

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

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### 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

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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 similarities with 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

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

96

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

114

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

131

## 132 2. Material and methods

133

### 134 2.1. Sampling site, animal collection and specimen preservation

135 The mytilid bivalves were collected in June 2007 at a cold-seep site in the north-east  
136 Central Basin of the Marmara Sea (40°51.27'N - 28°10.19'W, Figure 1) at a depth of  
137 1120 m using the manned submersible *Nautilie* deployed from the R/V l'Atalante. This  
138 cold-seep site was characterised by upward fluid flows and carbonate crust

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

160

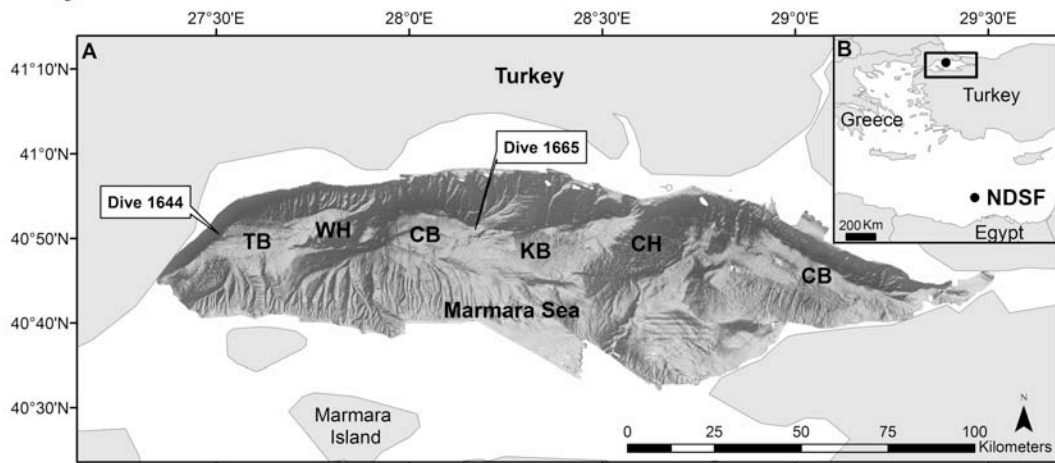


Figure 1. (A) The Marmara Sea showing the succession of the different basins and the location of the dives 1644 and 1665 during the MarNaut cruise (2007). (B) General map of the eastern Mediterranean Sea with the sampling sites (black dots) in the Nile Deep-Sea Fan (NDSF) explored during the NAUTINIL 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.

161

## 162 2.2. Morphology and morphometry

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

172

173 Size (length) frequency distribution was analysed to determine the different  
 174 distribution modes that might correspond to different settlement events  
 175 (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<sub>70</sub> or A<sub>96</sub>=alcohol 70% or 96% respectively, F<sub>10</sub>=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 <sub>96</sub>
Symbiont diversity			1 Fz
FISH analyses			1 A <sub>70</sub> → Ind. A 1 FISH/Fz → Ind. B
Stable isotopes			3 Fz/3 TC
Biomass	60 F <sub>10</sub>		152 F <sub>10</sub> / 55 A <sub>70</sub>
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*<sup>2</sup>) 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<sup>®</sup> 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



219 law (C, four categories) and a proportion of invariants (I) was used for both genes  
220 and adjusted with respect to data partition. The mutation rate was set to 1 to get  
221 branch lengths in units of substitution per site. The tree was rooted on *Modiolus*  
222 *modiolus* according to Samadi et al. (2007). Four parallel analyses starting from  
223 distinct coalescent trees were run over 20 million generations and sampled each  
224 1000 steps. After analysing the results with Tracer v1.4.1 and discarding the first 50%  
225 of the samples as a burn-in, independent runs were pooled and resampled each  
226 4000 steps. The maximum clade credibility tree was drawn from these pooled results  
227 (10,000 samples). Posterior probabilities of its nodes and mean branch lengths were  
228 calculated from the rest of trees (i.e. all Bayesian trees sampled after posterior  
229 distribution reached stationary).

230

### 231 2.3.3. Symbiont characterisation

232 The 16S rRNA sequences were compared to sequences available in databases  
233 using the BLAST search program (<http://blast.ncbi.nlm.nih.gov/Blast>); (Altschul et al.,  
234 1990), aligned with the SINA Web Aligner (Pruesse et al., 2007) and edited in the  
235 BioEdit v7.0.5 programme (Hall, 1999). Phylogenetic trees were estimated using the  
236 Maximum Likelihood heuristic search using the PHYLIP software (Felsenstein, 1989).  
237 Rarefaction curves were calculated for the 16S rRNA clone library using the RarFac  
238 programme (<http://www.icbm.de/pmbio>) and gene library coverage was calculated  
239 using the following formula:  $C=[1-(n_1/M)] \times 100$ , where  $n_1$  is the number of unique  
240 OTUs and  $N$  the number of clones in the library (Singleton et al., 2001). Sequences  
241 from different symbiont types observed in other mussels from the literature have  
242 been included in our dataset, including those associated with *Idas* aff.  
243 *modiolaeformis* from the eastern Mediterranean Sea (Duperron et al., 2008b).

244

#### 245 2.4. Fluorescence *in situ* hybridisation

246 Two mussels from carbonate crust CC3 were dissected. One (individual A, Table 1)  
247 had been fixed in unbuffered 10% formalin, naturally buffered by the carbonates, and  
248 transferred after 48h in 70% alcohol. Another (individual B, Table 1) had been frozen  
249 and stored several months before post-fixation of the gills for FISH analyses (2 hours  
250 in unbuffered 4% formalin, two washes in 1X phosphate-buffered saline (PBS), and  
251 storage in PBS:ethanol 1:1). Gill fragments were dehydrated in increasing ethanol  
252 series and embedded in polyethylene glycol distearate: hexadecanol-1 (9:1) wax. 10  
253 µm-thick transverse sections were cut with a microtome (Jung, Germany) and  
254 collected on SuperFrost Plus slides. The wax was removed with ethanol and samples  
255 were rehydrated in decreasing ethanol series. Hybridisations were performed for 3  
256 hours at 46°C as described previously using a hybridisation buffer containing 30%  
257 formamide (5M NaCl, 1 M Tris-HCl 20% SDS, 30% formamide in (Duperron et al.,  
258 2008b). Seven oligonucleotide probes were used to test the presence of different  
259 bacterial groups (Table 2). Probes were labelled with Fluoresceine (FITC), Cyanine  
260 Cy3 or Cyanine Cy5. The general bacteria probe EUB338 was used as a positive  
261 control. After hybridisation, slides were washed (5 M NaCl, 1 M Tris-HCl, 0.5 M  
262 EDTA, 20% SDS) at 48°C for 15 minutes, rinsed with MilliQ water, and mounted in a  
263 SlowFade medium. Sections were observed under an epifluorescence microscope  
264 (Olympus, Japan).

265

266

267

268

**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	Gamma proteobacteria	Manz et al., (1992)
ImedM-138	ACCATGTTGTCCCCACTAA	138	Methanotrophic bacteria	Duperron et al., (2008b)
BangM-138	ACCAGGTTGTCCCCACTAA	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<sub>2</sub> for nitrogen as follows:

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

283

## 284 3. Results

285

### 286 3.1. Morphological description

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

296

297 *Idas*-like nov. sp. differed markedly from most described Mediterranean and Atlantic  
298 species, namely *Idas argenteus*, *Idas simpsoni*, *Idas dalmasi*, *Idas ghisotti* and *Idas*  
299 *cylindricus*, in having a modioliform shell shape, thick and dark brown periostracum,  
300 and no periostracal hair. These characters and the occurrence of a fringe on the  
301 boundary of the inhalant siphon (not shown) of specimens studied by Duperron et al.  
302 (2008b) and Lorion et al. (2012) has also allowed the distinction between *Idas*-like  
303 nov. sp. and *Idas* aff. *modiolaeformis*. However, shell morphology of *Idas*-like nov.  
304 sp. (Figures 2A, C, E) was very close to that of the large type specimen of *Idas*  
305 *modiolaeformis* described by Sturany (1896).

306

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

310

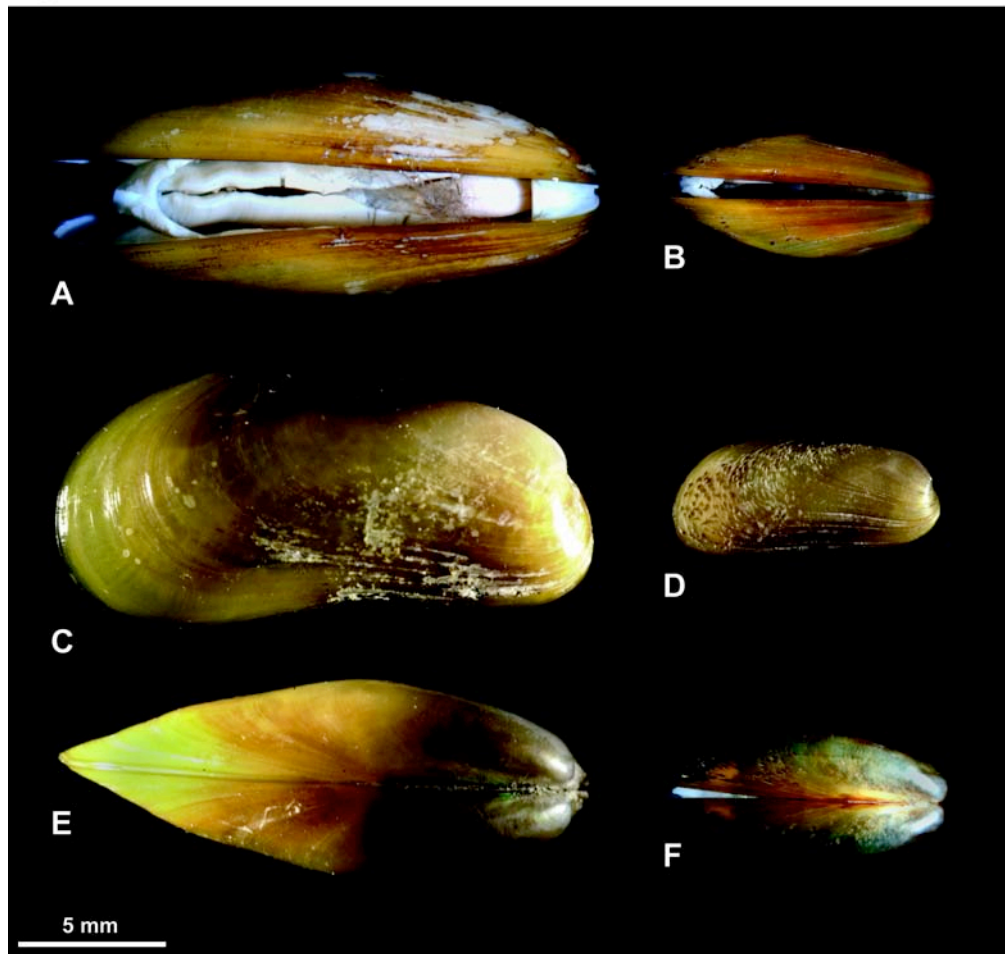


Figure 2. Photographs of external views of the mytilid *Idas*-like nov. sp. (A, C, E) and *Idas* aff. *modiolaeformis* from the eastern Mediterranean Sea (B, D, F): ventral view (A, B), right valve (C, D), and dorsal view (E, F). All specimens were sampled during the MarNaut (2007) and NAUTINIL (2003)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

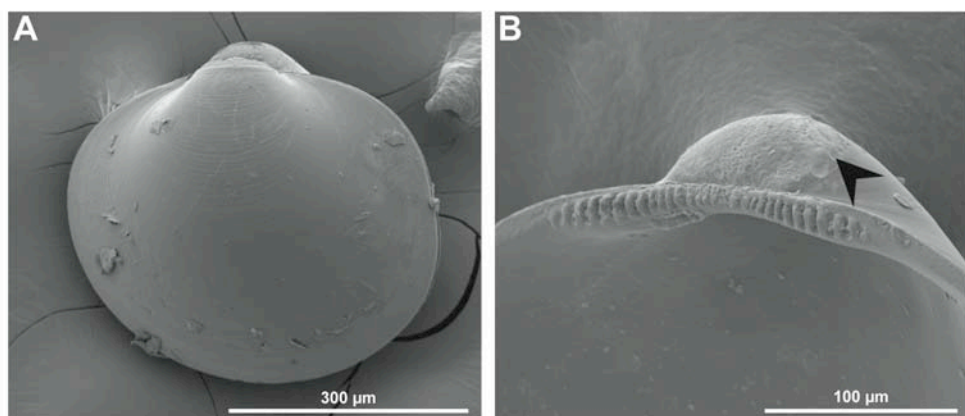


Figure 3 Scanning electron microscope imagery of (A) the prodissoconch II of a larvae sampled from sediments during the MarNaut cruise (2007) and (B) details of the hinge, the boundary between the prodissoconch I and prodissoconch II (black arrow).

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 ( $5.5 \pm 1.8$  mm) and  
318 height ( $2.7 \pm 0.9$  mm) than those from the Marmara Sea with a mean shell length of  
319  $15.5 \pm 3.2$  mm and a mean shell height of  $7.5 \pm 1.5$  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).

323

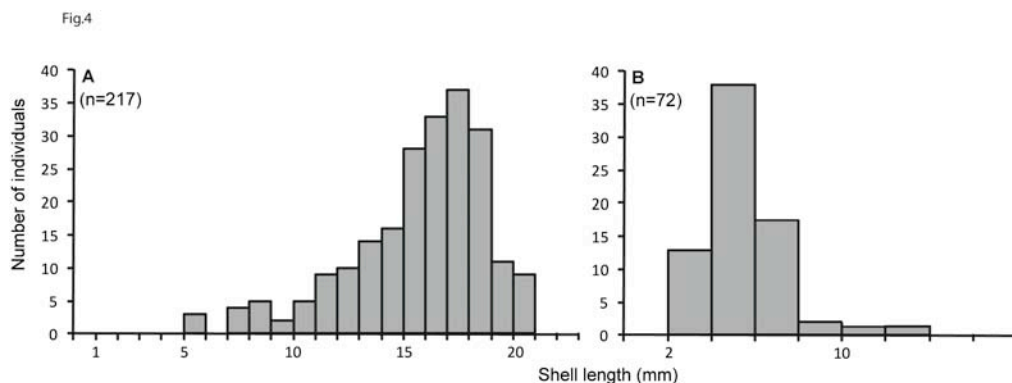


Figure 4. Length frequency distribution of (A) *Idas*-like nov. sp. sampled in June 2007 and (B) *Idas* aff. *modiolaeformis* from the eastern Mediterranean Sea sampled in September 2003. n=sample size.

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

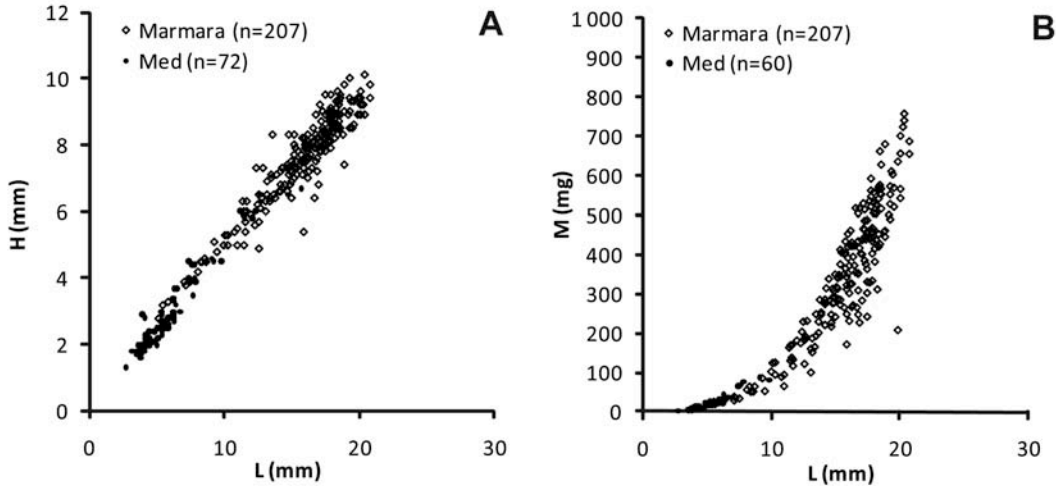


Figure 5. (A) Length-height and (B) length-mass relationships for the *Idas*-like nov. sp. (light diamonds) and *Idas* aff. *modiolaeformis* (black circles) from the eastern Mediterranean Sea. Total preserved wet biomass indicates total animal biomass (shell + tissue). The relation used is  $Y=a(X)^b$  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	<i>Idas</i> -like nov. sp.	$H=0.6942*L^{0.867}$	0.92	$F=754, p<0.001$
Med	<i>Idas</i> aff. <i>modiolaeformis</i>	$H=0.5014*L^{0.9895}$	0.88	$F=527, p<0.001$
Marmara	<i>Idas</i> -like nov. sp.	$M=0.16226*L^{2.7526}$	0.91	$F=2161, p<0.001$
Med	<i>Idas</i> aff. <i>modiolaeformis</i>	$M=0.1596*L^{2.8893}$	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

339 6) resulting from the Bayesian analysis of combined COI mtDNA and 28S rRNA gene  
 340 fragments was consistent with the results presented by Lorion et al. (2010).  
 341 Specimens from the Marmara Sea clustered within the clade that includes all  
 342 symbiont-bearing mussels except the genus *Benthomodiolus*. However, *Idas*-like  
 343 nov. sp. could not be included into any of the lineages discussed in Lorion et al.  
 344 (2010) and instead formed a long branch clustering within the multifurcation of those  
 345 lineages (Figure 6). The position of *Idas*-like nov. sp. within the Mytilidae tree could  
 346 thus not be further resolved.  
 347

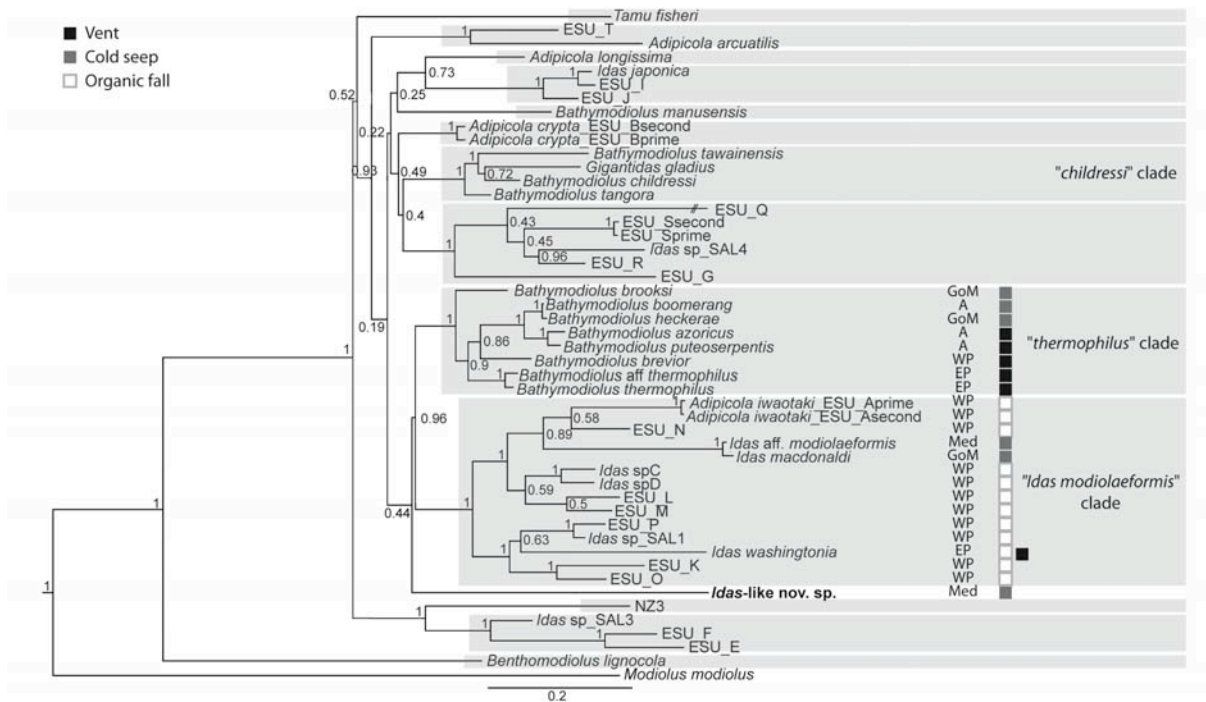


Figure 6. Maximum clade credibility phylogram obtained from Bayesian analyses of the sequences. Values above the nodes correspond to the posterior probabilities obtained from Bayesian analyses. The grey boxes correspond to lineages discussed in Lorion et al. (2010). The type of ecosystem inhabited by 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.

348

349



350 3.4. Diversity and phylogeny of associated bacteria

351 Of the 90 clones sequenced, the majority (87%) of the sequences were affiliated with  
352 the Gammaproteobacteria class, and highly similar to the sulphur-oxidising symbiont  
353 of cold-seep and hydrothermal vent mussels of the genera *Bathymodiolus* and *Idas*  
354 (>98% similarity; Figure 7). The 1 base pair differences between clones are  
355 potentially due to sequencing errors and therefore these sequences may represent  
356 the same phylotype as the one related to mussel-associated thiotrophic symbionts  
357 (Figure 7). Besides the Gammaproteobacteria, two sequences were affiliated with the  
358 Epsilonproteobacteria class, three to uncultured bacteria involved in the ANaerobic  
359 AMMonium OXidation reactions (or Anammox) and the last one did not have any  
360 clear phylogenetic affiliation. With a coverage value of 75% for the clone library and a  
361 rarefaction curve that shows saturation, it is unlikely we have missed any abundant  
362 symbionts (Electronic supplementary material 1). One clone of  
363 Gammaproteobacteria, the dominant phylotype, is used in the phylogenetic tree.  
364 Indeed, the low clone number of other types of bacteria suggests that they represent  
365 potential contaminants, free-living bacteria attached to the gills due to the filtration  
366 abilities of the mussels, or some rare symbionts.

367

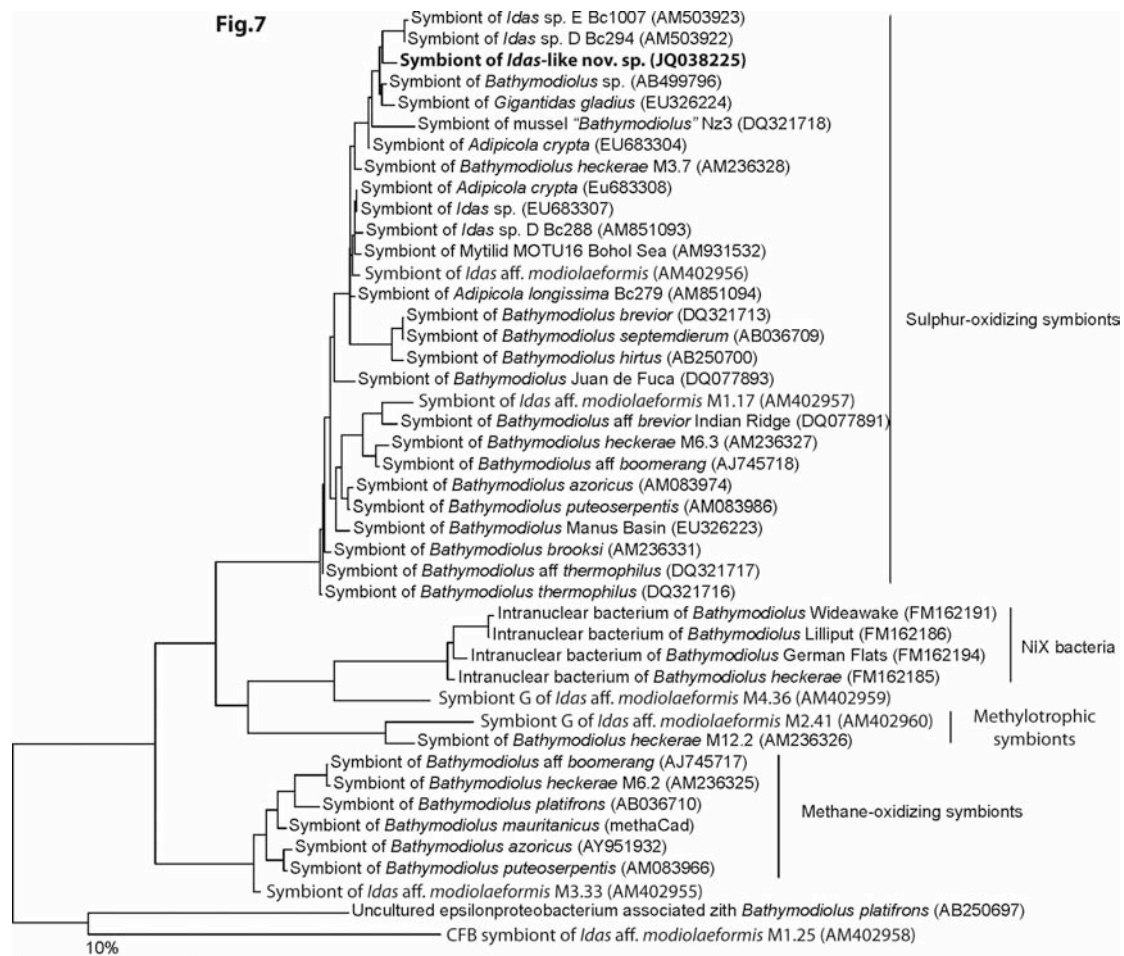


Figure 7. Phylogenetic tree displaying bacterial symbionts associated with *Idas-like nov. sp.* based on 16S rRNA gene sequences (in bold). Bacteroidetes (CFB) and uncultured epsilonproteobacteria are used as an outgroup to the gammaproteobacteria. Posterior probabilities are displayed as percentages. 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

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

389

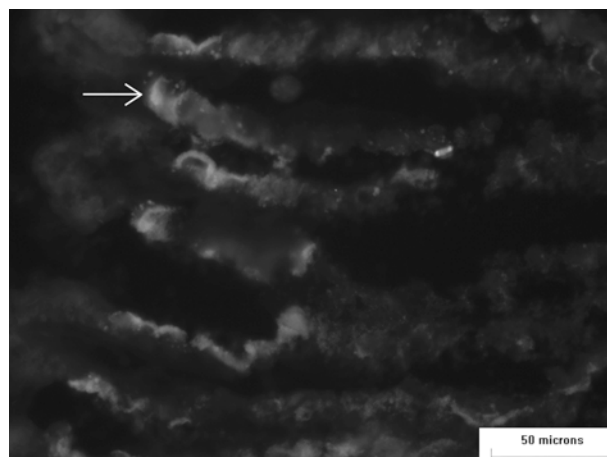


Figure 8. Fluorescence *in situ* hybridisation on transverse sections of gill filaments of *Idas*-like nov. sp. showing the distribution of thiotrophic symbiont (BangT) on the brightest part (pointed by the white arrow). Composite pictures in colour are reported in supplementary material with (A) BangT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

390

391 3.6. Isotopic signatures

392 Specimens from the Marmara Sea (n=3) displayed  $\delta^{13}\text{C}$  values between -37.4 and -  
 393 35.5‰ and  $\delta^{15}\text{N}$  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‰ ( $\delta^{13}\text{C}$ , Figure 9)  
 395 and 4.0‰ and 5.3‰ for  $\delta^{15}\text{N}$ . 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  $\delta^{13}\text{C}$  values varying from -64.1‰ to -44.1‰ (Figure 9).

398

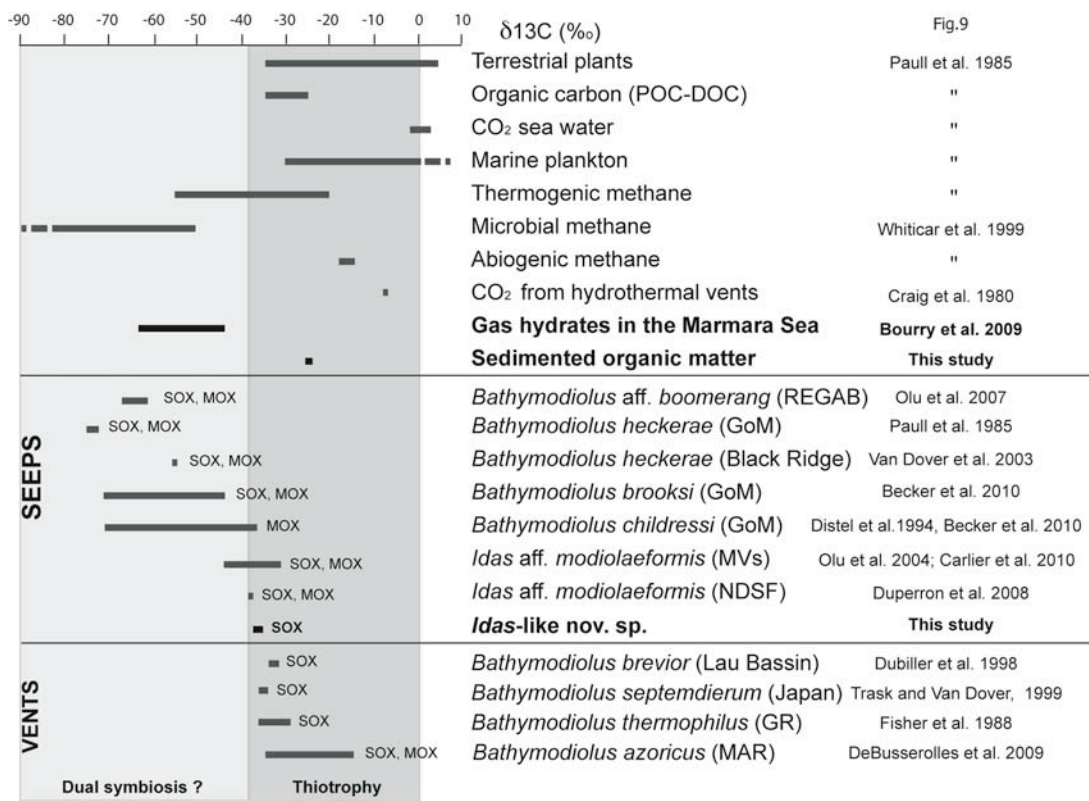


Figure 9.  $\delta^{13}\text{C}$  signatures of several marine and terrestrial carbon sources and a list of mussel species from vent and seep sites. Abbreviations: POC: particular organic matter, DOC: dissolved organic matter, SOX: sulphur-oxidising bacteria, MOX: methane-oxidising bacteria, GoM: Gulf 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 ( $\approx 1\%$ ; 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 1 to  
440 2% per million years, it appears that *Idas* aff. *modiolaeformis* diverged from its sister  
441 species *Idas macdonaldi* between 0.60 and 3.61 million 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. *modiolaefomis*.

453

454 Another interesting result is that *Idas*-like nov. sp. and *Idas* aff. *modiolaefomis* 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 vesicomid *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. *modiolaefomis*, however,  
460 has been reported in the Mediterranean cold-seep sites (Duperron et al., 2008b;  
461 Gaudon 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 *modiolaefomis* 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 *modiolaefomis* 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

475 The life cycle of deep-sea mytilids is still poorly known in terms of reproduction,  
476 growth rates, recruitment, and larval dispersal. However, the occurrence of distinct  
477 modes in several mussel species at deep hydrothermal vents indicates discontinuous  
478 episodes of massive larval settlement linked with discontinuous release of gametes  
479 and planktotrophic larval development (Comtet, 1994; Comtet and Desbruyères,  
480 1998; Dixon et al., 2006; Rhoads et al., 1981; Smith et al., 2000; Van Dover et al.,  
481 1996). The same pattern has been observed at cold-seeps for *Bathymodiolus*  
482 *childressi* in the Gulf of Mexico (Arellano and Young, 2009; Nix et al., 1995; Smith et  
483 al., 2000; Tyler et al., 2007). In the present study, the size-frequency distribution  
484 exhibits a single mode. Furthermore, a high abundance of prodissoconch II post-  
485 larval stages was observed in sediments close to the sampled carbonate crusts.  
486 These prodissoconch II from the Marmara Sea are in the same range of size (450  
487  $\mu\text{m}$ ) as those of species from vents and seeps (from 380 to 520  $\mu\text{m}$ ), supporting the  
488 idea of a planktonic phase (Arellano and Young, 2009; Comtet et al., 2000; Gaudron  
489 et al., 2012; Lorion et al., 2012; Lorion et al., in press; Lutz et al., 1980; Lutz et al.,  
490 1984). The abundance of these post-larvae and the absence of distinctive modes  
491 suggest the presence of a continuous recruitment. Several of these larvae were  
492 dead, raising questions about the cause of this massive mortality. High recruitment  
493 rates in hydrothermal *Bathymodiolus* mytilids were hypothesised to balance for  
494 episodic, important mortality resulting from natural changes in hydrothermal flow and  
495 tectonic activity (Comtet and Desbruyères, 1998). Competition with adults may be  
496 another explanation of the larval mortality at our sampling site. Indeed, post-larvae  
497 may have been excluded from the hard substratum they need to settle, by the  
498 presence of adult specimens already attached on the substratum. It is hypothesised  
499 that all of the mussels within a single patch have the same age and resulted from a



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

502

#### 503 4.3. A thiotrophic symbiosis

504 Thiotrophic symbioses are well-documented for many small mytilids associated with  
505 organic falls, including various *Idas* species from sunken woods in the eastern Pacific  
506 (Duperron et al., 2008a). In *Idas*-like nov. sp., thiotrophic bacteria unambiguously  
507 dominate the populations of gill-associated symbionts. This symbiotic association  
508 resembles that of many *Idas* spp., but greatly differs from that occurring in *Idas* aff.  
509 *modiolaeformis* which harbours up to six distinct bacterial phlotypes, including  
510 methane- and sulphur-oxidising bacteria (Duperron et al., 2008b). Also, the  
511 thiotrophs of these two mussel species are not very closely related, an observation  
512 that is often reported in not very closely related species of mussels (Duperron et al.,  
513 2009).

514

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

524

#### 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. septemdiarum* (-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 <sup>13</sup>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

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

558

#### 559 4.5. Conclusion

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

569

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

575 Marmara Sea species despite the presence of methane-enriched fluids (Ritt et al.,  
576 2010). However further analyses on a higher number of individuals is needed to  
577 define whether the SOX bacteria are truly the only type, or at least the very dominant  
578 type of symbionts living within *Idas*-like nov. sp. gills. Questions about the ability of  
579 this symbiotic species to cope with its seep environment, and the exact role of the  
580 symbionts in host nutrition compared to other potential sources, remain to be  
581 elucidated.

582

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584

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597

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599

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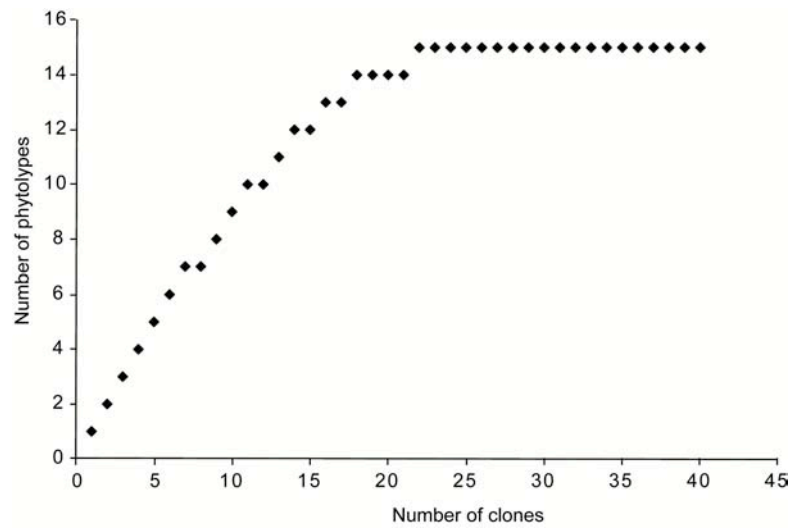
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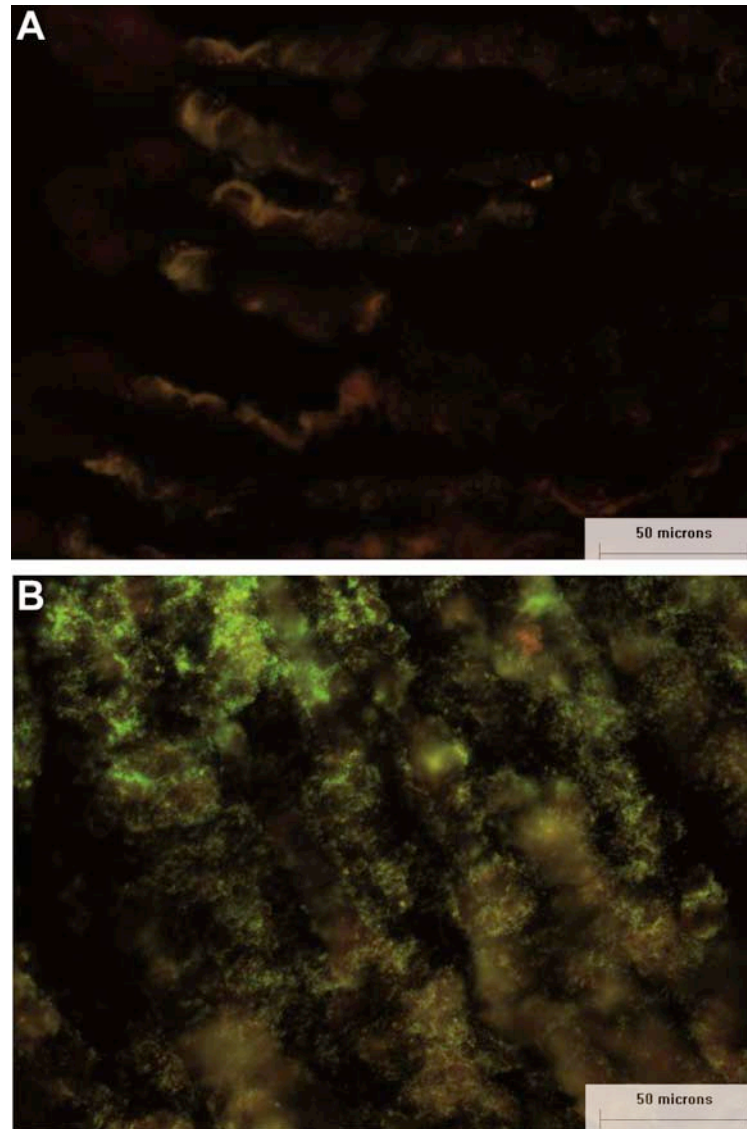
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Electronical supplementary 1. Rarefaction analysis of the overall, combined bacterial 16S rRNA gene clone library recovered from gills of the *Idas*-like nov. sp. Marmara specimen. The rarefaction curve, plotting the number of observed phylotypes as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