

## Evolutionary history of *Idas* sp. Med (Bivalvia: Mytilidae), a cold seep mussel bearing multiple symbionts

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**Abstract:** Small mytilids of the genus *Idas* are related to the large mussels found worldwide at deep-sea hydrothermal vents and cold seeps. They are therefore keys to a better understanding of the colonization of vents and seeps by symbiont-bearing organisms, but still little is known about their biology. For this study, specimens of a mytilid referred to the genus *Idas* were collected from various substrates in a cold seep area near the Nile deep sea fan. Based on molecular and morphological data, all specimens are confirmed to belong to a single species of the genus *Idas*, which was previously shown to host six distinct bacterial symbionts. Its larval shell characteristics indicate a long planktonic phase, which could explain its close relationship to a mussel species that occurs in the Gulf of Mexico. 3-D FISH indicates the dominance of sulfur-oxidizing, methane-oxidizing and methylotrophic symbionts in all specimens analysed.

**Résumé :** Histoire évolutive de *Idas* sp. Med (Bivalvia : Mytilidae), une moule de suintements froids associée à plusieurs bactéries symbiotiques. Les mytilidés du genre *Idas* forment un groupe monophylétique avec ceux associés aux sources hydrothermales et aux suintements froids. Ils sont donc la clé d'une meilleure compréhension du scénario de colonisation de ces environnements par les organismes symbiotiques. Pour autant, leur biologie est encore aujourd'hui fort peu connue. Pour la présente étude, des moules ont été collectées à partir de différents substrats aux alentours du delta profond du Nil. Nous utilisons ici une approche moléculaire et morphologique pour démontrer que ces spécimens appartiennent à une espèce du genre *Idas*, dont il a été montré précédemment qu'elle était en association symbiotique avec 6 lignées bactériennes, un cas sans précédent dans ce taxon. La coquille larvaire suggère une longue phase planctonique qui pourrait expliquer la parenté avec une espèce présente dans le Golfe du Mexique. Une approche de 3D-FISH indique la dominance de symbiontes de types sulfoxydants, méthanotrophes et méthylotrophes dans tous les individus analysés.

**Keywords:** *Idas* • Planktotrophy • Organic falls • Cold seeps • Symbiosis

## Introduction

Bathymodiolin mussels are among the dominant macrofauna occurring at hydrothermal vents and cold seeps worldwide (Olu-Le Roy et al., 2007). Distel et al. (2000) first showed that these deep-sea mussels cluster within a single clade that also includes other mussels living on sunken organic substrates such as wood and vertebrate bones. This extended bathymodiolin clade was later confirmed by other phylogenetic studies (Iwasaki et al., 2006; Samadi et al., 2007; Fujita et al., 2009; Kyuno et al., 2009; Miyazaki et al., 2010). Recently, improved sampling of mussel species associated with sunken organic substrates suggested that several habitat shifts occurred during the evolution of the bathymodiolin clade (Lorion et al., 2010). Apart from early morphological descriptions, the mussels associated with organic falls have received attention only recently (Deming et al., 1997; Gros & Gaill, 2007; Duperron et al., 2006, Duperron et al., 2008a; Samadi et al., 2007; Southward, 2008; Lorion et al., 2009; Tyler et al., 2009). Because there are many species of these mussels, an investigation of their biology is needed to improve our understanding of the evolution of the whole group and the adaptive processes that allowed the colonization of deep-sea hydrothermal vents and cold seeps.

The presence of symbiotic chemosynthetic bacteria in mussel gills is one of their most striking characteristics (reviewed in Duperron et al., 2009). Bacteria are mostly sulfur-oxidizing Gammaproteobacteria, which sustain part of the mussel nutrition and therefore represent a major adaptation to sulfide-rich deep-sea environments (Cavanaugh et al., 2006). Among deep-sea mussels, some species harbour several bacterial strains. The most common bacterial type in addition to sulfur oxidizers is methane-oxidizers, which are found in species sampled at methane-rich vent and seep sites (Cavanaugh et al., 1987). One may hypothesize that the acquisition of additional symbionts allows greater flexibility of the host in the use of substrates and therefore enhances adaptability (Cavanaugh et al., 2006). Moreover, some species display more than two lineages of bacteria in their gills, and it has been shown that these multiple associations are highly flexible (Distel et al., 1995; Halary et al., 2008).

To better understand how flexibility of symbiotic associations has influenced evolution of deep-sea mussels, the undescribed species so-called *Idas* sp. Med, sampled in the eastern Mediterranean, seems a relevant model. Indeed, *Idas* sp. Med has been collected from cold seeps and harbours six distinct symbionts, based on their 16S rRNA, although it belongs to a clade including mostly mussels from organic falls bearing only sulfur-oxidizing symbionts (Duperron et al., 2008a, Lorion et al., 2010). The six symbionts include five Gammaproteobacteria (one

methane- and two sulphur oxidizers, a methylotroph, and a bacterium of unknown metabolism) and a representative of the Bacteroidetes. The closest relative of *Idas* sp. Med is *Idas macdonaldi* Gustafson, Turner, Lutz & Vrijenhoek, 1998, which lives at cold seeps in the Gulf of Mexico and harbours sulfur-oxidizing bacteria (Won et al., 2008). It is striking that such a trans-Atlantic geographical pattern is also reported for other species complexes in the bathymodiolin clade, namely *Bathymodiolus boomerang* Cosel & Olu, 1998 and *Bathymodiolus mauritanicus* Cosel, 2002 (Olu-Le Roy et al., 2007; Génio et al., 2008). In these species complexes, the large scale at which occurs genetic structure is explained by the occurrence of highly dispersive larvae (Lutz et al., 1984; Tyler et al., 2007).

Until now only four specimens of *Idas* sp. Med from a single collection site have been analysed and this species remains poorly known (Duperron et al., 2008a). In particular, there is little or no data regarding (i) its taxonomic status (ii) its developmental mode (iii) the flexibility of its symbiotic associations and (iv) its evolutionary history. Recently, three cruises explored the eastern Mediterranean cold seeps and yielded additional specimens. These were collected from three substrates: authigenic carbonates, tubes of the siboglinid annelid *Lamellibrachia* sp., and wood colonization devices deployed for a year away from dark sediment patches, animal aggregates, or carbonate crusts (and thus hopefully from the direct influence of seepage). Here, morphological and molecular analyses are employed to test whether all specimens correspond to one species, *Idas* sp. Med, and discuss its similarity with species already described morphologically in the Mediterranean sea. Dispersal capabilities were evaluated by analysing larval shell characteristics. The presence of the six reported symbionts was tested in mussel specimens sampled on the different substrates (Duperron et al., 2008a), and symbiont relative abundances were estimated using the newly developed 3-Dimensional Fluorescence *In Situ* Hybridization (3D-FISH) technique (Halary et al., 2008). Finally, the evolutionary history of *Idas* sp. Med is discussed here in the light of the new biological and phylogenetic data.

## Material and Methods

### Sampling

The NAUTINIL (2003, chief scientist J. P. Foucher, specimens M1 to M4), BIONIL (2006, chief scientist A. Boetius, specimens M11 to M27) and MEDECO (2007, chief scientist C. Pierre, specimens M31 to M49-3) cruises from the EU Mediflux / HERMES program allowed the collection of 24 mussel specimens at three locations of the

Nile deep-sea fan (Table 1). Samples were recovered from the Central Zone, from the Amon mud volcano, and from Caldera (Kheops mud volcano) in the vicinity of the Nile deep-sea fan at depths between 1150 and 3000 m. Mussels were found attached to three types of substrates: authigenic carbonate crusts, tubes of the siboglinid tubeworm *Lamellibrachia* sp., and wood chips consisting of Douglas Fir deployed in experiments during the BIONIL cruise. For six specimens, one gill was fixed for *in situ* hybridization as described previously (Duperron et al., 2008b). Remaining tissues and the 17 other specimens were stored in 95% EtOH for genetic analyses.

#### *Morphological examination*

The shell morphology of 8 new specimens was compared with published descriptions and pictures of type specimens of *Idas* species reported from the Mediterranean Sea and Atlantic Ocean: *I. argenteus* Jeffreys, 1876, *I. modiolaeformis* (Sturany, 1896), *I. simpsoni* (Marshall, 1900), *I. dalmasi* Dautzenberg, 1927, *I. ghisotti* Warén & Carrozza, 1990, *I. macdonaldi* Gustafson, Turner, Lutz & Vrijenhoek, 1998 and *I. cylindricus* Pelorce & Poutiers, 2009. Type specimens of *I. simpsoni*, *I. ghisotti* and *I. cylindricus* were available for direct comparison. Larval shells of two specimens (M3 and M49.3) were investigated in detail. Valves were mounted on aluminium stubs, covered with a 700 Å layer of gold-palladium, and examined under a JEOL JSM 840A Scanning Electron Microscope (SEM).

#### *Host genetics*

DNA was extracted using the QIAamp® DNA Micro Kit (Qiagen). A fragment of the Cytochrome Oxidase I (COI) mitochondrial gene was amplified using the primers H691 (5'-GTRTTAAARTGRCGATCAAAAAT-3'), which was designed for deep-sea mussels, and LCO1490 (Folmer et al., 1994). Domains D1, D2 and D3 (Qu, 1986) of the 28S rRNA nuclear gene were amplified using primers C1' (5'ACCCGCTGAATTTAAGCAT3') and C4 (5'TCG-GAGGGAACCAGCTACTA3'). PCR reactions were performed in 25 µL final volume, containing approximately 3 ng template DNA, 1.5 mM MgCl<sub>2</sub>, 0.26 mM of each nucleotide, 0.3 µM of each primer, 5 % DMSO and 0.75 unit of Taq Polymerase (Qbiogene). Amplification products were generated by an initial denaturation step of 4 min at 94°C followed by 35 cycles at 94°C for 40 sec, 50°C (52°C for 28S rDNA) for 50 sec and 1 min at 72°C, and by a final extension at 72°C for 10 min. PCR products were purified and sequenced in both directions at the Genoscope (Evry, France). Sequences were deposited in the NCBI database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov), Table 1).

Sequences obtained were added to a dataset including COI mtDNA and 28S rRNA sequences from each species

and evolutionary significant unit (ESU) of the lineage which includes *Idas* sp. Med and *I. macdonaldi* (lineage L6, Lorion et al., 2010; Table 2). According to that study, sequences of *Bathymodiolus thermophilus* Kenk and Wilson 1985 and *B. aff. thermophilus* were also included and these two sister species were used as an outgroup. Datasets were aligned using the module Clustal W in Mega 4 (Tamura et al., 2007). Positions of the 28S rRNA alignment for which homology was ambiguous due to insertions and deletions were not considered in analyses. The best fitting models of nucleotide substitution were selected using JModeltest and the AICc criterion (Posada & Crandall, 1998). These models were set in Bayesian analyses performed with Beast 1.5.3 (Drummond & Rambaut, 2007). The Yule model was used as the tree prior and for each gene, the heterogeneity of the mutation rate across lineages was set under uncorrelated log-normal clocks for each gene. In an attempt to estimate the age of the divergence between *I. macdonaldi* and the species documented here, the COI mtDNA mutation rate was set under a normal prior whose 95% confidence interval ranged from 1% to 2% per million years, according to Won et al. (2003). For each dataset, four independent analyses, starting from distinct coalescent trees, were run over 40 million generations and sampled each 2,000 steps. After analyzing results with Tracer v1.4.1 and discarding 10% samples as a burn-in, independent runs were pooled and resampled each 8,000 steps. The maximum clade credibility tree, the posterior probabilities of its nodes, divergence estimates and corresponding 95% Highest Posterior Densities (95% HPD) were calculated from these pooled results. Kimura two-parameters (K2P) genetic distances were calculated with Mega 4.

#### *Symbiont relative abundances using 3D-FISH*

Gill tissues of specimens M31, 32, 33, 38-3, 45 and 46, representing distinct habitats (exposed crust, crust covered with dark sediment, and wood) were embedded in Steedman's wax as described in Duperron et al. (2008a). Transverse sections of gill filaments, 10 µm-thick, were cut using a microtome (Jung, Germany) and deposited on Superfrost Plus slides. Hybridizations were performed using protocol and probes from Duperron et al. (2008a). Individual hybridization with each of the six probes described in Duperron et al. 2008a was performed to test qualitatively the presence of each symbiont. To estimate the percentage of bacterial volume originating from methano- and methylotrophs, probes ImedM-138 (methanotrophs) and BhecM2-822 (methylotrophs) were both Cy3-labelled and tested against a Cy5-labelled Eub338 probe (Amann et al., 1990; Duperron et al., 2008a). To estimate the percentage of bacterial volume originating from sulfur oxidizers, probes Bthio-193 and ImedT2-193, specific for

**Table 1.** *Idas* sp. Med. Specimens sequenced for this study. Cruise, type of substrate, collection sites, coordinates and depth are indicated, as well as the COI haplotype number used in the text and Genbank accession numbers for COI mtDNA and 28S rRNA. Specimens whose shell was examined and those used for SEM examination of larval shell are marked with \* and \*\*, respectively. Specimens investigated using 3D-FISH are in bold. Mussels correspond to event labels NL-D1561-Panier-3 (M1 to M4), M70/2b\_798\_SFS-8 (M11, M13, M23), M70/2b\_805\_SFS-8 (M26, M27), MEDECO2-D338-BOX-3 (M31 to M33), MEDECO2-D339-BOX-6 (M38, M41) and MEDECO2-D343-PANIER-1 (M45 to M49-3) stored in the PANGAEA database ([www.pangaea.de](http://www.pangaea.de)).

**Tableau 1.** *Idas* sp. Med. Specimens analysés pour la présente étude. La campagne, le type de substrat, le site de collecte et la profondeur sont indiqués, ainsi que le numéro d'haplotype utilisé dans le texte et les numéros d'accès Genbank pour les séquences de COI mtDNA et 28S rRNA. Les spécimens dont la coquille a été examinée et ceux utilisés pour l'examen des coquilles larvaires en SEM sont respectivement marqués avec \* et \*\*. Les spécimens pour lesquels a été utilisée la méthode 3D-FISH sont marqués en gras. Les spécimens correspondent aux numéros d'événements NL-D1561-Panier-3 (M1 à M4), M70/2b\_798\_SFS-8 (M11, M13, M23), M70/2b\_805\_SFS-8 (M26, M27), MEDECO2-D338-BOX-3 (M31 à M33), MEDECO2-D339-BOX-6 (M38, M41) et MEDECO2-D343-PANIER-1 (M45 à M49-3) enregistrés dans la base de données PANGAEA ([www.pangaea.de](http://www.pangaea.de)).

Specimen	Substrate	Location	Long lat	Depth	Haplotype	COI mtDNA	28S rRNA
M1	Carbonate	Central zone	N 32°38 E29°55	2129 m	Hap 1	FJ158587	FJ159557
M2, M3**, M4*	Carbonate	Central zone	N 32°38 E29°55	2129 m	Hap 2	FJ158584-6	FJ159554-6
M11*	Carbonate	Amon mud volcano	N 32°22 E 31°42	1153 m	Hap 2	FJ158583	FJ159553
M13*, M23*	Carbonate	Amon mud volcano	N 32°22 E 31°42	1153 m	Hap 5	FJ158581-2	FJ159551-2
M26, M27	Tube of <i>Lamellibrachia</i>	Amon mud volcano	N 32°22 E 31°42	1157 m	Hap 2	FJ158579-80	FJ159549-50
<b>M31, M32</b>	Carbonate	Central zone, site 2A	N 32°32 E30°21	1700 m	Hap 2	FJ158577-8	FJ159547-8
M38-2*, -4*, -6	Wood	Central zone, site 2A	N 32°32 E30°21	1700 m	Hap 2	FJ158573,4,6	FJ159543,4,6
<b>M33</b>	Carbonate	Central zone, site 2A	N 32°32 E30°21	1700 m	No data	No data	No data
<b>M38-3</b>	Wood	Central zone, site 2A	N 32°32 E30°21	1700 m	Hap 4	FJ158575	FJ159545
M41	Carbonate	Central zone, pockmarks site 2A	N 32°32 E30°21	1700 m	Hap 2	FJ158572	FJ159542
<b>M45</b>	Carbonate with dark sediment	Caldera, Kheops mud volcano	N 32°08 E 28°09	2995 m	Hap 5	FJ158571	FJ159541
<b>M46</b>	Carbonate with dark sediment	Caldera, Kheops mud volcano	N 32°08 E 28°09	2995 m	Hap 3	FJ158570	FJ159540
M47	Carbonate with dark sediment	Caldera, Kheops mud volcano	N 32°08 E 28°09	2995 m	Hap 2	FJ158569	FJ159539
M48	Carbonate with dark sediment	Caldera, Kheops mud volcano	N 32°08 E 28°09	2995 m	Hap 5	FJ158568	FJ159538
M49-1	Carbonate with dark sediment	Caldera, Kheops mud volcano	N 32°08 E 28°09	2995 m	Hap 1	FJ158567	FJ159537
M49-2, -3**	Carbonate with dark sediment	Caldera, Kheops mud volcano	N 32°08 E 28°09	2995 m	Hap 2	FJ158565-6	FJ159535-6

**Table 2.** *Idas* sp. Med. Specimens used for this study from Genbank. Species (or ESU) name, habitat, known depth range and geographic distribution are indicated, as well as accession numbers for COI mtDNA and 28S rRNA.

**Tableau 2.** *Idas* sp. Méd. Spécimens de la base de données Genbank utilisés dans cette étude. Le nom d'espèce (ou d'ESU), l'habitat, la gamme de profondeur et l'aire de distribution connue sont indiqués, ainsi que les numéros d'accès pour les séquences COI mtDNA et 28S rRNA correspondantes.

Species / ESU	Habitat	Depth range	Known distribution	COI mtDNA	28S rRNA
<i>B. thermophilus</i> Kenk & Wilson, 1985	Vent	2,460-2,747 m	Eastern Pacific Rise	GU966639	GU966640
<i>I. washingtonia</i> Bernard 1978	Wood, bone	960-2420 m	Eastern Pacific	AY275546	AY781146
<i>I. macdonaldi</i> Gustafson, Turner, Lutz & Vrijenhoek, 1998	Seep	650 m	Garden Banks (Gulf of Mexico)	AY649804	AY781145
<i>A. iwaotakii</i> Habe 1958 (ESU A' and A'')	Wood, bone	440-1760 m	Off Japan, Philippines, New Caledonia & Vanuatu	EU702322 EU702330	EU683295 EU683290
<i>A. arcuatilis</i> Dell, 1987	Bone		Off New-Zealand	FJ937036	GU065880
<i>B. aff. thermophilus</i>	Vent	2,331 m	Eastern Pacific Rise	AF456317	AY781140
<i>Idas</i> sp. Med	Seep	2,129 m	Nile fan (Eastern Mediterranean)	EF210072	FJ159534
<i>Idas</i> sp. SAL1	Wood, bone	450-900 m	Off Solomon islands & New Caledonia	DQ340790	DQ863944
<i>Idas</i> sp. ESU C	Wood, bone	290-800 m	Off Solomon islands & Vanuatu	EU702375	EU683261
<i>Idas</i> sp. ESU D	Wood	760-1760 m	Off Japan & Philippines	EU702350	EU683281
ESU K	Wood	500-540 m	Off Philippines	FJ937192	GU065868
ESU L	Wood	480-990 m	Off Philippines, Vanuatu & Solomon islands	FJ937193	GU065772
ESU M	Wood	590-630 m	Off Vanuatu	FJ937202	FJ159552
ESU O	Wood	600-900 m	Off Vanuatu, Chesterfields and Solomon islands	FJ937208	GU065766
ESU P	Wood	180-1390 m	Off Philippines	FJ937214	GU065853

the two sulfur-oxidizing symbionts, were both Cy3-labelled and used along with Cy5-labelled Eub338 probe (Duperron et al., 2008a). Probes CF319 and ImedG-193 were tested, but yielded very few positive signals.

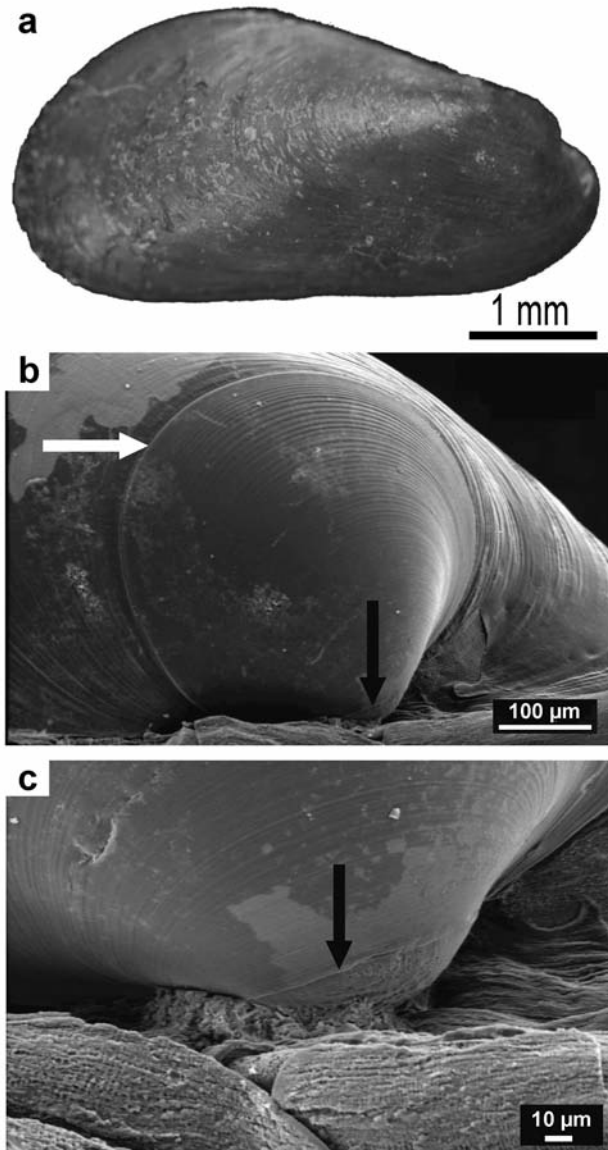
Image stacks were acquired for Cy3 and Cy5 fluorochromes using an Olympus BX61 epifluorescence microscope (Olympus Optical Co., Tokyo, Japan). Images were acquired every 0.3 µm over the whole thickness of sections using the Optigrid™ system (QiOptic, Rochester, NY, USA). The percentage of voxels corresponding to specific signals versus general eubacterial signal was computed using the SymbiontJ plug-in implemented into ImageJ as described elsewhere (Halary et al., 2008).

## Results and Discussion

### *Species identification and molecular taxonomy*

Shells were similar for all 8 examined specimens: yellowish, thin and fragile, often displaying periostracal hairs and ranging from 3 to 10 mm in length. Shape was rhomboidal, umbos were sub-terminal, ventral margins were straight and formed an angle with dorsal margins (Fig. 1a). Shells were highly similar to those of *I. macdonaldi*, and of the three Mediterranean species *I. ghisotti*, *I. simpsoni* and *I. modiolaeformis*. Only *I. modiolaeformis* was collected from cold seeps (mud volcanoes) in the area where our specimens were collected. These may therefore be attributed to that species. However, shape and small size correspond to the morphogroup II defined by Lorion et al. (2010), for which species identification using only shell characters was considered not reliable. Moreover, the original description of *I. modiolaeformis* was very brief and based on two shell valves (Sturany, 1896, p. 20, figs 34-35, 37-38; see also Warén, 1991). Therefore the current identification should be taken cautiously, and that further illustrates the “shell handicap” of the mytilid systematics (Lorion et al., 2010).

We obtained 23 COI mtDNA sequences of 579 base pairs, among which 5 haplotypes were identified. The most frequent haplotype was represented on all three substrate types sampled and was the same as obtained from the specimen analysed previously (Duperron et al., 2008a). The COI mtDNA dataset included overall 39 sequences of 579 base pairs, of which 213 were variable. For the 28S rRNA gene, we obtained 24 sequences of 1004



**Figure 1.** *Idas* sp. Med. (a) Lateral view of a specimen representative of samples collected near the Nile deep-sea fan. (b) and (c) SEM views of the larval shell of the specimen M3. Pictures b and c correspond to magnification of  $\times 190$  and  $\times 500$ , respectively. The white arrow indicates the boundary between dissoconch and prodissoconch, while black arrows indicate the boundary between prodissoconch I and prodissoconch II.

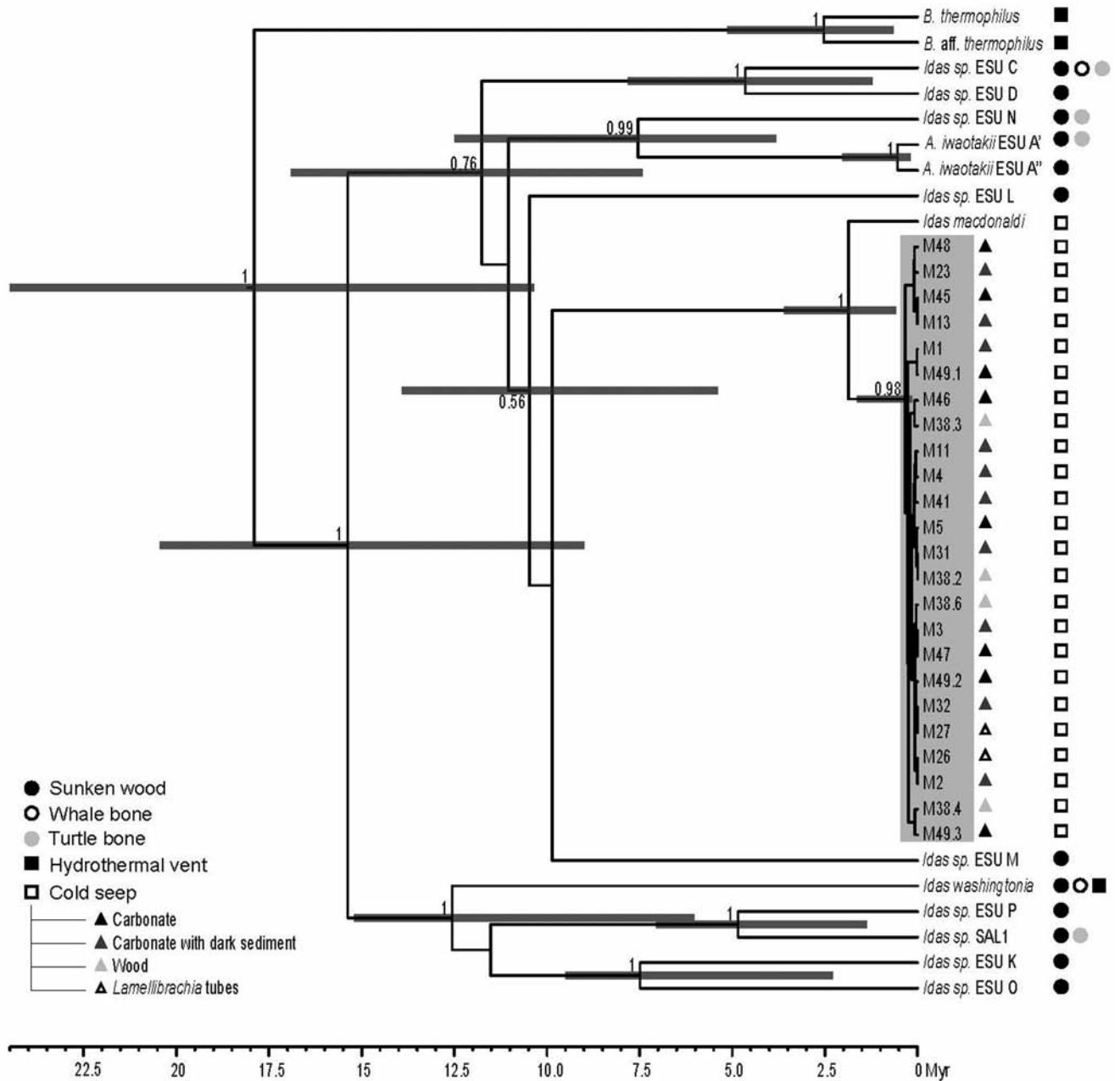
**Figure 1.** *Idas* sp. Med. (a) Vue latérale d'un spécimen représentatif des échantillons collectés aux alentours du delta du Nil. (b) et (c) Vue en SEM des coquilles larvaires du spécimen M3. Les photos C et D correspondent aux grossissements  $\times 190$  et  $\times 500$ , respectivement. La flèche blanche indique la limite entre dissoconque et prodissoconque, et les flèches noires indiquent la limite entre prodissoconque I et prodissoconque II.

base pairs. All were identical to the 28S rRNA sequence of the specimen analysed by Duperron et al. (2008a). The specimen M33 was not included in analyses because its COI mtDNA sequence was missing. The 28S rRNA aligned dataset included 39 sequences and 1029 base pairs, but positions 900 to 905, 429 to 425, 408 to 417 and 386 to 390 were excluded because of uncertainties on their positional homology. The final 28S rRNA alignment included 1003 positions, of which 69 were variable. In the Bayesian tree, specimens from this study formed a well-supported clade (Fig. 2). The mean K2P genetic distance calculated from COI sequences was 0.1% within the clade, which is very close to genetic distances usually reported within other mussels species from deep-sea chemosynthetic environments (Lorion et al., 2010; Won et al., 2003). Both the monophyly and the small genetic distances confirmed that all specimens belong to a single species.

Mussels from the Eastern Mediterranean were closely related to *I. macdonaldi* (Gulf of Mexico), from which COI mtDNA and 28S rRNA differed in mean by 15 substitutions (K2P = 3.3%) and two 1 base pair indels (K2P = 0), respectively. However, only one 28S rRNA sequence was available for *I. macdonaldi*, which prevented the testing of putative introgression patterns that are well-known in mussels despite high levels of mitochondrial divergence (Faure et al., 2009). The current dataset therefore did not allow testing whether *I. macdonaldi* and *I. modioliformis* are two allopatric populations of a single species, or two recently differentiated species. As no molecular data is available for *I. ghisotti*, *I. simpsoni*, *I. argenteus*, *I. cylindricus* and *I. dalmasi*, their relationships with *I. macdonaldi* and *I. modioliformis* remain unclear.

#### Larval shells

All 8 examined specimens exhibited similar larval shells (protodissoconch), approximately 500  $\mu\text{m}$ -diameter, displaying a red colour and clearly distinct from the post-larval shell (dissoconch). These were also observed in SEM-examination of two specimens, which showed that the most distal part of larval shells displayed numerous concentric lines, whereas the part closest to the umbo consisted of a granulated structure, roughly 100  $\mu\text{m}$ -diameter (Figure 1b and 1c). Similar larval shells were reported in *I. argenteus* and in other species associated with hydrothermal vents and cold seeps (Lutz et al., 1984; Dean, 1993). They are interpreted as evidence of a planktotrophic larval development. The small size of prodissoconch I results from the absence of yolk reserves in the eggs, while the well-developed prodissoconch II suggests a long free-swimming feeding phase.



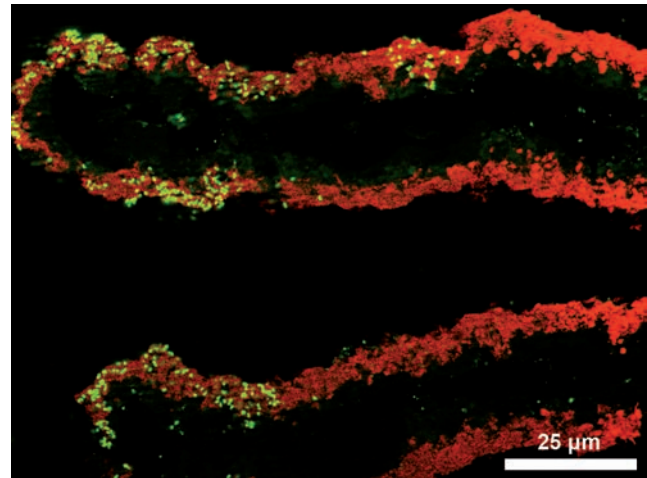
**Figure 2.** *Idas* sp. Med. Chronogram obtained from the bayesian analysis of the combined dataset (COI mtDNA and 28S rRNA). Substrates of specimens for this study and environments of other species are indicated. Dark grey bars at nodes indicate 95% Highest Posterior Densities (HPD) of divergence estimates in million years (Myr). Values above nodes correspond to posterior probabilities.

**Figure 2.** *Idas* sp. Med. Chronogramme obtenu à partir de l'analyse bayésienne du jeu de données combinées (COI mtDNA et 28S rRNA). Le substrat utilisé par les spécimens et leur environnement sont indiqués. Les barres gris sombre aux noeuds indiquent les intervalles comprenant les intervalles de confiance bayésiens à 95% (HPD) des temps de divergence en millions d'années (Myr). Les valeurs aux nœuds correspondent aux probabilités postérieures.

### Symbiotic associations

Analysis of 12 to 35 image stacks per specimen suggested that methylo- and methanotrophs (together referred to as MOX) represented between 23.3 and 32.5% of bacterial volume in gills of specimens collected from exposed crusts (M31, 32, 33) and 17.1 and 22.2% in specimens from the crust half buried in sulfidic sediment (M45 and M46, Table 3, Fig. 3). With respect to the number of stacks analysed, these results are quite reliable. Overall, 3D-FISH indicated that MOX are much less abundant in the new specimens investigated than observed in our earlier study (compare Fig. 3 with Fig. 3 from Duperron et al., 2008a). Due to low FISH signal intensity, the image analysis tool SymbiontJ could analyse successfully only a single image stack from the wood specimen M38-3, yielding 55.6% MOX. This is the first evidence of methanotrophic and methylotrophic bacteria occurring in gills of mussels collected from wood substrate.

Sulfur oxidizers (together referred to as SOX) represented 68.2 to 79.7% of bacteria in specimens from carbonates and 27% in the wood specimen M38-3. The estimation of volume occupied by sulfur oxidizers was rendered difficult by the low-intensity FISH signals obtained, despite attempts, and very few (0 to 4) useable image stacks were obtained per specimen. However, proportions of SOX and MOX in specimens M32, 45 and 46, from which both were estimated, sum up to around 100%. Thus the large proportions of SOX in these three specimens are likely realistic, and clearly visible on FISH images. SOX were also present in the wood specimen.



**Figure 3.** *Idas* sp. Med. Fluorescence *in situ* hybridization on a section of two gill filaments from specimen M45. Sulfur-oxidizing symbionts appear in red and are more abundant than methanotrophs and methylotrophs which both appear in green.

**Figure 3.** *Idas* sp. Med. Hybridation *in situ* à l'aide de sondes fluorescentes sur une section de deux filaments branchiaux du spécimen M45. Les symbiotes sulfo-oxydants sont en rouge et sont plus abondants que les symbiotes méthanotrophes et méthylotrophes, qui apparaissent en vert. Barre d'échelle = 25  $\mu$ m.

Very low numbers of bacteria, and in some sections none at all, hybridized with the probes targeting Bacteroidetes and Gammaproteobacteria G, two groups previously documented as possible symbionts in *Idas modiolaeformis*. This suggests that they might not be associated with all

**Table 3.** *Idas* sp. Med. Number of 3D-FISH stacks analyzed per symbiont type and specimen collected on three distinct substrates (carbonate crust 1 exposed to seawater, carbonate crust 2 half buried in dark and sulfidic sediment, and surface of a wood log). MOX includes methane- and methylotrophs, SOX includes sulfur-oxidizers T1 and T2 (*sensu* Duperron et al., 2008b). Values are provided as percentages  $\pm$  standard deviations. (-) indicates absence of data. Some SOX stacks could not be analysed because of low FISH signal intensities.

**Tableau 3.** *Idas* sp. Med. Nombre de coupes 3D-FISH analysées pour chaque type de symbiote et pour les trois types de substrats (croûte carbonatée exposée à l'eau de mer, croûte carbonatée à moitié enfouie dans un sédiment noir riche en sulfures, et surface de la pièce de bois). MOX inclue les bactéries méthylotrophes et méthanotrophes, SOX inclue les bactéries sulfoxydantes des lignées T1 et T2 (*sensu* Duperron et al., 2008b). Les résultats sont fournis sous forme de pourcentages  $\pm$  déviation standard. (-) indique l'absence de données. Certains empilements d'images SOX n'ont pu être analysées en raison d'un niveau d'intensité insuffisant du signal FISH.

Substrate	specimen	MOX		SOX		MOX+SOX (% of Eub338)
		Stacks analysed/ Total	Volume occupied $\pm$ std dev (% of Eub338)	Stacks analysed /total	Volume occupied $\pm$ std dev (% of Eub338)	
Crust 1 – exposed	M31	19/19	32.5 $\pm$ 22.9	0/7	-	32.5
	M32	12/12	23.3 $\pm$ 4.2	3/9	68.2 $\pm$ 38.4	91.5
	M33	35/35	31.1 $\pm$ 9.6	0	-	31.1
Crust 2 – dark and smelly	M45	22/22	22.2 $\pm$ 14.5	2/11	79.7 $\pm$ 8.8	101.9
	M46	17/17	17.1 $\pm$ 17.0	1/12	68.8	85.9
Wood	M38-3	1/10	55.6	4/17	27 $\pm$ 2.1	82.6



specimens, and questions their significance as symbionts. In particular, the hypothesis that Bacteroidetes could help digest the wood (Duperron et al., 2008a) is not supported because these were absent from the specimen collected on wood.

All specimens investigated here harboured both MOX and SOX in significant amounts, a combination which supposedly allows a greater flexibility with regards to the use of reduced compounds (Distel et al., 1995). Only sulfur-oxidizing symbionts are reported from other *Idas*-like mussels documented from the group including *Idas* sp. C, *Idas* sp. D (Duperron et al., 2008b, based on molecular analyses and FISH), *I. washingtonia* (Southward, 2008, based on electron micrographs) and *I. macdonaldi* (Won et al., 2008, based on molecular analyses). It is thus reasonable to assume that in this clade, methanotrophs and methylotrophs were only acquired in *I. modiolaeformis*. This multiple symbiosis could have helped *I. modiolaeformis* to adapt to the local environments associated with the different substrates.

#### Evolutionary history

The ingroup presented in the tree of Fig. 1B includes only small *Idas* mussels belonging to the L6 clade defined by Lorion et al. (2010). Applying a COI mtDNA mutation rate ranging from 1% to 2% per million years indicated that the clade L6 started diversifying around 15 million years ago (95% HPD: 9-20 Myr, figure 1B). The oldest fossil records of small *Idas*-like mussels are from late Eocene, which range from about 40 to 34 Myr ago (Kiel & Goedert, 2006 & 2007; Amano et al., 2007; Kiel et al., 2010). As our phylogenetic tree includes only a sub-clade of *Idas*-like mussels, the current results are consistent with paleontological data. Using that 1-2%/Myr COI mutation rate also allowed estimating that *I. macdonaldi* and *I. modiolaeformis* diverged about 1.84 million years ago (95% HPD: 0.60-3.61 Myr), well after the Messinian salinity crisis (5.96-5.33 Myr ago) (Duggen et al., 2003). We thus cannot conclude whether the Mediterranean basin played a role in the divergence between *I. macdonaldi* and *I. modiolaeformis* or if these two diverged across the Atlantic. Such geographical patterns are already reported in at least two amphi-Atlantic species complexes of cold seep mussels, namely the “*childressi*” and “*boomerang*” species complexes (Olu-Le Roy et al., 2007; Génio et al., 2008). It was suggested that these complexes are the result of a progressive colonization using intermediate habitats as stepping stones (Olu-Le Roy et al., 2007). Such a scenario is plausible for *I. modiolaeformis*, given its larval development, its wide depth distribution (1150 to 3000 m, table 1) and the occurrence of cold seep areas in the Eastern Atlantic, such as for example in the Gulf of Cadiz. *I. macdonaldi* and *I. modiolaeformis* thus represent the third

example of amphi-Atlantic species or sister species of mussels, and the first among smaller bathymodiolins from the genera *Idas*, *Adipicola* and *Benthomodiolus*.

*I. macdonaldi* and *I. modiolaeformis* belong to a clade which includes mostly species with a strong affinity to sunken wood and bone. As suggested by a statistical reconstruction of ancestral habitats, these two species likely colonized cold seeps from wood-living ancestors (Lorion et al., 2010; see also Figure 1B). The four specimens of *I. modiolaeformis* collected here from wood substrates deployed meters away from fluid seepages suggest that sunken wood could still act as stepping stones for the dispersal of the *I. modiolaeformis* / *I. macdonaldi* group. However confirming this hypothesis will require further experimentation. Indeed, without measurements of reduced compounds around the experimental area, the possible influence of neighbouring seep fluids cannot be completely ruled out.

## Conclusion

Specimens examined in this study were assigned to *Idas modiolaeformis*, although the ambiguity of shell-based identification and the lack of molecular data for other mussel species described from the eastern Mediterranean stress the need for a taxonomic revision of *Idas*-like mussels. This species and *I. macdonaldi*, which are closely related and diverged recently, display an amphi-Atlantic distribution that could be explained by highly dispersive larvae, and by the existence of intermediate habitats. Examination of symbiont relative abundances using 3D-FISH confirmed that multiple symbiont strains occurred in all *I. modiolaeformis* specimens analysed, and emphasized the dominance of sulfur oxidizers, methylotrophs and methanotrophs as symbionts. The later two being absent in other members of the clade, it can be suggested that they were acquired recently, after the divergence with *I. macdonaldi*. Gammaproteobacteria G and Bacteroidetes on the other hand were rarely seen, questioning their significance as symbionts.

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