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Integrative study of a new cold-seep mussel (Mollusca: Bivalvia) associated with chemosynthetic symbionts in the Marmara Sea

Bénédicte Ritt[a, b, *], Sébastien Duperron[c, d], Julien Lorion[e], Cassandre Sara Lazar[f, g], Jozée Sarrazin[a, *]

[a] Ifremer, Centre de Brest, DEEP/LEP, BP 70, 29280 Plouzané, France
[b] Biology Department, Temple University, BL 1900N 12th St., Philadelphia, PA 19122, USA
[c] Université Pierre et Marie Curie, Bâtiment A, 7 quai St Bernard, 75005 Paris, France
[d] UMR 7138 (UPMC CNRS IRD MNHN), Systématique, Adaptation, Evolution, 75005 Paris, France
[e] Japan Marine Science and Technology Center (JAMSTEC), Marine Ecosystems Research Department, 2-15 Natsushima, Yokosuka 237-0061, Japan
[f] Ifremer, Centre de Brest, DEEP/LM2E, BP 70, 29280 Plouzané, France
[g] Department of Marine Sciences, University of North Carolina, Chapel Hill, NC, USA

*: Corresponding authors : Bénéditcte Ritt, +1 215 204 8858 ; fax: +1 215 204 6646 ;
email address : benedicte.ritt@temple.edu
Jozée Sarrazin, Tel.: +33 1 98 22 43 29 ; email address : jozee.sarrazin@ifremer.fr

Abstract:

Recently, small *Idas*-like mussels have been discovered living on carbonate crusts associated with cold-seeps in the Marmara Sea. These mussels, here referred to as *Idas*-like nov. sp., differ morphologically and genetically from another species identified as *Idas* aff. *modiolaeformis*, living in the same type of ecosystem in the Nile Deep-Sea Fan (eastern Mediterranean Sea). A phylogenetic analysis confirms the distinction between the two species, which belong to highly divergent lineages. Carbon stable isotope values, as well as the detection of thiotroph-related bacteria in the gill tissue, support the presence of a symbiotic, thiotroph-derived nutrition. In contrast, *Idas* aff. *modiolaeformis* displays six different types of symbionts. Finally our size-frequency data suggest that the recruitment is continuous in the examined area. The present study extends the documented distribution of symbiont-bearing mussels to the Marmara Sea, and contributes to the characterisation of biological communities in this recently explored area.

Highlights

► First description of a thiotrophic mussel species discovered associated with cold-seep ecosystems in the Marmara Sea. ► *Idas*-like nov. sp. is morphologically different from *Idas* aff. *modiolaeformis* of the eastern Mediterranean Sea. ► *Idas*-like nov. sp. represents a new lineage in the Mytilidae tree. ► Both *Idas* species diverged a long time before both species colonised the Mediterranean Sea seeps.

Keywords : Mytilidae ; *Idas*-like Cold-seeps ; Marmara Sea ; Phylogeny ; Symbiosis ; Stable isotopes

1. Introduction

Mytilid bivalves are ubiquitous metazoans in the marine environment, occurring from shallow waters to the abyssal zone in the oceans worldwide. Species exclusively observed in deep-sea chemosynthesis-based ecosystems, such as hydrothermal vents, cold-seeps, and organic falls, were traditionally referred to the sub-family Bathymodiolinae Kenk and Wilson, 1985 (*i.e.* genera *Bathymodiolus*, *Gigantidas*, *Tamu* and *Vulcanidas*). The taxonomy of these deep mussels, however, is under discussion especially since the discovery of small *Adipicola*Dautzenberg, 1927 and *Idas*Jeffreys, 1876 reported from cold-seeps and organic falls and classified within the sub-family Modiolinae (Lorion et al., 2009). Indeed, the monophyly of the Bathymodiolinae clade is no longer supported by molecular results (Samadi et al., 2007), which suggest that (1) the Bathymodiolinae are rooted within the Modiolinae Keen, 1958; and (2) the symbiont-bearing mussels are monophyletic mussels within the family Mytilidae ([Kenk and Wilson, 1985], [Duperron et al., 2007], [Miyazaki et al., 2010] and [Von Cosel and Marshall, 2010]). Thus, we consider it more convenient to refer to the Marmara Sea mussels as *Idas*-like due to their similaritieswith small symbiont-bearing species assigned to the *Idas* genus (*sensu stricto*) that have been previously reported from vents, seeps and organic falls. The purpose of this study is not to re-evaluate the classification of mytilids but to describe the new species found in Marmara cold seeps and the identity of its symbionts.

At vents and seeps, bivalves occur in dense beds and their distribution patterns appear to be strongly related to substratum types and chemical gradients (particularly methane and sulphides, see reviews in [Duperron et al., 2009], [Levin, 2005] and [Sibuet and Olu, 1998]). Their adaptations to these extreme environments, which are inhospitable to many other invertebrates because of low oxygen and high hydrogen sulphide concentrations, include their association with symbiotic bacteria. These symbionts are localised in gill tissues, use diverse carbon sources and derive their energy from the oxidation of reduced compounds present in the fluids emitted at the seafloor ([Felbeck et al., 1981], [Cavanaugh, 1983], [Fisher, 1990] and [Duperron, 2010]). To date, the most frequent types of associations within symbiont-bearing mussels involve thiotrophic (sulphur-oxidising: SOX) and methanotrophic (methane-oxidising: MOX) bacteria (see reviews in [Dubilier et al., 2008] and [Duperron et al., 2009]).

Symbiont-bearing mussels from deep-sea chemosynthetic ecosystems have been intensively studied. Phylogenetic studies suggest that organic falls served as “stepping-stones” allowing the shallow ancestors to colonise deep-sea vents and cold-seeps ([Distel et al., 2000], [Samadi et al., 2007], [Lorion et al., 2009] and [Lorion et al., 2010]). Although stimulating, this hypothesis is still debated, in particular because of a sampling bias, as very few species associated with organic falls were investigated compared to those from vents and seeps (Lorion and Samadi, 2010). Authors are

also faced with nomenclatural issues arising from early species descriptions, which where based on morphological shell characters from few individuals and published before the advent of molecular methods. Moreover, some common features found in mollusc taxa, such as allometric growth, environmental plasticity and crypticism, were not often taken into account (Baker et al., 2003; Von Cosel and Olu, 1998; Won et al., 2003). Therefore, the use of anatomical characters alone introduced some ambiguities in the definition of species and even genera.

In spite of these difficulties, new symbiont-bearing mussel species are regularly sampled and described from different deep-sea ecosystems. A recent study of a small mussel from cold-seep sites in the eastern Mediterranean Sea, tentatively attributed to *Idas modiolaeformis*, indicated that it occurred in low densities, mostly associated with carbonate crusts while its close relatives were associated with sunken organic remains (Duperron et al., 2008b; Lorion et al., 2012). Unexpectedly, this *Idas* aff. *modiolaeformis* was shown to harbour six types of symbionts including sulphur- and methane-oxidising bacteria, representing the highest diversity of symbionts reported in mussels so far. These results suggest that mytilids can associate with a wider diversity of bacteria than previously thought (Duperron, 2010). More recently, a global re-assessment of deep-sea mussels using molecular tools has been initiated with the addition to the Mytilidae tree of 25 mussel species from organic falls in the Pacific Ocean (Duperron et al., 2008a; Lorion et al., 2010; Lorion et al., 2009). These studies highlighted the complexity and multiplicity of colonisation events among vents, seeps and organic falls and substantially challenge earlier hypotheses. Their conclusions emphasise the fact that the history of the whole group is still poorly understood.

During the MarNaut cruise (2007), the exploration of new cold-seep sites in the deep Marmara Sea, the easternmost semi-enclosed basin of the Mediterranean Sea, led to the collection of new *Idas*-like mytilid specimens, referred to herein as *Idas*-like nov. sp. This Marmara Sea mytilid species presented similarities with *Idas* aff. *modiolaeformis* from the eastern Mediterranean in terms of colonised substratum (carbonate crust), depth range (between 1000-2000 m) and morphology. Hence, in this study, we aimed at determining: (1) whether the mussel sampled in the Marmara Sea is the same species as *Idas* aff. *modiolaeformis* recently collected in the eastern Mediterranean Sea, (2) how these two species are related, and, (3) whether they have a similar group of symbiotic bacteria. Mussel morphology and symbiont type were characterised using morphological, microscopic and molecular methods. Carbon-nitrogen stable isotope compositions of tissues were also investigated to estimate the contribution of bacterial symbionts to the host's nutrition. The present study extends the documented distribution of symbiont-bearing mussels to the Marmara Sea, and contributes to the characterisation of biological communities in this recently explored area.

2. Material and methods

2.1. Sampling site, animal collection and specimen preservation

The mytilid bivalves were collected in June 2007 at a cold-seep site in the north-east Central Basin of the Marmara Sea (40°51.27'N - 28°10.19'W, Figure 1) at a depth of 1120 m using the manned submersible *Nautile* deployed from the R/V l'Atalante. This cold-seep site was characterised by upward fluid flows and carbonate crust

precipitations forming outcrops where mussel beds were observed (Ritt et al., 2010). During dive 1665, three fragments of carbonate crusts (CC1, CC2 and CC3, see details in Ritt et al. (2010)) were sampled. The fauna was removed from the crusts and 220 mussels were fixed for a variety of analyses (Table 1). Tube cores (30 cm long, 5.4 cm inner diameter) were also taken in reduced sediments (n=3) located a few meters away from the sampled carbonate crust for carbon and nitrogen stable isotope analyses of the sedimented organic matter (SOM). In the laboratory, the lengths and heights of 217 unbroken mussel shells were measured according to Kenk and Wilson (1985). The total preserved wet weight (shell + tissue) was also measured for 207 individuals (including only individuals preserved in formalin and alcohol, not the frozen ones). Moreover, two additional specimens were also sampled on carbonate crusts from a second location explored during dive 1644 on the western slope of the Tekirdağ Basin (40°50'N - 27°30'E) at 1068 m depth (Figure 1A). These individuals were used for DNA extraction to determine whether one or several species could occur in the different basins of the Marmara Sea. To complete our study, we used 72 individuals that were sampled in the eastern Mediterranean Sea during the NAUTINIL cruise (2003) at 2130 m depth in the Nile Deep-Sea Fan (NDSF, Figure 1B; 32°38.4'N - 29°55.0'E), a site described in Bayon et al. (2009) and Huguen et al. (2009). The specimens tentatively identified as *Idas* aff. *modiolaeformis* by Lorion et al. (2012) were measured in the same way as those from the Marmara Sea.

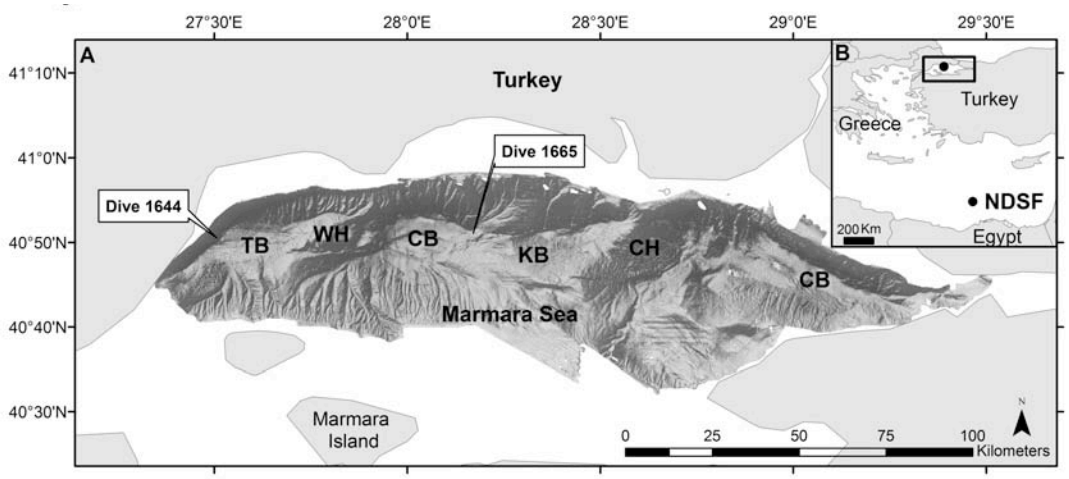

Figure 1. (A) The Marmara Sea showing the succession of the different basins and the location of the dives 1644 and 1665 during the MarNaut cruise (2007). (B) General map of the eastern Mediterranean Sea with the sampling sites (black dots) in the Nile Deep-Sea Fan (NDSF) explored during the NAUTINIL cruise (2003) and the Marmara Sea. Abbreviations from west to east: TB, Tekirdağ Basin; WH, Western High; CB, Central Basin; KB, Kumburgaz; CH, Central High: Basin CB, Çinacik Basin.

2.2. Morphology and morphometry

The morphology of the sampled mussels was examined and compared with descriptions of the seven species from the genus *Idas* reported from the Mediterranean Sea and Atlantic Ocean, namely *Idas argenteus* Jeffreys, 1876; *Idas modiolaeformis* Sturany, 1896; *Idas simpsoni* (Marshall 1900); *Idas dalmasi* Dautzenberg, 1927; *Idas ghisotti* Warén and Carrozza, 1990 *Idas macdonaldi* Gustafson et al. (1998) and *Idas cylindricus* Pelorce and Poutiers, 2009. Type specimens of *Idas simpsoni*, *Idas ghisotti* and *Idas cylindricus* and specimens of *Idas* aff. *modiolaeformis* studied by Duperron et al. (2008b) and Lorion et al. (2012) were also available for direct comparison.

Size (length) frequency distribution was analysed to determine the different distribution modes that might correspond to different settlement events (Bhattacharya, 1967). In this analysis, the magnitude of size classes was chosen so

176 that at least 30 individuals cluster in the main classes (i.e. with the highest number of
177 individuals). Thus, the class-sizes were delimited according to an interval of 1 mm for
178 *Idas*-like nov. sp. and 2 mm for *Idas* aff. *modiolaeformis*.

179

180 To test for the effect of preservation method on mussel biomass, a non-parametric
181 Mann-Whitney *U* test was performed on average wet weights between mussels
182 preserved in alcohol (n=55) and formalin (n=152).

183

Table 1
Collection information as well as number of individuals and preservation type for each analysis performed in this paper. Abbreviations: A_{70} or A_{96}=alcohol 70% or 96% respectively, F_{10}=formalin 10%, Fz=frozen at -80°C, FISH=properly prepared for Fluorescence *In Situ* Hybridization analyses, TC= top layer of Tube Core.

Year	2003	2007	2007
Cruise-dive #	Nautinil-1551-1553	Marnaut-1644	Marnaut-1665
Sample reference		R4	CC1-2-3, BioBox
Morphometry	72		217
Host phylogeny		2 Fz	2 Fz / 1 A_{96}
Symbiont diversity			1 Fz
FISH analyses			1 A_{70} → Ind. A 1 FISH/Fz → Ind. B
Stable isotopes			3 Fz/3 TC
Biomass	60 F_{10}		152 F_{10} / 55 A_{70}
Total of individuals	83	2	220

184

185 The relationships between length (*L*), mass (*M*), and height (*H*) were estimated by
186 fitting coefficients *a* and *b* in a power function, $Y=a(X)^b$ where *X* is the length and *Y* is
187 alternatively the mass or the height (Huxley and Teissier, 1936). For *b*=3, it is
188 supposed that the growth is isometric, meaning that the growth in length occurs at
189 the same rate as the growth in height or mass. Size-mass relationships were
190 compared between sampling locations using non-parametric Mann-Whitney *U* test to
191 detect differences in the average biometric measurements (length, height, weight).
192 Finally, significance of the regression coefficient (R^2) between log-transformed [*log*
193 *(L) versus log (M)* or *log (H)*] measurements was also tested.

194

195 2.3. DNA analyses

196 2.3.1. Data acquisition

197 DNA was extracted from gills of one specimen for the symbiont analyses, and from
198 foot tissue of 5 specimens for the host analyses (Table 1) using the QIAamp® DNA
199 Micro Kit (Qiagen). For mussel taxonomy and phylogeny, DNA was extracted from
200 foot tissue to avoid the risk of sequencing paternal lineages, which are concentrated
201 in the gonads for those taxa. Fragments of the Cytochrome Oxidase subunit I
202 mitochondrial gene (COI mtDNA) and of the 28S rRNA nuclear gene were amplified
203 as described in Lorion et al. (2010). For symbiont characterisation, prokaryotic 16S
204 rRNA was amplified according to protocols described in Duperron et al. (2005)
205 including the application of 25 PCR cycles to minimise PCR biases. PCR and cloning
206 products were purified and sequenced in both directions at the Genoscreen facility
207 (Lille) and chromatograms were edited using Sequencher 4.1.4 (Gene Codes Co.).

208

209 2.3.2. Host taxonomy and phylogeny

210 Sequences of the *Idas*-like nov. sp. were added to the COI dataset #3 and 28S
211 dataset #4 analysed by Lorion et al. (2010), which are representative of all symbiont-
212 bearing mussel lineages currently known. The datasets obtained were aligned and
213 K2P genetic distances were calculated with Mega 4 (Tamura et al., 2007).
214 Phylogenetic relationships were inferred from the combined dataset using the
215 Bayesian approach implemented in the Beast 1.5.4 package (Drummond and
216 Rambaut, 2007). The Yule speciation model was used as a tree prior and
217 heterogeneity of mutation rates across lineages was set under an uncorrelated log-
218 normal relaxed clock. A Generalised Time Reversible (GTR) model with a gamma

law (C, four categories) and a proportion of invariants (I) was used for both genes and adjusted with respect to data partition. The mutation rate was set to 1 to get branch lengths in units of substitution per site. The tree was rooted on *Modiolus modiolus* according to Samadi et al. (2007). Four parallel analyses starting from distinct coalescent trees were run over 20 million generations and sampled each 1000 steps. After analysing the results with Tracer v1.4.1 and discarding the first 50% of the samples as a burn-in, independent runs were pooled and resampled each 4000 steps. The maximum clade credibility tree was drawn from these pooled results (10,000 samples). Posterior probabilities of its nodes and mean branch lengths were calculated from the rest of trees (i.e. all Bayesian trees sampled after posterior distribution reached stationary).

2.3.3. Symbiont characterisation

The 16S rRNA sequences were compared to sequences available in databases using the BLAST search program (http://blast.ncbi.nlm.nih.gov/Blast); (Altschul et al., 1990), aligned with the SINA Web Aligner (Pruesse et al., 2007) and edited in the BioEdit v7.0.5 programme (Hall, 1999). Phylogenetic trees were estimated using the Maximum Likelihood heuristic search using the PHYLIP software (Felsenstein, 1989). Rarefaction curves were calculated for the 16S rRNA clone library using the RarFac programme (http://www.icbm.de/pmbio) and gene library coverage was calculated using the following formula: C=[1-(n_1/N)] x100, where n_1 is the number of unique OTUs and N the number of clones in the library (Singleton et al., 2001). Sequences from different symbiont types observed in other mussels from the literature have been included in our dataset, including those associated with *Idas* aff. *modiolaeformis* from the eastern Mediterranean Sea (Duperron et al., 2008b).

2.4. Fluorescence *in situ* hybridisation

Two mussels from carbonate crust CC3 were dissected. One (individual A, Table 1) had been fixed in unbuffered 10% formalin, naturally buffered by the carbonates, and transferred after 48h in 70% alcohol. Another (individual B, Table 1) had been frozen and stored several months before post-fixation of the gills for FISH analyses (2 hours in unbuffered 4% formalin, two washes in 1X phosphate-buffered saline (PBS), and storage in PBS:ethanol 1:1). Gill fragments were dehydrated in increasing ethanol series and embedded in polyethylene glycol disterate: hexadecanol-1 (9:1) wax. 10 µm-thick transverse sections were cut with a microtome (Jung, Germany) and collected on SuperFrost Plus slides. The wax was removed with ethanol and samples were rehydrated in decreasing ethanol series. Hybridisations were performed for 3 hours at 46°C as described previously using a hybridisation buffer containing 30% formamide (5M NaCl, 1 M Tris-HCl 20% SDS, 30% formamide in (Duperron et al., 2008b). Seven oligonucleotide probes were used to test the presence of different bacterial groups (Table 2). Probes were labelled with Fluoresceine (FITC), Cyanine Cy3 or Cyanine Cy5. The general bacteria probe EUB338 was used as a positive control. After hybridisation, slides were washed (5 M NaCl, 1 M Tris-HCl, 0.5 M EDTA, 20% SDS) at 48°C for 15 minutes, rinsed with MilliQ water, and mounted in a SlowFade medium. Sections were observed under an epifluorescence microscope (Olympus, Japan).

Table 2
Oligonucleotide probes used in this study. The position in the 16S rRNA gene of *Escherichia coli* is given.

Probe	Sequence (5' - 3')	Position	Target	References
EUB-338	GCTGCCTCCCGTAGGAGT	338	Most eubacteria	Amann et al., (1990)
Bthio-193	CGAAGATCCTCCACTTTA	193	Thiotrophic bacteria	Duperron et al., (2007)
BangT-642	CCTATACTCTAGCTTGCCAG	642	Thiotrophic bacteria	Duperron et al., (2005)
EPY-549	CAGTGATTCCGAGTAACG	549	Epsilonproteobacteria	Manz et al., (1992)
CF-319	TGGTCCGTGTCTCAGTAC	319	Bacteroides	Manz et al., (1996)
GAM-42	GCCTTCCACATCGTTT	42	Gammaproteobacteria	Manz et al., (1992)
ImedM-138	ACCATGTTGTCCCCCACTAA	138	Methanotrophic bacteria	Duperron et al., (2008b)
BangM-138	ACCAGGTTGTCCCCCACTAA	138	Methanotrophic bacteria	Duperron et al., (2005)

269

270

271 2.5. Stable isotope analyses

272 Nitrogen and carbon stable isotope signatures were measured in soft tissue of three
273 individuals, and in the 0-1 cm layer of three tube core samples (Sedimented Organic
274 Matter, SOM). According to the small size of the animals, the whole soft tissues of
275 the frozen mussels (Table 1) were lyophilised overnight and homogenised in a fine
276 powder using a mortar and pestle. Sediment samples were treated as described in
277 Carlier et al. (2010). All samples were analysed at ISO-Analytical Laboratory
278 (Cheshire, UK) using the elemental analysis-isotope ratio MS method. The isotopic
279 composition was expressed as the relative difference between isotopic ratios in the
280 sample and that in conventional standards, PDB (Pee Dee Belemnite) for carbon and
281 air N_2 for nitrogen as follows:

282 $\delta^{13}C$ or $\delta^{15}N$ (‰) = $[(R_{sample} / R_{standard}) - 1] \times 1000$ where R = $^{13}C/^{12}C$ or $^{15}N/^{14}N$.

283

284 3. Results

285

286 3.1. Morphological description

The shells of all 220 *Idas*-like mussels from the Marmara Sea were modioliform, smooth, yellow to brown in colour, and devoid of periostracal hair unlike *Idas modiolaeformis* (Figures 2A, C, E). Morphological variability was observed on the ventral margin, which was occasionally straight (Figure 2C), but most of the time curved with an inflexion point in the middle of the ventral margin (Figure 2C). The anterior was usually narrower than the posterior. Antero-posterior lengths ranged from 5.2 to 20.8 mm (mean 15.5 ± 3.2 mm) and heights varied between 2.8 and 10.1 mm (mean 7.5 ± 1.5 mm). The boundary of the inhalant siphon was smooth (not shown).

Idas-like nov. sp. differed markedly from most described Mediterranean and Atlantic species, namely *Idas argenteus, Idas simpsoni*, *Idas dalmasi*, *Idas ghisotti* and *Idas cylindricus*, in having a modioliform shell shape, thick and dark brown periostracum, and no periostracal hair. These characters and the occurrence of a fringe on the boundary of the inhalant siphon (not shown) of specimens studied by Duperron et al. (2008b) and Lorion et al. (2012) has also allowed the distinction between *Idas*-like nov. sp. and *Idas* aff. *modiolaeformis*. However, shell morphology of *Idas*-like nov. sp. (Figures 2A, C, E) was very close to that of the large type specimen of *Idas modiolaeformis* described by Sturany (1896).

One post-larval shell, observed by SEM, was 450 µm in diameter (prodissoconch II stage, Figure 3A). The shell exhibited concentric lines except near the umbo, which is a granulated structure corresponding to the prodissoconch I stage (Figure 3B).

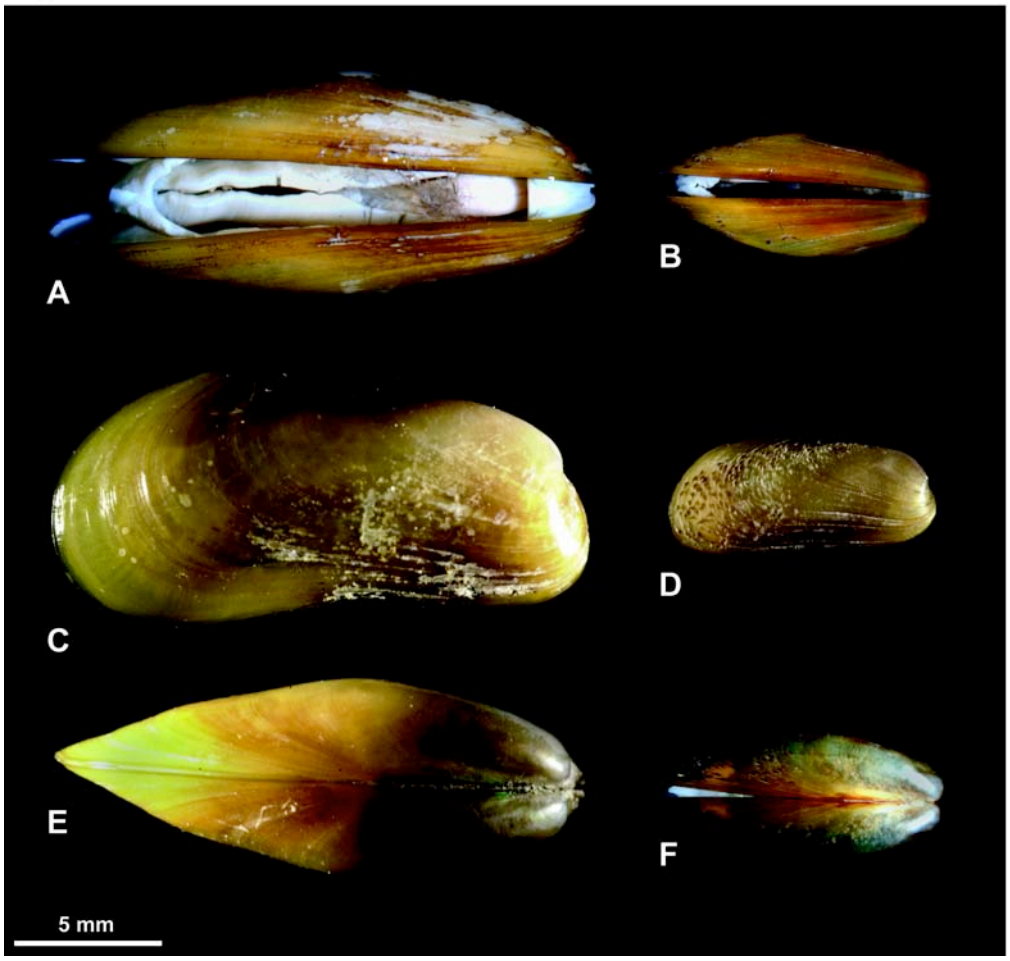

Figure 2. Photographs of external views of the mytilid *Idas*-like nov. sp. (A, C, E) and *Idas* aff. *modiolaeformis* from the eastern Mediterranean Sea (B, D, F): ventral view (A, B), right valve (C, D), and dorsal view (E, F). All specimens were sampled during the MarNaut (2007) and NAUTINIL (2003) cruises.

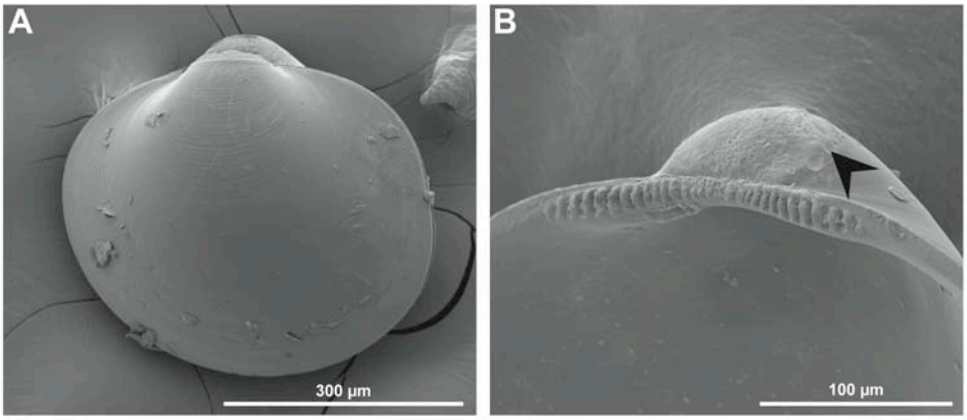

Figure 3 Scanning electron microscope imagery of (A) the prodissoconch II of a larvae sampled from sediments during the MarNaut cruise (2007) and (B) details of the hinge, the boundary between the prodissoconch I and prodissoconch II (black arrow).

311

312 3.2. Allometry and growth

313 Size-frequency distributions of both *Idas*-like nov. sp. and *Idas* aff. *modiolaeformis*
314 displayed a unimodal structure (Figure 4). In *Idas*-like nov. sp., the most abundant
315 size class was 17-18 mm (Figure 4), whereas smaller specimens (4-6 mm, Figure 4)
316 dominated the distribution in *Idas* aff. *modiolaeformis*. Specimens of *Idas* from the
317 eastern Mediterranean Sea were significantly smaller in length (5.5 ± 1.8 mm) and
318 height (2.7 ± 0.9 mm) than those from the Marmara Sea with a mean shell length of
319 15.5 ± 3.2 mm and a mean shell height of 7.5 ± 1.5 mm (Mann-Whitney test on shell
320 length, W=15365, p<0.05; height, W=17573, p<0.05). According to length-height
321 relationships (Figure 5A), the shell height increased more slowly than the cube of the
322 shell length during the growth in both groups (b<3; Table 3).

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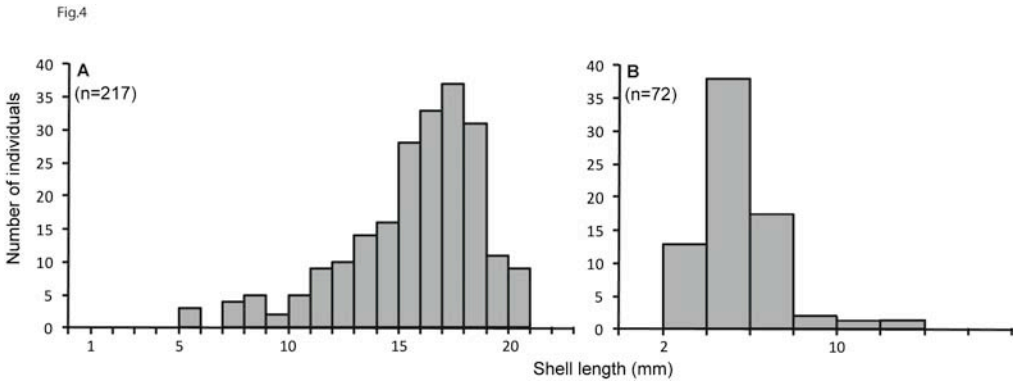

Figure 4. Length frequency distribution of (A) *Idas*-like nov. sp. sampled in June 2007 and (B) *Idas* aff. *modiolaeformis* from the eastern Mediterranean Sea sampled in September 2003. n=sample size.

324

325 There was no significant difference (Mann-Whitney, W=4048, p=0.73) between total
326 wet biomass measures of specimens preserved in alcohol (n=55) or formalin
327 (n=152). Thus, the length-mass relationship was analysed using all individuals from

328 the Marmara Sea (n=207) and showed that the total mass increased more slowly
329 than the cube of the shell length for both species (*b*<3; Table 3; Figure 5B).
330

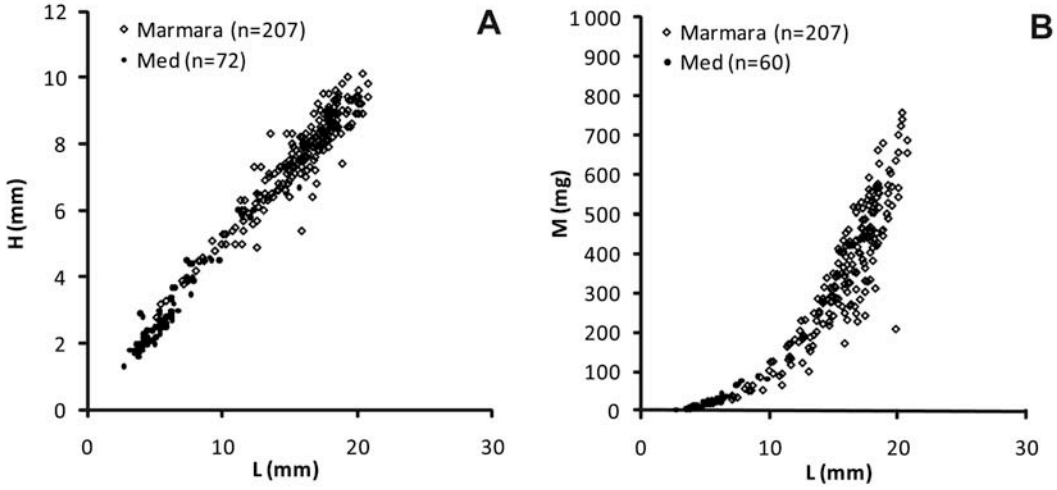

Figure 5. (A) Length-height and (B) length-mass relationships for the *Idas*-like nov. sp. (light diamonds) and *Idas* aff. *modiolaeformis* (black circles) from the eastern Mediterranean Sea. Total preserved wet biomass indicates total animal biomass (shell + tissue). The relation used is $Y=a(X)^b$ where Y is the height of the mass and X is the length. The relations with the parameters *a*, *b* and R^2 and all relationships are significant with $p<0.001$.

331

Table 3
Summary of the equations, coefficient of regression R^2 and *F* test results on the morphometric measurements done on *Idas*-like nov. sp. and *Idas* aff. *modiolaeformis*. Abbreviation: Med=Mediterranean Sea.

	Species	Equation $Y=a(X)^b$	R^2	*F* test
Marmara	*Idas*-like nov. sp.	$H=0.6942*L^{0.867}$	0.92	$F=754$, $p<0.001$
Med	*Idas* aff. *modiolaeformis*	$H=0.5014*L^{0.9895}$	0.88	$F=527$, $p<0.001$
Marmara	*Idas*-like nov. sp.	$M=0.16226*L^{2.7526}$	0.91	$F=2161$, $p<0.001$
Med	*Idas* aff. *modiolaeformis*	$M=0.1596*L^{2.8893}$	0.93	$F=754$, $p<0.001$

332

333 3.3. Molecular taxonomy and phylogeny of the host

334 The COI mtDNA and 28S rRNA sequences were obtained from four specimens of
335 *Idas*-like nov. sp. (Table 1). All specimens displayed a single 1001 bp 28S rRNA
336 allele, while 0 to 3 bp (mean K2P: 0.3%) were variable among 579 bp sequenced for
337 COI mtDNA. The COI sequences differed from those of other deep-sea mussels by
338 K2P genetic distances ranging from 17.3% to 30.1%. The phylogenetic tree (Figure

6) resulting from the Bayesian analysis of combined COI mtDNA and 28S rRNA gene fragments was consistent with the results presented by Lorion et al. (2010). Specimens from the Marmara Sea clustered within the clade that includes all symbiont-bearing mussels except the genus *Benthomodiolus*. However, *Idas*-like nov. sp. could not be included into any of the lineages discussed in Lorion et al. (2010) and instead formed a long branch clustering within the multifurcation of those lineages (Figure 6). The position of *Idas*-like nov. sp. within the Mytilidae tree could thus not be further resolved.

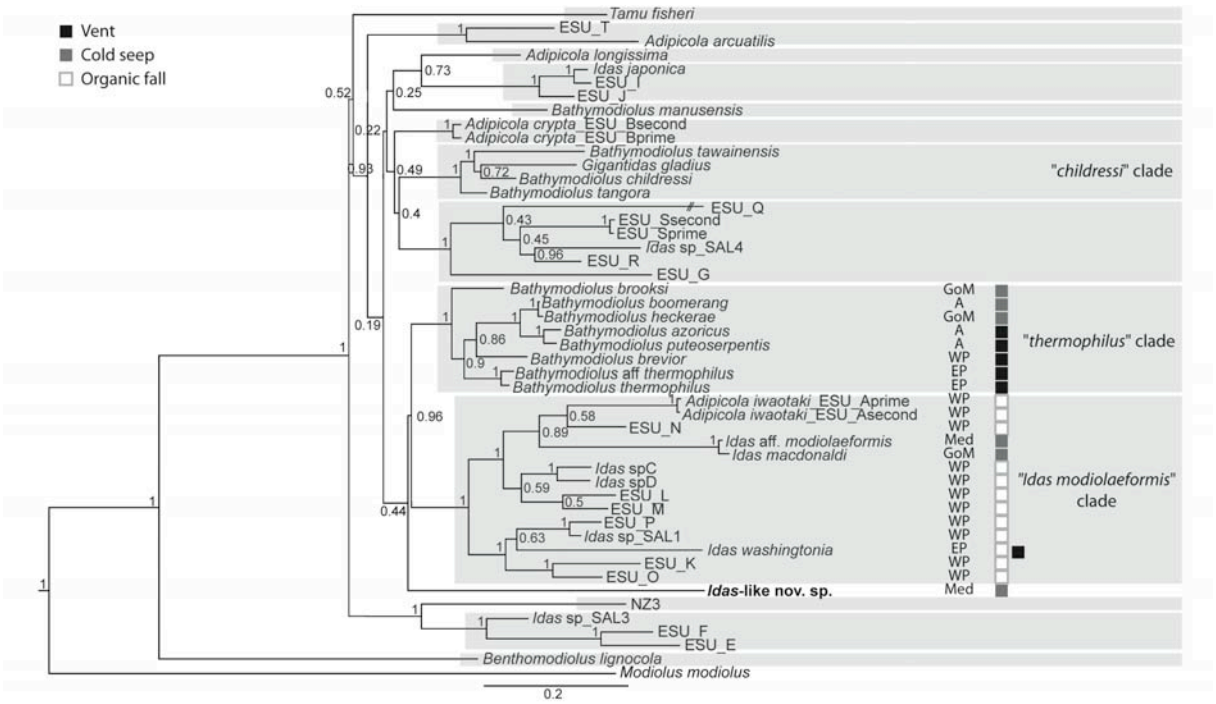

Figure 6. Maximum clade credibility phylogram obtained from Bayesian analyses of the sequences. Values above the nodes correspond to the posterior probabilities obtained from Bayesian analyses. The grey boxes correspond to lineages discussed in Lorion et al. (2010). The type of ecosystem inhabited by the species from the "thermophilus" clade and the clade including *Idas* aff. *modiolaeformis* are reported. The scale bar represents 20% estimated base substitution. Abbreviations: GoM=Gulf of Mexico, A=Atlantic, Med=Mediterranean Sea, WP=Western Pacific and EP=Eastern Pacific.

3.4. Diversity and phylogeny of associated bacteria

Of the 90 clones sequenced, the majority (87%) of the sequences were affiliated with the Gammaproteobacteria class, and highly similar to the sulphur-oxidising symbiont of cold-seep and hydrothermal vent mussels of the genera *Bathymodiolus* and *Idas* (>98% similarity; Figure 7). The 1 base pair differences between clones are potentially due to sequencing errors and therefore these sequences may represent the same phylotype as the one related to mussel-associated thiotrophic symbionts (Figure 7). Besides the Gammaproteobacteria, two sequences were affiliated with the Epsilonproteobacteria class, three to uncultured bacteria involved in the ANaerobic AMMonium OXidation reactions (or Anammox) and the last one did not have any clear phylogenetic affiliation. With a coverage value of 75% for the clone library and a rarefaction curve that shows saturation, it is unlikely we have missed any abundant symbionts (Electronic supplementary material 1). One clone of Gammaproteobacteria, the dominant phylotype, is used in the phylogenetic tree. Indeed, the low clone number of other types of bacteria suggests that they represent potential contaminants, free-living bacteria attached to the gills due to the filtration abilities of the mussels, or some rare symbionts.

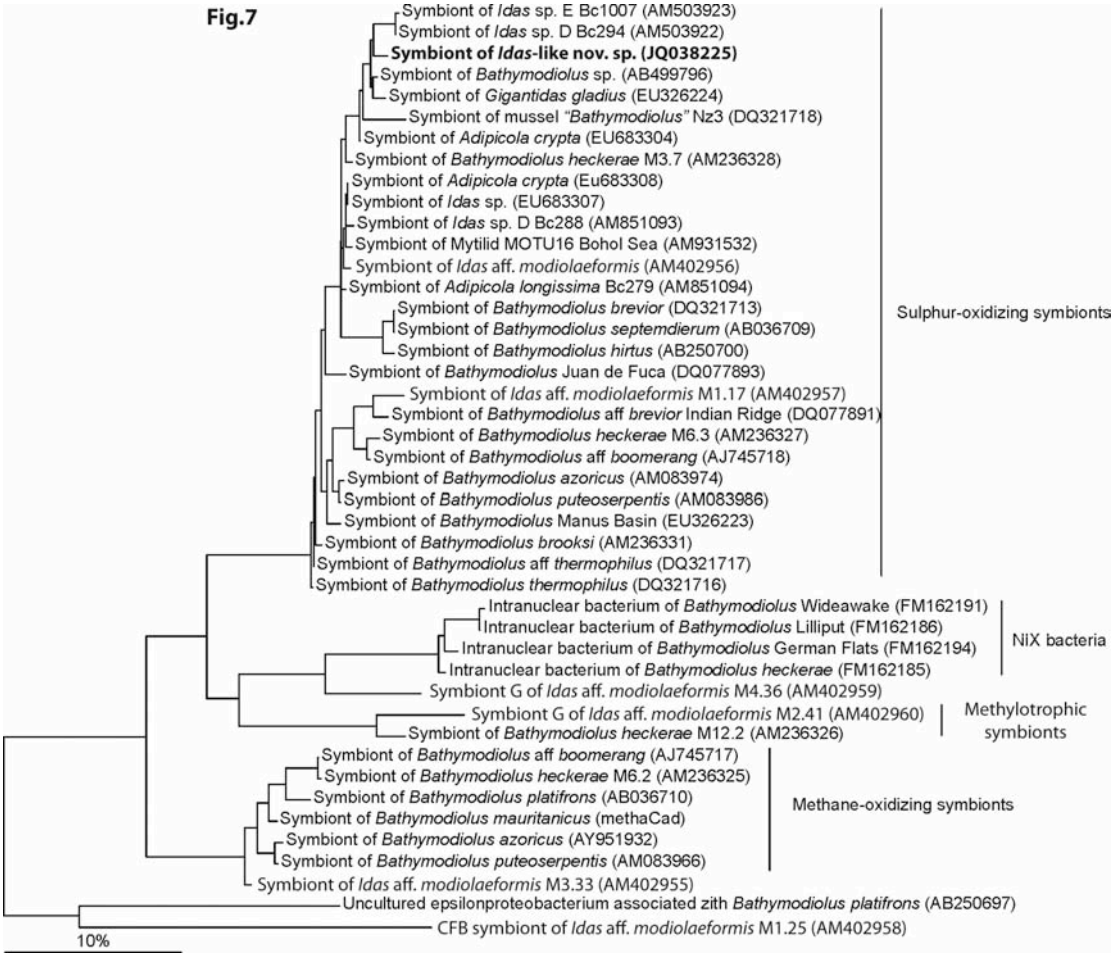

Figure 7. Phylogenetic tree displaying bacterial symbionts associated with *Idas*-like nov. sp. based on 16S rRNA gene sequences (in bold). Bacteroidetes (CFB) and uncultured epsilonproteobacteria are used as an outgroup to the gammaproteobacteria. Posterior probabilities are displayed as percentages. Scale bar represents 10% estimated base substitution. Abbreviation: NIX: Nuclea Inclusion X.

368

369 ## 3.5. Localisation of associated bacteria

370 Because the use of FISH was not anticipated, fixation and preservation of the
371 organisms was not ideal; FISH analyses usually require gill fixation directly on board
372 after recovery of the mussels. As a consequence, low signal intensities and good
373 morphology (formalin-fixed tissue; Figure 8 and Electronic supplementary material
374 2A) or stronger signal with disrupted host cells (freezing before fixation for FISH,
375 Electronic supplementary material 2B) were observed. However, the combination of
376 these results allowed reliable identification of FISH signals. Positive signals were

observed on hybridised gill sections with probe Eub-338 targeting all bacteria (not shown) whose signal overlapped the signal observed with BangT-642 (Figure 8). We also observed a signal with probes BThio-193. Both the probes (BThio-193 and BangT-642) target known deep-sea mussel thiotrophic symbionts. The latter probe hybridised despite a one base mismatch with the identified dominant 16S rRNA phylotype, which could be explained by the moderately stringent conditions used for hybridisation (30% formamide). Overlays of the Eub-338 and BThio-193 signals confirmed that bacteria in the gills were mostly thiotrophs. Few FISH signals were observed with the Bacteroidetes probe CF-319. Other probes, including ImedM-138 targeting methanotrophic symbionts, did not yield any signal. Despite being present in the clone library, Epsilonproteobacteria and Anammox bacteria were not detected, suggesting that these most likely represented environmental bacteria.

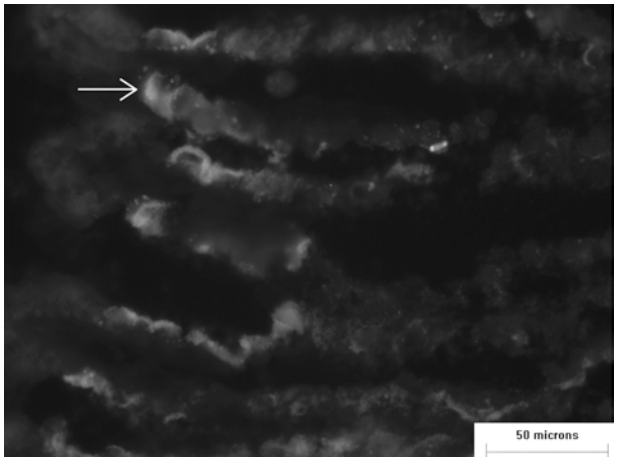

Figure 8. Fluorescence *in situ* hybridisation on transverse sections of gill filaments of *Idas*-like nov. sp. showing the distribution of thiotrophic symbiont (BangT) on the brightest part (pointed by the white arrow). Composite pictures in colour are reported in supplementary material with (A) BangT in green and Eub in red in individual A and (B) Bthio in green and BangT in red in individual B.

391 3.6. Isotopic signatures

392 Specimens from the Marmara Sea (n=3) displayed $\delta^{13}C$ values between -37.4 and -
393 35.5‰ and $\delta^{15}N$ values between 5.7‰ and 6.0‰ (Figure 9). Signatures of the SOM
394 at the sediments surface (n=3) ranged between -27.6‰ and -24.6‰ ($\delta^{13}C$, Figure 9)
395 and 4.0‰ and 5.3‰ for $\delta^{15}N$. Unfortunately, the signature for the methane source
396 was not determined, but gas hydrates and gas bubbles sampled in different basins of
397 the Marmara Sea exhibited $\delta^{13}C$ values varying from -64.1‰ to -44.1‰ (Figure 9).

398

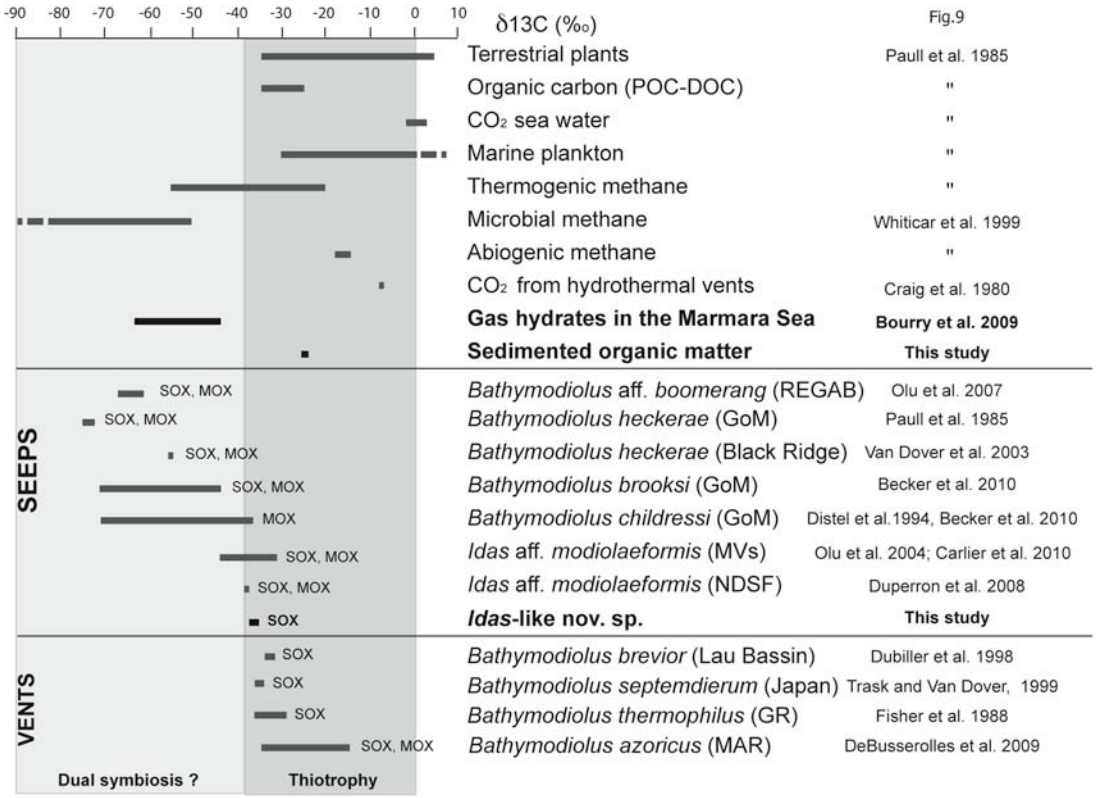

Figure 9. $\delta^{13}C$ signatures of several marine and terrestrial carbon sources and a list of mussel species from vent and seep sites. Abbreviations: POC: particular organic matter, DOC: dissolved organic matter, SOX: sulphur-oxidising bacteria, MOX: methane-oxidising bacteria, GoM: Gulf of Mexico, Med: eastern Mediterranean Sea, NDSF: Nile Deep-Sea Fan, MVs: Napoli and Amsterdam mud volcanoes, GR: Galápagos ridge, MAR: Mid-Atlantic ridge. Data related to this study are highlighted in bold.

399

400

401 4. Discussion

402

403 4.1. First symbiont-bearing mussel observed in the Marmara Sea: *Idas*-like nov. sp.

404 Based on both morphological and molecular data, we first assessed the systematic
405 status of our specimens. Mitochondrial K2P genetic distances of 0.3% and the
406 presence of a single 28S rRNA allele for all *Idas*-like nov. sp. individuals clearly
407 confirmed that our specimens coming from both the Central and Tekirdağ basins of
408 the Marmara Sea belong to a single species. Indeed, such genetic distances are well
409 within the range of intra-specific variability previously reported in mytilid species
410 associated with organic falls, vents and cold-seeps (≈ 1%; Lorion et al., 2010;
411 Miyazaki et al., 2004; Won et al., 2003). Additionally, specimens from the Marmara
412 Sea differed from all other mussels included in our tree by genetic distances above
413 17%, such values being in the range of interspecific differences (Lorion et al., 2010).
414 Some characters, such as length, shell and siphon shapes can be used to
415 consistently distinguish our specimens from most species described in the Atlantic
416 and Mediterranean Sea and also from *Idas modiolaeformis* (Lorion et al., 2012;
417 Lorion et al., in press). *Idas*-like nov. sp., however, was morphologically very close to
418 the largest type specimen of *Idas modiolaeformis,* a species that was succinctly
419 described in the 19th century on the basis of two empty shells (Sturany, 1896).
420 However, shell information alone has already proved irrelevant to species
421 identification in the absence of other anatomical and/or molecular information (Lorion
422 et al., 2010). The current study therefore further illustrates how old type specimens,
423 which often consist of a limited number of dried empty shells, should be treated with
424 caution when considering the taxonomy of bivalves having highly plastic shell
425 shapes. As a consequence, it is impossible to firmly conclude whether *Idas*-like nov.

426 sp. is a new species (Lorion et al., 2010). A complete re-assessment of the diversity
427 of the genus *Idas* in the Mediterranean basins, and of all symbiont-bearing mussels
428 worldwide, is ideally required to clarify the status of the various *Idas* species.
429
430 *Idas*-like nov. sp. represents a species that has not been previously included in
431 molecular phylogenies. It could not be included in any of the mussel lineages
432 reported by Lorion et al. (2010) and branched within the multifurcation that
433 encompasses all species found at vents, seeps and organic falls. The particular
434 history of the Mediterranean Sea nevertheless allows some inferences on its
435 evolutionary history. It is believed that the Messinian salinity crisis eradicated most
436 Mediterranean marine faunas between 5.33 and 5.96 million years ago and that
437 modern Mediterranean communities reflect recent re-colonisation events (Duggen et
438 al., 2003; Krijgsman et al., 1999; Popescu et al., 2008). In this context, the case of
439 *Idas* aff. *modiolaeformis* is quite clear. Using a COI mutation rate ranging from 1ù to
440 2% per million years, it appears that *Idas* aff. *modiolaeformis* diverged from its sister
441 species *Idas macdonaldi* between 0.60 and 3.61million years (Lorion et al., 2012).
442 Because *Idas macdonaldi* lives at cold-seeps in the Gulf of Mexico, it was suggested
443 that the colonisation of the Mediterranean Sea occurred from the Atlantic ocean.
444 Although a similar scenario seems a sound hypothesis to explain the occurrence of
445 *Idas*-like nov. sp. in the Marmara Sea, a more resolved tree and the identification of
446 close relatives of this species are needed to really test it properly. In any case, the
447 high divergence between *Idas*-like nov. sp. and *Idas* aff. *modiolaeformis* clearly
448 supports the hypothesis that those two species diverged a long time before they
449 colonised the Mediterranean basins. It is striking to note that, while such highly
450 divergent lineages of small *Idas*-like mussels occur in the Mediterranean basins,

451 species of the "*thermophilus*" lineage do not, despite being phylogenetically closer to
452 *Idas* aff. *modiolaeformis*.
453
454 Another interesting result is that *Idas*-like nov. sp. and *Idas* aff. *modiolaeformis* do not
455 co-occur despite the fact that seep settings, habitat characteristics, and depth ranges
456 at which they were collected are similar. This is surprising given that other symbiont-
457 bearing species such as the vesicomyid *Isorropodon perplexum* and the lucinid
458 *Lucinoma kazani* have been observed at both Marmara and eastern Mediterranean
459 Sea cold-seeps (Ritt et al., 2011; Ritt et al., 2010). *Idas* aff. *modiolaeformis*, however,
460 has been reported in the Mediterranean cold-seep sites (Duperron et al., 2008b;
461 Gaudonr et al. 2010; Ritt et al., 2011) but not in the Atlantic Ocean nor the western
462 Mediterranean Sea. Furthermore, a recent study hypothesised that *Idas* aff.
463 *modiolaeformis* may have a planktonic phase between 4 weeks to 5 months
464 (Gaudron et al., 2012). This is unlikely that *Idas*-like nov. sp. or *Idas* aff.
465 *modiolaefomris* larvae could be able to pass through the Dardanelles strait, the
466 connection between the Marmara Sea and the Eastern Mediterranean Sea
467 (Besiktepe et al., 1994). This barrier to dispersal could explain the different
468 distribution of both species. However, the dispersal abilities of *Idas*-like nov. sp.
469 remain to be explored as our sampling effort was restricted to a single sampling
470 location. Further explorations of the Mediterranean and Marmara seeps are
471 necessary to really document the reproductive strategy, dispersal abilities, and
472 distribution, of both species.
473
474 4.2. Life cycle

The life cycle of deep-sea mytilids is still poorly known in terms of reproduction, growth rates, recruitment, and larval dispersal. However, the occurrence of distinct modes in several mussel species at deep hydrothermal vents indicates discontinuous episodes of massive larval settlement linked with discontinuous release of gametes and planktotrophic larval development (Comtet, 1994; Comtet and Desbruyères, 1998; Dixon et al., 2006; Rhoads et al., 1981; Smith et al., 2000; Van Dover et al., 1996). The same pattern has been observed at cold-seeps for *Bathymodiolus childressi* in the Gulf of Mexico (Arellano and Young, 2009; Nix et al., 1995; Smith et al., 2000; Tyler et al., 2007). In the present study, the size-frequency distribution exhibits a single mode. Furthermore, a high abundance of prodissoconch II post-larval stages was observed in sediments close to the sampled carbonate crusts. These prodissoconch II from the Marmara Sea are in the same range of size (450 µm) as those of species from vents and seeps (from 380 to 520 µm), supporting the idea of a planktonic phase (Arellano and Young, 2009; Comtet et al., 2000; Gaudron et al., 2012; Lorion et al., 2012; Lorion et al., in press; Lutz et al., 1980; Lutz et al., 1984). The abundance of these post-larvae and the absence of distinctive modes suggest the presence of a continuous recruitment. Several of these larvae were dead, raising questions about the cause of this massive mortality. High recruitment rates in hydrothermal *Bathymodiolus* mytilids were hypothesised to balance for episodic, important mortality resulting from natural changes in hydrothermal flow and tectonic activity (Comtet and Desbruyères, 1998). Competition with adults may be another explanation of the larval mortality at our sampling site. Indeed, post-larvae may have been excluded from the hard substratum they need to settle, by the presence of adult specimens already attached on the substratum. It is hypothesised that all of the mussels within a single patch have the same age and resulted from a

single recruitment event. However, our data are not sufficient to reveal the reproductive strategy or recruitment patterns of this species.

4.3. A thiotrophic symbiosis

Thiotrophic symbioses are well-documented for many small mytilids associated with organic falls, including various *Idas* species from sunken woods in the eastern Pacific (Duperron et al., 2008a). In *Idas*-like nov. sp., thiotrophic bacteria unambiguously dominate the populations of gill-associated symbionts. This symbiotic association resembles that of many *Idas* spp., but greatly differs from that occurring in *Idas* aff. *modiolaeformis* which harbours up to six distinct bacterial phylotypes, including methane- and sulphur-oxidising bacteria (Duperron et al., 2008b). Also, the thiotrophs of these two mussel species are not very closely related, an observation that is often reported in not very closely related species of mussels (Duperron et al., 2009).

Most Mytilidae from deep-sea environments live with thiotrophic bacteria and only some of them, mostly chemosynthetic ecosystems, host methanotrophic bacteria as observed in *Idas* aff. *modiolaeformis* (Duperron, 2010; Duperron et al., 2008b). In the present study it is intriguing that no methane-oxidisers were detected despite the high concentration of methane at the study area (Ritt et al., 2010). The bacteria that were only occasionally detected in *Idas*-like nov. sp. may either represent potential contaminants, corresponding to free-living bacteria attached to the gills, or some rare symbionts. In any case, their very low abundance suggests a limited role in the animal's nutrition.

525 4.4. Nutrition

526 Measured carbon stable isotope signatures of *Idas*-like nov. sp. (from -37.4‰ to -
527 35.5 ‰; Figure 9) were in the range of values measured in hydrothermal vent
528 species associated exclusively with sulphur-oxidising bacteria such as *Bathymodiolus*
529 *thermophilus* (from -37.3‰ to -29.2 ‰), *B. septemdierum* (-37 ‰), and *B. brevior*
530 (from -35.8‰ to -30.8 ‰; Figure 9; Dubilier et al., 1998; Fisher et al., 1988; Trask and
531 Dover, 1999) as well as the cold-seep species *Idas modiolaeformis* (-44.6‰ to -38.3
532 ‰; Carlier et al., 2010; Duperron et al., 2008a; Olu-Le Roy et al., 2004). The ^{13}C
533 signature of *Idas*-like nov. sp. were higher than values from *Bathymodiolus* species
534 from the Gulf of Mexico, the Blake Ridge and Western Africa, which are generally
535 below -37.5 ‰ and in which the symbioses involves methanotrophs, either alone or
536 in co-occurrence with sulphur-oxidisers (Becker et al., 2010; Distel and Cavanaugh,
537 1994; Olu-Le Roy et al., 2007; Paull et al., 1985; Van Dover et al., 2003). Despite a
538 limited dataset (i.e. only three measurements from a single area), our results support
539 a chemoautotrophic-based nutrition for *Idas*-like nov. sp., mainly via the thiotrophic
540 pathway. Sulphide is one of the by-products of the anaerobic oxidation of methane
541 (AOM; Boetius et al., 2000; Carlier et al., 2010; Losekann et al., 2008). The values
542 obtained for sedimented organic matter indicate that is a mixture of photosynthesis-
543 derived and chemoautotrophic-derived carbon, as observed in other seep sites from
544 the Mediterranean Sea (Carlier et al., 2010). Carbon input from filter feeding cannot
545 be excluded, as mixotrophy (i.e. symbiosis in conjunction with filter feeding and
546 particle ingestion) has already been documented in several vent and seep
547 *Bathymodiolus* species (Page et al., 1991; Page et al., 1990; Riou et al., 2010).
548 Therefore, the carbon signatures of *Idas*-like nov. sp. may reflect the assimilation of
549 various food sources including: (i) oceanic DIC, (ii) free bacteria such as

methanotrophic or epsilonproteobacteria present in our clone libraries, (iii) surface-derived, photosynthetic carbon, and potentially (iv) some light methane-derived DIC. Nitrogen isotope ratios reflect trophic status; *Idas*-like nov. sp. exhibits $\delta^{15}N$ values higher than those of the sedimented organic matter, the potential food source. These relatively high $\delta^{15}N$ (≤ 6‰) values suggests the presence of chemoautotrophic symbionts. They are close to the limit established by Levin and Michener (2002) for species with symbionts. They also suggest the utilisation of organic matter from photosynthetic origin as suggested by the $\delta^{13}C$.

4.5. Conclusion

Here we present the first description of a thiotrophic mussel species, *Idas*-like nov. sp., associated with cold-seep deep-sea ecosystems in the Marmara Sea. Based on morphological characters of empty shells, we cannot firmly conclude that *Idas*-like nov. sp. is different from *Idas modiolaeformis* (Sturany, 1896) of the eastern Mediterranean Sea. However, according to molecular data *Idas*-like nov. sp. branches separately in phylogenetic reconstructions, far from any other documented "*Bathymodiolus*" and "*Idas*" suggesting that (1) it represents a new lineage and, (2) it diverged from *Idas* aff. *modiolaeformis* long before both species colonised the Mediterranean Sea seeps.

As well as molecular and morphological differences, both *Idas* species also present distinct types of symbiotic association. *Idas*-like nov. sp. harbours thiotrophic symbionts in its gills, a symbiosis comparable to that described in several small mussels from organic falls and vents (Duperron, 2010; Duperron et al., 2009), while *Idas* aff. *modiolaeformis* has six symbiont types. No methanotroph was found in the

Marmara Sea species despite the presence of methane-enriched fluids (Ritt et al., 2010). However further analyses on a higher number of individuals is needed to define whether the SOX bacteria are truly the only type, or at least the very dominant type of symbionts living within *Idas*-like nov. sp. gills. Questions about the ability of this symbiotic species to cope with its seep environment, and the exact role of the symbionts in host nutrition compared to other potential sources, remain to be elucidated.

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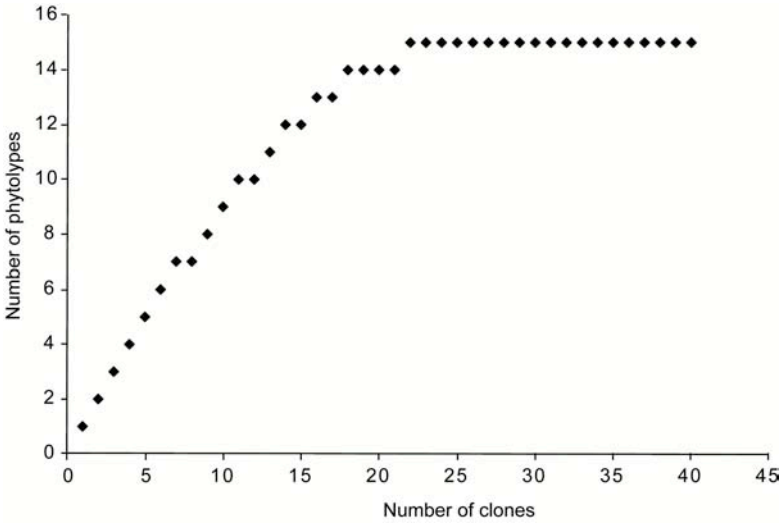
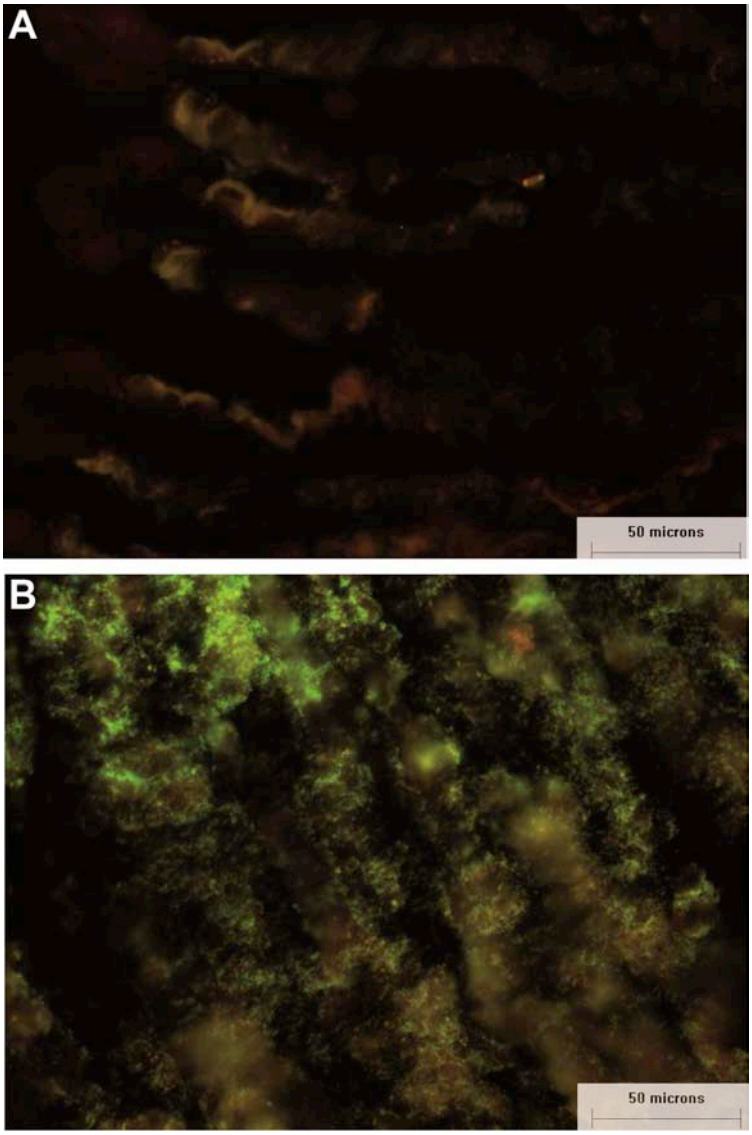


Electronical supplementary 1. Rarefaction analysis of the overall, combined bacterial 16S rRNA gene clone library recovered from gills of the *Idas*-like nov. sp. Marmara specimen. The rarefaction curve, plotting the number of observed phylotypes as a function of the number of clones, was computed by estimates.



Electronical supplementary 2. Fluorescence *in situ* hybridization on transversal sections of gill filaments of a *Idas*-like nov. sp. (A) Composite picture showing the distribution of thiotrophic symbionts in green (BangT) and Eubacteria in red in the individual A. (B) Composite picture showing the distribution of two types of thiotrophic symbionts in green (Bthio) and in red (BangT) in the individual B. Differences in the quality of the signal (favoured in B) and in the preservation of the gill ultra-structure (favoured in A) is noticeable.

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