004). As in previous analyses, our phylogenetic results were not fully concordant with current classificatory schemes (Krylova and Sahling 2006 2010). Some lineages are referred to as a complex (e.g., *pacifica/lepta* and *gigas/kilmeri*), because the taxonomy of the family remains uncertain (Krylova and Sahling 2010).

Nevertheless, it is possible to assign generic names to some of the main clades recovered: Abyssogena s.s. to the phaseoliformi complex, and Calvptogena s.s. to the pacifica/lepta complex (Figs. 2, 3). The Abyssogena clade is a remarkable lineage, composed of 4 haplotypes. A recent description of this genus (Krylova et al. 2010) allowed DNA sequences in GenBank to be related to species reported for this genus. The Calyptogena clade is well-defined and composed of 7 forms: Cal. gallardoi, Cal. fausta, and Cal. pacifica, 3 unidentified species referred to as Cal. sp. mt II, C. sp. mt III, and Cal. mt V, and a 4th sequence assigned to 'lepta'. The 3 unidentified forms could correspond to the following species: mt II to Cal. starobogatovi and Cal. rectimargo, mt III to Cal. costaricana, and mt V to Cal. peruviana (E. Krylova, pers. commun.).

The large clade earlier referred as to the *gigas/kilmeri* complex (Peek et al. 1997) is formed of 4 main clades, which can be assigned to the genera *Akebiconcha*, *Archivesica*, *Ectenagena*, and *Phreagena* (Figs. 2, 3). However, relationships among these genera are yet unresolved. In addition, species assigned to these genera are in need of revision (Krylova and Sahling 2010). In spite of this, our results, in accordance with that obtained in previous molecular phylogenetic

studies (Kojima et al. 2004), suggest that new genera may be needed to accommodate the large phylogenetic diversity seen in this clade.

Two consistent evolutionary lineages shared taxonomic similarities with the genus *Pliocardia* (Krylova and Sahling 2010, Martin and Goffredi 2011). However, the molecular results suggested that these 2 clades may represent different genera. Awaiting a comprehensive revision, we refer to them as '*Pliocardia*' I for the clade containing haplotypes of the species *krylovata* and *packardana*, and '*Pliocardia*' II for the clade containing haplotypes of the species *crenulomarginata* and *kuroshimana* (Figs. 2, 3). However, the genus *Pliocardia* will remain problematic as long as no living representatives are precisely established (Martin and Goffredi 2011).

# Pliocardiinae from the CMSA

The genetic evidence obtained in the present study for the 2 species of the CMSA analyzed, *Cal. gallardoi* and *Archivesica* sp. 1, helps place them in the vesicomyid tree and thus advances a preliminary hypothesis accounting for their biogeographic history.

Calyptogena gallardoi falls in the monophyletic group Calyptogena s.s. (Fig. 2), corroborating the morphological assessment of Sellanes and Krylova (2005). Given the taxonomic uncertainty surrounding most vesicomyd species (but see advances summarized in Krylova and Sahling 2010), the latter conclusion is relevant. Meanwhile, the form *Archivesica* sp. 1 was placed in the clade of *Archivesica* (Figs. 2, 3). The CMSA form of *Archivesica* differs from its sister species, *Arc. gigas* (type species of the genus), by a genetic distance of p = 0.037; this large value suggests that the CMSA form belongs to an undescribed species. This hypothesis should be further tested by detailed morphologically based studies.

The phylogenetic results clearly show that at least 2 distantly related vesicomyid lineages independently reached the CMSA.

### Evolutionary history of the subfamily Pliocardiinae

Specialization of pliocardiins to inhabit sulfiderich reducing habitats is due to the incorporation of chemoautotrophic endosymbiotic bacteria in the branchial cysts, which provide clams with required nutrients (Boss and Turner 1980, Fisher

1990, Levin 2005). This way of obtaining energy may have led to degeneration of the digestive system, which is still present but is generally non-functional (Cosel and Salas 2001, Krylova and Sahling 2006, Amano and Kiel 2007, Cosel and Olu 2008). It is feasible that early Eocene oceanic conditions, with high levels of sulfides in oceanic bottoms (Kiel and Littlel 2006), might have favored vesicomyid diversification. Those anoxic environments probably furnished ideal conditions for chemosynthesis-based communities, which at the time, were presumably the cause of the demise/extinction of several other marine species (Riebesell et al. 2000, Zachos et al. 2005). Anoxic environments could have acted as a "chemosynthetic bridge", triggering specialization of some non-chemosynthetic taxa in this kind of ecosystem (see also Carney 1994, Jacobs and Lindberg 1998), and probably vesicomyids are an example of this, due to physiological adaptations, allowing them to exploit reducing environments.

It was also suggested that the rapid dispersion of several evolutionary lineages which appeared in the mid-Eocene-Oligocene (Fig. 3), such as, Abyssogena, Calyptogena, and the gigas/kilmeri complex, was facilitated by the presence of whale falls, and the finding of fossils of such systems supports this theory (Baco et al. 1999, Smith and Baco 2003); however, Kiel and Goedert (2006) argued against this scenario given that Eocene-Oligocene whale fossils lack vesicomyds. Even so, an outstanding fossil record of a chemosynthetic system supported by a plesiosaurus carcass from the Cretaceous (Kaim et al. 2008) revealed that such systems facilitated by large organic falls were not restricted to whales. This finding assumes that previous to the apparition of whales during the late Eocene (Gingerich et al. 2001), and during the early evolution of the group, carcasses of other large marine animals could have helped sustain chemosynthetic ecosystems. The enhanced amount of carcasses during the massive extinctions at the end of the Mesozoic could have also been a facilitating factor for the dispersion and diversification of this biota during its early evolutionary history. However, it is important to remark that the fossil record and our molecular clock estimates (Fig. 3) suggest that current vesicomyid diversity would have taken advantage of dispersion of only whale carcass and not of other large carcasses that were available in earlier ages. Moreover, the chronogram of pliocardiine cladogenetic events indicates that there was no marked pulse of diversification; instead, it seems

that lineages have accumulated at a regular pace since the Eocene. Lineages leading to most living genera appeared in the Oligocene and Miocene.

The subfamily Pliocardiinae occurs exclusively in sulfide-rich reducing habitats, and is distributed worldwide at depths of 100-6400 m. It may be formally divided into 4 groups, characterized by the following distribution patterns: (1) transoceanic with mainly a bathyal/abyssal distribution and a near-continental range type.(2) a transoceanic bathyal/abyssal distribution with a panthalassic range type, (3) a regional mainly upper-bathyal distribution with a nearcontinental range type, and (4) regional lowbathval/upper-abyssal distribution with a oceanic range type (Krylova and Sahling 2010). The large geographical distances between close evolutionary lineages (e.g., Cal. gallardoi and C. fausta; and Archivesica sp. 1 and Arc. gigas) (Table 1) suggest a complex pattern with several events of largedistance dispersion during their evolutionary history.

Kojima et al. (2004) suggested that multiple migrations between the Northeast and Northwest Pacific occurred during the evolutionary history of the subfamily Pliocardiinae. In addition to these, the austral sequences generated in the present study suggest at least 2 dispersal events from the North to the South Pacific. It is important to note that another vesicomyid was reported from the Southeast Pacific: "Calyptogena" (Ectenagena) australis. This species was reported from 2 specimens collected off Mocha I. (about 100 km south of the CMSA) at a depth of 1400 m (Stuardo and Valdovinos 1988). Preliminary morphological observations suggested that "Cal." australis is a different species from Archivesica sp. 1, and no molecular data for the former exist. The inclusion of this species in the new phylogenetic analysis is mandatory to clarify if an additional dispersal event from the North to the South Pacific has to be invoked to explain vesicomyid historical biogeography. The continental margin of the Pacific Ocean could have been one the main dispersal routes southwards. Although few seep sites were reported for the Southeast Pacific, the geologic and environmental conditions suggest that the area is certainly rich in this kind of habitat. Moreover, the area is a renowned whale migration route, thus providing carcasses that could be used as "stepping stones", further supporting this scenario of high dispersion capacity for vesicomyids along the Pacific continental margin (Baco et al. 1999, Smith and Baco 2003, Kojima et al. 2004).

Our results indicate that the Southeast Pacific species Cal. gallardoi and Archivesica sp. 1 originated in the late Miocene; a period in which large cetaceans were abundant in all of the world's oceans, especially on the Pacific margin (McGowen et al. 2009). The dispersion of these 2 lineages from the North Pacific towards the south could have been facilitated by this fact, and fossil evidence of whale-fall chemosynthetic communities is also available for the same period (Baco et al. 1999, Smith and Baco 2003, Amano and Kiel 2007, Kiel et al. 2008). The incorporation of the Southeast Pacific lineages into a phylogenetic framework provides new evidence about the taxonomy, evolution, and dispersion of vesicomyids during their history. The emerging picture is complex, and we speculate that as new surveys uncover new vesicomyid forms, the scenario will grow in complexity. In this sense, the analysis of nuclear DNA sequences would be of much help in proposing a more-robust phylogenetic hypothesis for the family and reducing uncertainty around our time estimates.

**Acknowledgments:** We are indebted to E. Krylova, Shirshov Institute of Oceanology (Moscow, Russia), who kindly revised and helped improve an early version of the manuscript. S. Kiel, University of Göttingen (Göttingen, Germany), clarified distinct aspects of the vesicomyid fossil record. A. Parada, Univ. Católica de Chile (Santiago, Chile), assisted with estimating divergence times. This work was funded by projects FONDECYT 1100166 to JS, and FONDECYT 1110737 and MECESUP AUS0805 to GD. Additional support from the Center of Oceanographic Research in the Eastern South Pacific (COPAS) of the Univ. of Concepción (Concepción, Chile) is also acknowledged.

#### REFERENCES

- Amano K, S Kiel. 2007. Fossil vesicomyid bivalves from the North Pacific region. Veliger 49: 270-293.
- Baco AR, CR Smith, AS Peek, GK Roederick, RC Vrijenhoek. 1999. The phylogenetic relationships of whale-fall vesicomyid clams based on mitochondrial COI DNA sequences. Mar. Ecol. Progr. Ser. **182**: 137-147.
- Boss KJ, RD Turner. 1980. The giant white clam from the Galapagos Rift, *Calyptogena magnifica* species novum. Malacologia **20:** 161-194.
- Carney RS. 1994. Consideration of the oasis analogy for chemosynthetic communities at Gulf of Mexico hydrocarbon vents. Geo-Mar. Lett. 14: 149-159.

Cosel RV, K Olu. 2008. A new genus and new species of

Vesicomyidae (Mollusca, Bivalvia) from cold seeps on the Barbados accretionary prism, with comments on other species. Zoosystema **30**: 929-944.

- Cosel RV, C Salas. 2001. Vesicomyidae (Mollusca: Bivalvia) of the genera *Vesicomya, Waisiuconcha, Isorropodon* and *Callogonia* in the eastern Atlantic and the Mediterranean. Sarsia **86:** 333-366.
- DNASTAR. 2003. Lasergene: expert sequence analysis software. Vers. 5. Madison, WI: DNASTAR, Inc.
- Donrung P, S Tunkijjanukij, P Jarayabhand, S Poompuang. 2011. Spatial genetic structure of the surf clam *Paphia undulata* in Thailand waters. Zool. Stud. **50**: 211-219.
- Drummond AJ, SY Ho, MJ Phillips, A Rambaut. 2006. Relaxed phylogenetics and dating with confidence. PLoS Biol. 4: 699-710.
- Drummond AJ, A Rambaut. 2003. BEAST vers. 1.4.3. Available at http://beast.bio.ed.ac.uk/software/Main\_Page Accessed 29 Oct. 2012.
- Farris JS. 1982. The logical basis of phylogenetic analysis. Adv. Clad. 2: 7-35.
- Fisher CR. 1990. Chemoautotrophic and methanotrophic symbioses in marine invertebrates. Rev. Aquat. Sci. 2: 399-436.
- Folmer O, M Black, W Hoeh, R Lutz, R Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3: 294-299.
- Gingerich PD, M Haq, IS Zalmout, H Khan, MS Malkani. 2001. Origin of whales from early artiodactyls: hands and feet of Eocene Protocetidae from Pakistan. Science 293: 2239-2242.
- Goffredi SK, LA Hurtado, S Hallam, R Vrijenhoek. 2003. Evolutionary relationships of deep-sea vent and cold seep clams (Mollusca: Vesicomyidae) of the 'pacifica/lepta' species complex. Mar. Biol. 142: 311-320.
- Jacobs D, D Lindberg. 1998. Oxygen and evolutionary patterns in the sea: onshore/offshore trends and recent recruitment of deep-sea faunas. Proc. Natl. Acad. Sci. USA **95**: 9396-9401.
- Kaim A, Y Kobayashi, H Echizenya, R Jenkins, K Tanabe. 2008. Chemosynthesis-based associations on Cretaceous plesiosaurid carcasses. Acta Palaeontol. Polon. 53: 97-104.
- Keane T, T Naughton, J McInerney. 2007. MultiPhyl: a highthroughput phylogenomics webserver using distributed computing. Nucleic Acids Res. doi:10.1093/nar/gkm35.
- Kiel S, K Amano. 2010. Oligocene and Miocene vesicomyid bivalves from the Katalla District, southern Alaska, USA. Veliger **31**: 76-84.
- Kiel S, K Amano, RG Jenkins. 2008. Bivalves from Cretaceous cold-seep deposits on Hokkaido, Japan. Acta Palaeontol. Polon. 53: 525-537.
- Kiel S, JL Goedert. 2006. Deep-sea food bonanzas: early Cenozoic whale-fall communities resemble wood-fall rather than seep communities. Proc. R. Soc. Biol. Sci. 273: 2625-2631.
- Kiel S, C Little. 2006. Cold seep mollusks are older than the general marine mollusk fauna. Science 313: 1429-1431.
- Kluge AG, JS Farris. 1969. Quantitative phyletics and the evolution of anurans. System. Zool. **18:** 1-32.
- Kojima S, K Fukikura, T Okutani. 2004. Multiple trans-Pacific migrations of deep-sea vent/seep-endemic bivalves in the family Vesicomyidae. Mol. Phylogen. Evol. 32: 396-406.
- Krylova E, H Sahling. 2006. Recent bivalve molluscs of the

genus *Calyptogena* (Vesicomyidae). J. Mollusc. Stud. **72:** 359-395.

- Krylova E, H Sahling. 2010. Vesicomyidae (Bivalvia): current taxonomy and distribution. PLoS ONE 5, e9957. doi: 10.1371/journal.pone.0009957.
- Krylova E, H Sahling, R Janssen. 2010. Abyssogena: a new genus of the family Vesicomyidae (Bivalvia) from deep water vents and seeps. J. Mollusc. Stud. 76: 107-132.
- Levin LA. 2005. Ecology of cold seep sediments: interactions of fauna with flow, chemistry and microbes. Oceanogr. Mar. Biol. Annu. Rev. **43:** 1-46.
- Mantelatto FL, LG Pileggi, I Miranda, IS Wehrtmann. 2011. Does *Petrolisthes armatus* (Anomura, Porcellanidae) form a species complex or are we dealing with just one widely distributed species? Zool. Stud. **50:** 372-384.
- Martin A, S Goffredi. 2011. 'Pliocardia' krylovata, a new species of vesicomyid clam from cold seeps along the Costa Rica Margin. J. Mar. Biol. Assoc. UK 1-11. doi:10.1017/S0025315411000713.
- McGowen M, M Spaulding, J Gatesy. 2009. Divergence date estimation and a comprehensive molecular tree of extant cetaceans. Mol. Phylogen. Evol. 53: 891-906.
- Okutani T, T Koshi-ichi, T Sato, T Imai, C Kato. 2009. Vesicomyid Fauna in the Chishima (Kurile) Trench: Occurrences of a New Taxon and *Calyptogena extent*. Venus **68:**15-25
- Peek AS, RA Feldman, R Lutz, RC Vrijenhoek. 1998. Cospeciation of chemoautotrophic bacteria and deep sea clams. Proc. Natl. Acad. Sci. USA Physical Sci. 95: 9962-9966.
- Peek AS, BS Gaut, RA Feldman, JP Barry, RE Kochevar, RA Lutz, RC Vrijenhoek. 2000. Neutral and nonneutral mitochondrial genetic variation in deepsea clams from the family Vesicomyidae. J. Mol. Evol. **50**: 141-153.
- Peek AS, RG Gustafson, RA Lutz, RC Vrijenhoek. 1997. Evolutionary relationships of deep-sea hydrothermal vent and cold-water seep clams (Bivalvia: Vesicomyidae): results from the mitochondrial cytochrome oxidase subunit I. Mar. Biol. **130:** 151-161.
- Rambaut A, AJ Drummond. 2003. Tracer vers. 1.2. Available at http://tree.bio.ed.ac.uk/software/tracer/ Accessed 29 Oct. 2012.
- Rambaut A, AJ Drummond. 2006. LogCombiner v1.4. Available at http://beast.bio.ed.ac.uk/LogCombiner Accessed 29 Oct. 2012.
- Riebesell U, I Zondervan, B Rost, P Tortell, R Zeebe, F Morel. 2000. Reduced calcification of marine plankton in

response to increased atmospheric  $CO_2$ . Nature **407**: 364-367.

- Sellanes J, E Krylova. 2005. A new species of *Calyptogena* (Bivalvia: Vesicomyidae) from a recently discovered methane seepage area off Concepción Bay, Chile (~36°S). J. Mar. Biol. Assoc. UK **85:** 969-976.
- Sellanes J, E Quiroga, VA Gallardo. 2004. First direct evidences of methane seepage and associated chemosynthetic communities in the bathyal zone off Chile. J. Mar. Biol. Assoc. UK 84: 1065-1066.
- Sellanes J, E Quiroga, C Neira. 2008. Megafauna community structure and trophic relationships at the recently discovered Concepción Methane Seep Area, Chile, 36°S. ICES J. Mar. Sci. 65: 1102-1111.
- Sibuet M, K Olu. 1998. Biogeography, biodiversity and fluid dependence of deep-sea cold-seep communities at active and passive margins. Deep-Sea Res. Part II 45: 517-567.
- Smith CR, AR Baco. 2003. Ecology of whale falls at the deepsea floor. Oceanogr. Mar. Biol. Annu. Rev. 41: 311-354.
- Stewart FJ, R Curtis, CR Young, CM Cavanaugh. 2008. Lateral symbiont acquisition in a maternally transmitted chemosynthetic clam endosymbiosis. Mo. Biol. Evol. 25: 673-687.
- Stuardo J, C Valdovinos. 1988. A new bathyal *Calyptogena* from off of the coast of central Chile (Bivalvia: Vesicomyidae). Venus **47:** 241-250.
- Swofford DL. 2002. PAUP: phylogenetic analysis using parsimony (and other methods), vers. 4.0. Sunderland, MA: Sinauer Associates.
- Tamura K, D Peterson, N Peterson, G Stecher, M Nei, S Kumar. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. 28: 2731-2739.
- Thompson JD, DG Higgins, T Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**: 4673-4680.
- Van Dover CL, P Aharon, JM Bernhard, E Caylor, M Doerries, W Flickinger et al. 2003. Blake Ridge methane seeps: characterization of a soft-sediment, chemosynthetically based ecosystem. Deep-Sea Res. PT. I. 50: 281-300
- Zachos JC, U Röhl, S Schellenberg, A Sluijs, D Hodell, D Kelly et al. 2005. Rapid acidification of the ocean during the Paleocene-Eocene thermal maximum. Science **308**: 1611-1615.

1164